BARD

FINAL REPORT
PROJECT NO. US-2090-91

**The Study of Primordial Germ Cell Development as a
Tool for Gene Transfer in Chickens**

J.N. Petitte, H. Eyal-Giladi, M. Ginsburg

1996

Date: April 30, 1996

BARD Project Number: US-2090-91

Project Title:

THE STUDY OF PRIMORDIAL GERM CELL DEVELOPMENT AS A TOOL FOR GENE TRANSFER IN
CHICKENS

Investigators:
(Principal first)

James N. Petitte

Hefzibah Eyal-Giladi

Malka Ginsburg

Affiliated Institution:

North Carolina State University

Hebrew University of Jerusalem

Hebrew University of Jerusalem

Project Start Date: October 1, 1991

Type of Report: 1st Annual _____ 2nd Annual _____ Final XXX

Signature:

Principal Investigator

Signature:

Authorizing Official, Affiliated Institution

DR. CHARLES G. MORELAND
VICE CHANCELLOR FOR RESEARCH,
OUTREACH, EXTENSION AND
ECONOMIC DEVELOPMENT

Abstract:	2
Introduction	4
Objectives of Original Research Proposal	7
Objective 1:	7
Materials and Methods:	8
Results and Discussion:	12
Objective 2:	19
Materials and Methods:	20
Results and Discussion:	25
Objective 3:	29
Materials and Methods	29
Results and Discussion:	31
Description of Cooperation:	32
Evaluation of the Research Achievement:	33
Conclusions:	35
Publications:	35
References:	36
Plate Legends:	43

Abstract:

The Study of Primordial Germ Cell Development as a Tool for Gene Transfer in Chickens

The ability to introduce novel genetic material into the genome of commercial poultry has been impeded by a lack of knowledge regarding the origin in the early embryo of the target cell of interest, namely, the germ cell. Hence, this project investigated the emergence of primordial germ cells (PGCs) during the early development of the avian embryo to aid in efforts to produce transgenic poultry on a routine basis. The strategy was to introduce foreign DNA into the area of the unincubated embryo that is destined to give rise to the germ line. The objectives of this project were: 1) to identify and localize a subpopulation of cells in the early embryo which will give rise to PGCs, 2) to determine the best location and stage of development to transfer donor cells for efficient germline chimerism, and 3) to transfect donor cells to produce transgenic/germline chimeric embryos. We show that by using the monoclonal antibody SSEA-1 and by various cell culture techniques that germ cells appear to segregate from the somatic lineages at St. X., a process that is gradual and continues through St. XIV. Using microsurgical transplantation between quail and chick embryos, we demonstrated that the inner 1/3 of the *area pellucida* between Stages X-XII gives rise to about 2/3 of the germ cell population at the time of their residence in the germinal crescent. Because of the non-localized emergence of PGCs, attempts

to introduce foreign DNA into clonal precursors of germ cells through liposome-mediated transfection yielded unacceptable levels of efficiency. However, through our investigation of germ cell origins, an *in vitro* model of germ cell differentiation was developed that could offer a means of determining the factors required for the long term culture of avian PGCs thereby providing a convenient means of manipulating the avian genome.

Introduction - Description of the Problem

During the past few years considerable interest has emerged regarding the application of recombinant DNA technology to animal agriculture. Much of this interest in biotechnology has focused upon gene transfer as a means of creating novel genotypes to improve animal performance. In the past few years, several methods of gene transfer devised for laboratory animals are now being applied to livestock. These include: microinjection, retroviral infection, and the development of transgenic/chimeras using embryonic stem cell lines. However, the use of these techniques for the production of transgenic poultry has been complicated by the peculiarities of the avian reproductive system.

