

DEVELOPMENT OF AVIAN PRIMORDIAL GERM CELLS
IN VIVO AND IN VITRO

by

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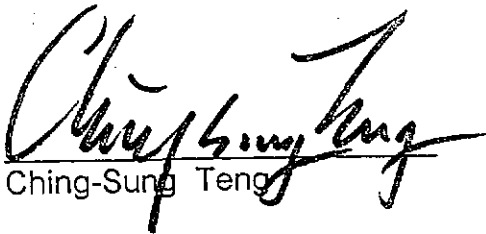
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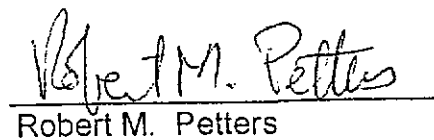
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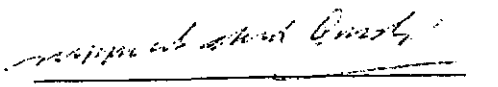
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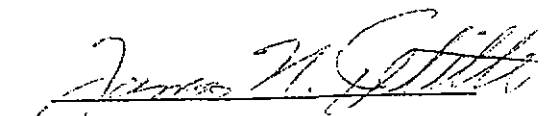
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ABSTRACT

KARAGENÇ, LEVENT. Development of Avian Primordial Germ Cells *In Vivo* and *In Vitro*. (Under the direction of James N. Petitte).

The temporal and spatial patterns of the origin of chick primordial germ cells (PGCs) between stages IX - XIV were investigated using immunohistological analysis of whole mount embryos and histological sections with a monoclonal antibody (MC-480) that recognizes Stage Specific Embryonic Antigen-1 (SSEA-1), and using the culture of dispersed blastoderms at stages IX - XIV with and without STO feeder cells, a **SIM** mouse embryo-derived, Thioguanine- and Oubain-resistant fibroblast cell line. The carbohydrate epitope recognized by MC-480 first appeared at stage X on cells in association with polyingressing cells on the ventral surface of the epiblast and later on the dorsal surface of the hypoblast. The SSEA-1-positive hypoblast cells gave rise to chicken PGCs when injected into the sub-germinal cavity of quail blastoderms or when cultured on a feeder-layer of quail blastodermal cells. When individual stage IX-XIV embryos were dispersed and cultured without a feeder-layer, 25-45 PGCs/embryo were detected using stages X-XIV, but not with stage IX blastoderms. Similar results were obtained when blastodermal cells were cultured either on gelatin-coated tissue-culture plastic or on feeder-layers of CV-1 cells. However, the culture of blastodermal cells obtained from the area *pellucidae* of stages IX-XIII using feeder-layers of STO cells yielded about 110-

170 PGCs/embryo. The addition of STO cell-conditioned medium (STO-CM) to blastodermal cells cultured either on gelatin-coated plastic or on feeder-layers of CV-1 cells resulted in a significant increase in the number of PGCs compared to cultures carried out without STO-CM. Blastodermal cells cultured on feeder-layers of CV-1 cells in the presence of recombinant avian stem cell factor (SCF) (100 ng/mL) yielded a significantly higher number of PGCs after 48 hr of culture compared to those cultured in the absence of SCF. Conversely, the supplementation of blastodermal cell cultures carried out on feeder-layers of either STO or CV-1 cells with basic fibroblast growth factor (bFGF) (10 and 100 ng/mL) significantly reduced the number of PGCs. The inhibitory effect of bFGF on the culture of PGCs on STO feeder-layers was associated with the down-regulation of SSEA-1 and was concomitant with the up-regulation of *brachyury*, a general marker for early mesodermal cells. In accordance with the possible involvement of SCF in the development of avian PGCs, mRNA transcripts for SCF and its tyrosine kinase receptor, c-kit, were detected at pre-streak stages of development. *In situ* hybridization analyses demonstrated that SCF was expressed in epithelial cells forming the gonadal ridge, the final destination of PGCs, while c-kit was expressed by PGCs themselves.

On the basis of these observations, it is proposed that 1) the segregation and development of avian PGCs is a gradual process associated with the translocation of SSEA-1-positive cells from the ventral surface of the *area pellucida* at stage X to the dorsal side of the hypoblast at stages XI-XIV and 2) the differentiation of avian PGCs during the formation of *area pellucida* is not a

cell autonomous process and is the result of specific local cell-cell interactions which might be mediated by peptide growth factors and their corresponding receptors, such as the SCF/c-kit ligand-receptor complex.

(*Key words:* primordial germ cells, chicken, embryo, SSEA-1, basic fibroblast growth factor, stem cell factor, *brachyury*)

DEDICATION

This thesis is dedicated to Prof. Dr. Aydın Evren for introducing me to the world of cells and embryos and to my family for loving me unconditionally.

BIOGRAPHY

Levent Karagenç was born December 13, 1967 in Develi, Turkey, where he completed his primary school education. He completed his middle and secondary school education in Mustafa Kemal Lisesi in Ankara, the capital city of Turkey. He received his Doctor of Veterinary Medicine (DVM) degree from Ankara University in 1989. He became a research assistant in the Department of Animal and Poultry Science the same year. He was awarded a scholarship by the Higher Educational Council of Turkey to pursue a degree of Doctor of Philosophy. To this end, he enrolled in the Ph. D. program in Physiology with a minor in Biotechnology at North Carolina State University in the spring of 1994 under the supervision of Dr. J. N. Petitte in the Department of Poultry Science. Following the completion of his degree, the author has three immediate goals to accomplish: 1- to marry to Tulin Ilhan, a wonderful person and a great friend; 2- to learn how to swim; and 3- to initiate a research program in Turkey to study the origin of primordial germ cells in vertebrates.

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LITERATURE REVIEW

The developmental program leading to the formation of oocytes or spermatozoa begins with the segregation of primordial germ cells (PGCs) from somatic cell lineages at early stages of embryonic development. In vertebrates, this process is temporarily linked to the specification of the body plan in which the four distinguishing features of chordates, viz. the presence of a notochord, a dorsal hollow nerve cord, gill slits and a post-anal tail, are generated. In order to analyze germ line development in relation to events leading to the establishment of this general body plan, it is essential to give an overview of two significant developmental events, namely the formation of the embryonic axes and endomesoderm induction.

The establishment of the vertebrate common body plan can be considered as a process which begins with the formation of an animal-vegetal axis of the oocyte. In all vertebrate groups, the animal-vegetal axis appears to be fixed during oogenesis (Gurdon, 1992).

SPECIFICATION AND FORMATION OF EMBRYONIC AXES IN BIRDS

The plasma membrane of the huge avian egg is supplemented by a vitelline membrane that is composed of an inner (or perivitelline layer) and an outer layer. The inner layer is secreted by the granulosa cells in the ovarian follicle,

whereas the outer layer is secreted by the cells lining the infundibulum. As the ovum descends down the oviduct of the hen, it is surrounded first by a layer of albumen and then by the shell membranes (Etches, 1990).

The first axis that is gained by the avian oocyte is the animal-vegetal axis. Unlike in amphibians, however, the animal-vegetal axis of the oocyte later corresponds to the dorsal-ventral axis of the embryo. This polarity is imprinted on the oocyte while it is still in the follicle.

In birds, the secondary axis corresponds to the anterior-posterior (cranio-caudal) axis, which manifests itself as the primitive streak forms. This is in contrast to the amphibian development, where the secondary axis to be formed is the dorsal-ventral axis. In addition, the formation of the secondary axis takes place at a multicellular stage, when the egg is in the reproductive tract of the hen. In order to obtain a clear understanding of the symmetrization process in the avian embryo, it is essential to analyze the sequence of events that take place from the fertilization of the egg up to the establishment of the anterior-posterior axis.

EARLY DEVELOPMENT OF THE AVIAN EMBRYO

The fertilization of the oocyte takes place in the infundibulum, the anterior region of the oviduct, as the outer layer of the vitelline membrane is laid down. Although several spermatozoa penetrate the perivitelline layer and enter the

germinal disc, only one spermatozoon fuses with the female pronucleus (Perry, 1987). This pattern of fertilization, i.e. physiological polyspermy, appears to be very similar to that observed in urodele amphibians (Elinson, 1997). Soon after fertilization, cleavage divisions begin.

The early development of the chicken embryo from the first cleavage division until the determination of the anterior-posterior axis has been divided into three developmental periods, namely cleavage, formation of the *area pellucida*, and formation of the hypoblast (Eyal-Giladi and Kochav, 1976; Kochav *et al.*, 1980). The cleavage divisions and formation of the *area pellucida* take place while the egg is still in the uterine environment; whereas the formation of the hypoblast requires the incubation of a freshly-laid egg at 37-38 °C for about 6-8 hours. These three developmental periods are further divided into fourteen developmental stages, which are represented by the Hamburger and Hamilton (1951) staging system as a single stage, stage 1. In order to distinguish the developmental stages from those designated by Hamburger and Hamilton (1951), Roman numerals have been used to classify the development of the chicken embryo up to the appearance of the primitive streak. The development of the avian embryo from the time of laying up to the formation of the primitive streak is summarized in Fig. A.

Cleavage

The cleavage period, encompassing stages I to VI, starts some 5 hours following the fertilization as the egg enters the uterus. The first cleavage division of the zygote roughly coincides with the onset of shell membrane formation. The cleavage pattern is very variable from one embryo to the next. Due to the large amount of yolk present in the egg, initial cleavage furrows, running in parallel to the animal-vegetal axis, are incomplete. Hence, blastomeres at the ventral and lateral sides of the blastodisc remain open to the yolk. Until about the 16-cell stage, all the cleavage cells are open (Eyal-Giladi and Kochav, 1976). Cells which are located in the center of the blastodisc are the first to be surrounded by a cell membrane. By the 64-cell stage, only the peripheral cells remain open to the yolk. When the blastoderm consists of a few hundred cells, a cavity forms beneath the blastodisc, separating it from the underlying yolk (Eyal-Giladi and Kochav, 1976; Eyal-Giladi, 1984). This non-yolky cavity is referred to as the subgerminal cavity. After about 10-11 hours from the entrance of the egg to the uterus, the entire cytoplasmic mass of the blastodisc is cleaved, resulting in the formation of an opaque, some five to six cell-thick, embryo. This marks the end of the cleavage period, and from this stage on, the blastodisc is referred to as the blastoderm.

Formation of the *area pellucida* and the process of symmetrization

The formation of the *area pellucida* is a continuous process which begins at stage VII and is completed at stage X when the egg is laid (Eyal-Giladi and Kochav, 1976). This is the first morphological evidence of the symetrization process.

The *area pellucida* forms as cells are shed from the lower part of the blastoderm into the subgerminal cavity (Eyal-Giladi, 1984). The pattern of this rather peculiar cell shedding process is not random. Conrad and Philips (1938) note that the blastoderm acquires an oblique position as a result of the rotation of the egg in the uterus. The cell shedding begins at stage VII from the uppermost portion of the blastoderm, which foretells the posterior side of the embryo. The shedding of cells advances gradually towards the lowermost part of the blastoderm, which foretells the anterior side of the embryo. Therefore, it appears that it is the oblique position of the blastoderm during the process of cell shedding that correlates with the eventual orientation of the anterior-posterior axis. This hypothesis was tested experimentally. If eggs were removed from the hen's uterus and the blastoderm was forced into an oblique position, the highest part of the blastoderm always became the future posterior side of the developing embryo (Kochav and Eyal-Giladi, 1971). This would demonstrate that the ultimate force determining the anterior-posterior polarity is the gravitational field acting on the egg as cell shedding process takes place (Kochav and Eyal-Giladi, 1971; Olszanska *et al.*, 1984). Although the mechanism by which the gravity transforms a radially-symmetric blastodisc into a bilaterally-symmetric

blastoderm is not known, it appears that this is a gradual process since the orientation of the axis can be altered experimentally between 14-16 hour of the uterine age (Eyal-Giladi and Fabian, 1980).

As the cell shedding process continues in a posterior to anterior direction, the multilayered blastoderm is transformed into one to a few cells-thick epithelium surrounded by a peripheral ring of cells attached to the yolk, i.e. *area opaca* (Watt *et al.*, 1993). It is at this stage of development, designated as stage X (Eyal-Giladi and Kochav, 1976) that the process of cell-shedding is completed and the egg is laid. If the blastoderm is cleaned of yolk and viewed under a dissection microscope at this stage, two regions can be distinguished: A translucent, inner region, referred to as the *area pellucida* and an outer, opaque region, referred to as the *area opaca*. The junctional area separating the two regions is referred to as the marginal zone (Fig. A). It should be noted that the marginal zone of the chick embryo is not a homologous structure to the marginal zone of the amphibian embryo.

In a certain percentage of stage X blastoderms a crescent-shaped ridge, referred to as the Koller's sickle, can be distinguished at the posterior side of the marginal zone. This region of the embryo has been demonstrated to be extremely important in patterning the avian embryo (Khaner, 1993). According to Eyal-Giladi (1991), the posterior marginal zone consists only of an epiblastic layer. On the other hand, Stern (1990) accepted the existence of an epiblastic layer, but took notice of the presence of a deep, non-epiblastic layer within this

region, referred to as the deep endodermal layer (Stern, 1990). Cells of the deep endodermal layer are characterized by their expression of the HNK-1 epitope (Canning and Stern, 1988) and their rather loose attachment to the epiblast.

Formation of the hypoblast

The formation of the hypoblast, which requires the incubation of a freshly-laid egg at 37-38 °C for about 6-8 hours, represents the second morphogenetic process in avian embryo development. This period of development is analyzed in three subsequent stages, viz. stages XI to XIII (Eyal-Giladi and Kochav, 1976).

Shortly after the initiation of incubation, the hypoblast begins to form at the posterior (caudal) side of the *area pellucida* as an advancing front of cells (stage XI) and gradually extends anteriorly until it comes to lie beneath the central portion of the *area pellucida* (stage XIII). As the hypoblast forms, the avian blastoderm is transformed into a bilayered-structure. The narrow space between the upper epiblast layer and the lower hypoblast layer is referred to as the blastocoel cavity. This represents the blastula stage of the chick embryo (Khaner, 1993). At this stage of development, the marginal zone is delineated distinctly as a peripheral ring between the inner border of the *area opaca* and the hypoblast. The epiblast, including the marginal zone, will form the embryo

proper, while the hypoblast will give rise to the yolk sac endoderm (Rosenquist, 1966; Wolk and Eyal-Giladi, 1977).

It is generally accepted that at least two distinct cell types contribute to the formation of the hypoblast. One of these cell types is already present in a stage X embryo when the egg is laid as small islands of cells that are loosely attached to the ventral surface of the *area pellucida*. These cells are referred to as the polyingressing cells, since they are believed to arise from the epiblast by ingression (Eyal-Giladi, 1984). The polyingressing cells represent the earliest component of the hypoblast, and therefore, sometimes are referred to as the *primary* hypoblast (Stern, 1990).

A second population of cells that will contribute to the formation of the hypoblast appears shortly after the start of incubation as an advancing front of cells from the posterior marginal zone and Koller's sickle (Weinberger and Brick, 1982). Since the appearance of these cells is secondary to the appearance of the polyingressing cells, they are referred to as the *secondary* hypoblast (Stern, 1990). There are two different opinions as to the origin of this secondary component of the hypoblast. The difference in opinion relates to the definition of the posterior marginal zone and its cellular composition. According to Stern (1990), the secondary hypoblast derives exclusively from the deep portion of the posterior marginal zone, viz. deep endodermal layer. On the other hand, Eyal-Giladi and Debby (1992), who doubt the presence of this deep endodermal

layer, maintain that the epiblast of the posterior marginal zone is the only source of the secondary hypoblast.

The cellular mechanism by which the two cell types contribute to the formation of the hypoblast appears to be different. Cells derived from the deep portion of the posterior marginal zone are considerably more motile than polyingressing cells, whose translocation towards the anterior of the embryo evidently depend entirely on the movements of the secondary hypoblastic cells (Stern, 1990).

ENDOMESODERM INDUCTION

It is generally accepted that the formation of the body axis is a two step process that involves formation of mesoderm and then organization of this newly formed mesoderm into axial structures during the process of gastrulation. The term gastrulation is used to define morphogenetic movements by which vertebrate embryos become a three-layered structure, consisting of an ectoderm, a mesoderm, and an endoderm (Slack, 1991). It is also shortly after the process of gastrulation that the phylotypic body plan common to all vertebrates is obtained.

At the morphological level, gastrulation appears dramatically different among various vertebrate classes. Nevertheless, the end product of the vertebrate gastrulation, i.e. the phylotypic body plan, is strikingly similar among vertebrates.

Therefore, it has been assumed that the mechanisms underlying the process of gastrulation are similar among various vertebrate groups. Molecular studies in recent years have led to the identification of several genes that are involved in events that take place during the process of gastrulation (De Robertis *et al.*, 1994). It is only through a comparative analysis of the expression pattern and function of these genes that it has become possible to reveal some of the common themes that govern vertebrate gastrulation.

ENDOMESODERM INDUCTION IN AMPHIBIANS

In amphibians, the blastocoelic cavity divides the embryo into two different sections, a yolk-rich vegetal half and a cap-shaped animal half. Between the vegetal and animal halves lies a ring-shaped equatorial region that is destined to form the entire endo-mesoderm.

On the basis of several embryological observations, the sequence of events leading to the formation and patterning of the mesoderm in *Xenopus* has been explained using a model known as the "three-signal" model (Slack, 1991). According to this model, signals originating from the ventral vegetal pole cells induce ventral mesodermal structures such as mesenchyme, lateral plate mesoderm and blood islands. On the other hand, a "dorsalizing" signal originating from a group of cells forming the so called Nieuwkoop Center induce equatorial cells in the dorsal marginal zone to form an *organizer* tissue known as

the Spemann *organizer*, i.e. dorsal mesoderm. Therefore, under normal circumstances the establishment of the Nieuwkoop Center precedes the formation of the organizer tissue. It has been recently demonstrated that the activation of the Wnt/ β -catenin signaling pathway is a prerequisite for the establishment of the Nieuwkoop Center in *Xenopus* (Fogotto *et al.*, 1997). Once the Spemann *organizer* is brought into existence, more lateral regions of the marginal zone are induced to become muscle under the influence of the organizer itself. This “horizontal” signaling is mediated by factors such as *folistatin*, *noggin* and *chordin* (Smith, 1995; Conlon *et al.*, 1996). However, the evidence obtained in recent years indicate that the formation and patterning of the mesoderm is more complicated than it appears from the “three-signal” model. Apparently, the activity of dorsalizing signals originating from the Spemann organizer is counterbalanced by a gradient of ventralizing signals, established by *X-Wnt8*, *BMP-4*, *vent-1* and *vent-2* (Grunz, 1997). Therefore, in *Xenopus*, different mesodermal cell types are formed from yet unspecified marginal zone cells under the influence of “dorsalizing” and “ventralizing” signals.

Despite a relatively high level of resolution reached in understanding the molecular mechanisms surrounding mesoderm induction and patterning in the amphibian embryo, the molecular identities of endogenous signals which are responsible for the formation of various mesodermal cell types remain largely unknown. Nevertheless, several advances have been made in elucidating the

molecular and cellular interactions that lead to the formation of the dorsal mesodermal structures (Heasman, 1997).

ENDOMESODERM INDUCTION IN BIRDS

The primitive streak is the sole source of the endodermal and mesodermal structures in the avian embryo, much like the blastopore of the amphibian embryo. As the primitive streak begins to form at the posterior side of the *area pellucida*, the embryo is transformed from a bilayered-structure into a three-layered structure, consisting of ectoderm, mesoderm and endoderm from which the entire body of the embryo will develop. Two significant tissues are thought to be involved in the formation of the primitive streak: the hypoblast and the posterior marginal zone.

The role of the hypoblast in the induction of the primitive streak

It has been hypothesized that the induction of the primitive streak in the chicken embryo is an event analogous to the induction of the dorsal blastopore in amphibians. The evidence in support of this hypothesis came first from the pioneering experiments of Waddington (1933). If the hypoblast is rotated 180° in such a way that the anterior part of the hypoblast lays under the posterior site of the epiblast, a new primitive streak forms at a site corresponding to the posterior

side of the hypoblast. On the basis of these observations, Waddington (1933) suggested that the hypoblast induces morphogenetic movements of the epiblast cells that lead to the formation of an ectopic primitive streak. Later, Azar and Eyal-Giladi (1981) extended Waddington's experiments. Hypoblasts from stage XIII to stage 2 embryos were separated from the epiblast and rotated 90° - 180° relative to the posterior marginal zone. These experiments revealed that the induction of the primitive streak requires the action of an interactive system consisting of the epiblast (competent tissue) and the hypoblast (inductive tissue). The inductive potential of the hypoblast was found to be highest at stage XIII. Moreover, the posterior side of the hypoblast appeared to be more potent in inducing a primitive streak than the anterior and the lateral sides of the hypoblast. The competence of the epiblast coincided with the inductiveness of the hypoblast, both temporally and spatially at stage XIII. According to Azar and Eyal-Giladi (1981), if the polarity of the hypoblast is brought into a conflict with that of the epiblast, as it happens in the rotation experiments, it is the polarity of the hypoblast that determines the polarity of the primitive streak.

If single cells obtained from a hypoblast are allowed to form a sheet in culture, a process which disrupts the polarity of the hypoblast, and then combined with an intact epiblast, the polarity of the epiblast coincides with the polarity of the primitive streak (Mitrani and Eyal-Giladi, 1981). In contrast, if apolarized epiblast explants are combined with a normal hypoblast, the hypoblast appears to dictate the polarity of the streak (Mitrani and Eyal-Giladi,

1984). Furthermore, if the polarity of both the hypoblast and the epiblast is disrupted, and these tissues are combined together axial mesoderm still forms, although in a disorganized manner (Mitrani and Eyal-Giladi, 1984). This would suggest that the function of the hypoblast in the formation of the primitive streak is two fold: formation of the axial mesoderm, and the organization of this newly-formed mesoderm into axial structures.

What is the cellular basis of the polarity that is assigned to the inductiveness of the hypoblast? According to Azar and Eyal-Giladi (1979), a central epiblastic disc of a stage XIII embryo is capable of forming an embryonic axis only if it contains a marginal zone or a hypoblast. However, in the absence of one of these tissues an embryonic axis does not form. Furthermore, despite the fact that the central epiblastic disc can form a lower layer, without the contribution from the marginal zone, this newly formed hypoblast is not capable of inducing a primitive streak (Azar and Eyal-Giladi, 1979). Therefore, it appears that the hypoblast gains its inductive potential as the cells from the posterior marginal zone are incorporated into this tissue. In fact, cell-labeling experiments indicate that the posterior marginal zone cells that contribute to the formation of the hypoblast reside primarily at the posterior side of the hypoblast, where the inductive potential of the hypoblast was suggested to be the highest (Eyal-Giladi and Debby, 1992).

It would appear from the foregoing discussion that the formation of the primitive streak actually represents the formation of the axial mesoderm and that

this process requires the inductive action of the hypoblast acting on the overlying epiblast. It is through this inductive action of the hypoblast that the competent cells of the epiblast change their fate to form a primitive streak from which axial mesoderm and the endoderm will derive. Surprisingly then, a re-examination of the inductive potential of the hypoblast by Khaner (1995) revealed that, in the chicken embryo, the rotation of the hypoblast does not initiate the formation of an ectopic axis and that it is the basic polarity of the overlying epiblast that determines the polarity of the embryonic axis. These recent observations not only cast shadows on the assumption that the hypoblast is the inducer of the primitive streak, but also call for a re-evaluation of the issues that relate to the mesoderm induction and the primitive streak formation in the chick embryo (Khaner, 1995).

The role of the posterior marginal zone in the induction of the primitive streak

Initial studies of Azar and Eyal-Giladi (1979) on the function of the marginal zone in the formation of the primitive streak were later extended by Khaner *et al.* (1985) and by Khaner and Eyal-Giladi (1986). It was demonstrated that if a fragment of the posterior marginal zone (PMZ), including Koller's sickle, is exchanged with a lateral marginal zone (LMZ) of a stage X embryo, only one primitive streak forms from the site where the PMZ fragment had been

transplanted (Khaner and Eyal-Giladi, 1986). Similarly, the transplantation of the marginal zone with its Koller's sickle 90° relative to the posterior side of the central disc results in the formation of an ectopic primitive streak at a site corresponding to the side of the PMZ (Khaner and Eyal-Giladi, 1986). These results clearly indicate that the formation of the primitive streak is initiated where the PMZ resides. Unfortunately, in these experiments cell-labeling was not employed. Therefore, it is not evident whether the forming primitive streak is derived from the central epiblast or from the posterior marginal zone itself. It is important to make this distinction, since in another series of experiments it was demonstrated that the PMZ of a stage X embryo is capable of forming a rudimentary primitive streak in the absence of the central disc (Khaner *et al.*, 1985) which would suggest that the PMZ of a stage X embryo has the developmental potential to form a primitive streak. Thus, it is not possible to conclude with certainty that the function of the PMZ is to induce the formation of the primitive streak.

How is it possible that the rotation of the PMZ shifts the site of primitive streak formation? An answer to this question might come from a critical evaluation of the expression pattern of *gooseoid* (*gsc*) in the chicken embryo. In the chicken embryo, *gsc*-expressing cells are first detected in the posterior marginal zone in association with the Koller's sickle (Izpisua-Belmonte *et al.*, 1993) well before the formation of the hypoblast at stage XIII. It is generally accepted that these cells represent the earliest cell population qualified to be the

functional homologue of the Spemann *Organizer* in the chicken embryo (Izpisua-Belmonte *et al.*, 1993). Therefore, it is possible that the ability of a PMZ fragment to dictate the site of primitive streak formation stems from this small population of $\overline{gsc}$ -expressing cells residing between the epiblast and the hypoblast. Since in amphibians the Spemann *Organizer* is established by signals emanating from a dorsalizing center, *viz.* the Nieuwkoop Center, it is pertinent to ask if there is a dorsalizing center in the chicken embryo whose function would be to establish a small population of *gsc*-expressing cells?

Since *gsc*-expressing cells are located first in the posterior marginal zone region, such a dorsalizing center would be expected to reside within this region. Before an attempt is made to speculate on the possible source of a chicken dorsalizing center, it is important to define the Nieuwkoop Center in morphological, functional and molecular terms. However, for the purpose of this discussion, it would suffice to mention two distinguishing features of the Nieuwkoop Center. First, cells comprising the Nieuwkoop Center do not contribute to the formation of axial structures, but maintain their original endodermal fates. The second most distinguishing feature of the Nieuwkoop Center is the specific localization of a newly-identified transcription factor, *siomoi*s, within this region (Lemaire *et al.*, 1995). Could the deep endodermal portion of the PMZ be the dorsalizing center in the chicken embryo? This hypothesis draws support from several observations. First, the deep endodermal portion of the PMZ does not contribute to the formation of axial

structures, but only to the formation of the yolk-sac endoderm (Stern, 1990). Second, it is in close proximity to the *gsc*-expressing cells in such a way that it can induce the expression of *gsc* in a small population of cells. Third, CWnt8-C is expressed in the epiblast portion of the PMZ (Hume and Dodd, 1993). Moreover, CWnt8-C and *gsc* are expressed by different cell populations (Hume and Dodd, 1993). Therefore, it is possible that CWnt8-C might mediate the establishment of a small population of *gsc*-expressing organizer cells in the PMZ by activating a Wnt/ β -catenin signaling pathway in a similar manner to the establishment of the Nieuwkoop Center in *Xenopus* (Fogotto *et al.*, 1997). If these assumptions are correct, cells forming the deep endodermal layer would be expected to express the transcription factor *siomais*.

GENES THAT ARE INVOLVED IN THE FORMATION AND PATTERNING OF MESODERM

Several genes have been demonstrated to be involved in mesoderm induction and patterning. Among these are *goosecoid*, *brachyury*, *fork-head*, *nodal*, and *nodal*-related genes. An emerging concept from these studies was the similarity in the expression pattern of these genes among various vertebrate groups (De Robertis *et al.*, 1994). This evolutionary conserved pattern of expression also led to the expectation that some of these genes might as well share functional similarities. Since only *goosecoid* and *brachyury* have been

studied in the chicken embryo, only these two genes will be the subject of this discussion.

goosecoid

The *goosecoid* (*gsc*) gene encodes a transcription factor which is homologous to the *bicoid* and *gooseberry* genes of *Drosophila*. In mouse, *gsc*-expressing cells are first detected at the posterior end of the egg cylinder at 6.4 d.p.c. (Blum *et al.*, 1992), and later it is expressed primarily by cells located at the tip of the node, which has been demonstrated to have organizing activity in mouse (Beddington, 1994) and, more dramatically, in *Xenopus* embryos (Blum *et al.*, 1992).

In *Xenopus laevis*, *gsc* mRNA is already present in the unfertilized egg (De Robertis *et al.*, 1992). Before gastrulation, it is expressed primarily by the organizer cells. As the endo-mesodermal cells involute through the dorsal blastoporal lip, *gsc* is down-regulated in many cells, except in those forming the prechordal plate or head mesoderm (Steinbeisser and De Robertis, 1993).

In the chick embryo, *gsc*-expressing cells are first detected in the posterior marginal zone in association with Koller's sickle (Ipsizula-Belmonte *et al.*, 1993) well before the formation of the hypoblast. Cells expressing *goosecoid* are demonstrated to have organizing properties in that they can induce the formation of an ectopic primitive streak (Izpisua-Belmonte *et al.*, 1993).

It would appear that *gsc* marks cells that have organizer activity in vertebrates. These cells form the Spemann's Organizer in *Xenopus*, the node in the mouse, Koller's sickle and later Hensen's node in the chicken (Lemaire and Kodjabachian, 1996).

brachyury

Brachyury is a member of a family of diverse genes that all share a conserved region of about 200 amino acids which includes a DNA binding domain designated the "T-box" (Knezevic *et al.*, 1997). The *brachyury* gene, abbreviated T, was first identified using semidominant mouse mutants characterized by short tails (Beddington *et al.*, 1992). Homozygous mutant animals fail to form a notochord and are characterized with the absence of posterior mesoderm, although anterior axis formation appears to be normal (Herrmann, 1996). Transcripts of the T-gene are first detected in cells ingressing through the primitive streak. At later stages of development, it is down-regulated in the majority of ingressing cells except in those that will form the notochord (Wilkinson *et al.*, 1990).

In *Xenopus*, transcripts of *Xbra* are first detected in the dorsal marginal zone cells, but later expression occurs throughout the marginal zone of the embryo, indicating that *brachyury* marks all mesodermal cell types (Smith *et al.*, 1991). As cells involute during gastrulation, *Xbra* is down-regulated in all cells except

those whose progeny will form the notochord and the tailbud (Smith *et al.*, 1991). The evidence obtained from overexpression studies in *Xenopus* indicate that the expression of *Xbra* is a prerequisite for cells to differentiate into mesodermal cell types (Conlon *et al.*, 1996).