Transgenic mice, and more recently domestic cattle, sheep and pigs, have been produced by microinjection of DNA into the pronuclei of newly fertilized ova (Gordon *et al.*, 1980; Palmiter and Brinster, 1985; Pursel *et al.*, 1989). Such experimental manipulation is impractical in birds since the newly fertilized ovum is technically inaccessible without killing the hen and the large vitellus is very fragile. In addition, fertilization in birds is polyspermic and the supernumerary male pronuclei are indistinguishable from the male pronucleus that will contribute to the zygote (Harper, 1904; Perry, 1987). To circumvent these problems, an *in vitro* culture system has been devised using a combination of shell-less culture and surrogate-eggshell culture (Rowlett and Simkiss, 1987; Perry, 1988; Naito *et al.*, 1990). This system has allowed the microinjection of DNA into the cytoplasm of the avian egg at about the time of the first cleavage divisions. In most cases, the DNA remains episomal and is expressed only transiently in the embryo (Sang and

Perry, 1989). However, one transgenic rooster has been developed using direct DNA injection (Love *et al.*, 1994).

Retroviral infection of the early embryo has been the most frequently used method to produce transgenic poultry (Souza *et al.*, 1984; Shuman and Shoffner, 1986; Salter *et al.*, 1986, 1987; Bosselman *et al.*, 1989). The advantages of using retroviral vectors include the efficiency of infection and the incorporation of single copies of the vector into chromosomal DNA (Temin, 1989). However, even with the use of replication defective-vectors, the birds may become viremic. This possibility limits the use of retroviral vectors for commercial applications. In addition, retroviral constructs may be unstable and the size of the DNA that can be packaged into the vector is limited.

The major disadvantage in the use of retroviruses or microinjection is that the incorporation of the exogenous DNA into the genome will occur at random. This requires a large amount of screening and an extensive evaluation of transgene expression before deciding which birds will be useful to establish commercial breeding stock. Transgenic birds are most likely to be of value to developmental biologists and the poultry industry if the modifications of the genome occur in a specific, site-directed manner rather than by a shotgun approach. Targeting of introduced genes to specific sites in the chromosome can be achieved using gene constructs that are capable of undergoing homologous recombination, in which exogenous and native DNA sequences recombine within regions of homology. The attraction of this approach lies in its potential for modification of endogenous genes *in situ* to enhance or eliminate expression, to alter tissue-specificity, or to alter developmentally regulated expression.

The application of "gene-targeting" technology towards the development of transgenic poultry requires the ability to produce avian germline chimeras and the establishment of avian embryonic stem cell lines. Simkiss *et al.* (1989) and Wentworth *et al.* (1989) have produced avian gonadal chimeras by the intraspecific transfer of germinal crescent or vascular primordial germ cells (PGCs). Since PGCs are committed to the germ cell lineage, the transfer of these cells will result in chimerism that is restricted to the eggs or spermatozoa. Hence, manipulation and transfection of PGCs is seen as one way to target the avian genome. Recently, Simkiss *et al.* (1990) reported gene insertion into the embryonic gonad by retroviral transfection of PGCs before transfer. Nevertheless, the demonstration of germline transmission in sexually mature birds has not been consistent. Wentworth *et al.* (1989) noted the recovery of the donor genotype in the offspring of a single quail. However, Wentworth *et al.* (1989) and Petite *et al.* (1991) could not demonstrate germline transmission after PGC transfer in chickens. Some of the inconsistencies surrounding these results may be due to the technical difficulties of PGC isolation and transfer or to mixed-sex chimerism (Wentworth *et al.*, 1989; Petite *et al.*, 1991). In addition to PGC transfer, Petite *et al.* (1990) reported that the transfer of blastodermal cells from the unincubated Stage X (Eyal-Giladi and Kochav, 1976) embryo to a recipient of the same age can give rise to somatic and germline chimerism in the chicken. Recent experiments show that the frequency of chimerism can be increased from 10 to 35% by using donor cells from the central disk of the *area pellucida* (Petitte *et al.* 1993, Carscience, *et al.* 1993). In addition, Brazolot *et al.* (1991) have shown that blastodermal cells can be efficiently transfected and that after injection into the Stage