In the chick embryo, transcripts of the chick homologue of the mouse *brachury/T* gene, designated as Ch-*T*, are first detected in the posterior epiblast of stage XIII embryos. As Ch-*T*-expressing cells ingress through the primitive streak, Ch-*T* is downregulated except in cells forming the notochord (Kispert *et al.*, 1995; Knezevic *et al.*, 1997). Recently, two chicken *T*-box genes, namely Ch-Tbx7 and Ch-Tbx6L, have been isolated and characterized (Knezevic *et al.*, 1997). Transcripts of the Ch-Tbx7 are first detected at stage 3 at the posterior end of the primitive streak. Transcripts of Ch-Tbx6L on the other hand, are already present at the posterior marginal zone in a stage X embryo (Knezevic *et al.*, 1997). Although the exact function of chicken *T*-box genes remains to be determined, it is suggested that Ch-*T* and Ch-Tbx6L mark cells that are destined to form the primitive streak (Knezevic *et al.*, 1997).

Treatment of *Xenopus* animal caps with activin, Vg-1, BMP-4 and FGF induces the expression of *XBra*. In the case of FGF, this occurs in the absence of protein synthesis, indicating that the expression of *Xbra* is an immediate-early response to the induction of mesoderm by FGF (Smith *et al.*, 1991). In the chicken embryo, activin and FGF-4 upregulate Ch-*T* and Ch-Tbx6L expression in epiblast cells in culture (Knezevic *et al.*, 1997). Activin has also been

demonstrated to upregulate the expression of Ch-T when applied locally to whole embryos (Kispert *et al.*, 1995).

MESODERM INDUCING FACTORS

In elucidating the molecular mechanisms surrounding the mesoderm induction and patterning in amphibians, two functional assays have been employed, namely mesoderm induction and ectopic axis formation. In the mesoderm induction assay, isolated animal caps are cultured *in vitro* in the presence of putative mesoderm inducing substances and then scored for the presence mesodermal structures using molecular markers. Animal caps cultured in the absence of a mesoderm inducing factor form a solid ball of epidermis.

In the ectopic axis formation assay, molecules to be tested for their ability to induce mesoderm of dorsal type are injected into a cell whose progeny will form ventral mesodermal structures, and then embryos are examined for the presence of an ectopic axis. In a similar approach, UV-irradiated embryos, which are incapable of forming an axis, are injected with putative molecules and then scored for axis development. Using these assays, several molecules and genes have been demonstrated to be involved in mesoderm induction and axial patterning in *Xenopus*. Such factors include members of the transforming growth factor beta superfamily, such as activin, Vg1 and perhaps BMP-4, and members of the fibroblast growth factor family (Heasman, 1997).

Unfortunately, in the chick embryo a direct assay for mesodermal differentiation has been absent. This was mainly due to the fact that, unlike the *Xenopus* animal caps, the chick epiblast forms various mesodermal tissues — including mesenchyme and blood cells spontaneously (Mitrani and Eyal-Giladi, 1982). One drawback of this intrinsic ability of the chick epiblast to form non-axial mesodermal structures is that the induction of non-axial mesoderm can not be examined in the chick embryo directly. Therefore, the end point in an assay following treatment is the formation of axial mesodermal structures rather than mesoderm as such. Furthermore, due to the absence of molecular markers, potential mesoderm inducers had been assessed according to whether embryos deprived of a hypoblast and a marginal zone form a complete embryonic axis. However, it should be noted that the interpretation of results using this criterion is fraught with difficulty, particularly in the light of the evidence that the hypoblast does not induce the formation of a primitive streak (Khaner, 1995). Nevertheless, as the molecular markers for various mesodermal cell types have become available in recent years, it has become possible to examine mesoderm induction and axial patterning in the avian embryo at the molecular level.

Members of the TGF-beta superfamily and mesoderm induction

The TGF- β superfamily includes TGF- β and structurally related molecules such as activin, Vg1, and bone morphogenic proteins (BMPs). Transforming

growth factor betas are homodimeric, disulphide-bonded polypeptides. In the chicken embryo, transcripts of TGF- β 2, 3, and 4 can be detected using *in situ* hybridization analysis as the formation of the primitive streak is completed (Jakowlew *et al.* 1992, 1994). Sanders and Wride (1994) examined the distribution of TGF- β isoforms at early stages of development, viz. prior to the formation of the primitive streak, using a polyclonal antibody which recognizes TGF- β 1 and TGF- β 3. In the unincubated embryo, cells expressing TGF- β 1 or TGF- β 3 are localized in the *area opaca* and the marginal zone in a radially symmetric pattern. At stages XII-XIII, a few immunoreactive cells are observed in association with the hypoblast. Although it is possible that TGF- β s are involved in mesoderm induction, especially in the transformation of epiblast cells into mesodermal cell types during primitive streak formation (Sanders and Prasad, 1991), the experimental evidence is not conclusive as to the involvement of TGF- β s in mesoderm induction (Mitrani and Shimoni, 1990).

Activins, a group of molecules within the TGF- β superfamily, are formed either by a disulfide-linked homodimer of the inhibin β A or β B subunits, designated as activin A and activin B, respectively, or by heterodimerisation of inhibin β A and inhibin β B subunits, forming activin AB.

In *Xenopus*, several lines of evidence indicate that activin is a potent mesoderm inducer (Smith, 1995). Nevertheless, it is not certain if activin is an endogenous player in mesoderm induction during normal development. This

uncertainty comes mainly from the fact that mouse embryos with defects either in activin A, or in both activin A and B (Matzuk *et al.*, 1995) form mesoderm and develop to term, suggesting that activin is not essential for mesoderm induction and patterning in the mouse embryo, a feature which may apply to other vertebrates.

In the chick embryo, activin represents the most extensively studied member of the TGF-beta superfamily. When isolated epiblast explants are cultured in the presence of activin B, they are capable of forming normal axial structures despite the absence of the marginal zone and the hypoblast (Mitrani *et al.*, 1990a). Activin is also capable of inducing an ectopic axis when applied locally at 90° from the original posterior region of the blastoderm (Ziv *et al.*, 1992). Similarly, when small grafts of cells expressing activin A are applied 180° from the original posterior marginal zone, an ectopic primitive streak forms at the site of treatment (Cooke *et al.*, 1994). Apparently, the ectopic axis inducing ability of activin is potentiated by Wnt (Cooke *et al.*, 1994). In the same line with these observations, epiblast cells treated with activin in culture appear to mimic the behavior of endo-mesodermal cells ingressing through the primitive streak (Cooke, 1993). Moreover, activin can induce a range of mesodermal cell types in explants of *area opaca* cells from a stage XII embryo in a dose dependent manner (Stern *et al.*, 1995). It was also demonstrated that activin can induce the expression of *Gnot1*, an organizer-associated chick homeobox gene, in epiblast cells (Knezevic *et al.*, 1995). Interestingly, the highest level of *Gnot1* expression

in epiblast corresponds to the posterior side of the blastoderm, where the hypoblast forms as an advancing front of cells. It is possible that the upregulation of *Gnot 1* in the posterior region of the epiblast is due to a signal, perhaps activinB (Mitrani *et al.*, 1990a), originating from the hypoblast.™

Altogether, these observations support the view that activin might be a major player in axial patterning in the chicken embryo. However, neither activin B itself, nor its two receptors, cActR-IIA and cActR-IIB, are expressed at developmental stages when axis formation is believed to be initiated. Therefore, it would appear that the induction of axial mesoderm by activin is mediated via receptors other than cActR-IIA and cActR-IIB (Stern *et al.*, 1995).

Vg-1 is another member of TGF- β superfamily that has been implicated in the mesoderm induction. In *Xenopus*, Vg1 mRNA is present in the oocyte and early embryos (Rebegliati *et al.*, 1985). Furthermore, a secreted form of Vg1 called BMP-Vg1 induces the formation of a complete axis when applied ectopically (Kesler and Melton, 1995). Recently, chicken Vg1 (cVg1) has been demonstrated to have an ectopic-axis forming ability when applied locally to the epiblast of early chicken embryos (Shah *et al.*, 1997). This would suggest that cVg1 is an active player in mesoderm induction and axis formation in the chicken embryo. Several observations support this hypothesis. First, cVg1 is already expressed by epiblast cells within the posterior marginal zone, well before the formation of the primitive streak (Shah and Dodd, 1995; Seleiro *et al.*, 1996). Secondly, cVg1 can induce the expression of both *brachury*, a general

mesodermal marker, and a range of organizer-specific genes such as *gooseoid*, *chordin*, and *noggin* in *Xenopus* animal cap assays. Thirdly, if a graft of COS-cells expressing cVg1 is applied locally to whole epiblasts an ectopic primitive streak forms (Shah *et al.*, 1997).

Members of the fibroblast growth factor family and mesoderm induction

There is a substantial body of evidence indicating that FGFs can act as inducing agents during early embryonic development. In *Xenopus*, FGF can induce ectodermal cells to differentiate into ventral mesodermal cell types in isolated animal caps (Slack, 1994). This observation is substantiated by the fact that animal caps obtained from blastula-stage embryos that overexpress a dominant-negative FGF-receptor construct do not respond to FGF (Amaya *et al.*, 1993). In such embryos, gastrulation movements are also impaired (Amaya *et al.*, 1993). In *Xenopus*, the upregulation of the transcription factor *Xbra* is an immediate-early response to FGF-mediated mesoderm induction (Smith *et al.*, 1991). However, it is not known if FGF functions in a similar manner during normal development, since embryos which overexpress a truncated activin receptor fail to develop any form of mesodermal structures including ventral mesoderm (Hemmati-Brivanlou and Melton, 1992). In *Xenopus*, this and other observations (see Smith, 1995) cast doubts on the function of FGF as an endogenous mesoderm inducer.

In the chicken embryo, basic FGF (FGF-2) is expressed in the blastoderm from stage XI onwards (Mitrani *et al.*, 1990b). At these early stages of development, bFGF is localized primarily in the nucleus of cells (Dono and Zeller, 1994). If heparin and suramin, substances which inhibit the function of FGFs, are applied to early chicken embryos, the formation of mesodermal structures are inhibited (Mitrani *et al.*, 1990b). There are two additional studies indicating that FGF is biologically active at these early stages of development. First, FGF induces *brachury* expression in epiblast cells obtained from stage X blastoderms (Knezevic *et al.*, 1997). Second, uncommitted cells of stage X blastoderms can be induced either by hypoblast tissue or by bFGF itself to proceed along a haemoglobin differentiative pathway in culture (Gordon-Thomson and Fabian, 1994). This experiment also provided perhaps the most telling data as to the involvement of FGF in mesoderm induction at early stages of the avian embryo development. Gordon-Thomson and Fabian (1994) proposed that the segregation of the erythropoietic cell lineage, i.e. red blood cells, occurs by induction of uncommitted cells by bFGF in the posterior marginal zone before stage XII and that the source of the inductive signal is in the newly emerging hypoblast. According to these authors, blastodermal cells at stage X do not yet enter the erythropoietic cell lineage, but can be induced to do so by bFGF.

Taken together, the establishment of a common vertebrate body plan is the result of two interrelated phenomena, namely gastrulation and endomesoderm

induction, and that there exists a considerable amount of variation in the types of movements observed during gastrulation among various vertebrates. For example, in birds and mammals the main gastrulation movement is the ingression of cells through a linear primitive streak. In *Xenopus*, on the other hand, endomesodermal cells pass through a circular blastopore (De Robertis *et al.*, 1994).

In addition to a body plan common to all vertebrates, the segregation of a small set of cells known as primordial germ cells from somatic cell lineages at an early stage of development is another feature that is shared by all vertebrate embryos. Before the realization of this dichotomous decision in development, however, the embryo consists of multipotent or pluripotent cells whose progeny will contribute not only to the germ cell lineage, but also to the formation of the body plan. Furthermore, it will become evident from the following discussion on the origin of the germ cell lineage in various vertebrates that the segregation of PGCs takes place before the establishment of the common body plan. Therefore, the differentiation of PGCs must be in coordination with that of somatic cells as the embryo is transformed into a complex multilayered body plan. In addition, a mechanism must exist so that the earliest precursors of PGCs are not recruited into cell lineages other than the germ cell lineage during the process of gastrulation.

THE DEVELOPMENT OF THE GERM LINE

The development of the germ line can be analyzed in three subsequent periods. The segregation of primordial germ cells(PGCs), migration of PGCs into the gonadal primordium, and the establishment of a meiotic cell population in the gonads.

The establishment of the germ cell lineage begins with the specification of early embryonic cells to differentiate into PGCs, embryonic precursors of mature gametes. This is the first phase of germ line development. It will become evident that there is a considerable amount of variation as to the temporal and spatial aspects of this process among various vertebrate groups.

In all vertebrates, PGCs first appear at a site away from the region of the embryo where gonads ultimately form. This extragonadal location confronts PGCs with the task of searching for the gonadal primordium as soon as they are determined. This corresponds to the second phase of the germ line development. Once the formation of the gonadal tissue is completed, primordial germ cells undergo a differentiation program leading to the formation of mature gametes. This process corresponds to the third phase of the germ line development. The following discussion will focus on the first two phases of the germ line development, namely the origin and migration of primordial germ cells in amphibians, mammals and birds.

ORIGIN OF PRIMORDIAL GERM CELLS

Anuran amphibians

In vertebrates the specification of the germ cell lineage by means of cytoplasmic determinants, a well documented phenomenon in invertebrates, was reported first by Bounoure who studied the origin of PGCs in anuran amphibians. (Nieuwkoop and Sutasurya, 1979). Bounoure was able to detect patches of a cytoplasmic substance, identified by their staining properties, within the vegetal cortical region of *Rana temporaria* eggs. This cytoplasmic substance, referred to as the "germinal plasm", was later confined to a few cells within the endodermal mass. At later stages of development, cells containing the germinal plasm were located in the gonadal ridge. Later studies not only verified Bounoure's observations in *Rana temporaria*, but also led to the realization that a similar germ cell-specific cytoplasmic substance exists in eggs and embryos of a number of anuran species (Smith and Williams, 1979). In anurans, this intimate association of "germinal plasm" with germ cells throughout embryonic development not only provided a reliable means to trace the origin of the germ cell lineage back to the earliest stages of development, but also led to the assumption that it is through the determinative action of the "germinal plasm" that a few endodermal cells are specified to become primordial germ cells.

The ontogeny of the germ plasm, and therefore, of the germ cell lineage is particularly well understood in South African frog, *Xenopus laevis*. In examining the structural features of the germ plasm, it became evident from electron-

microscopic studies that this substance is composed of numerous mitochondria and electron-dense structures (Eddy, 1975; Nieuwkoop and Sutasurya, 1979). The latter are referred to as the germinal granules and appear to consist of electron-dense foci, embedded in a matrix of fine fibrils. According to Heasman *et al.* (1984), the germ plasm originates from the mitochondrial cloud, also known as the Balbiani body. This structure is initially associated with the germinal vesicle at perivitellogenic stages of oogenesis. During vitellogenesis, the cloud breaks down into smaller clumps that will eventually reside at the vegetal pole of the oocyte. Recent studies have indicated that the germ plasm contains several mRNA molecules such as *Xcat-2* (Foristall *et al.* 1995; Zhou and King, 1996), *Xlsirt* (Klock *et al.*, 1993). However, it is currently unknown if any of these mRNA molecules play a role in the establishment of the germ cell lineage in *Xenopus*.

In examining the dynamic behavior of the germ plasm in *Xenopus* and *Rana pipiens* eggs, Savage and Danilchik (1993) categorized the movements of the germ plasm into four phases. During the first cell cycle, the islands of the germ plasm move along with the vegetal yolk mass towards the future ventral side of the embryo. At the end of the cortical rotation, individual islands begin to fuse with each other. This local aggregation, a microtubule-dependent process (Robb *et al.*, 1996), results in a dramatic decrease in the number of islands by the end of the first cleavage division (Savage and Danilchik, 1993). As the local aggregation process takes place, the islands of germ plasm are carried to the vegetal pole with surface contraction waves. This constitutes the second

aggregation phase. Aggregates of the germ plasm then ingress into the interior of the embryo. By the end of the first two cleavage divisions, patches of germ plasm form large aggregates and are partitioned equally between the first four blastomeres. During subsequent cleavage divisions, the germ plasm is segregated to only one daughter cell of each pair (Whittington and Dixon, 1975). As a result of this asymmetrical division, the germ plasm is distributed among only a few endodermal cells, despite the exponential increase in the number of vegetal blastomeres. As the germ plasm moves from its polar position to a perinuclear position, this substance is distributed equally among daughter cells (Whittington and Dixon, 1975). It is at this stage of development, *viz.* gastrula stage, that the germ cell lineage is believed to be established. At later stages of development, cells containing the germ plasm divide clonally until they reach the gonadal ridge.

The hypothesis that the germ plasm contains some deterministic factor(s) by which a few endodermal cells are specified to become the earliest precursors of the germ cell lineage has been tested in experiments aimed at either removing the germ plasm at a very early stage of development or through destruction of the possible determinants by UV irradiation. The embryos challenged by these methods are later scored for the presence of PGCs in the gonadal ridge. Several lines of evidence obtained from such studies indicate that the germ plasm contains a determinant(s) for the germ cell lineage. First, when incisions are made at the vegetal pole of 2-cell and 4-cell embryos, about 30 % of the

surviving eggs are sterile, i.e. they have gonads which are completely devoid of any germ cells (Blackler, 1970). Second, UV-irradiation of the vegetal hemisphere of 1-, 2-, and 4-cell embryos (wavelength 253.7 nm), but not the animal hemisphere, also results in complete sterility (Smith, 1966; Tanabe and Kotani, 1974). Third, the degree of UV-induced sterility relates to the position of the germ plasm during embryogenesis in such a way that as the germ plasm ingresses into the embryo, the severity of sterility diminishes (Smith, 1966; Tanabe and Kotani, 1974). Fourth, a positive correlation exists between the intrinsic amount of the germ plasm at the 8-cell stage and the number of PGCs that reside in the gonadal ridge at later stages of development (Akita and Wakahara, 1985). Such a correlation would appear to support the previous observations which indicated that the intrinsic number of PGCs can be augmented experimentally by administering exogenous "germ plasm" to eggs and embryos at early stages of development (Smith, 1966; Wakahara, 1977).

The notion that the germ plasm is indeed the causal agent in the determination of the germ cell lineage is challenged by the finding that the apparent sterility of irradiated embryos is simply due to delayed migration of PGCs to the gonadal ridge (Zust and Dixon, 1977; Smith and Williams, 1979). Nevertheless, it is evident that the germ plasm is important in the establishment of the germ cell lineage in anurans. However, its function might not be one of specification. One possible function of the germ plasm would be related to the migratory behavior of PGCs, and that the destruction and/or elimination of a

component(s) within this substance would incapacitate presumptive PGCs to migrate to the gonadal ridge (Zust and Dixon, 1977; Dixon, 1981). This hypothesis draws support from the observation that the difference in the spatial distribution of PGCs between the UV-irradiated and normal embryos becomes evident only at later stages of development, when PGCs are believed to migrate actively (Kamimura *et al.*, 1976). Therefore, it is possible that the germ plasm is simply involved in the migration of PGCs. In this context, it is pertinent to note that *Drosophila nanos*, a component of the germ plasm, has been demonstrated to be essential for proper migration of pole cells, the germ line precursors (Kobayashi *et al.*, 1996). In *Xenopus*, *Xcat2*, a putative RNA-binding protein related to *Drosophila nanos*, has been shown to be a component of the germ plasm in *Xenopus* (Forristall *et al.* 1995; Zhou and King, 1996), and might play a similar role in the establishment of the germ cell lineage of anurans.

Urodele amphibians

In urodeles, as in mammals and birds, a specific cytoplasmic substance, similar to the germ plasm of anurans has not been detected at early stages of development. Therefore, the primary site of the origin of PGCs or their immediate precursors could not be examined directly using conventional cytological techniques. Inevitably, the absence of such early markers required the use of classical embryological techniques, such as transplantation, deletion

and recombination (Wakahara, 1996). Several lines of evidence obtained from such studies indicate that PGCs are not of endodermal origin in urodeles. First, neither the removal of the vegetal pole plasm at the 1-cell stage, nor UV-irradiation of the vegetal pole alters the number of PGCs at later stages of development (Nieuwkoop, 1949, 1991). Second, in recombination experiments in which labeled animal ectodermal cells are combined with the unlabeled endoderm, PGCs are always of ectodermal origin (Sutasurya and Nieuwkoop, 1974).

In contrast to the absence of evidence for an endodermal origin of PGCs in urodeles, there is a substantial body of evidence suggesting a mesodermal origin of PGCs in this order of amphibians. Early experiments of Humphrey (1929) indicated that PGCs were associated with the lateral plate mesoderm. To test if the lateral plate mesoderm is the primary site of the origin of PGCs, extirpation and transplantation experiments were performed (Humphrey, 1929). It was observed that the removal of the lateral plate mesoderm at stages 21-25 results in complete absence of PGCs at the operated side. Furthermore, when these mesodermal strips were grafted into the latero-ventral body wall of another embryo and cultured for 10 to 30 days, PGCs were observed within the graft (Humphrey, 1929). On the basis of these observations, it was concluded that PGCs or their immediate precursors originate in the lateral plate mesoderm in urodeles.

In an attempt to clarify the relationship between the mesodermal cell lineages and the germ cell lineage further, a series of experiments were performed in which the presumptive lateral plate mesoderm (ventro-lateral marginal zone) were removed and operated embryos were examined for the presence of PGCs at a later stage of development. It became evident from such studies that the removal of the presumptive lateral plate mesoderm (ventro-lateral marginal zone) results in the absence of PGCs in the developing larvae (Nieuwkoop, 1949, 1991), proving unequivocally that PGCs are indeed mesodermal in origin. These observations not only substantiated the earlier observations (Humprey, 1929), but also indicated that during normal development, PGCs arise from ventro-lateral marginal zone under the influence of signals originating from the endodermal cells within this region. Several additional lines of evidence support this conclusion. First, endodermal cells from the ventral region of the embryo can induce ectodermal cells to differentiate into mesodermal cells, including PGCs (Mauroid and Capuran, 1985). Second, any region of the ectodermal hemisphere appears to have the potential to differentiate into PGCs when combined with the ventral vegetative yolk mass (Sutasurya and Nieuwkoop, 1974; Michael, 1984).

It can be concluded from these studies that both the origin of PGCs and the mechanism(s) by which the germ cell lineage is specified are fundamentally different in urodele and anuran amphibians. In anurans, the specification of the germ cell lineage appears to be a cell-autonomous process that takes place at

the gastrula stage of development. The specification of the germ cell lineage in this manner is referred to, perhaps erroneously, as the "preformistic mode" of germ line segregation. On the other hand, in urodeles, it is evident that the specification of the germ cell lineage is not a cell-autonomous process and that it appears to be mediated via an inductive event by which ventro-lateral marginal zone cells are induced to differentiate into PGCs along with ventral mesodermal cell types. The specification of the germ cell lineage via such an active signaling event is referred to as the "epigenetic mode" of germ line segregation.

Mammals

A germ plasm-like substance that could serve as a convenient marker to trace the early ontogeny of germ cells has not been detected in mammalian PGCs. Therefore, studies on the ontogeny of the germ cell lineage in mammals, and especially in mouse, have relied heavily on their high alkaline phosphatase content (Chiquoine, 1954). The type of alkaline phosphatase that is expressed by mouse PGCs is tissue non-specific alkaline phosphatase (TNAP) (MacGregor *et al.*, 1995). Using this feature of mouse PGCs, Chiquoine (1954) and others (Ozdzinski, 1967; Tam and Snow, 1981) were able to identify a cluster of cells first at the base of the allantois in 8 day embryos, i.e. 8.5 days *post coitum* (*d.p.c.*), and later along the wall of the hindgut. Cells with similar morphology and staining properties were later located along the dorsal mesentery (Mintz and

Russell, 1957). Recently, it has been demonstrated that if whole mount mouse embryos at 7-8 *d.p.c.* are stained for alkaline phosphatase activity, a cluster of AP-expressing cells are detected posterior to the primitive streak in association with the extraembryonic mesoderm (Ginsburg *et al.*, 1990). The number of AP-positive cells increases from about 8 at 7 *d.p.c.* to about 125 at 8 *d.p.c.* (Ginsburg *et al.*, 1990).

Despite the absence of experimental evidence indicating that alkaline phosphatase expressing cells at the base of the allantois are the only precursors of PGCs, it is generally accepted that these cells do indeed represent the earliest cells which will eventually form the germ line. Perhaps the most convincing evidence which is in support of this hypothesis comes from the observation that the number of AP-expressing cells is greatly reduced in two sterile mouse mutants, *White Spotting (W)* and *Steel (Sl)*, which are known to lack germ cells in the gonads (Mintz and Russell, 1957).

It has been established through transplantation studies between blastocysts, that mouse primordial germ cells are derived from the epiblast (Gardner *et al.*, 1985). Furthermore, single epiblast cells have the potential to contribute to both somatic and germ cell lineages indicating that PGCs are not determined during pre-implantation development (Gardner *et al.*, 1985). According to fate mapping studies, these multipotent/pluripotent epiblast cells might reside at the proximal half of the epiblast cup at the onset of the gastrulation, corresponding to 6.5 *d.p.c.* (Lawson *et al.*, 1991).

Recently, the timing of the segregation of the germ cell lineage at pre-gastrula and early-gastrula stages of development has been addressed by cell lineage analysis in the mouse (Lawson and Hage, 1994). When individual proximal epiblast cells were injected with fluorescent dextran and their progeny followed at a later stage of development, it was found that the precursors of PGCs are scattered along the proximal epiblast, close to the extraembryonic ectoderm prior to the formation of the primitive streak. Interestingly, a significant proportion of labeled cells gave rise not only to the PGCs, but also to extraembryonic mesodermal structures (Lawson and Hage, 1994). On the basis of these observations, along with those of Gardner *et al.* (1985), it can be concluded that in the mouse, the segregation of the germ cell lineage does not take place at pre-streak stages of development.

In labeling experiments of Lawson and Hage (1994), the progeny of the distal epiblast cells did not differentiate into PGCs, but mainly into cells forming the neuroectoderm and surface ectoderm. Nevertheless, a recent experiment by Tam and Zhou (1996) clearly demonstrated that even distal epiblast cells have the potential to contribute to the germ cell lineage, when transplanted to proximal sites. On the other hand, proximal epiblast cells whose progeny would normally give rise to PGCs and extraembryonic mesodermal cell types, form predominantly neuroectoderm when transplanted to distal sides (Tam and Zhou, 1996). These observations strongly suggest that local cell-cell interactions

mediated possibly by growth factors and their corresponding receptors are involved in the segregation of mouse PGCs (Tam and Zhou, 1996).

Birds

The biology of avian PGCs has been investigated mainly in chicken. Swift (1914) was the first to show the presence of PGCs in an extraembryonic region located at the border between the *area pellucida* and the *area opaca*. This region of the embryo is referred to as the germinal crescent. On the basis of this observation, which used only morphological characteristics of the PGCs, it was suggested that the germinal crescent is the primary site of the origin of the germ line in the chicken embryo. This hypothesis was later tested and substantiated on both experimental and descriptive grounds.

If the chick blastoderm is dissected into fragments and then cultured on the chorioallantoic membrane, PGCs could be observed only in fragments that contained a region of the germinal crescent (Willier, 1937). Furthermore, irradiation of the germinal crescent region with either UV-light (Reynaud, 1969) or laser beams (Mims and McKinnell, 1971) or the removal of this region (Reagan, 1916; Goldsmith, 1935) results in complete sterility of the developing embryo. In 1960, Meyer took advantage of the high glycogen content of germ cells and used PAS-staining to identify PGCs in the germinal crescent and at subsequent developmental stages. These studies also revealed that there

exists a significant amount of variation in the number of PGCs within the germinal crescent region.

These observations clearly demonstrated that, in birds, PGCs are of extra gonadal in origin much like in other vertebrates, and argued strongly against a long-standing hypothesis, advocated first by Waldayer (see Nieuwkoop and Sutasurya, 1979), and later by Firket (1920), that germ cells derive from the gonadal epithelium. Nevertheless, the histo-morphological criteria, including PAS-staining, used in identifying PGCs from their appearance in the germinal crescent region until their settlement in the gonadal primordium have been proved insufficient in tracing the origin of these cells back into the earlier stages of development. Therefore, studies concerning the temporal and spatial aspects of the origin of avian PGCs had to rely heavily on classical embryological techniques. In these studies, the unincubated blastoderm is dissected into fragments and each fragment is cultured further to be scored later for the presence of PGCs using morphological criteria and periodic acid Schiff staining (PAS) for glycogen. Accordingly, if the unincubated blastoderm is cut into pieces, perpendicular or longitudinal to the axis of the embryo and cultured *in vitro* (Dubois and Croiselle, 1970; Eyal-Giladi *et al.*, 1976) or *in ovo* (Rogulska, 1968), PGCs could be detected in any of the fragments, indicating that each fragment, i.e. anterior, posterior or lateral, had initially contained cells which have the potential to give rise to definitive PGCs. Therefore, it appeared that the earliest precursors of the germ cell lineage are located within the embryo well

before the formation of germinal crescent and that the germinal crescent is a secondary location in avian germ cell development.

In an attempt to pinpoint the location of presumptive PGCs in the unincubated chicken embryo, Dubois and Croiselle (1970) transected the unincubated blastoderm perpendicular to the axis of the embryo to obtain three transversal bands, i.e. posterior, central and anterior fragments. When these fragments were cultured for 48 hours and scored for the presence of PGCs, it became evident that majority of PGCs were located within the central fragment, with a small number of PGCs present in anterior fragments. In examining the origin of PGCs, experiments similar to those conducted by Dubois and his colleagues were later performed by Ginsburg and Eyal-Giladi (1987). In these experiments, embryos from stage X to XII were dissected first into three circular fragments, i.e. a peripheral *area opaca*, an intermediate marginal zone and a central disc. Central disks were dissected further into three equal fragments, longitudinally or transversely, and each fragment was then cultured separately and examined for the presence of PGCs, using PAS staining. The evidence obtained from these studies substantiated previous observations in that a significant proportion of PGCs is derived from the central disc, with a small contribution from the *area opaca* and the marginal zone. It also became evident from these studies that differentiation of embryonic cells into PGCs could take place even in the absence of the formation of an embryonic axis (Dubois and Croiselle, 1970; Ginsburg and Eyal-Giladi, 1987). This would suggest that the segregation of the

germ cell lineage is independent of the formation of an embryonic axis, at least at the morphological level.

Other studies carried out with fragments of unincubated chicken embryos revealed a close association of PGCs with the hypoblast and suggested that PGCs originate from the hypoblast (Dubois, 1969). Furthermore, it was proposed that the displacement of PGCs towards the germinal crescent was due to the morphogenetic movements of the lower layer (Vakaet, 1970; Dubois and Croiselle, 1970). In support of this hypothesis, it was observed that if the morphogenetic movements of the lower layer were mechanically blocked, PGCs could not migrate to anterior regions of the embryo, and remained mainly in the central area *pellucida* (Dubois and Croiselle, 1970).

The hypothesis that avian PGCs originate from the hypoblast was tested by Eyal-Giladi *et al.* (1981) using interspecific transplantation of stage XIII hypoblasts between chicken and quail embryos. It was observed that only a small portion of the PGC population, corresponding to 5 % of total number of PGCs per embryo, was derived from the hypoblast, leading to the conclusion that avian PGCs are of epiblastic origin. Although the experimental details reported by Eyal-Giladi *et al.* (1981) have been criticized on technical grounds (see Callebaut *et al.*, 1997), it is now generally accepted that avian PGCs are indeed of epiblastic origin.

Studies in which hypoblasts were separated from the epiblast layer and cultured independently indicated that PGCs or their immediate precursors

originating in the epiblast gradually translocate to the hypoblast where they are carried anteriorly to the germinal crescent as a result of morphogenetic movements during gastrulation (Ginsburg and Eyal-Giladi, 1986). Nevertheless, particular details of the developmental mechanisms by which they appear in the germinal crescent remain unexamined.

Despite a substantial body of indirect evidence indicating that the segregation of the germ cell lineage might take place as early as stage X, the temporal and spatial aspects of this phenomenon could not be examined directly due to the absence of molecular markers that could identify PGCs or their immediate precursors at early stages of development. Nevertheless, several attempts have been made to show the presence of PGCs via direct immunohistochemistry at these early stages. Urven *et al.* (1988) was the first to examine the origin of PGCs in the chick embryo using the monoclonal antibody EMA-1. EMA-1, embryonic mouse antigen-1, antibody is raised against a glycoprotein cell-surface antigen of the Nulli SCC1 cells (Hahnel and Eddy, 1982). The antibody recognizes fucosylated polylactosamine carbohydrate groups and has been shown to label murine PGCs (Hahnel and Eddy, 1986). Urven *et al.* (1988) demonstrated that EMA-1 could identify what appeared to be PGCs as early as stage XII. In addition to the presence of EMA-1 positive cells throughout the epiblast, some of the immunoreactive cells were found to be in close association with the hypoblast. Cells with similar morphology and staining properties were located along the migratory route of avian PGCs, viz. in the

germinal crescent region, in the circulatory system, and finally in the gonadal ridges. On the basis of the observation that cells forming the hypoblast did not immunoreact with the antibody, Urven *et al.* (1988) argued that there exists a more common lineage between PGCs and cells of the epiblast, than between PGCs and cells of the hypoblast. This observation has also reinforced the view that avian PGCs are epiblastic in origin. More recently, Loveless *et al.* (1990) reported that FC10.2, an oligosaccharide antigen, is also a reliable marker of chicken PGCs at stages 1 - 17.

In examining the origin of PGCs in the quail embryo, Pardanaud *et al.* (1987a) used the monoclonal antibody QH-1 which recognizes the quail hemangioblastic lineage, i.e., endothelial and hemopoietic cells (Pardanaud *et al.*, 1987b). According to Pardanaud *et al.* (1987a), a small number of immunoreactive cells were detected first in the unincubated quail blastoderm, corresponding to stages X-XI. At a slightly later stage of development, i.e. stages XII-XIII, about 20 immunoreactive cells were scattered in the central region of the blastoderm. With the formation of the primitive streak, immunoreactive cells were located mainly in the germinal crescent region, indicating that cells expressing the QH-1 epitope are indeed PGCs. The identification of only a few immunoreactive cells at stages X-XIII appear to reinforce the conclusion derived from classical embryological studies (Dubois and Croiselle, 1970; Ginsburg and Eyal-Giladi 1987) that the segregation of avian germ cell lineage begins at stages X-XIII.

Although it is generally agreed that PAS cannot be used as a reliable marker for PGCs or their precursors before the end of gastrulation (Urven *et al.* 1988), Muniesa and Dominguez (1990) carried out a detailed examination of the spatial distribution of PGCs between stage X and stage 2 using periodic acid - Schiff (PAS) staining and ultrastructural morphology. According to Muniesa and Dominguez (1990), PGCs could be detected first at stage X. At stages X-XIII, the majority of PGCs were randomly distributed within the central region of the *area pellucida*. At slightly later stages of development, stages XIV and stage 2, most PGCs were located in the anterior region of the embryo. On the basis of these observations, it was concluded that the segregation of the germ cell lineage takes place as early as stage X (Muniesa and Dominguez, 1990). Nevertheless, the verification of these results using PAS and ultrastructural criteria is difficult. However, the results obtained from this study are in general agreement with those obtained by Pardanaud *et al.* (1987a) in the quail embryo using QH-1, particularly the spatial distribution of PGCs and their numbers at pre-streak stages of development.

Unfortunately, none of these experiments provided direct evidence indicating that the cells recognized by these antibodies are, in fact, PGCs or their immediate precursors, since no experimental cell lineage analysis was performed.

MIGRATION OF PRIMORDIAL GERM CELLS

In all vertebrates, PGCs arise at relatively early stages of development, and at a site away from the region of the embryo where the gonads ultimately form. This, apparently evolutionary conserved (Denis, 1994) feature of the germ line development confronts PGCs with the task of searching for the gonadal primordium as soon as they are determined. This is accomplished by the migration of PGCs from their site of initial appearance towards gonads.

Apparently in all vertebrates, the displacement of PGCs leading to their settlement in the gonadal primordium occurs in two phases: a "passive" migratory phase during which PGCs do not migrate actively but rather are carried away by morphogenetic movements of embryonic tissues in which they arise. This "passive" displacement phase by which PGCs are brought into the vicinity of the gonadal primordium is followed by an active migratory phase, during which PGCs migrate by their amoeboid movements towards the gonadal ridge.

The migratory route taken by PGCs during their passive phase of migration appears to be dictated by morphogenetic movements of tissues where these cells are first located. In anuran amphibians, this site corresponds to the endodermal cell mass at the blastula-gastrula stage. A few endodermal cells containing "germinal plasm" which will become PGCs are incorporated into the larval gut, along with the rest of the endodermal cell population. It is believed that the passive displacement of presumptive PGCs (pPGCs) is mediated via morphogenetic movements that take place during blastula and gastrula stages of

development. It is through such movements that pPGCs are swept into the central portion of the endodermal mass (Nieuwkoop and Sutasurya, 1979). The migration of pPGCs from the center of the endodermal mass towards the gonadal ridge takes place as a result of the active migration of these cells through the dorsal mesentery (Kamimura *et al.*, 1976).

In urodele amphibians, PGCs derive from the blastomeres in the ventral marginal zone rather than from endodermal cells. Therefore, instead of being incorporated into the hindgut, they are incorporated into the lateral plate mesoderm (Nieuwkoop and Sutasurya, 1979). Because of this difference in the spatial distribution of pPGCs, it is generally accepted that urodele PGCs follow a different migratory pathway than their anuran counterparts. Unfortunately, the details of this process remain ill-defined.

In the mouse, PGCs first appear as a distinct cell population at 7 *d.p.c.* at the base of the allantois, near the endoderm that will give rise to the hind-gut (Ginsburg *et al.*, 1990). As the hind-gut forms, PGCs are incorporated into the hind-gut wall where they are found at 9 *d.p.c.* At this stage of development, PGCs show no morphological or structural features characteristic of actively migrating cells. At 10.5 *d.p.c.*, PGCs begin to migrate out of the hindgut and into the dorsal mesentery where they migrate into the paired gonadal primordia by active amoeboid movements. This active phase of migration is completed at 12.5-13.5 *d.p.c.* (Gomberts *et al.*, 1994)

In birds, PGCs originate from the epiblast (Eyal-Giladi *et al.*, 1981). PGCs originating in the epiblast gradually translocate to the hypoblast, where they are carried anteriorly to the germinal crescent as a result of morphogenetic movements during gastrulation (Ginsburg and Eyal-Giladi, 1986).

Nieuwkoop and Sutasurya (1979) classified the migration of chick PGCs from the germinal crescent towards the gonadal ridge in four phases (Fig. B). In the first phase, PGCs residing on the hypoblast segregate from this tissue and accumulate between endoderm and ectoderm. In the second phase, PGCs are associated with the vascular network as blood islands differentiate to form the vitelline circulation. Shortly after this process, PGCs are found in the extra-embryonic blood vessels and later in the embryo proper. In the last phase of migration, most of the PGCs circulating in the blood leave the blood vessels within the vicinity of the gonadal anlage and migrate actively into the gonadal epithelium through the dorsal mesentery. In this second, active phase of migration, chemotactic signals released from the gonad (Dubois and Croiselle, 1970; Kuwana *et al.* 1986), extracellular matrix components (Lee *et al.* 1982; Urven *et al.* 1989) and the anatomical arrangement of the vascular system surrounding the gonadal epithelium (Nakamura *et al.* 1988) are thought to be important.

One set of molecules that have been indicated in the migration of PGCs is extracellular matrix proteins. Among them, fibronectin and laminin are present in the migratory route of PGCs in *Xenopus* and in mouse (Gomberts *et al.*,

1994), and in birds (England, 1985; Urven *et al.*, 1989). Apart from fibronectin and laminin, chondroitin sulphate and collagen IV (Urven *et al.*, 1989) and recently, tenascin (Anstrom and Tucker, 1996) have been suggested to mediate germ cell migration in birds.

Several lines of evidence obtained from various vertebrate groups indicate that the gonadal ridge exerts a chemoattractive effect on the migratory PGCs (Dubois and Croiselle, 1970; Kuwana *et al.*, 1986). It is even possible that these chemoattractive agents are not species-specific, since mouse PGCs grafted near the site of the gonadal ridge of a chicken embryo are capable of finding their way to the host gonadal ridge, along with the chicken PGCs (Rogulska *et al.*, 1971). In the mouse, the chemoattractive effect of the gonadal ridge might be mediated partly by TGF β 1 (Godin and Wylie, 1991). Stem cell factor (SCF) may also provide a chemotactic stimulus, since this factor is expressed along the migratory pathway of mouse PGCs with the highest concentration in the gonadal ridge (Keshet *et al.*, 1991). Nevertheless, the same factor does not appear to influence the direction of PGC migration *in vitro* (Godin *et al.*, 1991). Furthermore, most of cells in mouse embryos homozygous for SCF mutations do migrate normally (Buehr, 1997).

CYTOKINES THAT ARE INVOLVED IN PRIMORDIAL GERM CELL DEVELOPMENT

Knowledge of the involvement of cytokines in the ontogeny of the germ cell lineage comes mainly from studies carried out with mouse PGCs. As discussed above, in the mouse PGCs can be identified first at 7 *d.p.c.* at the base of the allantois as a small cluster of alkaline phosphatase positive cells (Ginsburg *et al.*, 1990). This is probably the time that the germ line is segregated in the mouse. At a slightly later stage of development, PGCs are incorporated into the hind-gut on their way to the gonadal primordium. During this active phase of migration and until shortly after their settlement in the gonadal ridge, PGCs divide clonally in such a way that a population of about 25,000 cells resides in the gonad by 13.5 *d.p.c.* (Tam and Snow, 1981). The proliferative phase of PGCs ends as they enter meiosis in the female and mitotic arrest in the male embryo. Therefore, it is almost certain that the initiation, maintenance, and control of germ cell proliferation is tightly regulated during normal development.

Studies directed towards a molecular understanding of this undoubtedly complex phenomenon have been carried out mainly in the mouse, where several mutant phenotypes affecting germ cell development are available. Some of these mutations include Hertwig's microcytic anemia, *teratoma*, *artichokes*, *germ cell deficient*, and most alleles of the *Dominant White Spotting* (*W*) and *Steel* (*S/*). Among these, mutations at the *W* and *S/* loci are most extensively studied in terms of germ cell development.

In *W* and *S/* mutants, the development of three stem cell populations, namely melanoblasts, hematopoietic stem cells, and germ cells is impaired.

Homozygote mutants (W/W , S/S), which are characterized by the absence of pigmentation of skin and hair, die before birth due to the severe anemia. Another distinguishing feature of W - and S - mutants is the lack of germ cells in the gonad leading to sterility (Mintz and Russell, 1957).

Although both W - and S - mutant phenotypes are characterized by similar pleiotropic effects, transplantation experiments between normal and W - and S - mutant mice revealed that the defect in W - mutants is intrinsic to the stem cell, while that in S -mutants is intrinsic to a stromal cell component of the microenvironment necessary for stem cell development (Fleishman, 1993). It is now established that W -locus encodes c-kit, a receptor tyrosine kinase, whereas S locus encodes for a transmembrane growth factor which acts as the ligand for the c-kit receptor (Copeland *et al.*, 1990; Flanagan and Leder, 1990; Zsebo *et al.*, 1990; Williams and Lyman, 1991). It is because of this receptor-ligand relationship that both W - and S - mutant phenotypes have similar pleiotropic effects.

The product of the c-kit gene is a transmembrane tyrosine kinase receptor with homology to the receptors for colony stimulating factor1 and platelet-derived growth factor receptors α and β (Chabot *et al.*, 1988). In addition to three stem cell populations affected by the W - and S - mutants, *viz.* melanoblasts, hematopoietic stem cells, and germ cells, various embryonic cell types including interstitial cells of Cajal (ICC) in the small intestine, endothelial, epithelial and endocrine cells are known to express c-kit (Motro *et al.*, 1991; Bernex *et al.*,

1996). The kit receptor contains an extracellular region, thought to be critical for ligand binding, a transmembrane region, and an intracellular region. The tyrosine kinase domain of the c-kit receptor is located in the intracellular segment, which is divided into two subdomains by a hydrophilic insert sequence. In the mouse, two isoforms of c-kit, designated Kit⁺ and Kit A⁺, are produced as a result of alternative splicing (Fleishman, 1993).

The cDNA for chicken c-kit encodes a protein with a 505 amino acid extracellular domain, a 25 amino acid membrane-spanning segment, and a 430 amino acid cytoplasmic domain (Sasaki *et al.*, 1993). The deduced amino acid sequence of the chicken c-kit shows 63 % homology to mouse kit (Sasaki *et al.*, 1993).

The *Steel* locus encodes a peptide growth factor known as stem cell factor (SCF), mast cell growth factor (MGF), *Steel* factor (SL) and kit-Ligand (KL) (Copeland *et al.*, 1990; Flanagan and Leder, 1990; Zsebo *et al.*, 1990). SCF exists in two isoforms generated by alternative splicing: a membrane-bound form and a soluble form (Flanagan *et al.*, 1991). Soluble SCF is rapidly released from the cell surface by an unidentified protease which recognizes a consensus cleavage site in exon 6. The alternatively spliced transcript lacking exon 6 does not contain the consensus proteolytic cleavage site and produces a membrane bound protein.

The deduced amino acid sequence of the chicken SCF, a 287 amino acid polypeptide, shows 52 % and 53 % homology to human and mouse SCF,

respectively (Zhou *et al.*, 1993). In chicken tissue, SCF is transcribed as a 6.4 kb mRNA, corresponding to the soluble form of the mammalian SCF. Recently, both the soluble and membrane-anchored forms of the SCF have been cloned in the quail (Petitte and Kulik, 1996). The long or soluble form of the quail SCF (qSCF) shows 98 % and 53 % homology to chicken and various mammalian SCFs, respectively (Petitte and Kulik, 1996). It was demonstrated that qSCF is expressed as a 5.9 and a 2.7 kb transcript in several quail tissues, including spleen, heart, brain, and ovary (Petitte and Kulik, 1996).

It has been shown that the number of PGCs at the base of the allantois is about the same between *W/W*, *W/+*, and wild-type embryos (Buehr *et al.*, 1993), suggesting that in homozygous mutants the segregation of PGCs is not affected. However, the absence of PGCs in mutant animals becomes evident at the end of the active migratory phase, as only a few PGCs are detected in the gonadal ridge of *W/W* embryos (Mintz and Russell, 1957). The few PGCs that reach the gonad apparently do not survive. On the basis of these observations, it would appear that the effect of SCF/c-kit signaling pathway is primarily on the proliferation of PGCs as they migrate towards the gonadal primordium and on the survival of germ cells shortly after their settlement in gonads.

In an ongoing effort to examine not only the function of SCF/c-kit signaling system but also of several other cytokines, feeder-dependent culture systems have been employed in examining PGC development in the mouse. In these studies, migratory or post-migratory PGCs are cultured *in vitro* on feeder layers

of mitotically inactive somatic cells such as STO, NIH3T3, and TM4. In such feeder-dependent cultures, PGCs are seeded onto feeder cells along with mitotically active stromal cells. Therefore, the culture system is composed of three components: mitotically inactive feeder cells, mitotically active embryonic somatic cells and PGCs. The intrinsic complexity of the system is augmented further by the fact that the response of PGCs is feeder cell-type specific (Donovan, 1994, Buehr, 1997). For example, while mouse PGCs can survive and proliferate on feeder layers of STO cells, NIH3T3 cells could only support the survival and not proliferation (Dolci *et al.*, 1991). Thus, it is most often very difficult to pinpoint the specific function of a single compound in the system. This is especially true in distinguishing cell proliferation from cell survival. Although the rate of cell proliferation can be estimated using substances such as BrdU or tritiated thymidine, it is possible that a survival factor, which itself does not cause cell proliferation, can potentiate cells to respond to another mitogenic factor present in the culture system (Donovan, 1994). Nevertheless, the feeder-dependent culture systems have proved to be valuable not only as a powerful system to examine the effect of a variety of growth factors on PGCs, but also in the establishment of immortalized embryonic cell lines such as embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981).

In contrast to the observation that in *W*- and *S*⁻ mutants the main defect in the development of germ cells is the lack of proliferation during their active phase of migration, several lines of evidence obtained from *in vitro* studies

indicate that SCF/c-kit interaction is involved in survival but not proliferation of PGCs. First, CV-1 cells, which lack transcripts for SCF, do not support PGC development unlike STO and NIH-3T3 cells which do express a 6.5 kb SCF mRNA (Dolci *et al.*, 1991). However, CV-1 cells can support the survival of PGCs up to three days when reconstituted with the membrane-bound form of the SCF (Dolci *et al.*, 1991). In addition, SCF does not cause an increase in the proportion of mitotic PGCs on STO cultures as assessed by BrdU incorporation (Godin *et al.*, 1991), suggesting that SCF acts as a survival factor for PGCs in culture. Furthermore, it appears that the membrane-anchored form of SCF is more effective in supporting the survival of PGCs (Dolci *et al.*, 1991; Matsui *et al.*, 1991). This finding is supported by the observation that *Steel-Dickie* (*S^d*) mutant mice which express only the soluble form of the SCF are sterile (Dolci *et al.*, 1991; Godin *et al.*, 1991). In accordance with these observations, Pesce *et al.* (1993) report that SCF can suppress apoptosis of PGCs in culture.

The hypothesis that SCF acts as a survival factor is substantiated further by the observation that addition of recombinant SCF to cultures initiated on NIH-3T3 cells, which support the PGC survival but not proliferation, does not lead to the proliferation of PGCs (Dolci *et al.*, 1991).

While it would appear from these studies that SCF acts mainly as a survival factor, there is evidence indicating that the effect of SCF is mediated and specified via other factors present in the culture system. For example, in the

presence of leukemia inhibitory factor (LIF), SCF can act as a mitogen on PGCs (Matsui *et al.*, 1991).

LIF is a pleiotropic cytokine that can act either as a mitogen (Hilton *et al.*, 1988) or as a differentiation-inhibiting factor (Smith *et al.*, 1988). The function of LIF is mediated by LIFR which forms a heterodimeric high-affinity LIF receptor with gp130 capable of transducing signals in response to LIF (Gearing *et al.*, 1992).

LIF appears to act either as a mitogen, along with the SCF, (Dolci *et al.*, 1991; Matsui *et al.*, 1991) or as a survival factor for mouse PGCs (Pesce *et al.*, 1993; Dolci *et al.*, 1993) in culture. This apparent uncertainty surrounding the specific effect of LIF on PGCs might stem partly from the fact that STO cells themselves produce LIF (Matsui *et al.*, 1991; Cheng *et al.* 1994). However, according to Cheng *et al.* (1994), PGCs might respond to LIF directly, since PGCs express a low-affinity LIF-binding receptor, referred to as LIFR. This hypothesis is supported by the observation that the number of PGCs in STO cultures is dramatically reduced in the presence of a neutralizing antibody against LIFR (Cheng *et al.*, 1994).

It would appear from these observations that LIF can act directly on PGCs and enhance the survival and/or proliferation of PGCs. Nevertheless, the specific action of LIF on PGCs remains ill-defined, particularly in the light of the fact that the development of PGCs is normal in mice homozygous for a targeted disruption of both LIF (Stewart *et al.*, 1992) and the LIFR (Ware *et al.*, 1995). In

principle, this observation would suggest either that LIF is not involved in the development of germ cells *in vivo* or that alternative ligands capable of activating the gp130 signaling pathway, such as IL-6, oncostatin M (OSM) and ciliary neurotrophic factor (CNTF) (Gearing *et al.*, 1992) can substitute for LIF in controlling the survival and/or proliferation of PGCs *in vivo*. A common feature of these cytokines is that their signal transduction activity requires the presence of both LIFR and gp130. For example, LIFR/gp130 functions as a high affinity receptor not only for LIF, but also for OSM (Gearing *et al.*, 1992). Addition of a low affinity receptor for CNTF (CNTF α) to the LIFR/gp130 results in the formation of a tripartite, high-affinity receptor for CNTF (Davis *et al.*, 1993).

In addition to LIF and SCF, basic fibroblast growth factor (bFGF, FGF-2) can also stimulate the proliferation of PGCs in a dose-dependent manner, with a peak response at 1 ng/ml (Resnick *et al.*, 1992). More interestingly, in the presence of LIF and both soluble and membrane-bound forms of the SCF, bFGF stimulates long-term proliferation of PGCs, leading to the establishment of an immortalized, embryonic cell line (Resnick *et al.*, 1992; Matsui *et al.*, 1992). These cells, referred to as the embryonal germ (EG) cells, resemble ES cells. ES cells are pluripotent cells derived from the inner cell mass (Evans and Kaufman, 1981; Martin, 1981), and embryonal carcinoma (EC) cells are pluripotent cells derived from teratomas (Martin, 1975). Like ES and EC cells, EG cells express alkaline phosphatase and Stage Specific Embryonic Antigen-1 (SSEA-1) (Resnick *et al.*, 1992; Matsui *et al.*, 1992). EG cells can also form

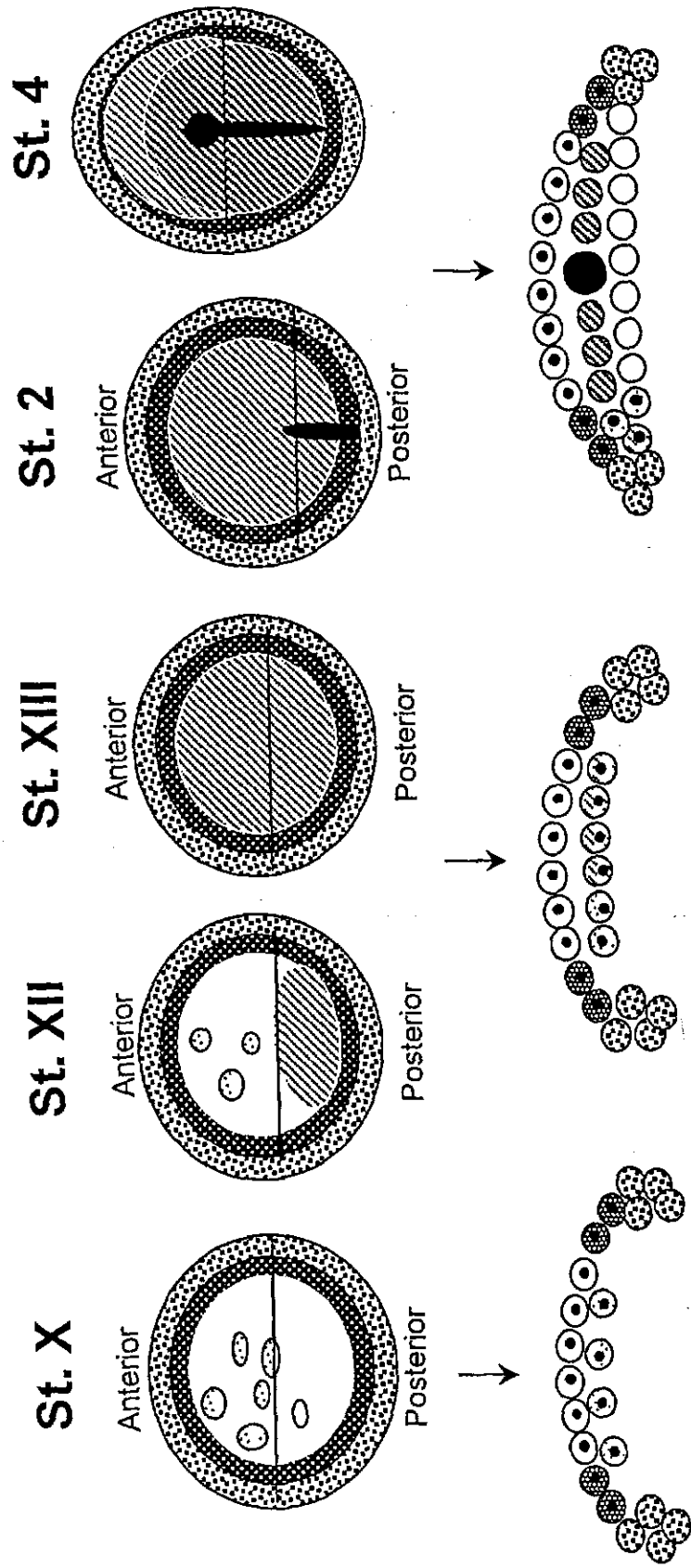
embryoid bodies which can differentiate into a variety of cell types and generate teratomas in histocompatible hosts (Matsui *et al.*, 1992). Finally, EG cells can give rise to both somatic and germ line chimeras when injected into blastocysts (Matsui *et al.*, 1992; Stewart *et al.*, 1994; Labosky *et al.*, 1994). —

There is a substantial body of evidence indicating that several cytokines, other than SCF, LIF, and bFGF may control the survival and proliferation of mouse PGCs *in vitro*. For example, IL-4 can act as a survival factor for cultured PGCs (Cooke *et al.*, 1996). The fact that both IL-4 and its receptors are expressed in the embryo at 8.5, 10.5, and 12.5 *d.p.c.* substantiates this hypothesis further. Retinoic acid (RA) is another compound which was demonstrated to stimulate the proliferation of PGCs (Koshimizu *et al.*, 1996). In the presence of RA (0.01 - 0.1 μ M), a significant increase in the number of PGCs is observed, apparently in a process independent of the feeder cells (Koshimizu *et al.*, 1996).

It also became evident from culture experiments that the mitogenic activity of PGCs is associated with an increase in the concentration of intracellular cAMP. There are two pieces of evidence in support of this hypothesis. First, when forskolin, a cAMP agonist is added to germ cell cultures, there is a significant increase in BrdU incorporation of PGCs (Dolci *et al.*, 1993). Second, pituitary adenylate cyclase activating peptides (PACAP) are able to stimulate the proliferation of PGCs by activating adenylate cyclase, which in turn leads to an increase in the concentration of intracellular cAMP (Pesce *et al.*, 1996).

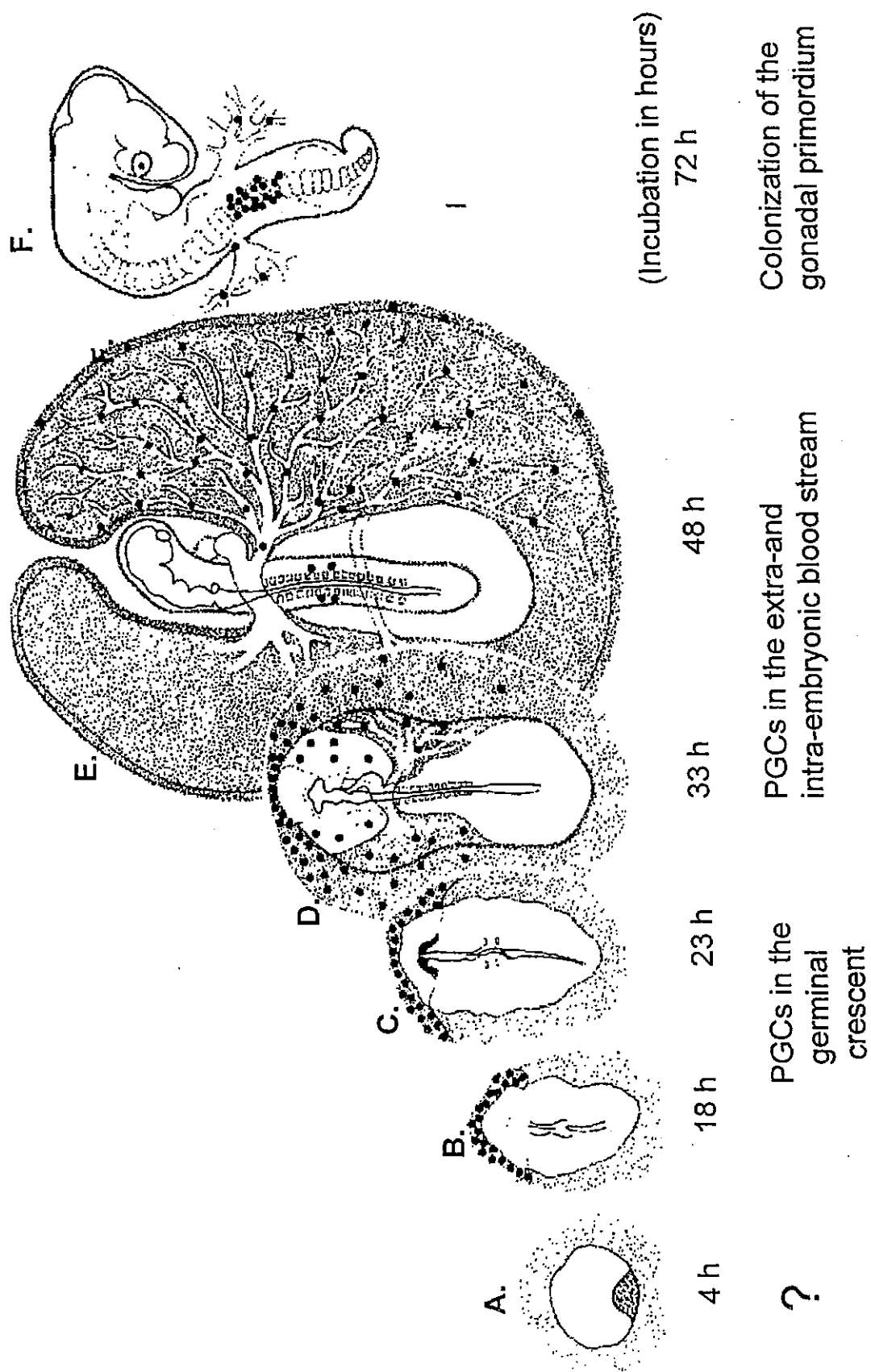
It is evident from the ongoing discussion that the initiation and maintenance of germ cell proliferation are complex phenomena regulated by a combination of cytokines such as SCF, bFGF and LIF acting either on their own or in combination with each other. Furthermore, it is currently unknown whether or not any of these factors are important in regulating PGC proliferation in the intact embryo.