X blastoderm, expression of the transgene can be observed in 24-65 hour embryos. Compared to PGC transfer, blastodermal cell isolation and transfer is relatively simple and the technique has been applied to the formation of interspecific chimeras (Naito *et al.*, 1991). This ability to form avian chimeras represents one of the first steps towards the development of transgenic poultry using embryonic stem cell technology. To date, the efforts to obtain transgenic poultry have been hampered by a lack of knowledge concerning the emergence of PGCs and the development of the avian embryo from the first cleavage divisions to the time that the egg is laid. Since PGCs represent the target-cell for any genetic modification of the germline, knowledge concerning their origin in the relatively undifferentiated embryo should assist in the development of a technical procedure to produce transgenic poultry using PGCs or their precursors.

Objectives of Original Research Proposal

- 1) the identification of a subpopulation of cells in the Stage X blastoderm which will eventually give rise to the germline.
- 2) to determine the best location and stage of development to transfer donor cells for efficient germline chimerism.
- 3) to transfect donor cells and produce transgenic/germline, chimeric embryos.

Objective 1: the identification of a subpopulation of cells in the Stage X blastoderm which will eventually give rise to the germline.

Materials and Methods:

Traditionally, primordial germ cells have been identified in the early embryo using morphological characteristics and periodic acid-Schiff (PAS) staining . These techniques cannot be used to detect PGCs or their precursors in the preprimitive streak embryo. Similarly, the use of immunological detection in quail embryos with a polyclonal antiserum cannot be used prior to gastrulation since several cell types are usually labeled (Ginsburg *et al.*, 1989). Until now, the only antibody that has been used to detect PGCs during Stages X-XIII has been the monoclonal antibody EMA-1 raised against a mouse embryonal carcinoma that cross-reacts with an epitope on chicken PGCs (Urven *et al.*, 1988). However, the authors did not verify that EMA-1 positive cells prior to gastrulation were in fact PGCs. Therefore, the identification of PGCs during pre-primitive streak development has not been investigated. To document the events surrounding germ cell emergence prior to gastrulation a suitable marker for PGCs must be found. Therefore, we attempted to produce monoclonal antibodies that would identify a sub-population of cells in the early embryo that would give rise to the germ cells lineage and to screen other known antibodies against PGCs.

Monoclonal Antibody Production (NCSU)

Six mice were immunized with cells from the *area pellucida* of freshly isolated Stage X embryos every three weeks. After the second immunization three mice died. Prior to the final

immunization, mouse serum was tested for the presence of antibodies against cells of the Stage X embryo. After obtaining a positive antibody titer, the remaining mice were immunized again and after three days the spleen cells were harvested and a portion was fused with myeloma cells and the remaining material was frozen. After growing the fusion under HAT selection for 14-21 days, the wells were screened for antibodies against Stage X cells and yolk using an enzyme-linked immunoassay. About 40 wells were positive against the embryo cells and not against yolk. Cells from these wells were expanded and frozen for further characterization. Unfortunately, none of the clones examined were suitable and attention focused on the evaluation of two monoclonal antibodies used to mark mouse PGCs.

Two antibodies were tested for the identification of PGCs, EMA-1 and SSEA-1. Embryos at the various stages were isolated and processed as whole mounts. On occasion, the hypoblast was removed from Stage XIII embryos. Using a 24-well plate, embryos were fixed in 1 ml of 4% paraformaldehyde in PBS, overnight at 4 C taking care that the embryos were fixed flat and submerged. The next day, fixative was removed (keeping the embryo flat) and the embryo washed in 2 ml of PBS, twice for 10 minutes. Non-specific binding was blocked by adding 2ml of diluted normal serum and incubated at RT for 30 minutes. Normal serum was removed and 0.5 ml of diluted primary antibody (for example, EMA-1 or SSEA-1) was added and incubated for 30 minutes. EMA-1 was provided as a 40X concentrate of hybridoma supernatant and was diluted to 1x with sterile water. SSEA-1 was diluted 1:1000 from ascites fluid in PBS containing normal goat serum as a carrier. The embryos were washed in 1-1.5 ml of PBS for 10 minutes. After removing the PBS, 0.5ml of diluted biotinylated second antibody was added and incubated for 30 minutes.