Fig. A. The early chick embryo development (after Stern, 1990; Petite and Karagenc, 1996). Roman numerals (X-XIV) are used to describe the pre-streak stages of development according to Eyal-Giladi and Kochav (1976). Each diagram shows the embryo viewed from its ventral surface, facing the yolk. The anterior (cephalic) end of the embryo is shown towards the top, and the posterior (caudal) end towards the bottom in each figure. A freshly laid egg often contains a stage X embryo which is characterized by the appearance of two distinguished regions: a translucent region, referred to as the *area pellucida* and an opaque region, referred to as the *area opaca*. The junctional area separating the two regions is referred to as the *marginal zone*. The ventral surface of the blastoderm contains cells, known as the polyingressing cells, that are loosely attached to the *area pellucida*. Shortly after the initiation of incubation at 37-38 °C, the hypoblast begins to form at the posterior site of the blastoderm as an advancing front of cells at stages XI-XII, and gradually extends anteriorly until it comes to lie beneath the central portion of the *area pellucida* at stage XIII. During this process, the polyingressing cells are incorporated into the hypoblast. At this stage of development, the avian embryo consists of two layers of cells: the epiblast (facing the egg white) and the hypoblast (facing the yolk). Shortly after the formation of the hypoblast is completed, primitive streak appears at the posterior side of the embryo. The appearance of the primitive streak is the whole mark of avian gastrulation (stages XIV-4 in the chick embryo) which results in the formation of an additional, middle layer of mesodermal cells.



- area opaca
- primary hypoblast (polyingressing cells)
- epiblast (central portion)
- epiblast (marginal zone)
- secondary hypoblast
- primitive streak, mesoderm
- definitive endoderm

Fig. B. Passive and active migration of avian primordial germ cells (after Nieuwkoop and Sutasurya, 1979). The diagram illustrates the migration of PGCs from their appearance in the germinal crescent until their settlement in the gonadal primordium. In the chick embryo, PGCs are of epiblastic in origin (Eyal-Giladi et al., 1981). However, their primary site of origin at pre-streak stages of development remains speculative (A). PGCs originating in the epiblast gradually translocate to the hypoblast where they are carried anteriorly to the germinal crescent as a result of morphogenetic movements that take place during the process of gastrulation (B and C). As blood islands differentiate to form the vitelline circulation within the germinal crescent region, PGCs are incorporated into the vascular network (D). These blood-borne PGCs are carried to the vicinity of the gonadal primordium passively through the embryonic circulation and a few PGCs begin to leave the vessels and migrate towards the gonadal epithelium actively (E). This active phase of migration is completed about 72 hr of incubation as the majority of PGCs settle within the gonadal epithelium (F).



OBJECTIVES OF THE CURRENT STUDY

The morphological aspects of the biology of chicken primordial germ cells from their appearance in the germinal crescent until their settlement in the gonadal primordium is relatively well known. However, the whereabouts of PGCs prior to the formation of the primitive streak remains uncertain. Furthermore, the cellular and molecular mechanisms governing the segregation and development of the germ cell lineage is currently unknown in the avian embryo. Therefore, the overall objectives of the current study were to examine the temporal and spatial aspects of the origin of PGCs at pre-streak stages of development and to elucidate the involvement of growth factors in the development of the avian germ line. The specific objectives of this thesis were:

- 1) To establish where and when PGCs first appear in the chick embryo.
- 2) To reveal patterns of the expression of stem cell factor (SCF) and c-kit in relation to the origin and migration of chicken PGCs.
- 3) To examine if SCF and basic fibroblast growth factor (bFGF, FGF-2) are involved in the development of chicken PGCs in culture.

CHAPTER 1

Origin of Primordial Germ Cells in the Prestreak Chick Embryo

ABSTRACT The temporal and spatial patterns of the origin of chick primordial germ cells (PGCs) between stages IX - XIV were investigated using immunohistological analysis of whole mount embryos and histological sections with a monoclonal antibody (MC-480) that recognizes Stage-Specific Embryonic Antigen-1 (SSEA-1), and through the culture of dispersed blastoderms at stages IX - XIV with and without an STO feeder-layer. When individual stage IX-XIV embryos were dispersed and cultured without a feeder-layer, 25-45 PGCs/embryo were detected using material from stages X-XIV, but not with stage IX blastoderms. However, the culture of blastodermal cells obtained from the *area pellucidae* of stages IX-XIII on feeder-layers of STO cells yielded about 150 PGCs/embryo. The carbohydrate epitope recognized by MC-480 first appeared at stage X on cells in association with polyingressing cells on the ventral surface of the epiblast and later on the dorsal surface of the hypoblast. The SSEA-1-positive hypoblast cells gave rise to chicken PGC when injected into the subgerminal cavity of quail blastoderms or when cultured on a feeder-layer of quail blastodermal cells. On the basis of these observations, it is proposed that the segregation and further development of avian PGCs is a gradual process associated with the translocation of SSEA-1-positive cells from

the ventral surface of the *area pellucida* at stage X to the dorsal side of the hypoblast at stages XI-XIV.

INTRODUCTION

The ontogeny of the germ cell lineage has been an active area of research for developmental biologist for almost a century. This long history of attention is well deserved since germ cells provide genetic continuity from generation to generation, a feature which makes them the cell type responsible not only for the perpetuation of the species but also the biological vehicle for evolutionary change. In all vertebrates, primordial germ cells (PGCs) appear during early stages of development in extragonadal sites; yet, their mode of development has been clarified only in anurans, urodeles (see Nieuwkoop and Sutasurya, 1979; Wakahara, 1996) and more recently in the mouse (Lawson and Hage, 1994).

In anurans, the specification of the germ cell lineage appears to be a cell-autonomous process that takes place at the gastrula stage of development (Bounoure, 1939; Whittington and Dixon, 1975). On the other hand, in urodeles, the specification of the germ cell lineage is believed to be the result of an inductive event by which ventro-lateral marginal zone cells are induced to differentiate into PGCs, along with ventral mesodermal cell types (Sutasurya and Nieuwkoop, 1974). In mammals, PGCs are epiblastic in origin (Gardner *et al.*, 1985) and first appear at 7 d.p.c. as a cluster of alkaline phosphatase-positive cells posterior to the primitive streak (Ginsburg *et al.*, 1990; Lawson and

Hage, 1994). Presumptive PGCs are located in the most proximal part of the extraembryonic mesoderm and restriction of the germ cell lineage takes place only after these cells leave the epiblast (Lawson and Hage, 1994).

In contrast to the above organisms, the developmental dynamics of the early segregation of the germ line in birds are largely unknown and attention has been focused on relatively late stages of development. Chicken PGCs can easily be identified after the formation of the primitive streak (Stages 4-10, Hamburger and Hamilton, 1951) in an extraembryonic region located anterior to the head fold, often referred to as the "germinal crescent" (Swift, 1914) using morphological criteria and periodic acid Schiff's (PAS) staining for glycogen (Meyer, 1960). These properties have allowed the elucidation of germ cell migration from the germinal crescent to their final residence in the gonadal ridge, a process which occurs through passive migration via the embryonic circulation followed by active migration to the gonadal ridge (Swift, 1914; Meyer, 1964; Dubois and Croiselle, 1970; Fujimoto *et al.*, 1976a,b; Kuwana *et al.*, 1986; Nakamura *et al.*, 1988). Nevertheless, such histo-morphological criteria have been insufficient in tracing the origin of these cells back to earlier stages of development. Therefore, studies concerning the temporal and spatial aspects of the origin of avian PGCs had to rely heavily on classical embryological techniques. In these studies, the unincubated blastoderm was dissected into fragments and each fragment is cultured and later scored for the presence of PGCs using morphological criteria and periodic acid Schiff staining (PAS)

(Rogulska, 1968, Dubois and Croiselle, 1970, Eyal-Giladi *et al.*, 1976). The results of such studies suggested that the earliest precursors of the germ cell lineage were located within the embryo well before the formation of germinal crescent and that the germinal crescent was a secondary location in avian germ cell development.

In an attempt to pinpoint the location of presumptive PGCs at earlier stages of development, the unincubated blastoderm was transected perpendicular to the axis of the embryo to obtain three transversal bands, i.e. posterior, central and anterior fragments (Dubois and Croiselle, 1970; Ginsburg and Eyal-Giladi, 1987). When these fragments were cultured for 48 hours and scored for the presence of PGCs, it appeared that the majority of PGCs were located within the central fragment with a small number of PGCs present in anterior fragments. These observations led to the conclusion that, in the chicken embryo, majority of presumptive PGCs are concentrated within the central portion of the *area pellucida* (Ginsburg and Eyal-Giladi, 1987).

Studies carried out with fragments of unincubated chicken embryos also revealed a close association of PGCs with the hypoblast and suggested that PGCs originate from the hypoblast (Dubois, 1969). This hypothesis was tested by Eyal-Giladi *et al.* (1981) using inter-specific transplantation of stage XIII (Eyal-Giladi and Kochav, 1976) hypoblasts between chicken and quail embryos. It was observed that only a small portion of PGC population corresponding to

about 5 % of the total number of PGCs per embryo was derived from the hypoblast, leading to the conclusion that avian PGCs are of epiblastic in origin.

Later work in which hypoblasts were separated from the epiblast layer and cultured independently suggested that PGCs or their immediate precursors originating in the epiblast gradually translocate to the hypoblast where they are carried anteriorly to the germinal crescent as a result of morphogenetic movements during gastrulation (Ginsburg and Eyal-Giladi, 1986).

Despite a substantial body of indirect evidence indicating that the segregation of the germ cell lineage might take place as early as stage X, the temporal and spatial aspects of this phenomenon could not be examined directly due to the absence of molecular markers that could identify PGCs or their immediate precursors at pre-streak stages of development. Nevertheless, several attempts have been made to locate PGCs prior to gastrulation using direct immunohistochemistry. Urven *et al.* (1988) and Pardanaud *et al.* (1987a) examined the origin of PGCs using the monoclonal antibodies EMA-1 in the chick and QH-1 in the quail embryo. In both cases, the recognition of PGCs at stages X - XI was inconsistent. More recently, Loveless *et al.* (1990) reported that FC10.2, an antibody recognizing an oligosaccharide antigen, was a reliable marker of PGCs at stages 1 - 17. Unfortunately, none of these experiments provided direct evidence indicating that the cells recognized by these antibodies were, in fact, PGCs or their immediate precursors, since no experimental cell lineage analysis was performed. Finally, Muniesa and Dominguez (1990)

claimed to detect PGCs between stage X and stage 2 on the basis of PAS staining and ultra-structural morphology. Since it is generally accepted that PAS could not be used to mark PGCs at these earlier stages of development (Urven et al., 1988), the verification of the results obtained by Muniesa and Dominguez (1990) using PAS and ultra-structural criteria is difficult. Therefore, the origin of avian PGCs in relation to the temporal and spatial development of the embryo prior to the formation of the primitive streak remains speculative and merits further study.

To enhance our current understanding of the segregation of the avian germ line, the temporal and spatial pattern of the origin of PGCs between stages IX - XIV were investigated through two experimental approaches:

- 1) Immunohistochemical analysis of whole mount embryos and histological sections using a monoclonal antibody (MC-480) which recognizes Stage Specific Embryonic Antigen-1 (SSEA-1), and
- 2) *In vitro* culture of dispersed chicken blastodermal cells at stages IX - XIV with and without an STO feeder-layer.

In this report, it is proposed that the formation of the *area pellucida* and the maintenance of cellular organization within this region are prerequisites for the segregation and further development of PGCs such that definitive PGCs begin to appear at stage X. The evidence obtained from cell lineage analyses indicates that a small population of SSEA-1 labeled cells scattered on the ventral surface of the *area pellucida* and later on the dorsal surface of the hypoblast is probably

the primary population of cells which will give rise to the germ line. Furthermore, it was demonstrated that cells obtained from stage IX embryos have the potential to give rise to definitive PGCs only when they are cultured on feeder layers of STO cells.

MATERIALS AND METHODS

Immunohistochemical staining of whole mount embryos using anti - SSEA-1

SSEA-1 is a carbohydrate antigen determined by galactose ($\beta 1 - 4$) N - acetylglucosamine ($\alpha 1 - 3$) fucose (Gooi *et al.*, 1981). It is expressed by murine PGCs during their migratory period and has been used as a marker of PGCs *in vivo* and *in vitro* (see Donovan, 1994).

For immunohistochemical staining of pre-primitive streak embryos, whole embryos at stages IX - XIV were fixed in 4 % paraformaldehyde (pH 7.2) overnight at 4 °C and washed three times in PBS (pH 7.4). The staining procedure was carried out using an avidin/biotin-conjugated-alkaline phosphatase system (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA) using anti-SSEA-1 (MC-480) ascites fluid diluted 1:1000 as the primary antibody. Cells expressing the antigen were detected using 0.168 mg/mL nitroblue tetrazolium, 0.088 mg/mL 5-bromo-4-chloro-3-indolyl phosphate

toluidinium salt and 3.75 mM levamisol in 100 mM Tris-HCl, pH 9.5. Embryos were mounted in an aqueous mounting media and the pattern of SSEA-1 expression was evaluated for each developmental stage under a light microscope. Some of the stained embryos were dehydrated, embedded in paraffin and sectioned at 12 μ m.

Immunohistochemical staining of thick sections using anti - SSEA-1

Thick (50 μ m) wax sections were prepared as described by Nieto *et al.* (1996). Paraffin sections were dewaxed with tetrahydronaphtaline three times (5 min each), washed in 100 % methanol three times (5 min each). Sections were rehydrated by 5 min washes in 75 %, 50 %, and 25 % methanol in PBS followed by three washes (5 min each) in PBS (pH 7.4), containing 1 % TritonX-100, 0.1 % Na-azide (PBST). The staining procedure was carried out using an avidin/biotin-conjugated-alkaline phosphatase system (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA) using anti-SSEA-1 (MC-480) ascites fluid diluted 1:1000 as the primary antibody. Cells expressing the antigen were detected using 0.168 mg/mL nitroblue tetrazolium, 0.088 mg/mL 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt and 3.75 mM levamisol in 100 mM Tris-HCl, pH 9.5. Sections were washed in PBST three times (5 min each), fixed in 4 % paraformaldehyde in PBS for 20 min, and equilibrated in 70 % glycerol in

PBST. For photomicroscopy of the pattern of staining, sections were placed on a slide in a small amount of 70 % glycerol and flattened under a cover-slip.

Periodic acid Schiff staining of whole embryos

Stage 22 (Hamburger and Hamilton, 1951) embryos were isolated in PBS and extraembryonic membranes were removed. Embryos were fixed in situ in Rossman's fluid (90 volumes of 100 % alcohol saturated with picric acid and 10 volumes of formalin) for 24-48 hours and were rehydrated by 15-20 min washes in 75 %, 50 %, and 25 % ethyl alcohol in distilled water followed by three washes (5 min each) in water. Embryos were treated with periodic acid in situ for 20 min at room temperature, rinsed in distilled water two times (5 min each) and incubated in Schiff's reagent for 30 min. The staining reaction was ended by washing embryos several times in distilled water. Stained embryos were dehydrated, embedded in wax, and sectioned according to routine histological procedures. The material was sectioned at 15 μ m.

Live immunostaining of a hypoblast cell suspension with anti-SSEA-1

This procedure was carried out as described by Cooke *et al.* (1993) with slight modifications. Hypoblasts peeled from ten to fifteen blastoderms at stages XII - XIV were pooled in Dulbecco's Minimal Essential Medium (DMEM),

supplemented with antibiotics, 0.2 mM L-Glutamine, and 10 % fetal bovine serum (DMEM-FBS), washed in PBS twice and incubated in 0.02 % EDTA for 10 minutes at 37 °C. The EDTA solution was gently removed and replaced with 500 µl PBS in which hypoblast fragments were dissociated by gentle tituration. The cells were centrifuged at 311g for 5 min and resuspended in PBS. Cells were washed in PBS again and incubated with anti-SSEA-1 for 1 hr using a 1:500 dilution of the ascites fluid. The cells were washed in PBS, centrifuged at 311g for 5 min and then incubated for 30 minutes in rhodamine - conjugated anti-mouse IgM (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. All of the above steps were carried out on ice. Cells were resuspended in DMEM-FBS at room temperature. SSEA-1-labeled, fluorescent cells were collected using a micro-manipulator under an inverted fluorescent microscope with a rhodamine filter set.

Chick - quail chimeras

About twenty five to thirty SSEA-1-positive, fluorescent chicken cells obtained from the hypoblast cell suspension as described above were collected in 3 - 5 µl medium and injected into the subgerminal cavity of unincubated, irradiated quail blastoderms as described by Petite *et al.* (1990) and Carscience *et al.* (1993). Parallel experiments were performed using similar numbers of SSEA-1 negative cells. Injected eggs were incubated for four to five days first in

quail and then in chicken surrogate shells according to Ono *et al.* (1994). Embryos were fixed *in situ* in Rossman's fluid, sectioned, and chicken PGCs identified by PAS staining as described by Nakamura *et al.* (1991, 1992).

In vitro culture of dispersed hypoblast cells

The development of SSEA-1 positive and negative chicken hypoblast cells was examined on a feeder layer of mitotically arrested quail embryo cells. Quail blastodermal cells obtained from unincubated, irradiated quail embryos were cultured in chamber slides (Nunc, 8 chamber, catalog # 177445, Naperville, IL) to form a feeder layer onto which 50-60 SSEA-1-positive fluorescent chicken hypoblast cells were seeded. Following 48 hours of culture, chicken PGCs were identified by PAS staining. Parallel experiments were performed using about 200-300 SSEA-1 negative hypoblast cells.

In vitro culture of dispersed stage IX - XIV embryos on glass cover-slips

The culture system employed in this experiment was a modification of that described by Ginsburg and Eyal-Giladi (1989). Embryos were isolated in phosphate - buffered saline (PBS, pH 7.4), the *area opaca* was discarded and the remaining *area pellucida* (including marginal zone) was dispersed enzymatically using 0.0125 % trypsin and 0.005 % EDTA in PBS for 10 minutes

at 37 °C. In another series of experiments, *area pellucidae* (including marginal zone) were dispersed mechanically by gentle tituration through a 1 mL-pipette tip. All cells obtained from individual embryos were cultured separately for 48 hours at 37 °C with 5 % CO₂ on 12 mm glass cover-slips in DMEM supplemented with antibiotics, 0.2 mM L-Glutamine, and 10 % fetal bovine serum (DMEM-FBS). After about six hours from the initiation of the culture, fresh medium was added slowly until the surface of the cover-slips was covered. After 48 hr, the cultures were fixed in Rossman's fluid, and PGCs were detected using the periodic acid Schiff staining (Meyer, 1960).

In vitro culture of dispersed stage IX - XIII embryos on feeder layers of STO cells

Feeder layers of STO cells were prepared in 96-well-microtiter plates as described by Cooke *et al.* (1993). The *area pellucidae* from embryos of the same developmental stage 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 tituration using a 200 µl pipette tip. The cells were centrifuged at 311g for 5 min and resuspended in fresh culture medium. The concentration of cells and the number of

blastodermal cells obtained per embryo were determined using a hemocytometer; 5000 cells/well were seeded onto an STO feeder layer, and cultured for 48 hours at 37 °C with 5 % CO₂ in DMEM-FBS. The cultures were fed after the first 24 hours by replacing 100µl spent medium with an equal amount of fresh medium. At the end of the culture period, the medium was removed and cells were fixed in 4 % paraformaldehyde (pH 7.2) overnight at 4 °C. Immunohistochemical staining of cells was carried out as described above for whole embryos and the number of PGCs per well determined. Since the number of blastodermal cells per embryo was known, it was possible to calculate the number of PGCs per embryo for each stage.

Statistical analysis

The data were analyzed for significance at the 5% level. Data collected from the *in vitro* culture of dispersed embryos on glass cover-slips were subjected to TTEST procedure of SAS (SAS Institute, 1990).

The data collected from *in vitro* culture of dispersed embryos on feeder-layers of STO cells were subjected to ANOVA utilizing the General Linear Model (GLM) procedure of SAS(SAS Institute, 1990), with the following model: PGC= date stage. Sources of variation for this data set were date and stage.

RESULTS

Expression of SSEA-1 in stage IX - XIV whole mount embryos

Chicken PGCs can readily be identified histomorphologically in the germinal crescent at stage 4-10, in the blood at stage 14, in the gonadal ridge at stage 22 (Hamburger and Hamilton, 1951) and in gonads at day six of incubation. Therefore, prior to using SSEA-1 as a marker for the identification of PGCs in whole mount stage IX - XIV embryos, SSEA-1 expression was initially tested on these tissues.

Anti-SSEA-1 recognized PGCs specifically in the germinal crescent (Fig. 1. 1 A, B), in the blood (Fig. 1. 1C) and the gonadal ridge (Fig. 1. 2A, B). It is most likely that cells recognized by anti-SSEA-1 within the gonadal ridge are germ cells because of their morphology and spatial distribution with respect to PAS-positive PGCs within this region (Fig. 1. 2C, D). Furthermore, double staining of dispersed gonadal cells using PAS and anti-SSEA-1 demonstrated that SSEA-1 was expressed only by PGCs with no expression in somatic cells (data not shown).

SSEA-1 immunoreactive cells were detected first at stage X (Fig. 1. 3B). No staining was observed at stage IX (Fig. 1. 3A). In most cases, the expression of SSEA-1 was confined mainly to the *area pellucida*, particularly to the central portion of this region, regardless of the developmental stage. Few cells were observed in the *area opaca*. Distinctly stained SSEA-1 positive cells were first detected at stage X on the ventral surface of the epiblast in association with the

polyingressing cells that did not express the epitope (Fig. 1. 3C). In whole mounts of stages XI - XIV, a substantial number of epiblast cells expressed SSEA-1 in a mosaic pattern such that immunoreactive cells were scattered among SSEA-1 negative cells (Fig. 1. 3D). It was also noted that the density of staining among SSEA-1 labeled epiblast cells varied significantly in a given embryo (Fig. 1. 3D). Cross sections taken from stained stage XIV embryos revealed that the expression of SSEA-1 was confined specifically to the dorsal (apical) side of the epiblast (Fig. 1. 4B).

When the whole hypoblasts were separated from the epiblast at stages XII, XIII and XIV and stained, the presence of distinct SSEA-1 labeled cells was revealed. These cells, which were located on the dorsal surface of the hypoblast, were morphologically similar to the immunoreactive cells observed in association with the polyingressing cells at stages X and XI (Fig. 1. 4A). The staining was uniformly intense among the labeled cells, some of which were found as clusters consisting of two to six cells and some as single cells (Fig. 1. 4A).

Staining of the epiblast separated from the hypoblast also revealed the presence of cells with high level of SSEA-1 expression on the ventral surface of the epiblast. All of these cells were loosely attached to the epiblast.

The high number of SSEA-1 expressing cells in the epiblast of whole mount embryos made the quantification impossible. However, it was possible to count the immunoreactive cells on the dorsal surface of the hypoblast. The number of

these cells increased from about 20 at stage XII to approximately 70 at stage XIV (Fig. 1. 5).

The analysis of the fate of SSEA-1 positive cells detected in the hypoblast

In the first series of experiments, seven putative quail-chicken chimeras were produced by injecting SSEA-1-positive fluorescent cells obtained from chicken hypoblast explants at stages XII - XIV into the subgerminal cavity of unincubated, irradiated quail embryos. Serial sections were taken from the germinal ridge region of the putative chimeras and stained for glycogen using PAS. In only one of the putative chimeras, two PAS-stained chicken germ cells could be identified in the dorsal mesentery (Fig. 1. 6A). In parallel experiments, SSEA-1 negative cells did not give rise to PGCs (data not shown).

In the second series of experiments, SSEA-1-positive fluorescent cells and SSEA-1-negative cells obtained from chicken hypoblast explants at stages XII - XIV were cultured in the presence of quail blastodermal cells. SSEA-1 negative hypoblast cells did not give rise to any PGCs (data not shown). On the other hand, about 10 PAS-stained chicken germ cells per well were detected in cultures that were initially seeded with 50-60 SSEA-1 expressing cells of the hypoblast (Fig. 1. 6B).

In vitro culture of dispersed stage IX - XIV embryos on glass cover slips

When dispersed stage IX-XIV embryos were cultured on glass cover-slips, definitive PGCs were detected from stage X onward. No PGCs were found in cultures initiated with stage IX embryos (Fig. 1. 7). The dispersion of embryos using trypsin/EDTA yielded a more uniform single cell suspension and a greater variation in the number of PGCs per embryo compared to mechanical dispersion. However, the number of PGCs obtained in a given stage did not differ significantly between the two methods of dispersion. In general, the number of PGCs/embryo increased from about 20 at stage X up to approximately 45 at stage XIV.

In vitro culture of dispersed stage IX - XIII embryos on feeder layers of STO cells

To further analyze the developmental potential of pre-primitive streak blastodermal cells to give rise to PGCs *in vitro*, we used mouse STO feeder cells. The STO feeder layer system provided the opportunity to dilute the concentration of embryo cells and minimize cell to cell contact among chicken cells.

A relatively small number of cells obtained from the dispersion of embryos at stages IX - XIII were cultured on feeder layers of STO cells. PAS staining proved to be an unsuitable marker for PGCs in culture, since STO cells were not completely PAS negative (data not shown). Therefore, PGCs were identified on

the basis of their morphology and SSEA-1 expression after 48 hr of culture. The estimated number of PGCs per embryo was relatively equal between stages including stage IX (Fig. 1. 8). A typical response of embryo cells cultured on feeder layers of STO cells was to form what appeared to be clonal patches of SSEA-1-positive and SSEA-1-negative cells after 48 hr of culture (Fig. 1. 9A, B). PGCs were observed most often in association with the SSEA-1 positive patches. Sometimes they were associated with SSEA-1-negative patches or with STO cells alone. Also, PGCs were found as clusters of SSEA-1 positive cells associated with the feeder cells (Fig. 1. 9C, D).

DISCUSSION

In this study, the temporal and spatial patterns of the segregation of the germ line were examined between stages IX-XIV of the chick embryo through immunohistological analysis of whole embryos and histological sections. To this end, the monoclonal antibody MC-480 that recognizes the stage specific embryonic antigen-1 (SSEA-1) was employed. MC-480 is known to identify murine (Hahnel and Eddy, 1986; Donovan *et al.*, 1987) and pig PGCs (Takagi *et al.*, 1997). In the chick embryo, EMA-1 was previously shown to identify germ cells prior to their appearance in the germinal crescent (Urven *et al.*, 1988; Karagenc *et al.*, 1996). The present study demonstrates that the monoclonal antibody MC-480 can be used for the same purpose (Fig. 1. 2, Fig. 1. 3).

It has been suggested that the antigenic determinant recognized by the monoclonal antibody EMA-1 is related to the mouse embryonic antigen SSEA-1 (Loveless *et al.*, 1990). In support of this hypothesis, a similar pattern of cell segregation is revealed by the two antibodies (Karagenc *et al.*, 1996). The expression of both SSEA-1 (present study) and EMA-1 (Urven *et al.*, 1988, Karagenc *et al.*, 1996) epitopes coincide with the completion of *area pellucida* formation at stage X. In subsequent stages, EMA-1 and SSEA-1-labeled cells are confined to the central portion of the *area pellucida*, excluding the marginal zone. Cells expressing SSEA-1 appear in a scattered fashion in such a way that no posterior-anterior orientation is detected. From stage XI onward, SSEA-1 positive cells with varying intensities of staining are peppered throughout the dorsal aspect of the central disc epiblast. A similar feature was described by Urven *et al.* (1988), using EMA-1. This differential pattern of expression is in agreement with several reports suggesting that the epiblast constitutes a heterogeneous population of cells even at these early stages of development (Canning and Stern, 1988; Stern and Canning, 1990; Riese *et al.*, 1995).

The staining of separated epiblasts and hypoblasts at stages XII - XIV revealed the presence of a small population of cells on the dorsal surface of the hypoblast with a uniform intensity of SSEA-1 staining. It was hypothesized that these cells represent PGCs or their immediate precursors on the basis of their expected number, spatial distribution and shared epitope with definitive PGCs in the blood and gonad. The evidence obtained from *in vivo* and *in vitro* cell

lineage analyses indicates that the labeled cells descending from the *area pellucida* to the hypoblast at stages X-XIV do indeed represent the earliest identifiable PGC population in the avian embryo. Although it was demonstrated that SSEA-1 positive cells of the hypoblast have the potential to give rise to PGCs *in vivo* and *in vitro*, the number of PGCs was always lower than the number injected or seeded. Two hypotheses can be put forward to account for these observations. First, it is possible that SSEA-1 positive cells of the hypoblast are not yet restricted to the germ cell lineage at stages XII - XIV and that these cells have the potential to differentiate into various cell types, other than PGCs, which can not be identified by PAS staining. Alternatively, the low numbers could be due to the inhibition of cell adhesion by the binding of anti-SSEA-1, a phenomenon which has been reported to interfere with the attachment of mouse PGCs to fibroblasts (Donovan *et al.*, 1987). Such an inhibitory effect would interfere with the active migration of PGCs on route to the gonadal ridge.

The culture of whole embryos and embryo fragments has been the conventional approach in examining the temporal and spatial aspects of the origin of PGCs in the avian embryo (Dubois and Croiselle, 1970; Eyal-Giladi *et al.*, 1976; Ginsburg and Eyal-Giladi, 1987). The results obtained from such studies suggested that the segregation of the germ cell lineage might take place as early as stage X and that the majority of PGCs are located within the central portion of the *area pellucida* (Dubois and Croiselle, 1970; Ginsburg and Eyal-

Giladi, 1987). Despite the ingenuity of these experiments, however, the specific details of the emergence of PGCs in relation to other developmental events have remained hypothetical. Nevertheless, recent experiments performed by M. Ginsburg and her colleagues at the Hebrew University of Jerusalem led to the realization that the development of the germ cell lineage might be linked to the formation of the *area pellucida*, the first morphological evidence of the symetrization process. In these studies the origin of PGCs was examined through *in vitro* culture of whole embryos and embryo fragments on vitelline membranes at stages VII-IX (Karagenc *et al.*, 1996). Accordingly, when peripheral and central fragments of stage VII - IX blastoderms were cultured separately, embryos did not develop in any of the isolated fragments. Nevertheless, this did not prevent the development of PGCs which were detected only in central fragments consisting of approximately one-third of the diameter of the blastodisc at stage VII and about one-half the diameter at stages VIII and IX. These central fragments always contained a partially formed *area pellucida*, suggesting that the formation of *area pellucida* is a prerequisite for normal development of PGCs (Karagenc *et al.*, 1996).

The detection of definitive PGCs in cultures of embryo fragments, however, did not indicate whether PGCs are set aside at a very early stage of development or differentiate from early precursors as a result of an inductive event in association with the formation of the *area pellucida*. The former hypothesis appears unlikely, since the culture of dispersed embryos on glass

cover-slips indicate that the blastodisc does not harbor committed PGCs at stage IX (Fig. 1. 7). This hypothesis draws support from the observation that no PGCs could be detected after extending the culture of dispersed stage IX embryos to 96 hours (data not shown).

An interesting observation associated with the culture of dispersed embryos on glass cover-slips was the absence of extensive cellular organization such as the formation of "dome"- like structures or the development of blood cells previously reported with the culture of fragmented stage X embryos (Sanders and Dickau, 1981; Ginsburg and Eyal-Giladi, 1989). It was suggested that formation of a trilaminar aggregate in culture consisting of an epiblastic-layer, a hypoblastic layer, and a mesenchyme-like middle layer is a prerequisite to support the differentiation of PGCs in culture (Ginsburg and Eyal-Giladi, 1989). According to Ginsburg and Eyal-Giladi (1989), the number of PGCs obtained in cultures carried out with dispersed blastoderms was a function of the initial cell population density, which, in turn, determined the level of differentiation and organization of somatic cells. These observations were interpreted such that the differentiation of PGCs in culture requires the formation of a three-layered structure and the translocation of presumptive PGCs from the upper layer to the lower layer. Since during normal development, PGCs originating in the epiblast gradually translocate to the hypoblast (Eyal-Giladi *et al.*, 1981; Ginsburg and Eyal-Giladi, 1986), the culture conditions reported by Ginsburg and Eyal-Giladi (1986) appeared to mimic the normal program of germ line segregation. It is

most likely that a minimum level of cellular organization is necessary for in vitro expansion of the population of PGCs beyond that already present. In the present study, 25-45 PGCs/embryo were detected in cultures initiated with stage X-XIV blastoderms (Fig. 1. 7). In addition, a gradual increase in the number of PGCs was observed between stages X to XIV with mechanically dispersed embryos (Fig. 1. 7). It should be noted that the numbers of PGCs detected at these stages are in relatively close agreement with the numbers of SSEA-1 labeled cells observed on the ventral surface of the area pellucida at stage X (Karagenc *et al.*, 1996) and then on the dorsal surface of the hypoblast at stages XII-XIV (Fig. 1. 5). Taken together, it would appear that the level of cellular organization reached in the present culture conditions was insufficient to promote the continued differentiation of PGCs and that the PGCs detected at stages X to XIV represent the population of cells committed to the germ cell lineage for each stage.

The results described above suggested that in the avian embryo the segregation of PGCs is not a cell autonomous process and that the differentiation and development of PGCs is an epigenetic event, requiring local cell-cell interactions. In an attempt to examine this phenomenon further, cells obtained from dispersed embryos at stages IX - XIII were cultured on feeder-layers of STO cells at a low density to help minimize possible cell to cell contact among other chicken cells (Fig. 1. 9A, B). PGCs were identified in feeder-dependent cultures initiated not only with stage X-XIII embryos as expected, but

also with stage IX embryos (Fig. 1. 8). Moreover, the number of PGCs at stages X - XIII was significantly higher compared to that observed in cultures carried out on glass cover-slips, suggesting that the micro-environment provided by STO cells compensated for a minimal level of cellular organization absent in cultures on glass cover-slips. Furthermore, it appears that the development of PGCs from dissociated blastoderms cultured on feeder layers of STO cells occurs according to the normal program of the germ line development. This hypothesis is supported by the observation that the pattern of the expression of SSEA-1 by non-PGCs is remarkably similar to that observed in the epiblast at stages XI-XIV (cf. Figs. 1. 3D, 1. 9B). These observations suggest that the culture of blastodermal cells on feeder-layers of STO cells provides a useful biological end point in examining the molecular basis of avian germ cell development.

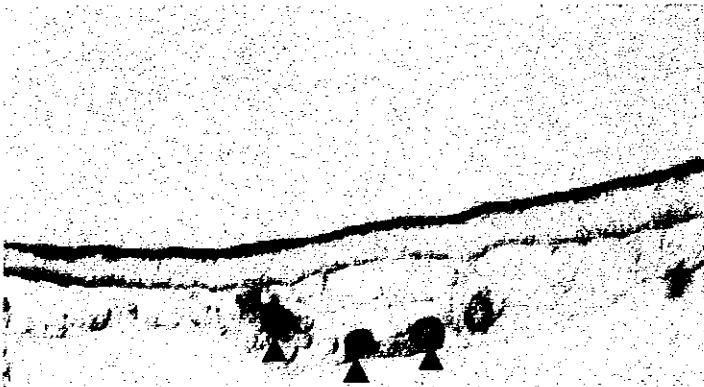
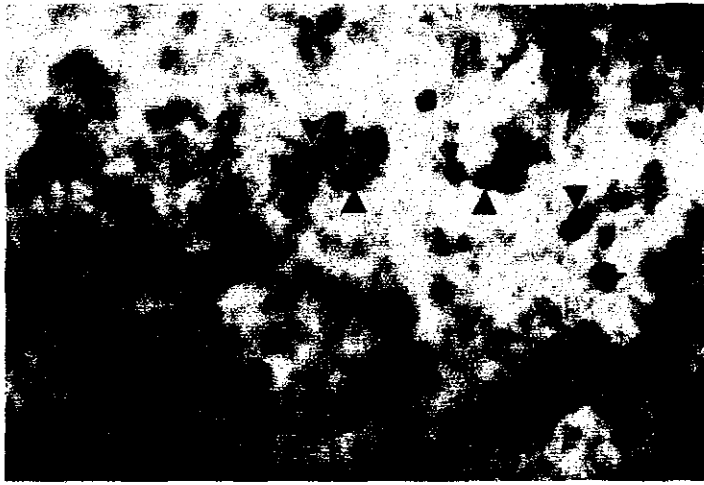
Very little is currently known about the peptide growth factors that may be involved in the development of the avian germ cell lineage. Some clues, however, are suggested from studies on mouse PGCs in culture. Several growth factors/cytokines have been reported to support the survival and/or proliferation of definitive murine PGCs (Dolci *et al.*, 1991; Godin *et al.*, 1991; Matsui *et al.*, 1991, 1992; Resnick *et al.*, 1992). Some of these factors include basic fibroblast growth factor (bFGF, FGF-2), stem cell factor (SCF), and leukemia inhibitory factor (LIF), which are produced by STO cells (Dolci *et al.*, 1991; Donovan, 1994). In the chicken embryo, FGF-2 is expressed at pre-primitive streak stages of development (Riese *et al.*, 1995) and has been

suggested to be involved in the specification of the erythropoietic cell lineage (Gordon-Thomson and Fabian, 1994). Although the data presented here do not provide direct evidence indicating the involvement of any of these cytokines in the determination and development of avian PGCs, it is likely that similar factors are involved.

Definitive PGCs were identified in cultures initiated with dispersed stage X-XIV embryos, coincident with the temporal pattern of SSEA-1 expression at these stages. The number of PGCs obtained in culture at stage X was about 20, in agreement with previous estimates by Muniesa and Dominguez (1990), using ultra-structural analysis. Furthermore, Pardanaud *et al.* (1987a) suggested that 20 PGCs were present at stages XII-XIII in the quail using the antibody QH-1.

It appears from the observations described in the present study that SSEA-1 positive cells detected first in the ventral surface of the epiblast and later on the dorsal surface of the hypoblast constitute the founding population of the germ cell lineage in the avian embryo. However, it remains to be seen whether the determination of the germ cell lineage takes place in the epiblast or after the translocation of SSEA-1 expressing cells to hypoblast.

Fig. 1. 1. The expression of SSEA-1 by PGCs within the germinal crescent region and during their passive migration through the circulation. Stage 10 (Hamburger and Hamilton, 1951) embryos were isolated, fixed in 4 % paraformaldehyde as whole mounts and stained with an avidin-biotin-conjugated alkaline phosphatase system, using the monoclonal antibody MC-480 which recognizes stage-specific embryonic antigen-1 (SSEA-1). Blood smears were prepared from stage 14 (H&H) embryos. Cells expressing SSEA-1 were detected using NBT-BCIP substrate. **A:** Expression of SSEA-1 by PGCs (arrow heads) within the germinal crescent region in a stage 10 (H&H) embryo stained as whole mount. **B:** Cross section taken from the germinal crescent region of a stage 10 (H&H) whole mount embryo after immunohistochemical staining with MC-480. Note intensely stained PGCs (arrow heads). SSEA-1 is also expressed on the apical side of the epiblast (arrow). **C:** Expression of SSEA-1 by blood borne PGCs (arrows) in a blood smear from a stage 14 (H&H) chicken embryo.



B



Fig. 1. 2. The identification of PGCs within the gonadal ridge by periodic acid Schiff (PAS) staining and the expression of SSEA-1. Thick sections obtained from stage 22 (H&H) embryos were stained with an avidin-biotin-conjugated alkaline phosphatase system, using the monoclonal antibody MG-480 which recognizes stage-specific embryonic antigen-1 (SSEA-1). SSEA-1 positive cells were detected using NBT - BCIP as the substrate. **A:** PGCs (arrow heads) recognized by MC-480 within the gonadal ridge. Note the absence of staining within other regions of the embryo. **B:** Higher magnification of the picture in A. **C:** PGCs (arrow heads) identified by PAS staining within the gonadal ridge. **D:** Higher magnification of the picture in C.

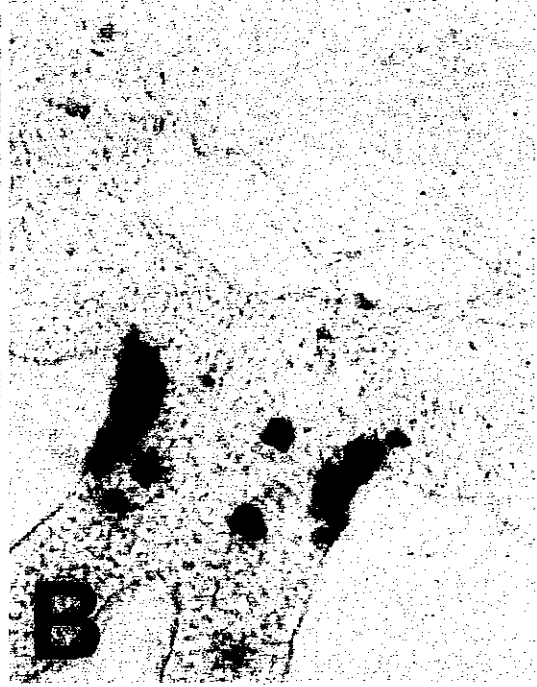


Fig. 1. 3. Immunohistochemical staining of pre-streak whole mount embryos using the monoclonal antibody MC-480 which recognizes stage-specific embryonic antigen-1 (SSEA-1). Embryos were isolated, fixed in 4 % paraformaldehyde as whole mounts and stained with an avidin-biotin-conjugated alkaline phosphatase system. Cells expressing SSEA-1 were detected using NBT-BCIP substrate. **A:** Ventral view of a stage IX embryo, showing the absence of staining. **B:** Ventral view of a stage X embryo. Note the SSEA-1 positive cells protruding from the *area pellucida*. **C:** Two labeled cells in association with SSEA-1-negative polyingressing cells. **D:** Ventral view of a whole stage XII embryo with hypoblast removed. Note the distribution of SSEA-1-labeled epiblast cells with various intensities of staining. This pattern on the epiblast was limited to the central disc.

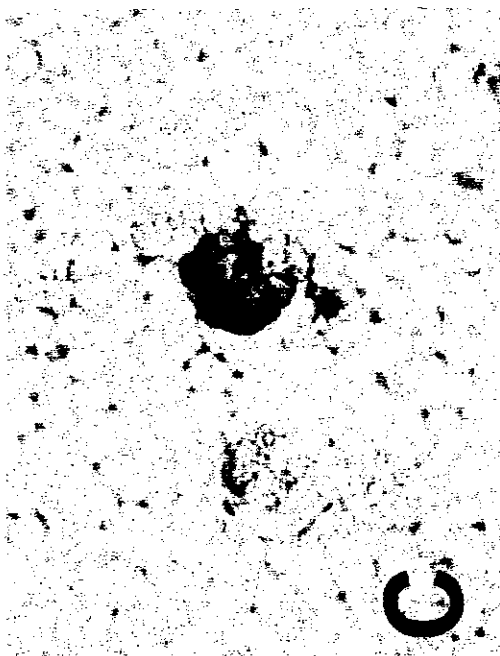
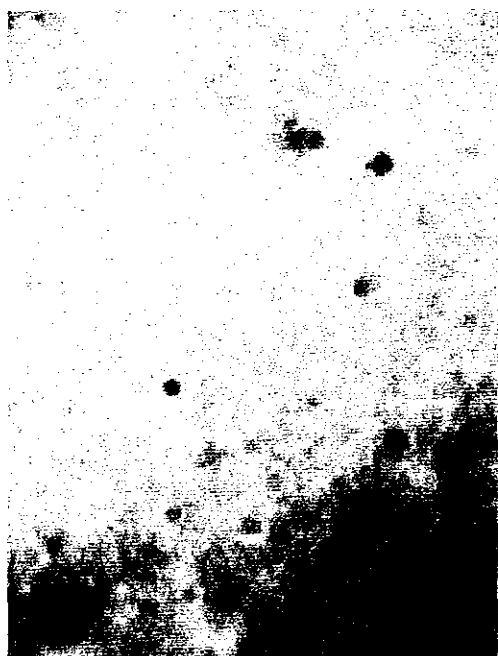
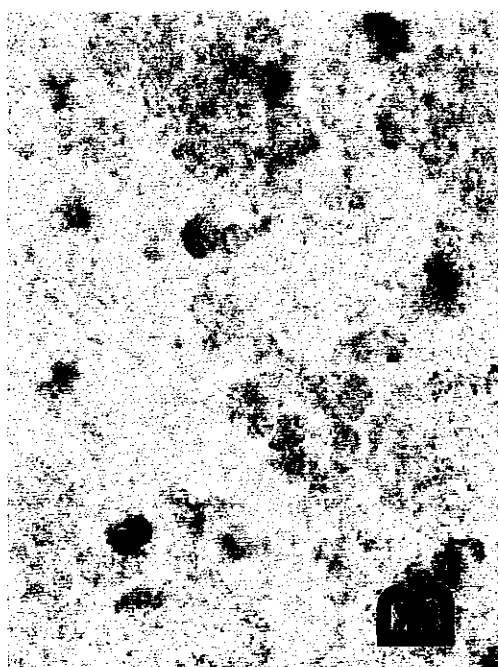


Fig. 1. 4. Immunohistochemical staining of a stage XIV hypoblast. Hypoblasts were separated from the epiblast, fixed in 4 % paraformaldehyde, and stained whole with an avidin-biotin-conjugated alkaline phosphatase system. Cells expressing SSEA-1 were detected using NBT-BCIP substrate. **A:** Dorsal view of a hypoblast from a stage XIV embryo. Note the collection of intensely stained single cells and clusters of 2-6 cells residing on the hypoblast. **B:** Transversal section taken from a stage XIV whole mount embryo after immunohistochemical staining with MC-480. Note intensely stained PGCs (arrow heads). SSEA-1 is also expressed on the apical side of the epiblast (arrow).

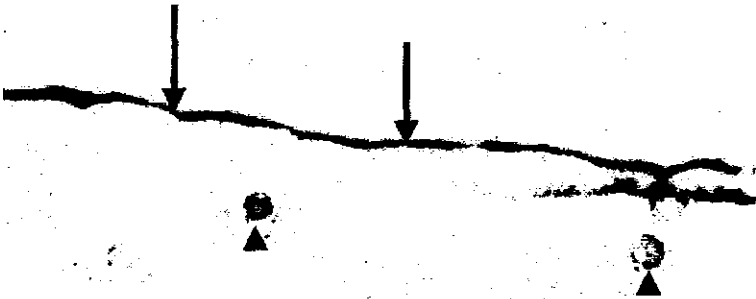
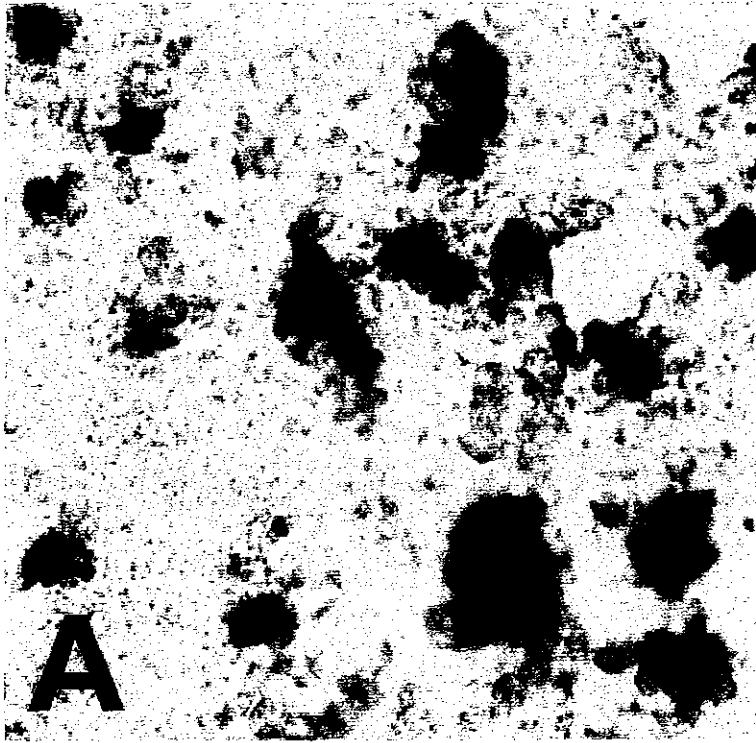


Fig. 1. 5. The number of SSEA-1 expressing cells detected on the dorsal surface of the hypoblast at stages XII - XIV (Mean $\pm$ S. E., $n = 4 - 10$). Embryos were isolated and staged according to Eyal-Giladi and Kochav (1976). Whole hypoblasts were removed, fixed in 4 % paraformaldehyde and stained with an avidin/biotin - conjugated-alkaline phosphatase system. SSEA-1 positive cells were detected using NBT - BCIP as the substrate.

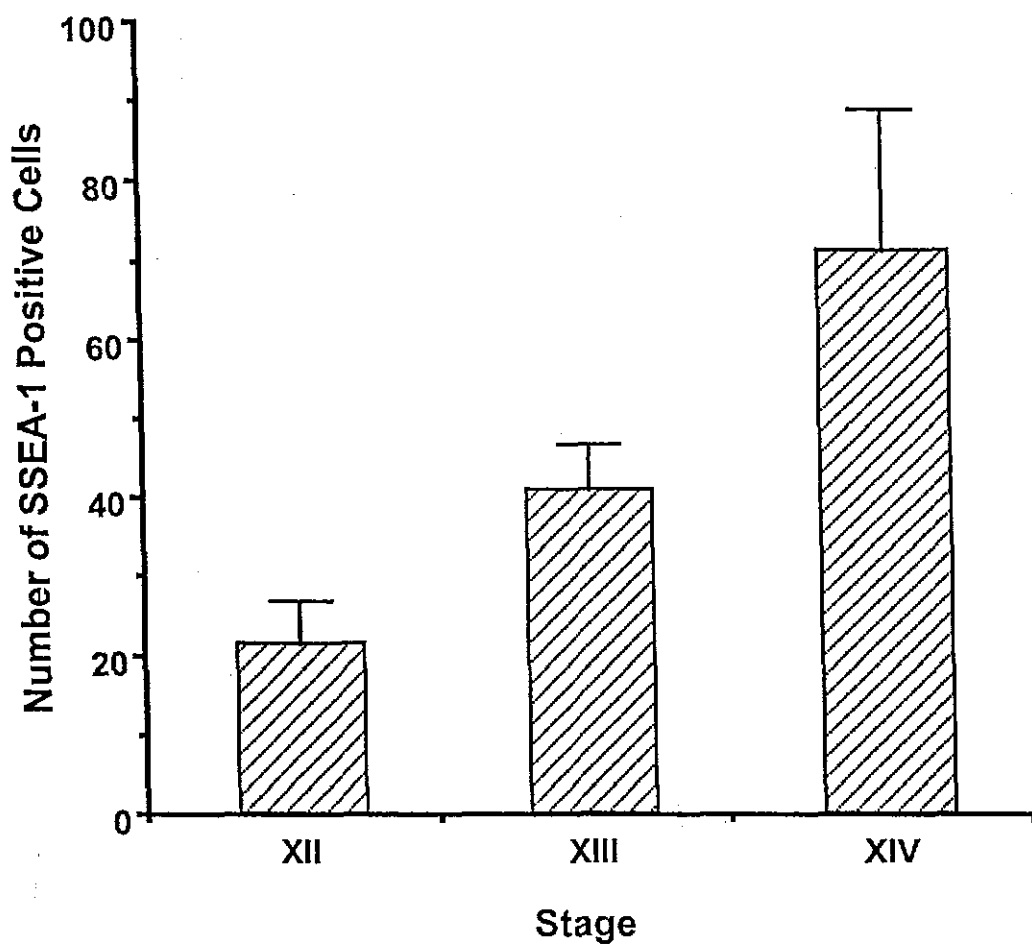


Fig. 1. 6. Chicken primordial germ cells identified by periodic acid-Schiff staining in a chick-quail chimera and on a feeder-layer of quail blastodermal cells. Chicken hypoblast cells from stages XII-XIV were live-stained using anti-SSEA-1 and a rhodamine-conjugated anti-mouse IgM. SSEA-1-labeled fluorescent cells were collected using a micromanipulator and 25-30 labeled cells were injected into the subgerminal cavity of unincubated, irradiated quail embryos. In another series of experiments, 50-60 SSEA-1-labeled cells were seeded onto a feeder-layer of mitotically arrested quail blastodermal cells. Putative chimeras were fixed at day 4, fixed *in situ* in Rossman's fluid, sectioned and stained by periodic acid-Schiff (PAS) staining. The identification of chicken PGCs in culture was carried out using PAS staining after 48 hours of culture. **A:** Two PAS-stained chicken PGCs (arrow) identified within the dorsal mesentery of a quail embryo. **B:** A cluster of chicken PGCs identified by PAS staining on a feeder-layer of quail blastodermal cells.



Fig. 1. 7. The number of PGCs in cultures initiated with dispersed St. IX - XIV embryos (Mean $\pm$ S. E., $n = 4 - 15$). Embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* discarded and the remaining *area pellucida* was dispersed into a single cell suspension either by trypsinization (0.0125 % trypsin, 0.005 % EDTA) or by gentle mechanical disruption of the embryo. Dispersed embryos were cultured individually for 48 hours at 37 °C / 5 % CO₂ on glass cover-slips in DMEM supplemented with antibiotics, glutamine and 10 % FBS. Cells were fixed in Rossman's fluid and the number of PGCs determined by periodic acid Schiff (PAS) staining. The number of PGCs obtained in a given stage did not differ significantly between the two methods of dispersion.

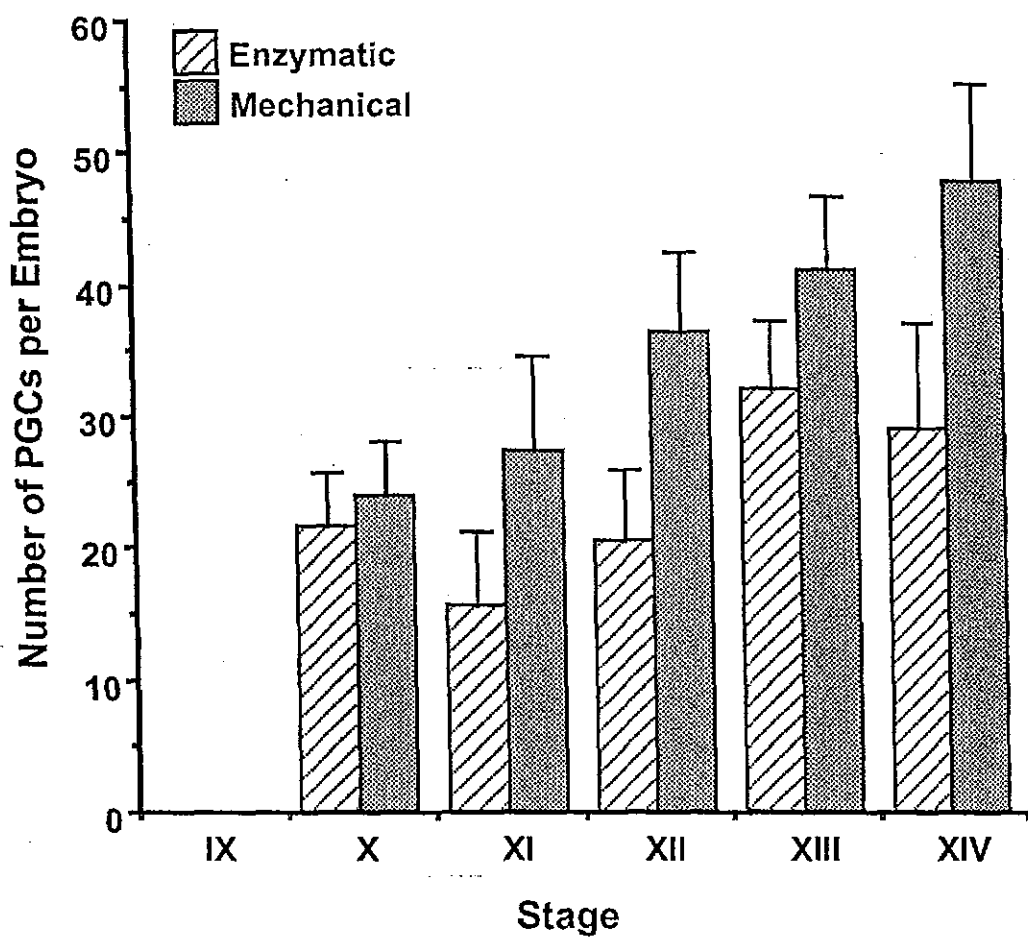


Fig. 1. 8. The number of PGCs detected in cultures of dispersed St. IX-XIII embryos on feeder-layers of STO cells. Each experiment was repeated with 6-30 wells, and numbers are the means $\pm$ S.E.M of at least two independent experiments. Embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded and the *area pellucida* was dissociated into a single cell suspension by trypsinization (0.0125 % trypsin and 0.005 % EDTA). The number of blastodermal cells obtained per embryo was estimated and approximately 5000 cells per well were seeded onto feeder layers of mitotically arrested STO cells in 96 - well microtiter plates. The cells were cultured for 48 hours at 37 °C / 5 % CO₂. The cultures were fixed in 4 % paraformaldehyde (pH 7.2) overnight and PGCs identified on the basis of morphology and SSEA-1 expression. Bars with different letters are statistically significant from one another.

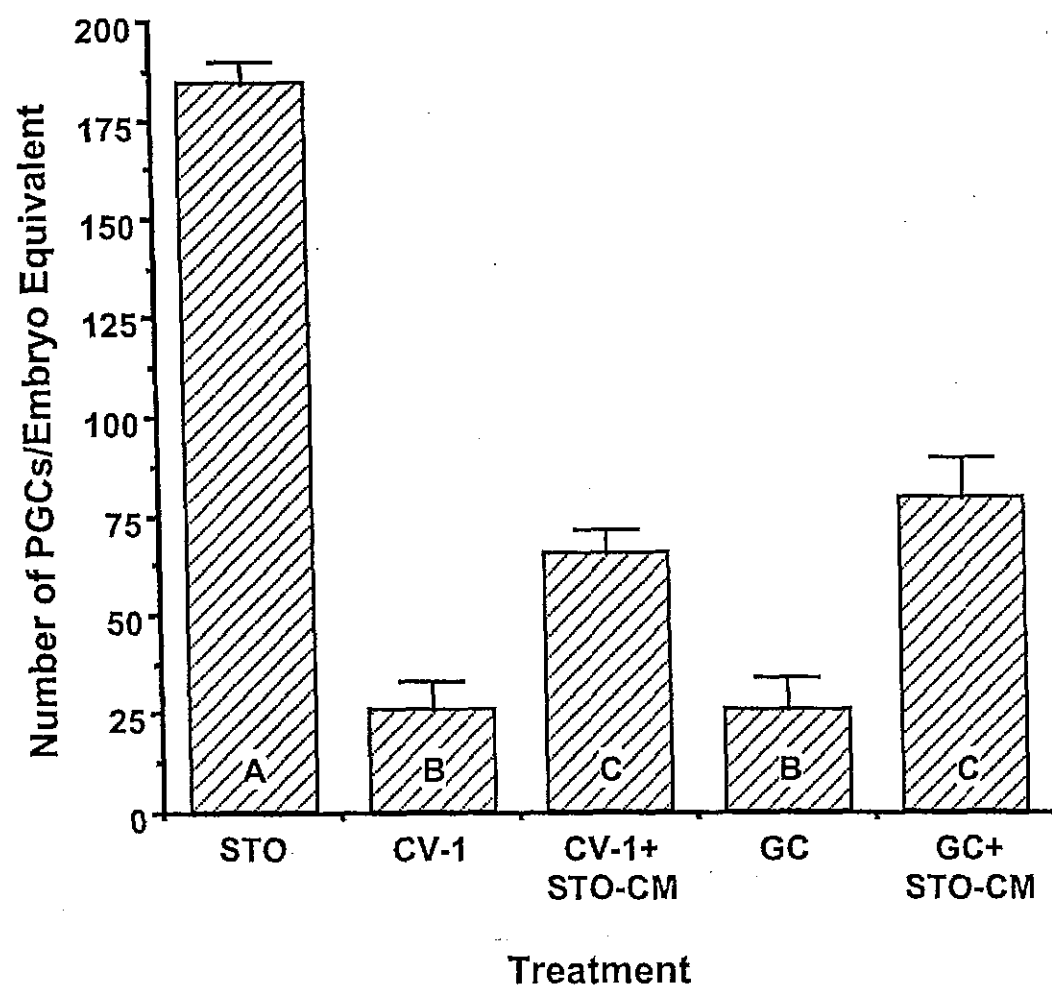
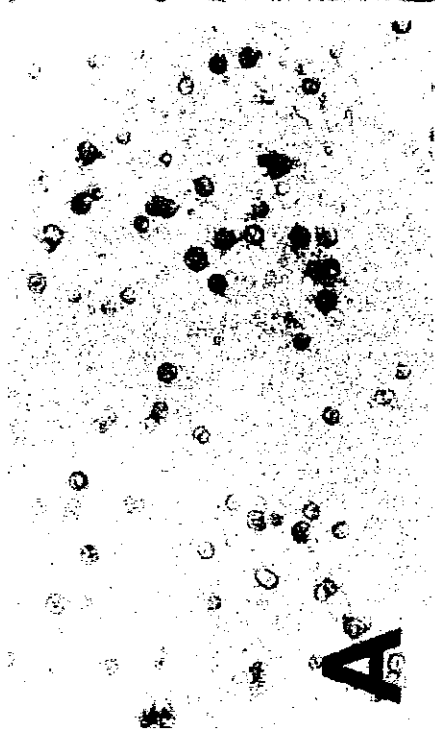


Fig. 1. 9. Development of PGCs in cultures of dispersed stage IX-XIII embryos on feeder-layers of STO cells. Embryos were isolated in phosphate-buffered saline (pH 7.4), the area opaca was discarded, and the area pellucidae were dissociated into a single-cell suspension. Cells (5000) were seeded onto mitotically-arrested STO cells. After 48 hr of culture, PGCs were identified on the basis of morphology and SSEA-1 expression. **A:** Distribution of freshly seeded blastodermal cells on an STO feeder-layer. **B:** Low magnification of a typical response of embryo cells on an STO feeder-layer. Cultures show SSEA-1 positive patches (arrows). PGCs (arrowheads) expressing SSEA-1 are scattered throughout. **C:** Higher magnification of the picture in B. Note the association of PGCs (arrowheads) with SSEA-1 positive patches of chicken cells. **D:** Typical clusters of 2-6 PGCs in association with STO cells alone.