Subsequently, the biotinylated second antibody was removed and the embryo washed in 2ml of PBS for 10 minutes. For detection, 0.5ml of ABC-alkaline phosphatase was added and incubated for 30 minutes and washed with PBS. The embryo was transferred to a new well and incubated with NBT/BCIP and levamisole in Tris-buffer (pH 9) for 10-15 minutes. Embryos were mounted in gelatin-based media or dehydrated and mounted in permount.

In vitro Culture of Embryo Fragments (HUJ) and Dispersed Embryos (NCSU)

Blastodiscs at the stages of *area pellucida* formation (Stages VII-IX) were isolated from eggs extracted manually from the hen and allowed to develop in vitro on a vitelline membrane using New's system of culture (New, 1955). The periphery of the *area pellucida* was delimited with carmine red granules. Since the embryos were cultured upside down, normal cell shedding associated with *area pellucida* formation could not proceed and the *area pellucida* remained only partially formed. Since polarity has already been established in the embryo by this time, the blastoderms were dissected into posterior, anterior, and central areas. These various isolated pieces were incubated for the time required to match 48h control embryos. PGCs were identified in the cultured fragments by PAS staining and morphology.

For culture of dispersed cells, embryos were isolated, cleaned and staged. The *area opaca* was discarded and the *area pellucida* was dispersed into a single cell suspension by either trypsin/EDTA or mechanically. Dispersed embryos were cultured separately for 48 hours 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 PAS staining and morphology. In addition to whole dispersed embryo culture, blastodermal cells obtained from dispersed St. IX - XIII (EG&K) embryos were cultured on feeder layers of STO cells, a mouse fibroblast cell line. In order to minimize cell to cell contact, a small number of cells (50000 cells/well in a 96 well plate) was seeded onto feeder layers of mitotically arrested STO cells. The culture conditions were the same as in the previous *in vitro* culture experiment. In this case PGCs were identified on the basis of their morphology and reactivity to anti-SSEA-1 since STO cells stain with PAS.

Cell Lineage Analysis *in vivo* and *in vitro* (NCSU)

The SSEA-1 positive cells found on the epiblast side of the hypoblast were assumed to be PGCs on the basis of expected number, morphological similarity to PGCs stained with anti-SEA-1 in the blood and gonad, and finally their association with the hypoblast. To our best knowledge, the SSEA-1 positive cells of the hypoblast represent the earliest identifiable *putative* PGC population reported in the avian embryo. Therefore, we tested the hypothesis that SSEA-1 positive cells of the hypoblast can give rise to the germ line *in vivo* and *in vitro* using the chick/quail chimera system. Since quail PGCs are PAS negative (Nakamura *et al.*, 1991 ; Nakamura *et al.*, 1992), it was possible to identify chicken PGCs by PAS staining in the chimeric gonad of recipient quail and also on a feeder layer of quail blastodermal cells. SSEA-1 positive cells gave rise to PGCs both *in vivo* and *in vitro*. Hypoblasts from Stage XIII-XIV chicken embryos were dispersed and labeled with SSEA-1 antibody followed by a rhodamine-conjugated second antibody at 4 C. Live fluorescent cells were individually picked with a micropipet and placed in a droplet of medium and stored under a layer of mineral oil. Approximately 50 cells were injected

into the subgerminal cavity of uncubated quail embryos. After five days of incubation, the quail embryos were fixed, embedded in paraffin, and serially sectioned. Chick PGCs were identified in the quail embryos using PAS staining and morphology. For *in vitro* analysis, SSEA-1 labeled chick cells from the hypoblast were seeded onto a feeder layer of cells from dispersed quail embryos. After 48 hours of culture, the chicken PGCs were identified using PAS and morphology.