CHAPTER 2

Expression of SCF and c-Kit in the chicken embryo and the development of chicken primordial germ cells in culture

ABSTRACT In the chicken embryo, the experimental evidence available is at variance with the hypothesis that the segregation of the germ cell lineage is a cell-autonomous process (Ginsburg, 1996; Karagenc *et al.*, 1996). Rather, it appears that the segregation and further development of PGCs is an epigenetic event, requiring local cell-cell interactions, which might be mediated by peptide growth factors (Karagenc *et al.*, 1996). The purpose of the present study was to examine if two such growth factors, namely stem cell factor (SCF) and basic fibroblast growth factor (bFGF), were involved in the development of avian PGCs. To this end, evidence was obtained indicating that both isoforms of SCF and its tyrosine kinase receptor c-kit are expressed at pre-streak stages of development. In situ hybridization analyses revealed that SCF is expressed in epithelial cells forming the gonadal ridge, the final destination of PGCs, while c-kit is expressed by PGCs themselves. In vitro culture experiments demonstrated that when blastodermal cells were cultured either on gelatin-coated tissue-culture plastic or on feeder-layers of CV-1 cells, the number of PGCs detected after 48 hr of culture was lower than expected. However, when blastodermal cells obtained from the same pool of embryos were cultured on feeder layers of STO cells, a marked increase in the number of PGCs was observed. Furthermore, the addition of STO cell-conditioned medium (STO-CM)

to blastodermal cells cultured either on gelatin-coated plastic or on feeder-layers of CV-1 cells resulted in a significant increase in the number of PGCs. When blastodermal cells were cultured on feeder-layers of CV-1 cells in the presence of avian SCF (100 ng/mL), a significant increase was observed in the number of PGCs after 48 hr of culture. Conversely, the addition of bFGF (10 and 100 ng/mL) to blastodermal cells cultured on feeder-layers of STO and CV-1 cells did not stimulate the emergence of PGCs. The inhibitory effect of bFGF on the emergence of PGCs on STO feeder-layers was associated with the down-regulation of SSEA-1 and with the up-regulation of *brachyury*. The implications of a possible causal relationship between these events are discussed within the context of the segregation of the germ cell lineage in the avian embryo.

INTRODUCTION

In vertebrates the segregation of the germ cell lineage is characterized by the appearance of primordial germ cells (PGCs) at an early stage of development. In avian embryos this process begins as early as stage X (Eyal-Giladi and Kochav, 1976) and is associated with the expression of EMA-1 and SSEA-1 epitopes (Urven *et al.*, 1988; Karagenc *et al.*, 1996). At this time, about 20 labeled cells are found in association with the polyingressing cells of the epiblast (Karagenc *et al.*, 1996). As the hypoblast forms between stages XI-XIV, SSEA-1/EMA-1 positive cells gradually descend to reside on the dorsal surface

of the hypoblast and give rise to definitive PGCs at later stages of development (Urven *et al.*, 1988; Karagenc *et al.*, 1996).

When individual stage IX-XIV embryos are dispersed and cultured on glass cover-slips, 25-45 PGCs/embryo are detected only with stage X-XIV, but not with stage IX blastoderms (Karagenc *et al.*, 1996). However, when the culture of blastodermal cells obtained from stages IX-XIII is carried out on feeder-layers of STO cells, PGCs can be identified in cultures initiated not only with stage X-XIII embryos as expected but also with stage IX embryos. Furthermore, the number of PGCs/embryo obtained on STO feeder-layers was much higher than that observed in cultures without feeder-layers (Karagenc *et al.*, 1996).

These observations suggested that the differentiation of avian PGCs is not a cell-autonomous process and that the segregation and development of PGCs is an epigenetic event requiring specific local cell-cell interactions. It is most likely that this process is mediated, at least in part, through peptide growth factors and their corresponding receptors. In mammals, a substantial body of evidence obtained from *in vitro* studies carried out with mouse PGCs point to the possibility that during normal development the survival, proliferation and migration of germ cells could be regulated by a complex set of growth factors/cytokines. Some of these growth factors include basic fibroblast growth factor (bFGF, FGF-2), stem cell factor (SCF) and leukemia inhibitory factor (LIF) (Dolci *et al.*, 1991, 1993; Godin *et al.*, 1991; Matsui *et al.*, 1991, 1992; Resnick *et al.*, 1992).

The analyses of the germ cell development in *W*- and *Sl*-mutants led to the hypothesis that SCF/c-kit signaling pathway would affect either the proliferation of PGCs as they migrate towards the gonadal ridge or the survival of germ cells shortly after their settlement in gonads (Donovan, 1994). The evidence obtained from culture experiments with mouse PGCs indicate that the SCF/c-kit system affects primarily survival, but not proliferation (Dolci *et al.*, 1991; Godin *et al.*, 1991). However, there is also evidence indicating that SCF can act as a mitogen in the presence of other factors such as LIF (Matsui *et al.*, 1991).

In chicken and quail, the cDNA for SCF has been cloned and like the mammalian counterparts, it encodes for a transmembrane protein that exists in a membrane-anchored form and a soluble form (Zhou *et al.*, 1993; Petite and Kulik, 1996). The percent amino acid homology between quail SCF and chicken SCF is 98 % (Petite and Kulik, 1996), while that between the avian and mammalian peptides is about 52-53 % (Zhou *et al.*, 1993; Petite and Kulik, 1996).

The product of the c-kit protooncogene is the only known receptor for SCF. The cDNA for chicken c-kit encodes a protein with a 505 amino acid extracellular domain, a 25 amino acid membrane-spanning segment, and 430 amino acid cytoplasmic domain (Sasaki *et al.*, 1993). The overall amino acid homology between the chicken and mammalian peptides is 63 % (Sasaki *et al.*, 1993). Nevertheless, the amino acid sequence identity of the kinase domains between the chicken and mouse c-kit is very high, viz. 94%.

In birds, c-kit (Sasaki *et al.*, 1993; Lecoin *et al.*, 1995) and SCF (Zhou *et al.*, 1993; Lecoin *et al.*, 1995, Petite and Kulik, 1996) are expressed in the brain, bursa of Fabricius, heart, kidney, spleen, thymus, ovary and testis. These patterns of expression of SCF and c-kit in the avian embryo are very similar to those observed in mouse embryos (Ray *et al.*, 1992). The fact that SCF and c-kit are expressed in similar cell types in avian and mouse embryos, along with the overall structural similarities between chicken/quail and mouse SCF/c-kit, would suggest that the biological roles played by the SCF/c-kit ligand-receptor complex might be evolutionary conserved between birds and mammals. However, the knowledge of the function of SCF in avian embryogenesis comes only from studies carried out with erythroid progenitors (Hayman *et al.*, 1993), neural crest cells (Lahav *et al.*, 1994; Lecoin *et al.*, 1995) and neural crest-derived sensory neurons (Carnahan *et al.*, 1994). The possibility that SCF is also involved in the development of the avian germ cell lineage has not been entertained.

In the chicken embryo, two types of erythroid progenitors reside in the bone marrow: a relatively frequent progenitor, expressing only c-kit, referred to as SCF progenitors, and a rare progenitor, co-expressing c-kit and cErbB, referred to as SCF/TGF α progenitors (Hayman *et al.*, 1993). Hayman *et al.* (1993) demonstrated that SCF progenitors undergo a limited number of self-renewal divisions in the presence of avian SCF. Likewise, when neural crest cells are cultured in the presence of SCF, there is a modest increase in the proportion of

BrdU-labeled cells (Lahav *et al.*, 1994). According to Lavah *et al.* (1994), SCF also mediates the differentiation of neural crest cells along the melanocyte differentiative pathway and leads to an increase in the number of melanocytes in the culture. On the other hand, SCF appears to act simply as a survival factor for sensory neurons, which are also derived from neural crest cells (Carnahan *et al.*, 1994).

Besides SCF, basic fibroblast growth factor (bFGF, FGF-2) is another peptide growth factor that can have profound effects on the proliferation of mouse PGCs cultured on various feeder-layers (Matsui *et al.*, 1992; Resnick *et al.*, 1992). In the presence of LIF and SCF, bFGF stimulates the long-term proliferation of PGCs, leading to the establishment of embryonal germ (EG) cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992). Nevertheless, it is currently unknown if bFGF has any physiological role in the establishment of the germ line in mouse. However, recent evidence indicates that mouse PGCs express receptors for bFGF and that bFGF can act directly on PGCs, independent of feeder cells (P. Donovan, personal communication). Therefore, it is most likely that bFGF has a role to play in regulating PGC proliferation in the intact mammalian embryo.

bFGF is a member of FGF family that currently consist of at least eleven separate gene products in mammals (Coulier *et al.*, 1997). In the chicken, three isoforms of bFGF are synthesized by alternative translation initiation from one coding region (Dono and Zeller, 1994). All isoforms of bFGF are expressed at

pre-primitive streak stages of development (Riese *et al.*, 1995). Interestingly, bFGF is localized primarily to the nucleus of embryonic cells at these early stages. As the primitive streak forms at the posterior side of the blastoderm, bFGF is translocated to the cytoplasm in patches of epiblast cells, while it remains localized to the nucleus of the hypoblast cells. The developmental significance of this dynamic distribution of bFGF is unknown, particularly in the light of the fact that it lacks a hydrophobic signal sequence.

The physiological functions of bFGF at early stages of avian embryo development remain to be examined. Nevertheless, Mitrani *et al.* (1990b) could demonstrate that if heparin and suramin, substances that interfere with the function of FGFs, are applied to early chicken embryos, the formation of mesodermal structures are inhibited (Mitrani *et al.*, 1990b). There are two lines of additional evidence indicating that FGF is biologically active at these early stages of development. First, treatment of epiblast cells obtained from stage X blastoderms in culture with FGF-4 leads to the upregulation of *brachury* (Knezevic *et al.*, 1997). Second, under certain culture conditions multipotent or pluripotent embryonic cells obtained from stage X blastoderms can be induced by bFGF to form blood cells in culture (Gordon-Thomson and Fabian, 1994). At later stages of development, bFGF has been implicated in various developmental events including controlling outgrowth of the chick limb bud (Fallon *et al.*, 1994) and growth of facial mesenchyme in culture (Richman and Crosby, 1990).

The biological effects of FGFs are mediated by the activation of specific cell surface receptors belonging to the tyrosine kinase receptor family. To date, four high affinity receptors for FGF have been identified in the chicken. These are named according to the order of their identification, i.e. FGFR1-4 (Jaye *et al.*, 1992). Chicken homologues of the mammalian FGFR1 (Pasquale and Singer, 1989), FGFR2 and FGFR3 (Pasquale, 1990), and a distantly related homologue of mammalian FGFR4, referred to as FREK (Marcelle *et al.*, 1994) have been cloned. The overall homologies between the chicken and human FGFRs 1, 2, and 3 are 92, 93, and 81 %, respectively (Marcelle *et al.*, 1994). Among the four FGF receptors, only the expression pattern of FREK has been characterized at early stages of avian embryo development. Accordingly, FREK is expressed by the epiblast cells whose progeny will form the future head and extraembryonic ectoderm (Marcelle *et al.*, 1994).

Based on the role that SCF and bFGF play in the development of mouse PGCs and the observation that bFGF is expressed in the chick embryo at pre-streak stages of development, we entertained the possibility that the same factors might play a role in the in the ontogeny of the germ cell lineage in the avian embryo. Therefore, the purpose of the present study was to examine 1) the expression of SCF and c-kit during avian germ cell development, and 2) the role of SCF and bFGF in the emergence of avian PGCs in blastodermal cell cultures.

MATERIALS AND METHODS

Reverse transcriptase polymerase chain reaction (RT-PCR)

Embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded, the *area pellucidae* of stages IX-X, and XI-XIII were pooled and embryos were lysed in Trizol™ for the extraction of RNA. To detect SCF and c-kit transcripts in feeder-dependent cultures, blastodermal cells obtained from freshly-laid, unincubated eggs were seeded onto feeder layers of mitotically arrested STO cells in 96 - well microtiter plates. The cells were cultured for 48 hours at 37 °C / 5 % CO₂, and lysed in Trizol™ reagent. Total RNA was extracted with Trizol™, as recommended by the manufacturer (Life Technologies). First strand cDNAs were synthesized using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Novagen) and oligo(dT) primers. The PCR was performed with an Idaho-thermal cycler (Model 1605) operating on a regimen of 96 °C for 1 min, 94 °C for 1s, 52 °C for 1s, and 72 °C for 30s for 35 cycles followed by 72 °C for 5 min. The primers for SCF, which flanked the extracellular proteolytic cleavage region absent in the shorter membrane-anchored form, were as follows: sense, 5'-ATGTCCCGAAGATGGATAGCC-3'; anti-sense, 5'-GCTGGAGCTGCTAATGAAGCC-3'. The PCR primers used in detecting chicken c-kit were: sense, 5'-

TCTCACTCATGGTCTGAATAAC-3'; anti-sense, 5'- TAGAAGTCCTCCCTCTCTC
AC-3'.

PCR products were electrophoresed through 1 % agarose gel containing ethidium bromide in 1X TBE buffer and subsequently visualized under UV illumination. The presence and identity of positive signals for SCF were confirmed by sequential Southern hybridization using a 27 bp oligonucleotide probe specific for each isoform of SCF. The probe specific to the longer or soluble isoform of SCF was 5'-AACACCAGAGAATGAAGAGGCACTTGG-3' and the probe specific for the membrane-anchored form of the SCF was 5'-TGACAGCATTGGCAGTAATACTAGCAG-3'. The probe that was used to verify the identity of the amplification product for c-kit was 5'-CTACAGATCATTCGGTGAGGATTAACCTCAG-3', complementary to a region within the expected amplification product. Southern blotting of PCR products onto Hybond N⁺ membrane was performed according to Sambrook *et al.* (1989). The hybridization, 3' end-labeling of oligonucleotides using digoxigenin-11-ddUTP, and the detection of the hybridized probe with anti-digoxigenin-AP fragments using CDP StarTM as a chemiluminescent substrate were carried out according to the manufacturer's instructions (Boehringer Mannheim).

In situ hybridization probes

A BamH I - Xba I cDNA fragment of 923 bp, which corresponds to nt 1-923 of the quail SCF sequence published by Petite and Kulik (1996) was subcloned

into pBS II (KS⁺) vector (Stratagene, U.S.A) to obtain pBSSCF. For SCF anti-sense and sense probes, pBSSCF was linearized with BamH I and Xba I, respectively.

To obtain a c-kit clone for use as a probe, total RNA was isolated from chicken spleen as described (Chomezynski, 1993). PCR primers were selected to amplify a fragment of c-kit cDNA corresponding to the nt 2353-2961 of the chicken c-kit sequence published by Sasaki *et al.* (1993). The forward primer was 5'-TCTCACTCATGGTCGAATAAC-3' and the reverse primer was 5'-TAGAAGTCCTCCCTCTCTCAC-3'. First strand cDNA was synthesized from 5 µg RNA using oligo-dT priming and Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Novagen). The "touchdown" PCR reaction was performed with 3 µl of the RT product and Taq DNA polymerase using an Idaho-thermal cycler (Model 1605) operating on a regimen of 96 °C for 5 min, 94 °C for 1 min, 75 °C for 1.5 min for 20 cycles with a 0.5 °C decrease every cycle and 72 °C for 1.5 min. This was followed by 15 cycles of 94 °C for 1 min, 55 °C for 1.5 min and a 72 °C for 1.5 min. A final extension of 7 min at 72 °C was used after cycling, followed by cooling to 4 °C prior to electrophoresis. After the RT-PCR, amplification products were cloned into the pT7Blue(R) vector using TA cloning according to the manufacturer's instructions (Novogen, WI). The identity of a clone containing the amplification product was verified by sequencing both strands using dideoxy sequencing and designated pT7cKit. pT7cKit was digested with BamH I and Xba I and a 608 bp c-Kit cDNA fragment was subcloned into pBS II (KS⁺) vector (Stratagene, U.S.A) to obtain pBSckIT. For c-kit anti-sense and sense probes, pBSckIT was linearized with BamH I and Xba

I, respectively. The plasmid pChT containing a 600 bp cDNA insert of chicken *brachyury* was a gift of Dr. Susan Mackem. For *brachyury* anti-sense and sense probes pChT was linearized with Xba I and Xho I, respectively. All anti-sense and sense probes were prepared by in vitro transcription (Boehringer Mannheim transcription kit) using T3 or T7 RNA polymerases with digoxigenin-11-dUTP.

In situ hybridization of thick sections

Thick (50 μ m) wax sections were prepared as described by Nieto *et al.* (1996). Paraffin sections were dewaxed with tetrahydronaphtaline three times (5 min each), washed in 100 % methanol three times (5 min each). Sections were rehydrated by 5 min washes in 75 %, 50 %, and 25 % methanol in PBS followed by three washes (5 min each) in PBS containing 0.1 % Tween-20 (PBT). Sections were treated with 10 μ g/mL proteinase K in PBT for 10 min at room temperature. The proteinase K solution was removed immediately, sections were re-fixed in freshly-prepared 4 % paraformaldehyde for 20 min and washed in PBT four times (5 min each), and incubated in zHYbe (Appendix A) hybridization buffer at 63 °C for 2-3 hours as described by Jowet (1997). The prehybridization solution was replaced with the zHybe buffer containing digoxigenin-labeled RNA probes at a concentration of 1 μ g/mL. The hybridization was carried out at 63 °C overnight. Sections were washed for 10 min at 63 °C in Solution 1 (50 % formamide, 5XSSC); in solution 1 diluted with

2XSSC at a ratio of (3:1), (1:1), (1:3), and then in 2XSSC. Sections were washed further two times (30 min each) at 63 °C in 0.2XSSC, 0.01 % Tween-20, then at room temperature for 10 min in 0.2XSSC diluted with PBT at a ratio of (3:1), (1:1), (1:3) finishing with a 10 min wash in PBT.

For immunolocalization of hybridized probes, sections were incubated at room temperature in a blocking solution (2 mg/mL BSA, 5 % sheep serum, 1 % DMSO in PBT) for 60 min and then in the blocking solution with 1:5000 diluted AP-conjugated antidigoxigenin antibody at 4 °C overnight. Sections were then washed six times (20 min each) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % TritonX-100, then equilibrated in NTMT buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1 % Tween20, 5mM levamisole) three times (5 min each), and stained in NTMT containing 4.5 µl/mL NBT (75 mg/mL in 70 % dimethylformamide) and 3.5 µl/mL BCIP (50 mg/mL in dimethylformamide) overnight at room temperature.

In situ hybridization of whole mount embryos

Whole mount embryos from stages X to 10 were fixed in 4 % paraformaldehyde overnight, rinsed in PBT, transferred to methanol and stored at -20 °C. Prior to the hybridization, embryos were rehydrated in a series of methanol:PBT (3:1, 1:1, 1:3 volume: volume), finishing with three volumes of PBT. Embryos were treated with proteinase K (20 µg/mL) for 1.5-20 min at room

temperature, rinsed with PBT for 5 min, re-fixed with 4 % paraformaldehyde, 0.2 % glutaraldehyde in PBS for 20 min at room temperature and washed twice (5 min each) in PBT. The procedures applied for in situ hybridization were as described by J wet (1997). Briefly, embryos were equilibrated in sHybe hybridization buffer (Appendix A) at room temperature for 5 min, incubated in sHybe at 63-65  C for 2-12 hr, and incubated in sHybe containing RNA probes at a concentration of 1  g/mL overnight at 63-65  C . Embryos were rinsed twice in pre-warmed sHybe, and washed in sHybe two times (30 min each) at 63-65  C, then in sHybe:maleic acid buffer (1:1) for 20 min, rinsed three times in MABT (100 mM maleic acid, 150 mM NaCl, 0.1 % Tween-20, pH 7.5) at room temperature, and washed two times (30 min each) in MABT. Embryos were washed further in MABTB (MABT containing 2 % blocking powder, Boehringer Mannheim) for 1 hr at room temperature, and incubated in MABTB containing 20 % goat serum for 2 hr. The solution was replaced with fresh MABTB which contains 20 % goat serum supplemented with a 1/2000 dilution of AP-conjugated anti-digoxigenin Fab fragments and embryos were incubated overnight at 4  C. In order to detect the hybridized probe, embryos were rinsed three times in MABT, washed three times (60 min each) in MABT, three times (10 min each) in NTMT buffer (100mM Tris-HCl pH 9.5, 100mM NaCl, 50 mM MgCl₂, 0.1 % Tween20, 5mM levamisole) and stained in NTMT containing 4.5  l /mL NBT (75 mg/mL in 70 % dimethylformamide) and 3.5  l/mL BCIP (50 mg/mL in dimethylformamide) for 6-24 hr at room temperature. Embryos were viewed

under a dissection microscope and fixed in 4 % paraformaldehyde in PBS overnight at 4°C.

In situ hybridization of cell cultures

Blastodermal cells were cultured for 48 hours at 37 °C / 5 % CO₂. At the end of the culture period, the cultures were fixed in 4 % paraformaldehyde (pH 7.2), washed two times (5 min each) in PBS containing 0.1 % Tween-20 (PBT). Cells were treated with proteinase K (10 µg/mL) for 5 min at room temperature, rinsed with PBT for 5 min, re-fixed with 4 % paraformaldehyde, 0.2 % glutaraldehyde in PBS for 20 min at room temperature and washed twice (5 min each) in PBT. The procedures applied for in situ hybridization and for immunolocalization of hybridized probes were as described for thick tissue sections.

Immunohistochemical staining of thick sections using anti - c-kit

Thick (50 µm) wax sections were prepared as described by Nieto *et al.* (1996). Paraffin sections were dewaxed with tetrahydronaphtaline three times (5 min each), washed in 100 % methanol three times (5 min each). Sections were rehydrated by 5 min washes in 75 %, 50 %, and 25 % methanol in PBS followed by three washes (5 min each) in PBS (pH 7.4), containing 1 % TritonX-100, 0.1 % Na-azide (PBST). The staining procedure was carried out using an

avidin/biotin-conjugated-alkaline phosphatase system (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA) using anti - c-kit supernatant (a gift of Dr. M. Hayman) diluted 1:100 as the primary antibody. Cells expressing the antigen were detected using 0.168 mg/mL nitroblue tetrazolium, 0.088 mg/mL 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt and 3.75 mM levamisole in 100 mM Tris-HCl, pH 9.5. Sections were washed in PBST three times (5 min each), fixed in 4 % paraformaldehyde in PBS for 20 min, and equilibrated in 70 % glycerol in PBST. For photographic visualization of the pattern of staining, sections were placed on a slide in a small amount of 70 % glycerol and flattened under a cover-slip.

Immunohistochemical staining of thick sections using anti - SSEA-1

Thick (50 μ m) wax sections were prepared as described by Nieto *et al.* (1996). Paraffin sections were dewaxed with tetrahydronaphtaline three times (5 min each), washed in 100 % methanol three times (5 min each). Sections were rehydrated by 5 min washes in 75 %, 50 %, and 25 % methanol in PBS followed by three washes (5 min each) in PBS (pH 7.4), containing 1 % TritonX-100, 0.1 % Na-azide (PBST). The staining procedure was carried out using an avidin/biotin-conjugated-alkaline phosphatase system (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA) using anti-SSEA-1 (MC-480) ascites fluid diluted 1:1000 as the primary antibody. Cells expressing the antigen were

detected using 0.168 mg/mL nitroblue tetrazolium, 0.088 mg/mL 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt and 3.75 mM levamisole in 100 mM Tris-HCl, pH 9.5. Sections were washed in PBST three times (5 min each), fixed in 4 % paraformaldehyde in PBS for 20 min, and equilibrated in 70 % glycerol in PBST. For photomicroscopy of the pattern of staining, sections were placed on a slide in a small amount of 70 % glycerol and flattened under a cover-slip.

Periodic acid Schiff staining of whole embryos

Stage 22 (Hamburger and Hamilton, 1951) embryos were isolated in PBS and extraembryonic membranes were removed. Embryos were fixed in situ in Rossman's fluid (90 volumes of 100 % alcohol saturated with picric acid and 10 volumes of formalin) for 24-48 hours and were rehydrated by 15-20 min washes in 75 %, 50 %, and 25 % ethyl alcohol in distilled water followed by three washes (5 min each) in water. Embryos were treated with periodic acid in situ for 20 min at room temperature, rinsed in distilled water two times (5 min each) and incubated in Schiff's reagent for 30 min. The staining reaction was ended by washing embryos several times in distilled water. Stained embryos were dehydrated, embedded in wax, and sectioned according to routine histological procedures. The material was sectioned at 15 μ m.

In vitro culture of dispersed embryos with and without feeder-layers

Feeder layers of STO and CV-1 cells were prepared in 96-well-microtiter plates as described by Cooke *et al.* (1993). The *area pellucidae* from stage X-XIII 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 tituration using a 200 µl pipette tip. The cells were centrifuged at 311g for 5 min and resuspended in fresh culture medium. The concentration of cells and the number of blastodermal cells obtained per embryo were determined using a hemocytometer; 5000 cells/well were seeded onto feeder layers of mitotically arrested STO and CV-1 cells in 96-well microtiter plates. In parallel experiments, blastodermal cells obtained from the same pool of embryos were cultured on gelatin-coated 96-well microtiter plates. STO cell-conditioned medium (STO-CM) was prepared by growing STO cells to confluency for 65 hr, the medium was harvested and was used after filtering through a 0.2 µm filter. Blastodermal cells were cultured for 48 hours at 37 °C / 5 % CO₂. At the end of the culture period, the cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression.

In vitro culture of hypoblast PGCs with feeder-layers

Hypoblasts peeled from nineteen blastoderms at stages XIII-XIV 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 hypoblast tissue was dispersed further to obtain a single cell suspension by gentle tituration using a 200 µl pipette tip. The cells were centrifuged at 311g for 5 min and resuspended in fresh culture medium. The concentration of cells and the number of cells obtained per hypoblast explant were determined using a hemocytometer; 3500 cells/well were seeded onto feeder layers of mitotically arrested STO and CV-1 cells in 96-well microtiter plates. Hypoblast cells were cultured for 24 hours at 37 °C / 5 % CO₂. At the end of the culture period, the cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression.

The effect of SCF and bFGF on the development of PGCs in culture

The isolation and culture of blastodermal cells in DMEM/FBS on feeder-layers of STO and CV-1 cells were performed as described above. Bovine bFGF (10-100 ng/mL) was obtained from R & D Systems (Minneapolis, MN). bFGF was reconstituted in sterile phosphate-buffered saline (pH 7.4) containing 0.5 % bovine serum albumen, and was stored at -70 °C in small aliquots to avoid repeated freeze-thaw cycles. Anti-FGF basic neutralizing antibody (10µg/mL)

was obtained from R & D Systems (Minneapolis, MN) and was reconstituted in sterile phosphate-buffered saline (pH 7.4) and stored at -70°C in small aliquots. Chicken recombinant soluble SCF (100 ng/mL) was expressed in *Escherichia coli* and purified as described by Bartunek *et al.* (1996). All factors were diluted in DMEM/FBS immediately before use and added to cultures subsequent to plating blastodermal cells onto feeder layers. Blastodermal cells were cultured for 48 hours at 37°C / 5 % CO_2 . At the end of the culture period, the cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression.

Statistical analysis

The data were analyzed for significance at the 5 % level. The data collected from *in vitro* culture of dispersed embryos were subjected to ANOVA utilizing the General Linear Model (GLM) procedure of SAS (SAS Institute, 1990). The model for the effect of bFGF on the number of PGCs in cultures initiated with blastodermal cells obtained from a pool of stage X-XIII embryos was: $\text{PGC} = \text{date stage}$. For this data set, variation among different dates and treatments was used as the error term. The model for the remaining experiments was: $\text{PGC} = \text{date stage date*stage}$. The variation originating from the interaction between date and stage was used as the error term. The treatment means were compared using LSMEANS procedure of SAS software (SAS Institute, 1990).

The data from in vitro culture of hypoblast PGCs on feeder-layers were subjected to TTEST procedure of SAS (SAS Institute, 1990).

RESULTS

Detection of SCF and c-kit transcripts at pre-streak stages of development

As a first step in examining the role of SCF and its tyrosine kinase receptor c-kit in the development of avian PGCs, RT-PCR amplification with primers specific for chicken SCF and c-kit was performed on RNA isolated from pooled stage IX-X and XI-XIII embryos. RNA from adult heart tissue was used as a positive control. Electrophoresis of the PCR products was performed after 35 cycles of amplification. The presence and identity of the positive signals arising from these tissues were confirmed following Southern hybridization with oligonucleotide probes specific for chicken SCF and c-kit. Amplification products diagnostic of SCF (Fig. 2. 1) and c-kit (Fig. 2. 2) could be detected in lanes corresponding to pooled stage IX-X and stage XI-XIII whole embryos and to blastodermal cells cultured on feeder-layers of STO cells.

Expression of SCF and c-kit in the developing embryo

The results described above indicated that transcripts of SCF and c-kit are present at pre-streak stages of development. To examine the expression pattern in the embryo, in situ RNA hybridization analyses were performed on tissue sections and whole mount embryos. In order to optimize conditions for in situ hybridization of whole embryos, anti-sense probes specific to the transcription factor *brachyury* were used as a positive control, since the expression pattern of this gene is well characterized in the early chicken embryo (Kispert *et al.*, 1995; Knezevic *et al.*, 1997). mRNA transcripts of *brachyury* could readily be detected in whole mount chick embryos with a pattern of expression identical to that described by Kispert *et al.* (1995) and Knezevic *et al.* (1997) (Fig. 2. 3).

In situ RNA hybridization performed on 50 μ m-thick sections taken from gonads at day 8 of incubation revealed that SCF is expressed by the gonadal epithelium (Fig. 2. 4A), while c-kit is expressed only by germ cells (Fig. 2. 4B). Fig. 2. 5A shows a 50 μ m-thick section taken from the gonadal ridge of a stage 22 (Hamburger and Hamilton, 1951) embryo, hybridized with anti-sense RNA probes specific for avian SCF. SCF expression is seen at its highest level within the gonadal ridge (Fig. 2. 5A, B), the final destination of migratory germ cells. Transcripts of SCF are also detected in lateral portions of the allantois, and less prominently in the notochord (Fig. 2. 5A, B). At the same developmental stage, c-kit expression is seen in a few cells along the dorsal mesentery and within the gonadal ridge (Fig. 2. 5C, D). c-kit expression was also detected in the dermatome where neural crest cells reside (Fig. 2. 5C, D) and in the limb apical

ectodermal ridge (data not shown). The availability of a monoclonal antibody recognizing avian c-kit allowed us to verify the expression pattern of c-kit at the translational level. The antibody staining revealed a more prominent pattern of expression for the protein (Fig. 2. 5D). The morphology and spatial distribution of immunoreactive cells located along the dorsal mesentery and gonadal ridge were similar to PAS-stained and SSEA-1-labeled PGCs within these regions (Fig. 2. 6A-C).

In vitro culture of dispersed embryos with and without feeder-layers

Previous studies suggested that the pattern of the emergence of PGCs on feeder layers of STO cells is similar, if not identical, to the pattern of the emergence of PGCs during normal development (Karagenc *et al.*, 1996). To test this hypothesis further and to see if the emergence of PGCs in blastodermal cell cultures carried out on feeder-layers of STO cells is a specific property of these cells, blastodermal cells obtained from dispersed stage X embryos were cultured on feeder-layers of CV-1 cells. It was previously demonstrated that CV-1 cells do not support the development of mouse PGCs (Dolci *et al.*, 1991). In parallel experiments, blastodermal cells obtained from the same pool of embryos were cultured on feeder-layers of STO cells and gelatin-coated tissue-culture plastic. PGCs were identified after 48 hr of culture, using the monoclonal antibody MC-480, recognizing SSEA-1. The pooled results obtained from three independent

experiments are illustrated in Fig. 2. 7. The number of PGCs obtained on feeder-layers of CV-1 cells was significantly less than that obtained on STO feeder-layers (Fig. 2. 7). In contrast, the number of PGCs obtained on CV-1 feeder-layers did not differ significantly than that obtained on gelatin-coated plastic ($p= 0.8$). Furthermore, the addition of STO cell-conditioned medium (STO-CM) to blastodermal cells cultured either on feeder-layers of CV-1 cells or on gelatin-coated plastic resulted in a significant increase in the number of PGCs after 48 hr of culture (Fig. 2. 7). The response of blastodermal cells to STO-CM did not differ significantly between cultures carried out on CV-1 feeder-layers and gelatin-coated plastic ($p= 0.2$).

The response of blastodermal cells was qualitatively different in cultures carried out on the two feeder-layers. A typical response of blastodermal cells cultured on STO cells was to form patches of SSEA-1 positive and SSEA-1 negative cells after 48 hr of culture. In contrast, blastodermal cells cultured on feeder-layers of CV-1 cells did not give rise to SSEA-1 positive patches, and formed isolated, small clumps consisting of SSEA-1 negative embryonic cells.

When dispersed hypoblast explants were cultured on feeder-layers of STO and CV-1 cells, the number of PGCs obtained on feeder-layers of CV-1 cells was significantly less than that obtained on STO feeder-layers (Fig. 2. 8).

The effect of SCF on the development of PGCs in culture

The evidence indicating that mRNA transcripts for both SCF and c-kit are present at pre-streak stages of development and that the patterns of expression seen for these genes are complementary during the active migratory phase of PGCs further suggested that SCF/c-kit system might be involved in avian germ cell development. This hypothesis was tested by culturing blastodermal cells obtained from dispersed stage X-XIII embryos on feeder-layers of CV-1 cells in the presence and absence of recombinant avian SCF. In parallel experiments, blastodermal cells obtained from the same pool of embryos were cultured on feeder-layers of STO cells. PGCs were identified after 48 hr of culture, using the monoclonal antibody MC-480. This experiment was performed six times. The pooled results obtained from six independent experiments are illustrated in Fig. 2. 9. In the presence of avian SCF (100 ng/mL), there was a significant increase in the number of PGCs in cultures using on feeder-layers of CV-1 cells compared to that observed with control cultures ($p=0.0001$).

The effect of bFGF on the development of PGCs in culture

Since under certain culture conditions bFGF can act as a mitogen on mouse PGCs (Resnick *et al.*, 1992; Matsui *et al.*, 1992) and that it is expressed in the chick embryo at pre-streak stages of development, we wanted to test if supplementing blastodermal cell cultures initiated on feeder-layers of STO and CV-1 cells with bFGF would have any effect on the emergence of PGCs. To this

end, bovine bFGF, which was demonstrated to be biologically active in the chicken embryo (Cooke and Wong, 1991; Cooke, 1993; Gordon-Thomson and Fabian, 1994), was added to blastodermal cells cultured on feeder layers of STO and CV-1 cells. In the first series of experiments, blastodermal cells obtained from pooled stage X-XIII embryos were used. The results obtained from four independent experiments are summarized in Fig. 2. 10.

In the presence of bFGF, a significant decrease in the number of PGCs was observed with STO feeder-layers after 48 hr of culture (Fig. 2. 10). Supplementation of cultures with bFGF (100 ng/mL) did not lead to an increase in the number of PGCs in cultures using feeder-layers of CV-1 cells. The addition of a neutralizing antibody to bFGF (10 μ g/mL) to cultures carried out on STO feeder-layers resulted in PGC numbers not significantly different from numbers that were obtained with the control medium ($p=0.89$, Fig. 2. 10).

In another series of experiments, blastodermal cells obtained from only stage X embryos were cultured on STO feeder-layers in the presence of bFGF at 10 and 100 ng/mL. The results obtained from three independent experiments are presented in Fig. 2. 11. Accordingly, the number of PGCs obtained after 48 hr of culture was found to be significantly less in the presence of bFGF at both 10 and 100 ng/mL with respect to control cultures. The effect of bFGF in terms of the number of PGCs obtained did not show a dose-response relationship ($p=0.6$).

The addition of bFGF to blastodermal cells cultured on feeder-layers of STO cells almost completely abolished the expression of SSEA-1 in the culture (Fig.

2. 12A). Instead, blastodermal cells formed patches consisting mainly of SSEA-1 negative embryonic cells (Fig. 2. 12B). PGCs were most often associated with these SSEA-1 negative patches of embryo cells (Fig. 2. 12B).

Since bFGF is well-known for its mesoderm inducing ability in *Xenopus* animal caps (Smith *et al.*, 1991), it was reasoned that bFGF could have a similar effect on avian blastodermal cells, leading to a decrease in the number of PGCs in association with the down-regulation of the SSEA-1 epitope. This hypothesis was tested by analyzing the expression of the transcription factor *brachyury* in cultures carried out on STO feeder-layers in the presence (100 ng/mL) and in the absence of bFGF. The *in situ* hybridization analyses revealed that *brachyury*, which is expressed by mesodermal cells ingressing through the primitive streak (Fig. 2. 3), is upregulated in patches of blastodermal cells cultured on STO feeder-layers with bFGF (Fig. 2. 13A). In contrast, the expression of *brachyury* could not be detected in cultures which were not supplemented with bFGF (Fig. 2. 13B).

DISCUSSION

In all vertebrates, the germ line stems from a small population of cells that are set aside at early stages of embryonic development. Yet, the mode of the segregation of the germ cell lineage appears to differ among various vertebrate groups. In anurans, for example, the differentiation of PGCs is a cell-

autonomous process that takes place at the gastrula stage of development (Dixon, 1981). On the other hand, in urodele amphibians, PGCs derive from yet unspecified marginal zone cells as part of the regional induction of the ventral mesoderm by signals emanating from the underlying endodermal cells (Nieuwkoop and Sutasurya, 1979). Similarly, cell-lineage analyses indicate that the earliest precursors of mouse PGCs are either multipotent or pluripotent epiblast cells whose progeny will not only give rise to PGCs, but also to extraembryonic mesodermal cells (Lawson and Hage, 1994). Furthermore, even distal epiblast cells of the mouse embryo that normally form neuroectodermal structures have the potential to give rise to PGCs when transplanted into the proximal epiblast, i.e. where the earliest precursors of PGCs normally reside (Tam and Zhou, 1996).

In the chicken embryo, the experimental evidence available does not support the hypothesis that the segregation of PGCs is a cell-autonomous process. For example, when blastodermal cells harvested from dispersed embryos are cultured without feeder cells, the number of PGCs detected is lower than expected after 48 hr of culture (Karagenc *et al.*, 1996). In the present study, similar results were obtained when blastodermal cells were cultured either on gelatin-coated tissue-culture plastic or on feeder-layers of CV-1 cells (Fig. 2. 7). However, when blastodermal cells obtained from the same pool of embryos were cultured on feeder layers of STO cells, a marked increase in the number of PGCs was observed (Fig. 2. 7). Furthermore, the addition of STO cell-

conditioned medium (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 PGCs (Fig. 2. 7). Therefore, STO cells provide a soluble factor or factors that enhance the development of chicken PGCs in culture. In principle, these unidentified factors could support either the survival and/or proliferation of PGCs 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. Nevertheless, these observations reinforce the view that the culture of blastodermal cells on STO cells provides a model system to examine the factor(s) associated with the development and proliferation of avian PGCs.

In any case, it is important to note that the response of blastodermal cells to STO-CM did not differ significantly between cultures carried out on CV-1 feeder-layers and without feeder cells (Fig. 2. 7). CV-1 cells do not support the survival of mouse PGCs (Dolci *et al.*, 1991) and some reports indicate that the inhibitory effect of CV-1 cells on the development of mouse PGCs relates directly to a failure in the activation of the SCF/c-kit signaling pathway (Dolci *et al.*, 1991; Pesce *et al.*, 1993). This hypothesis is supported further by the observation that the number of mouse PGCs detected on feeder-layers of CV-1 cells represent less than 1 % of PGCs detected on STO feeder-layers after 24 hours of culture (Dolci *et al.*, 1991).

Three observations indicate that CV-1 cells do not exert a direct inhibitory effect on chicken blastodermal cells under the present culture conditions. First, chicken PGCs detected on feeder-layers of CV-1 cells do not show morphological features characteristic of apoptosis (data not shown). Second, the addition of STO-CM to blastodermal cells cultured on CV-1 feeder-layers results in a significant increase in the number of PGCs (Fig. 2. 7). Third, when hypoblast cells along with SSEA-1 positive cells are cultured on feeder-layers of STO and CV-1 cells, the number of PGCs detected on CV-1 feeder-layers represent about 60 % of the PGCs detected on STO feeder-layers after 24 hours of culture (Fig. 2. 8). Therefore, it is unlikely that the failure of CV-1 cells to support the development of PGCs in blastodermal cell cultures is due to the selective death of PGCs.

In the mouse, the balance of evidence strongly suggests that SCF plays a key role in the survival and/or proliferation of PGCs (Donovan, 1994; Buehr, 1997). In the present study, three observations support the hypothesis that SCF/c-kit ligand-receptor complex is also involved in the development of avian PGCs. First, specific transcripts for both isoforms of SCF and c-kit are present in whole embryos at pre-streak stages of development and in blastodermal cell cultures performed on feeder-layers of STO cells (Fig. 2. 1, Fig. 2. 2). Second, the addition of recombinant avian SCF to blastodermal cells cultured on feeder-layers of CV-1 cells results in a significant increase in the number of PGCs after 48 hr of culture (Fig. 2. 9). Third, *in situ* hybridization analyses performed on

tissue sections reveal that SCF is expressed specifically by the gonadal epithelium (Fig. 2. 4A), while c-kit is expressed primarily by germ cells (Fig. 2. 4B) in the gonad at 8 day of incubation. As PGCs migrate actively towards the gonadal anlage, transcripts of SCF are detected most abundantly within the gonadal ridge (Fig. 2. 5A, B). At the same developmental stage, c-kit is expressed mainly by neural crest cells in the dermatome, in a few cells along the dorsal mesentery and within the gonadal ridge, where the expression of SCF is found to be at its highest level (Fig. 2. 5C, D). It is most likely that c-kit expressing cells along the dorsal mesentery and the gonadal ridge are germ cells because of their morphology and spatial distribution with respect to PAS-stained and SSEA-1-labelled PGCs within these regions (Fig. 2. 6).

Transcripts for both SCF and c-kit could not be detected through in situ hybridization at stages earlier than stage 22. However, mRNA transcripts of *brachyury* could readily be detected using the same hybridization conditions (Fig. 2. 3). Thus, it is unlikely that the failure to detect in situ transcripts of SCF and c-kit can be due to technical problems. In addition, several attempts to localize c-kit expressing cells at these early stages using a monoclonal antibody recognizing avian c-kit were unsuccessful. Taken together, it is most likely that SCF/c-kit expression is below the sensitivity of these procedures.

In the current study, the possibility that bFGF, along with SCF, might play a role in avian germ cell development was considered. Several lines of evidence supported this hypothesis. First, bFGF can act directly on mouse PGCs in

culture as a potent mitogen (Matsui *et al.*, 1992; Resnick *et al.*, 1992, P. Donovan, personal communication). Second, bFGF is expressed in the chicken embryo within the time frame that correlates with the segregation of PGCs (Riese *et al.*, 1995). Third, under certain culture conditions multipotent or pluripotent embryonic cells obtained from unincubated blastoderm can be induced by bFGF to form blood cells (Gordon-Thomson and Fabian, 1994). However, the addition of bFGF (10 and 100 ng/mL) to blastodermal cells cultured on feeder layers of STO or CV-1 cells does not increase the number of PGCs obtained after 48 hr of culture compared to that observed in control cultures (Fig. 2. 10, Fig. 2. 11). On the contrary, supplementation of cultures with bFGF leads to a 25-47 % decrease in PGC numbers (Fig. 2. 10, Fig. 2. 11). The addition of a neutralizing antibody to bFGF (10 µg/mL) to cultures carried out on feeder-layers of STO cells results in PGC numbers not significantly different from numbers that were obtained in control cultures (Fig. 2. 10). This observation would suggest that if any bFGF is present in the culture system, it is not sufficient to alter PGC numbers or that bFGF is not involved in the segregation of avian PGCs.

Supplementing blastodermal cell cultures with bFGF not only resulted in a significant decrease in PGC numbers, but also led to a dramatic decrease in the expression of SSEA-1 (Fig. 2. 12). Concomitant with the down-regulation of SSEA-1 in patches of blastodermal cells, the expression of the transcription factor *brachyury* was upregulated (Fig. 2. 13A). Since during normal

development *brachyury* is expressed specifically by early mesodermal cells that ingress through the primitive streak (Fig. 2. 3; Kispert *et al.*, 1995; Knezevic *et al.*, 1997), it is most likely that bFGF induces the differentiation of blastodermal cells towards the mesodermal cell lineage in the present culture conditions. In this context, it is interesting to note that the formation of mesodermal tissue is also associated with the down-regulation of SSEA-1 in the mouse embryo (Burdsal *et al.*, 1993; Klinowska *et al.*, 1994).

In accordance with the hypothesis that bFGF can act as a fate-determining factor on multipotent or pluripotent embryonic cells, it should be noted that in vitro differentiation of mouse embryonic stem (ES) cells through the formation of embryoid bodies is also associated with the up-regulation of *brachyury* (Savatier *et al.*, 1996). More importantly, the expression of *brachyury* in ES cell-derived embryoid bodies can be upregulated specifically by bFGF (Yamada *et al.*, 1994). Finally, P19 embryonal carcinoma cells were also reported to respond to bFGF in a similar manner (Vidricaire *et al.*, 1994).

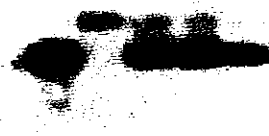
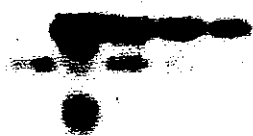
Hence, it would appear that under the present culture conditions, bFGF acts on either multipotent or pluripotent blastodermal cells to differentiate these cells towards a mesodermal lineage. However, it was noted that addition of bFGF (10 and 100 ng/mL) to blastodermal cell cultures on STO cells decreased the number of PGCs only by 25-47 %. Interestingly, the initiation of the culture with embryo cells obtained only from stage X blastoderms resulted in a greater reduction in PGC numbers with respect to cultures initiated with blastodermal

cells obtained from pooled stage X-XIII embryos (cf. Figs. 2, 10, 2, 11). These observations would suggest that a certain proportion of blastodermal cells destined to give rise to PGCs were refractory to the mesoderm inducing activity of bFGF and that such presumptive PGCs increase in number between stages X-XIII. This observation is in accordance with the concept that the segregation of avian PGCs occurs during a specific developmental window, a process which manifests itself as PGCs appear first in association with the polyingressing cells at stage X and then with the hypoblast at stages XI to XIII (Ginsburg and Eyal-Giladi, 1986; Urven *et al.*, 1988; Karagenc *et al.*, 1996). Since it is generally accepted that avian PGCs are of epiblastic in origin (Eyal-Giladi *et al.*, 1981), it would appear that the earliest precursors of PGCs are either multipotent or pluripotent epiblast cells as seen in the mouse (Tam and Zhou, 1996). It is most likely that the recruitment of these cells to somatic as well as germ cell lineages is the result of specific local cell-cell interactions, mediated in part by peptide growth factors and their corresponding receptors. In this context, it will be interesting to see if the differentiation of PGCs from early embryonic cells is coupled with the down-regulation of FGF receptor(s), perhaps providing a mechanism to uncouple the segregation of the germ cell lineage from that of somatic cell lineages during the course of specification and formation of embryonic axis.

Fig. 2. 1. Detection of membrane-bound and soluble forms of stem cell factor during PGC development *in vivo* and *in vitro*. Control lanes: **1:** Blank, **2:** plasmid containing a cDNA encoding the membrane-bound isoform of SCF, **3:** plasmid containing a cDNA encoding the soluble isoform of SCF, **4:** mRNA from adult chicken heart, **5:** mRNA from pooled stage IX and X *area pellucidae*, **6:** mRNA from pooled stage XI-XIII *area pellucidae*, **7:** mRNA from STO cells, **8:** mRNA from blastodermal cells cultured on feeder-layers of STO cells. PCR primers chosen flanked the extracellular proteolytic cleavage site absent in the shorter membrane-anchored form of SCF. PCR products were electrophoresed through a 1 % agarose gel and the presence and identity of positive signals for SCF were confirmed using 27 bp oligonucleotide probes specific for membrane-bound and soluble forms of stem cell factor. The 398 bp product is indicative of the shorter isoform.

1 2 3 4 5 6 7 8

1 2 3 4 5 6 7 8



— 500 bp
— 398 bp

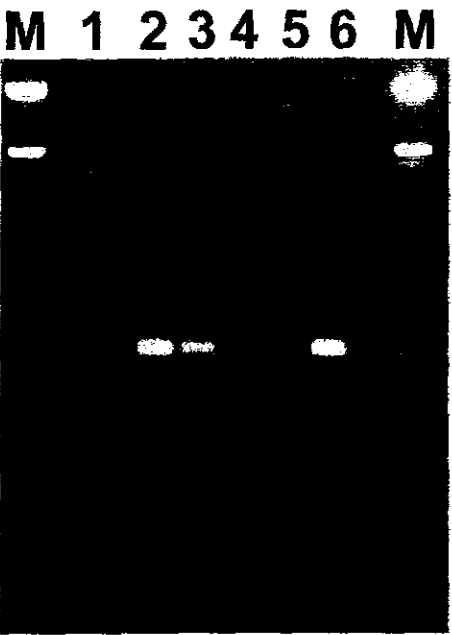
**Soluble
form**

**Membrane-bound
form**

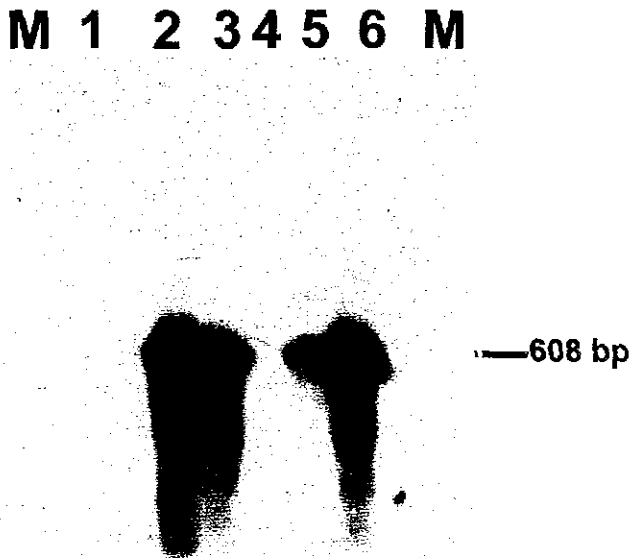
- 1- blank
- 2- pt7q8
- 3- pt7q17
- 4- heart
- 5- IX-X
- 6- XI-XIII
- 7- STO
- 8- STO/CBC

Fig. 2. 2. RT-PCR detection of c-kit transcripts during PGC development *in vivo* and *in vitro*. **M:** Marker, **1:** Blank, **2:** mRNA from pooled stage IX and X area *pellucidae*, **3:** mRNA from pooled stage XI-XIII area *pellucidae*, **4:** mRNA from STO cells, **5:** mRNA from blastodermal cells cultured on feeder-layers of STO cells, **6:** mRNA from adult chicken heart. The primers were: sense, 5'-TCTCACTCATGGTCGAATAAC-3'; anti-sense, 5'-TAGAAGTCCTCCC TCTCTCAC-3'. PCR products were electrophoresed through a 1 % agarose gel and the presence and identity of positive signals for c-kit were confirmed using a 29 bp oligonucleotide probe.

RT-PCR



Southern w/c-kit probe



- 1- blank
- 2- IX-X
- 3- XI-XIII
- 4- STO
- 5- STO/CBC
- 6- heart

Fig. 2. 3. Expression of chicken *brachyury* (Ch-*T*) mRNA transcripts in whole mount chick embryos as detected by in situ hybridization using digoxigenin labeled riboprobes. Whole mount embryos were fixed in 4% paraformaldehyde overnight, digested with proteinase K (10 µg/ mL) for 20 min at room temperature, and hybridized with digoxigenin labeled anti-sense RNA probes specific for chicken *brachyury* (Ch-*T*). Purple stain is indicative of the presence of transcripts. The expression of Ch-*T* is localized to the the primitive streak.



Fig. 2. 4. Expression of SCF and c-kit in gonads at day 8 of incubation as shown by whole mount in situ hybridization. Blue/purple stain is detection of transcripts. *In situ* hybridization analysis was performed on 50 μ m thick sections using digoxigenin-labeled riboprobes. **A:** *In situ* hybridization with SCF anti-sense RNA probes. SCF is expressed in the gonadal epithelium (arrow), **B:** *In situ* hybridization with c-kit anti-sense RNA probes. Note that c-kit is expressed by germ cells.

A



B

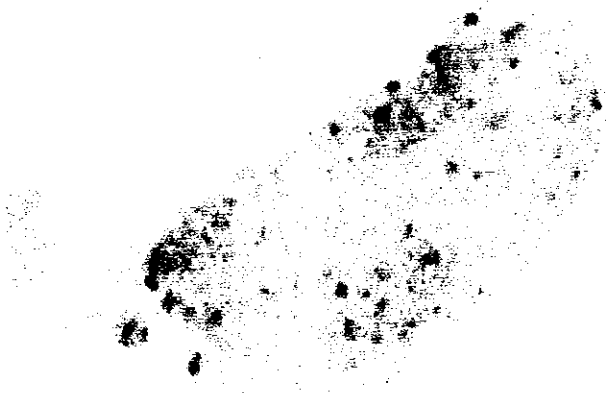


Fig. 2. 5. The expression of SCF and c-kit during active migration of PGCs towards the gonadal ridges shown by whole mount *in situ* hybridization. Blue/purple stain is indicative of the presence of specific transcripts. *In situ* hybridization analysis was performed on 50 μ m thick sections which were taken from a stage 22 chicken embryo (Hamburger and Hamilton, 1951). MRNA transcripts were detected with digoxigenin-labeled riboprobes. **A:** *In situ* hybridization with SCF anti-sense RNA probes. SCF expression is seen at its highest level within the gonadal ridge (arrow) and in lateral portions of allantois (arrow heads). **B:** Higher magnification of the picture in A. **C:** *In situ* hybridization with c-kit anti-sense RNA probes. c-kit is expressed by neural crest cells in the dermatome (arrows), and a few cells within the gonadal ridge. **D:** Immunohistochemical detection of c-kit expressing cells, using a monoclonal antibody which recognizes chicken c-kit. c-kit is expressed by neural crest cells in the dermatome (arrows). Note a few immunoreactive cells (arrow heads) along the dorsal mesentery and the gonadal ridge.



Fig. 2. 6. The identification of PGCs within the gonadal ridge by periodic acid Schiff (PAS) staining and the expression of SSEA-1 and c-kit. Thick sections obtained from stage 22 (H&H) embryos were stained with an avidin-biotin-conjugated alkaline phosphatase system, using the monoclonal antibody MC-480 which recognizes stage-specific embryonic antigen-1 (SSEA-1) and a monoclonal antibody which recognizes chicken c-kit. SSEA-1 positive cells were detected using NBT - BCIP as the substrate. **A:** c-kit expressing cells within the gonadal ridge (arrow heads), **B:** PGCs (arrow heads) recognized by MC-480 within the gonadal ridge. Note the absence of staining within other regions of the embryo. **C:** PGCs (arrow heads) identified by PAS staining within the gonadal ridge.

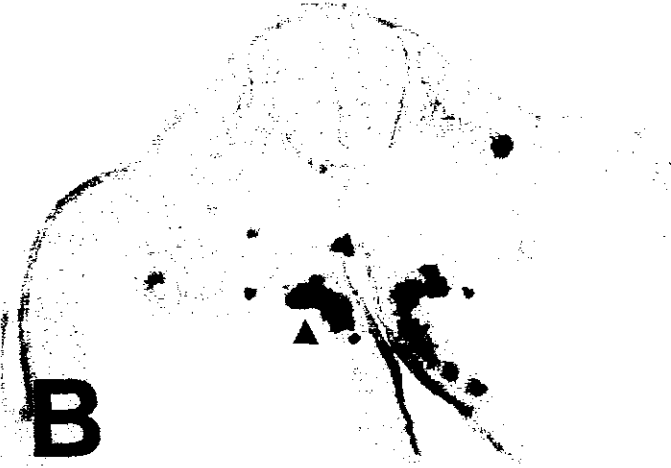


Fig. 2. 7. The number of primordial germ cells (PGCs) obtained after 48 hr culture of blastodermal cells on either feeder layers of STO and CV-1 cells or on gelatin-coated plastic. Each experiment was carried out with 4-20 wells, and numbers are the means $\pm$ S. E. M. of three independent experiments. Embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded and the *area pellucidae* were pooled and dissociated into a single cell suspension by trypsin/EDTA. The number of blastodermal cells obtained per embryo was determined and approximately 5000 cells per well were seeded onto feeder layers of mitotically arrested STO and CV-1 cells in 96 - well microtiter plates. In parallel experiments, blastodermal cells obtained from the same pool of embryos were cultured on gelatin-coated 96 - well microtiter plates. STO cell-conditioned medium (STO-CM) was prepared by growing STO cells to confluency for 65 hr. The cells were cultured for 48 hours at 37 °C / 5 % CO₂. The cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression. Bars with different letters are statistically significant from one another.

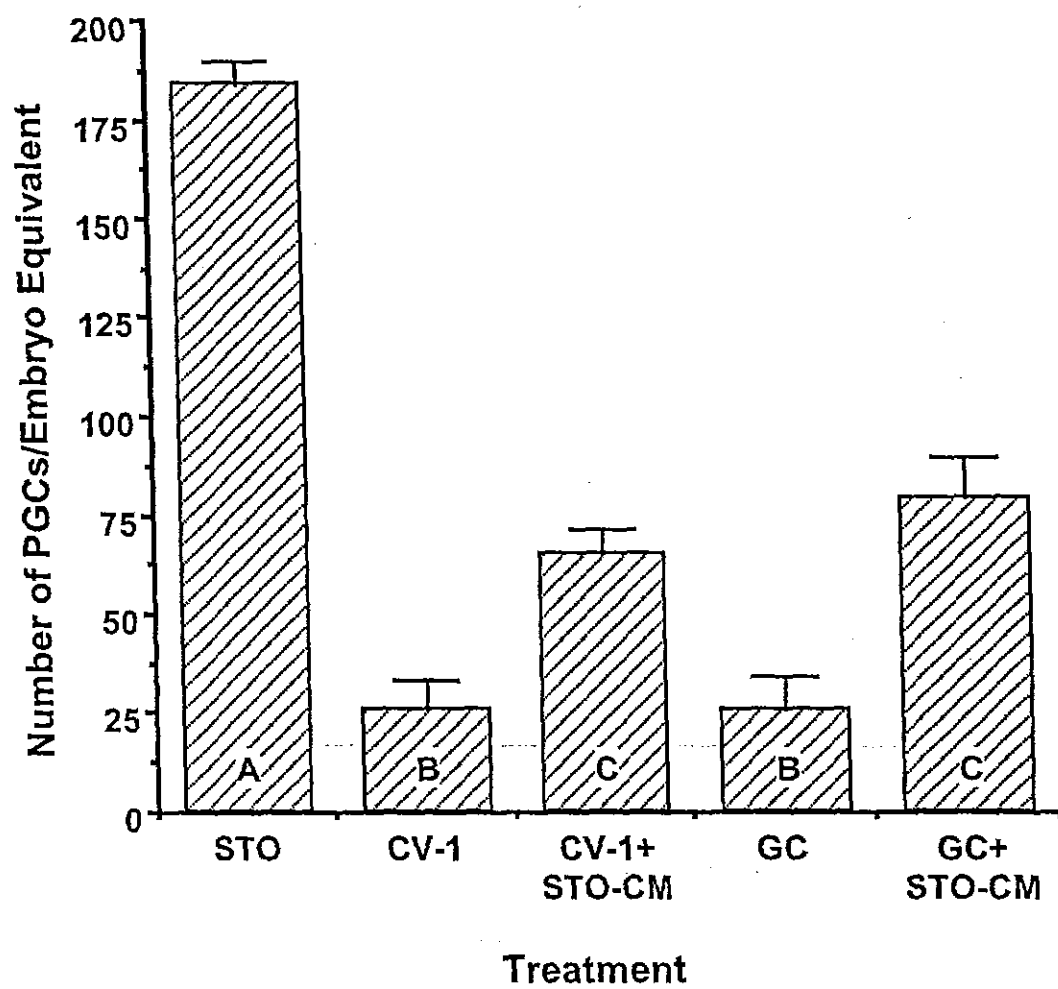


Fig. 2. 8. The number of primordial germ cells (PGCs) obtained after 24 hr culture of hypoblast cells on feeder layers of STO and CV-1 cells (Mean $\pm$ SE). Hypoblasts peeled from nineteen blastoderms at stages XIII-XIV were pooled in DMEM-FBS and were dissociated into a single cell suspension using trypsin/EDTA. The number of cells obtained per hypoblast explant was determined using a hemocytometer; 3500 cells/well were seeded onto feeder layers of mitotically arrested STO and CV-1 cells in 96-well microtiter plates. Hypoblast cells were cultured for 24 hours at 37 °C / 5 % CO₂. At the end of the culture period, the cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression. The number of PGCs detected after 24 h of culture was statistically significant between the feeder-layers of STO and CV-1 cells.