Results and Discussion:

EMA-1 was previously used to identify chicken PGCs after their migration to the germinal crescent, blood and residence in the gonad (Urven *et al.*, 1988). The other antibody examined was against Stage Specific Embryonic Antigen -1 (SSEA-1). This antigen is a cell-surface oligosaccharide expressed during preimplantation mouse development and by murine primordial germ cells in the allantoic stalk.

We confirmed that EMA-1 can identify PGCs during later stages of development. EMA-1 was tested on embryos at Stages X, XI, XII, and XIII using immunohistochemistry procedures on whole embryos. The pattern of staining appeared to be related to the stage of development. When the Stage X embryo was classified as early, middle, and late, onset of EMA-1 expression appeared to be temporally related to the stage of development of the embryo. Initial work using this antibody indicated that EMA-1 expression begins early in Stage X with a few positive cells at the level of the epiblast. By the middle of Stage X a mosaic of EMA-1 positive cells appears on the surface of the epiblast as well as the lower surface. For the most part, clusters of cells lying

beneath the epiblast are not stained except for the occasional large cell. A similar pattern was observed for late Stage X embryos. However, during the formation of the hypoblast (Stages XI-XIII), the amount of EMA-1 staining increased so much that correlations with PGCs emergence were not feasible. Thus, the expression of the EMA-1 epitope is clearly not limited to germ cells during preprimitive streak stages. This characteristic makes its use as a marker of the germ cell lineage prior to gastrulation rather limited.

On the other hand, the results for SSEA-1 were more specific than EMA-1. SSEA-1 is a carbohydrate antigen determined by galactose (1 - 4) N - acetylglucosamine (1 - 3) fucose (Gooi *et al.*, 1981). It is expressed by murine PGCs during migration to the gonad and has been used as a marker to identify mouse PGCs *in vivo* and *in vitro* (see Donovan, 1994 for review). Because chicken PGCs can be identified histomorphologically in the blood at St. 14-15 (H&H) and in the gonad at day 6 of incubation, SSEA-1 labeling was initially tested with these tissues. Anti-SSEA-1 specifically recognized PGCs in the blood and the gonad (Plate 1, 2). In order to confirm the specificity of SSEA-1 labeling, gonadal cells were stained with anti-SSEA-1 and PAS simultaneously. No PAS positive cells were detected among SSEA-1 negative cells. In embryo whole mounts prior to gastrulation, SSEA-1 labeling was first detected at St. X with no staining at St. IX (Plates 3,4,5,6). In all embryos, the staining was confined only to the *area pellucida* with none occurring in the *area opaca* regardless of the developmental stage. The most characteristic feature at St. X was the appearance of distinctly stained, large SSEA-1 positive cells in association with SSEA-1 negative polyingressing cells on the ventral side of the epiblast (Plate 7). From St. XI onwards, SSEA-1 positive cells with varying intensities were observed in the

dorsal aspect of the epiblast in a scattered pattern (Plate 8 & 9). This differential expression of the epitope is in agreement with several reports suggesting that the epiblast constitutes a heterogeneous population of cells (Canning and Stern, 1988 ; Stern and Canning, 1990; Riese *et al.*, 1995). Only after the removal of the hypoblast at St. XII, XIII, and XIV was it possible to reveal the presence of distinctively stained cells in the ventral surface of the epiblast and on the hypoblast (Plate 10). Unlike that of the epiblast, the density of staining among SSEA-1 positive cells of the hypoblast was uniform. Some cells were found as small aggregates of two to eight and some as single cells (Plate 11). These positive cells were always located on the epiblast side of the hypoblast often in a different plane among SSEA-1 negative cells. In addition to the hypoblast, SSEA-1 positive cells were also observed in the space between the epiblast and the hypoblast suggesting that a subpopulation of SSEA-1 positive cells in the epiblast translocated to the hypoblast both as individual cells and small aggregates. Although it was not possible to count the number of positive cells in the epiblast, the number of SSEA-1 labeled cells in the hypoblast showed a gradual increase between St. XII - XIV (Figure 1). It should be noted that the numbers of positive cells on the hypoblast at St. XII - XIV (EG&K) are in relatively close agreement with the culture of dispersed embryos (see Figure 2) and with those determined indirectly by Ginsburg and Eyal-Giladi (1986) for the corresponding stages. Lastly, we have also examined SSEA-1 staining in quail embryos and have found an equally impressive population of SSEA-1 positive cells on the hypoblast (data not shown).