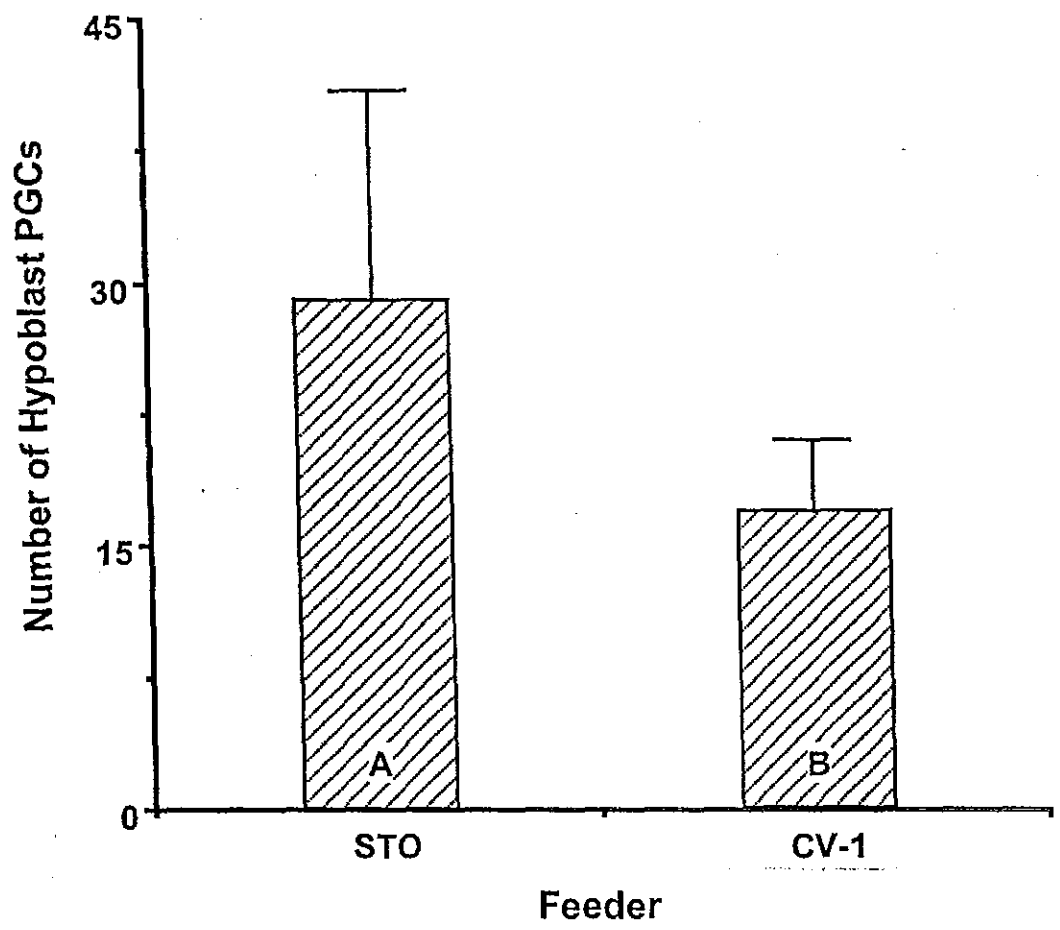


Fig. 2. 9. The effect of recombinant avian SCF on the number of primordial germ cells (PGCs) obtained in cultures carried out on feeder-layers of CV-1 cells.

Each experiment was performed with 9-10 wells, and numbers are the means $\pm$ S. E. M. of six independent experiments. Embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded and the *area pellucidae* were pooled and dissociated into a single cell suspension by trypsin/EDTA. The number of blastodermal cells obtained per embryo was determined and approximately 5000 cells per well were seeded onto feeder layers of mitotically arrested CV-1 cells in 96 - well microtiter plates. 100 ng/mL soluble cSCF was added at the start of the experiment. In parallel experiments, blastodermal cells obtained from the same pool of embryos were cultured on feeder layers of mitotically arrested STO cells. The cells were cultured for 48 hours at 37 °C / 5 % CO₂. The cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression. Bars with different letters are statistically significant from one another.

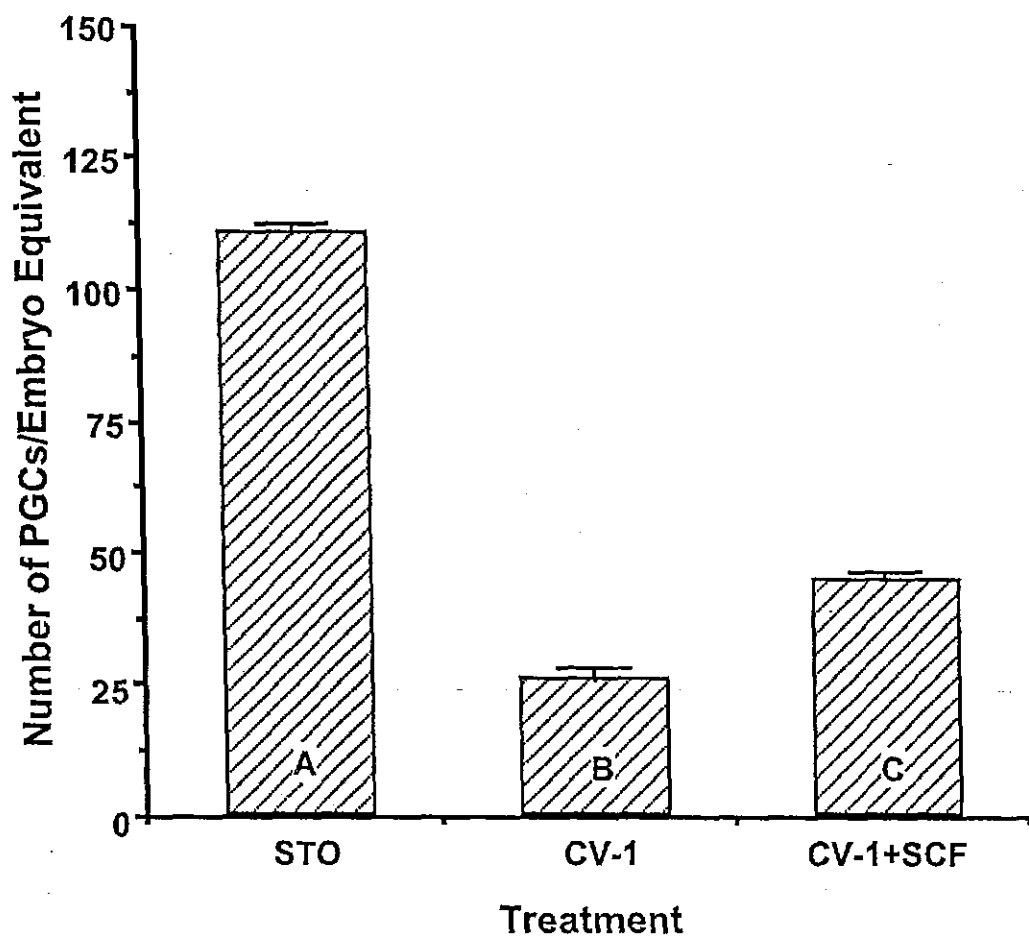


Fig. 2. 10. The effect of basic fibroblast growth factor (bFGF) on the number of primordial germ cells (PGCs) obtained in cultures initiated on feeder-layers of STO and CV-1 cells. Each experiment was performed with 4-8 wells, and numbers are the means $\pm$ S. E. M. of at least three separate experiments.

Stage X-XIII embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded and the *area pellucidae* were pooled and dissociated into a single cell suspension by trypsin/EDTA. The number of blastodermal cells obtained per embryo was determined and approximately 5000 cells per well were seeded onto feeder layers of mitotically arrested STO and CV-1 cells in 96 - well microtiter plates. 100 ng/mL bFGF or a neutralizing antibody to bFGF (10 μ g/mL) was added at the start of the experiment. The cells were cultured for 48 hours at 37 °C / 5 % CO₂. The cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression. Bars with different letters are statistically significant from one another.

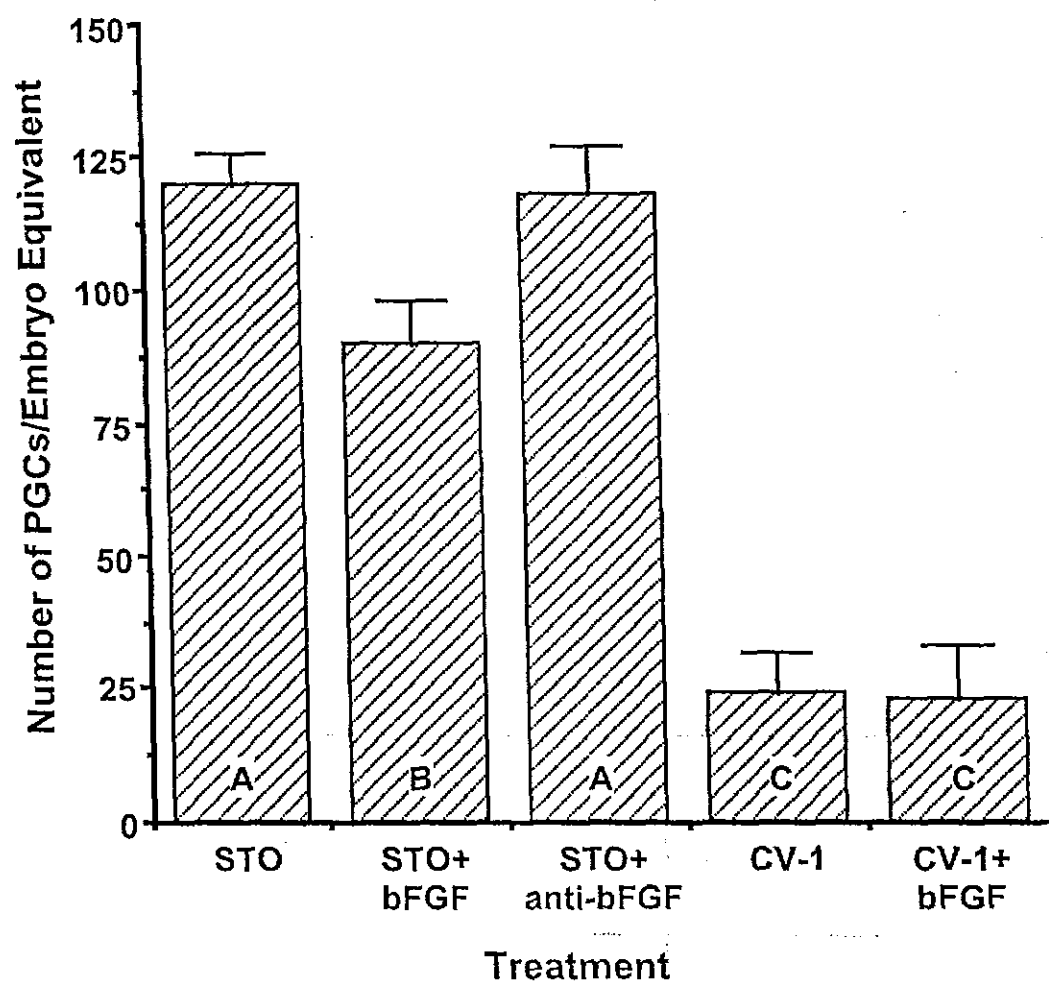


Fig. 2. 11. The effect of basic fibroblast growth factor (bFGF) on the number of primordial germ cells (PGCs) obtained in cultures carried out on feeder-layers of STO cells. Each experiment was performed with at least 4 wells, and numbers are the means $\pm$ S. E. M. of three separate experiments. Embryos obtained from freshly-laid, unincubated eggs were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded and the *area pellucidae* were pooled and dissociated into a single cell suspension by trypsin/EDTA. The number of blastodermal cells obtained per embryo was determined and approximately 5000 cells per well were seeded onto feeder layers of mitotically arrested STO cells in 96 - well microtiter plates. 10 or 100 ng/mL bFGF was added at the start of the experiment. The cells were cultured for 48 hours at 37 °C / 5 % CO₂. The cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression. Bars with different letters are statistically significant from one another.

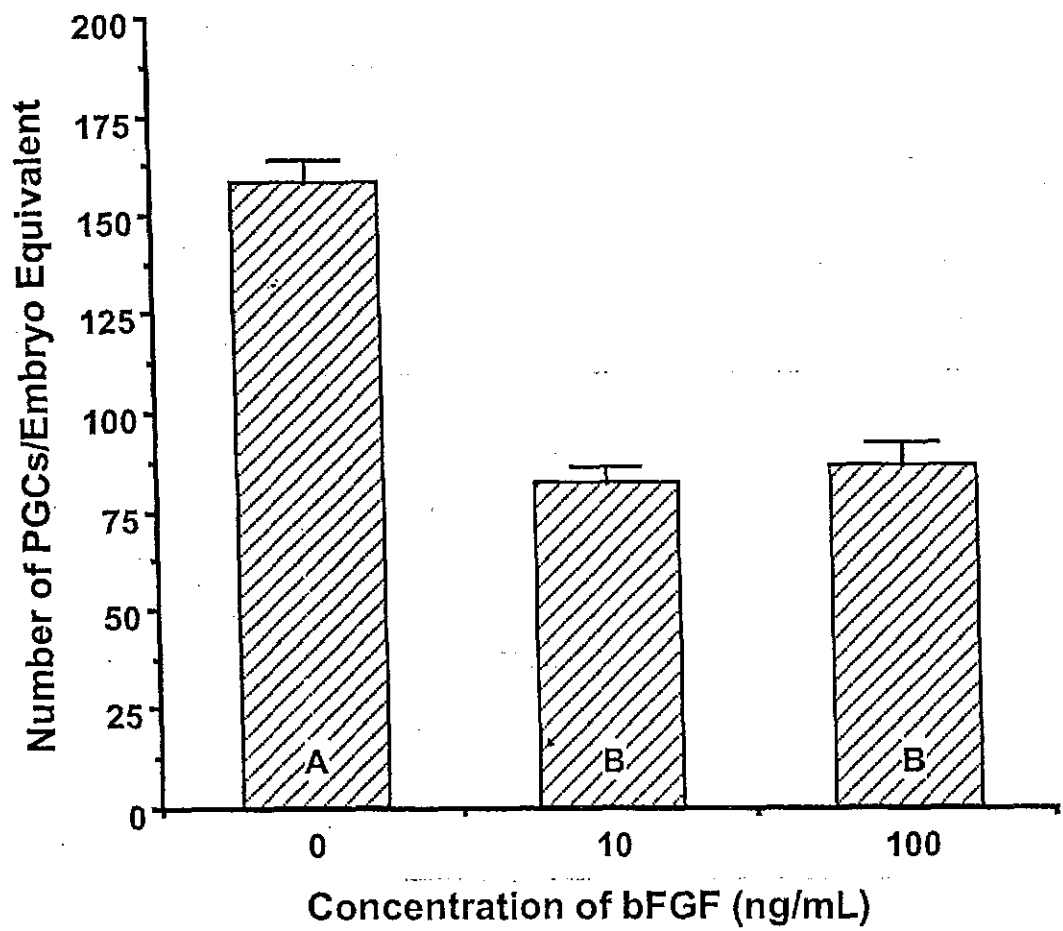


Fig. 2. 12. The effect of basic fibroblast growth factor (bFGF) on the pattern of the emergence of PGCs in blastodermal cell cultures carried out on feeder-layers of STO cells. Embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded and the *area pellucidae* were pooled and dissociated into a single cell suspension by trypsin/EDTA. The number of blastodermal cells obtained per embryo was determined and approximately 5000 cells per well were seeded onto feeder layers of mitotically arrested STO cells in 96 - well microtiter plates. 10 or 100 ng/mL bFGF was added at the start of the experiment. The cells were cultured for 48 hours at 37 °C / 5 % CO₂. The cultures were fixed in 4 % paraformaldehyde (pH 7.2), and PGCs identified on the basis of morphology and SSEA-1 expression. **A:** The emergence of PGCs on STO feeder-layers in the absence of bFGF. Cultures show patches of SSEA-1 positive cells (arrows). PGCs (arrow heads) expressing SSEA-1 are scattered throughout. **B:** The emergence of PGCs on STO feeder-layers in the presence of bFGF. Note patches consisting mainly of SSEA-1 negative cells (arrows) and PGCs (arrow heads) in association with the SSEA-1-negative patches.

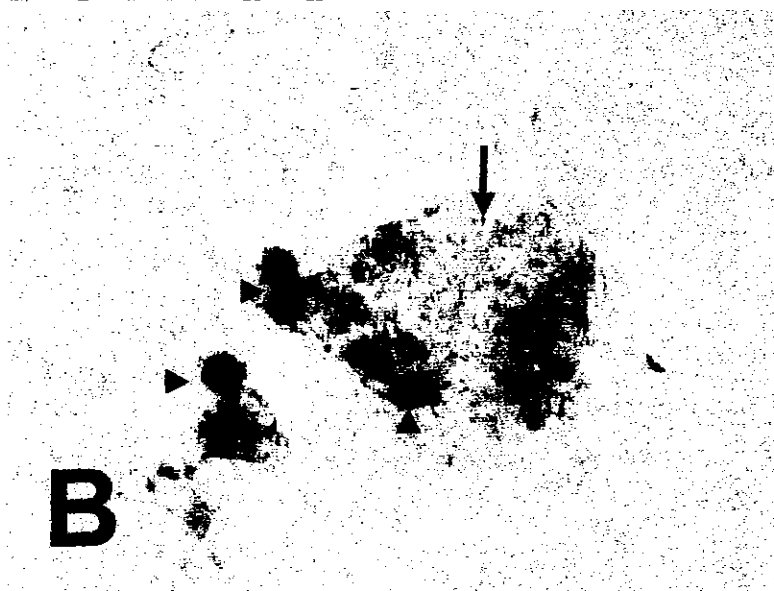
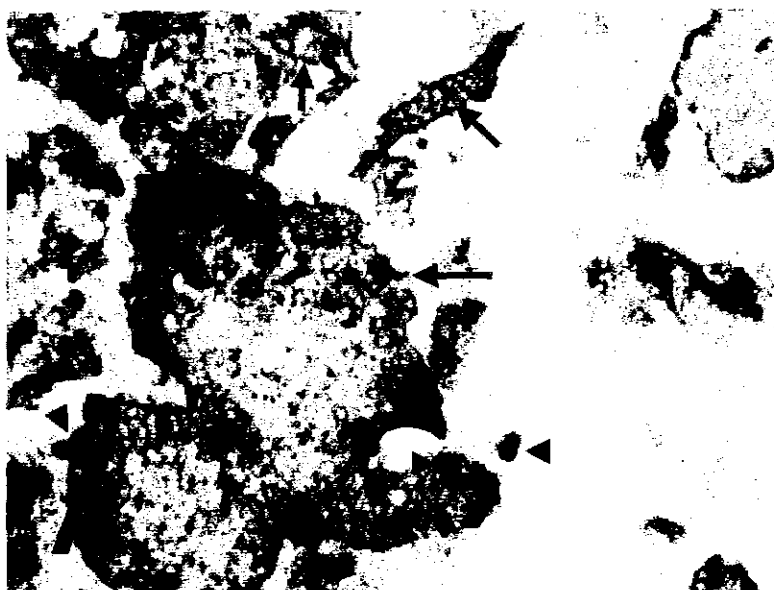


Fig. 2. 13. The effect of basic fibroblast growth factor (bFGF) on the expression of *brachyury* in blastodermal cells cultured on feeder-layers of STO cells.

Embryos were isolated in phosphate buffered saline (pH 7.4), the *area opaca* was discarded and the *area pellucidae* were pooled and dissociated into a single cell suspension by trypsin/EDTA. The number of blastodermal cells obtained per embryo was determined and approximately 5000 cells per well were seeded onto feeder layers of mitotically arrested STO cells in 96 - well microtiter plates. 100 ng/mL bFGF was added at the start of the experiment. The cells were cultured for 48 hours at 37 °C / 5 % CO₂. The cultures were fixed in 4 % paraformaldehyde (pH 7.2), and the expression of *brachyury* was detected by *in situ* hybridization analyses using anti-sense RNA probes specific for chicken *brachyury*. **A.** The expression of *brachyury* in +blastodermal cells cultured on STO feeder-layers in the presence of bFGF (100 ng/mL). Note that *brachyury* is highly expressed in patches of blastodermal cells. **B.** The expression of *brachyury* in blastodermal cells cultured on STO feeder-layers in the absence of bFGF.



A

B

GENERAL DISCUSSION

In all vertebrate embryos, certain cells are set aside from somatic cells early in development as progenitors of gametes. These primordial germ cells (PGCs), which are extragonadal in origin, first migrate to the developing gonads that will form the ovaries in females and the testes in males. After a period of mitotic proliferation, germ cells undergo meiosis, and differentiate into mature gametes, viz. spermatozoa in males and eggs in females. Later, the fusion of the spermatozoa with an egg initiates embryogenesis, with the subsequent production in the embryo of new PGCs, which begins the cycle again. The molecular mechanisms governing the specification of a small population of early embryonic cells to differentiate into PGCs is perhaps the least understood aspect of germ line development. In order to understand the nature of this dichotomous decision in development, it is essential to examine the point at which PGCs first appear in the embryo.

In the chicken embryo, PGCs can easily be identified through histomorphological criteria in an extraembryonic region, referred to as the germinal crescent (Swift, 1914). The balance of evidence, however, indicates that PGCs or their immediate precursors are present within the epiblast well before their appearance in the germinal crescent (Eyal-Giladi *et al.*, 1981). Nevertheless, the specific locus of the origin of PGCs within the epiblast at pre-streak stages of development could not be shown directly due to the absence of

molecular markers. Furthermore, it is currently unknown whether avian PGCs differentiate in a cell-autonomous manner mediated by the determinative action of a specific type of cytoplasm (germ plasm) as suggested for the segregation of PGCs in anuran amphibians (Nieuwkoop and Sutasurya, 1979), or derive from the induction of multipotent/pluripotent cells through extracellular signals as suggested for the segregation of PGCs in mouse (Tam and Zhou, 1996) and urodele amphibians (Sutasurya and Nieuwkoop, 1974).

The experimental evidence presented in this thesis has enhanced the current knowledge of the temporal and spatial aspects of the origin of chicken PGCs and suggested that the segregation of avian PGCs is an epigenetic event, requiring local cell-cell interactions, as opposed to being an autonomous phenomenon. Specifically, it was demonstrated that 1) the emergence of PGCs is associated with the expression of a differentiation antigen, known as stage specific embryonic antigen-1 (SSEA-1) after the formation of the *area pellucida* is completed at stage X, 2) PGCs appear in a scattered pattern, rather than confined to a specific region, within the central portion of the *area pellucida*, 3) SSEA-1 positive cells located on the dorsal surface of the hypoblast have the potential to give rise to PGCs *in vivo* and *in vitro*, 4) When blastodermal cells obtained from pre-streak stages of development are cultured without feeder cells, only 25-45 PGCs/embryo are detected with stage X-XIV blastoderms. However, when such cells are cultured on feeder-layers of STO cells, much higher number of PGCs could be obtained, 5) the ability of blastodermal cells to

give rise to PGCs in feeder-dependent culture systems can be altered by supplementing the culture with basic fibroblast growth factor (bFGF) and stem cell factor (SCF), suggesting the involvement of peptide growth factors in the development of avian PGCs *in vivo*, 6) c-kit is expressed by PGCs within the gonadal ridge, where the expression of SCF is found to be at its highest level.

On the basis of the observations described above, a simple model can be proposed for the segregation of PGCs in the chick embryo. The earliest precursors of PGCs that are peppered throughout the central portion of the *area pellucida* are suggested to be multipotent or pluripotent epiblast cells. These cells are most likely to be recruited to the germ cell lineage through specific cell-cell interactions, which take place as the formation of the *area pellucida* is completed, in a similar mode to the segregation of PGCs in mouse (Tam and Zhou, 1996) and urodele amphibians (Sutasurya and Nieuwkoop, 1974). Accordingly, a small set of uncommitted epiblast cells are specified to become PGCs through signals emanating from neighboring cells, perhaps polyingressing cells that are present on the ventral surface of the *area pellucida* in a stage X embryo. The specification of the germ cell lineage might be associated with the upregulation of a glycosyl transferase, responsible for the expression of the SSEA-1 epitope. A subpopulation of epiblast cells that express SSEA-1 reduce contact with the surrounding cells and leave this layer

by translocating ventrally through polyingressing cells. Lineage restriction might take place only after SSEA-1 positive cells leave the epiblast, as seen in the mouse embryo (Lawson and Hage, 1994). The enhanced expression of SSEA-1 in these presumptive PGCs might be coupled with the negative regulation of a signaling pathway leading to the expression of the transcription factor *brachyury*. Since *brachyury* is implicated in the migration of mesodermal cells through the primitive streak (De Robertis *et al.*, 1994), presumptive PGCs which do not express *brachyury* are likely to be excluded from ingressing through the primitive streak. This would provide a means for the earliest precursors of PGCs to become temporarily refractory to signals such as bFGF which favors the differentiation of cells towards the mesodermal cell lineage. Such consequences of the differential expression of *brachyury* might also provide a mechanism by which the segregation of the germ cell lineage is uncoupled from the segregation of somatic cell lineages during the process of gastrulation. As the hypoblast begins to form as an advancing front of cells from the posterior marginal zone and Koller's sickle, islands of polyingressing cells containing SSEA-1 positive cells are carried passively towards the anterior of the embryo to form the germinal crescent. It is probably during this period of development that SSEA-1 positive cells are committed to the germ cell lineage. This hypothesis would predict that the fate of SSEA-1 positive cells obtained from germinal crescent region can not be altered by bFGF in culture.

Germ cells are the only cell type that are endowed with the ability to pass on the full complement of genetic information present in the zygote from one generation to the next, a property that is denied to somatic cells of the body under normal circumstances. The fact that germ cells represent the actual line of heredity between generations defines the end point of transgenic technology, which is to introduce heritable changes into the genome of animals. Since PGCs are the forerunners of the germ line, it is the genome of these cells that needs to be modified. Various methods including microinjection into the unfertilized egg and retroviral-infection of the unincubated blastoderm have been employed to introduce foreign DNA into PGCs of the avian embryo (Etches *et al.*, 1993; Petitte *et al.*, 1997) with the aim of producing transgenic birds. However, the precise modification of the avian genome via homologous recombination can currently be accomplished only through the production of germ line chimeric intermediates. This approach requires the availability of cells that have the potential to colonize the germ line of the recipient embryo and form normal gametes following specific genetic manipulation and selection in culture. So far three embryonic cell types, namely blastodermal cells obtained from stage X embryos (Petitte *et al.*, 1990), PGCs (Yasuda *et al.*, 1992; Vick *et al.*, 1993; Naito *et al.*, 1994), and recently putative avian embryonic stem (ES) cells derived from stage X blastodermal cells (Pain *et al.*, 1996), have been demonstrated to be germ line competent when introduced into a recipient embryo. However, the use of blastodermal cells in obtaining germ line chimeras

is complicated in light of a growing body of evidence indicating that the *area pellucida* of a stage X embryo does not constitute a homegenous, pluripotent population of cells (Stern and Canning, 1990; Riese *et al.*, 1995; Karagenc *et al.*, 1996; Knezevic *et al.*, 1997) as once thought (Eyal-Giladi, 1984). In addition, the avian embryo already contains about 20-25 PGCs at stage X when the egg is laid (Pardanaud *et al.*, 1987a; Muniesa and Dominguez, 1990; Karagenc *et al.*, 1996). Therefore, it is not certain whether the germ line of the recipient embryo is colonized by multipotent/pluripotent blastodermal cells or by unipotent PGCs. It is important to make this distinction since if the competence for the germ cell lineage is restricted only to PGCs that are present in the unincubated embryo, then one can not expect to obtain a high and perhaps consistent efficiency of germ line chimerism, as only about 20-25 PGCs exist in a stage X chick embryo (Muniesa and Dominguez, 1990; Karagenc *et al.*, 1996). Alternatively, but not exclusively, multipotent/pluripotent blastodermal cells that are believed to exist in the early avian embryo might colonize the germ line of the recipient embryo as they give rise to PGCs when introduced into another embryo. How the germ line competence could be realized by such multipotent/pluripotent blastodermal cells is currently unknown. Since during normal development the majority of PGCs appear to originate from the most central portion of the *area pellucida* (Ginsburg, 1996), it is most likely that the colonization of the central portion of the *area pellucida* by donor cells is a prerequisite for for germ line competence.

Pluripotent embryonic stem cells such as ES cells derived from the inner cell mass of the mouse embryo (Evans and Kaufman, 1981; Martin, 1981) and EG cells derived from the long term culture of mouse PGCs (Matsui *et al.*, 1992; Resnick *et al.*, 1992) provide another means to access to the germ line. The use of germ line competent stem cell populations has facilitated the precise modification of the mammalian genome as these cells lend themselves to specific genetic manipulation and selection in culture before they are introduced into a blastocyst (Bradley and Robertson, 1986). Several attempts have been made to obtain such embryonic stem cell populations in various mammalian species (Gardner and Brook, 1997). Recently, Pain *et al.* (1996) reported the characterization of avian embryonic stem cells derived from the culture of chicken and quail stage X blastodermal cells. These putative avian ES cells have been demonstrated to resemble murine ES cells in features such as morphology, marker expression, and the ability to form embryoid bodies which can differentiate into a variety of cell types (Pain *et al.*, 1996). More importantly, these cells could give rise to both somatic and germ line chimeras when injected into the sub-germinal cavity of recipient embryos (Pain *et al.*, 1996). On the basis of the assumption that the *area pellucida* of a stage X embryo consists of multipotent or pluripotent cells, it was suggested that these putative ES cells are the functional equivalent of murine ES cells (Pain *et al.*, 1996). However, in the light of the evidence that progenitors of avian ES cells, i.e., stage X blastodermal cells, are already heterogenous in developmental potential, the possibility that

there exists a heterogeneity of cells within avian ES cell lines in competence to colonize the germ line should be considered.

In addition to embryonic stem cells derived from early blastodermal cells, PGCs obtained either from embryonic blood (Tajima *et al.*, 1993; Naito *et al.*, 1994) or from the gonadal anlage of 5-day old embryos (Chang *et al.*, 1995; Chang *et al.*, 1997; Tajima *et al.*, 1998) have been demonstrated to be able to populate the germ line when introduced into the embryonic circulation of the recipient embryo. This novel approach in accessing the germ line of the avian embryo is currently the most promising means towards the production of transgenic birds. However, the use of PGCs in altering the avian genome is hampered by the lack of knowledge regarding how the initiation and maintenance of germ cell proliferation is controlled in the intact embryo. Such basic information is required for the long term culture of avian PGCs. The current study is a modest effort towards a better understanding of the regulatory events that are involved in the development of avian PGCs.

Taken together, it is evident that a clear understanding of the cellular and molecular mechanisms governing the segregation and development of the germ cell lineage in the avian embryo will not only provide a useful model for vertebrate germ cell development, but also will help to develop strategies for the efficient development of transgenic birds.

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APPENDIX A

COMPOSITION OF IN SITU HYBRIDIZATION BUFFERS

Reagent (stock solution)	zHybe	For 10 ml zHybe	sHybe	For 10 ml zHybe
Formamide (100 %)	50%	5 ml	50 %	5 ml
SSC (20X)	5X	2.5 ml	1.3 X	0.65 ml
EDTA (0.5 M pH 8.0)	-	-	5 mM	0.1 ml
Yeast RNA (10 mg/ml)	50 µg/ml	25 µl	50 µg/ml	25 µl
Tween-20 (20 %)	0.1 %	50 µl	0.1 %	50 µl
CHAPS (10 %)	-	-	0.5 %	0.5 ml
Heparin (50 mg/ml)	50 µg/ml	5 µl	100 µg/ml	10 µl
Citric acid (1M)	to pH 6.0		to pH 5.0	
Water to volume		2.42 ml		3.64 ml