Figure 1. Mean $\pm$ s.e. of SSEA-1 labeled cells on the hypoblast according to stage. Embryos were isolated, cleaned and staged according to Eyal-Giladi and Kochav (1976) staging system. Whole hypoblasts were removed, fixed and stained with an ABC-AP based system (Vectastain ABC-AP kit, Vector Laboratories) using anti-SSEA-1 as the primary antibody. N=5-10.

When cells from embryos at uterine stages were cultured as fragments, PGCs appeared only in the fragments which were derived from the central area consisting of approximately one third of the diameter of blastodiscs at Stage VII and about half the diameter at Stages VIII and IX. The central areas in which PGCs developed always contained the partially formed *area pellucida*, indicating that the formation of the *area pellucida* is a prerequisite for the development of PGCs. No discrete location of the early PGCs could be demonstrated.

However, when embryos were dispersed into single cells and cultured, PGC development was restricted by developmental stage. PGCs were detected from St. X and onwards. No PGCs were found at St. IX (Figure 2). The difference in the number of PGCs between the mechanical and enzymatic methods was not statistically significant indicating that PGCs were not sensitive to enzymatic treatment. The average number of PGCs at St. X was found to be lower than that reported by Ginsburg and Eyal-Giladi (1989) who cultured fragments of embryos rather than completely dispersed blastodermal cells. The main difference between the dispersal study and

the cultured fragment work was the lower level of organization in the cultures initiated with dispersed embryos compared to that initiated with fragmented embryos. It should be noted that “dome-like” structures and blood cells, which were found to be closely associated with the emergence of PGCs in cultures of embryo fragments, were not present in the cultures initiated with dispersed embryos. However, extending the culture of dispersed St. IX embryos to 96 h did not yield any PGCs. When blastodermal cells were cultured on an STO feeder layer, germ cells were found after 48 hours of culture using embryonic cells from all stages, even St. IX (Figure

3).

Figure 2. Identification of PGCs from cultures of dispersed embryos (Mean $\pm$ s.e., $n=4-10$). Embryos were isolated in PBS, cleaned and staged according to Eyal-Giladi and Kochav (1976). The *area opaca* was discarded and the remaining *area pellucida* was dispersed into a single blastodermal cell suspension either by trypsin/EDTA or mechanically. Dispersed embryos were cultured separately for 48 hours at 37 °C/ 5 % CO₂ on glass coverslips in DMEM supplemented with antibiotics, glutamine and 10% FBS. Cells were fixed in Rossman's fluid and the number of PGCs determined by PAS staining. Difference between treatments was not statistically significant ($P<.05$)

This is in stark contrast to the number of PGCs without feeder layers of STO cells. Furthermore, the number of germ cells per 5000 blastodermal cells after 48 hours culture was relatively equal between stages suggesting that the results are due to the initiation of developmental processes *in vitro* and not simply to proliferation of unipotential PGCs. A typical response of dissociated

blastodermal cells cultured in this system was to form SSEA-1 negative and positive clonal patches (Plates 12, 13 and 14). PGCs were found to be in association with both types of patches. When examined on a per embryo basis, the number of PGCs was comparable to that usually found in the germinal crescent (Figure 3). Thus, these data are consonant with several studies suggesting an embryonic program by which the number of PGCs is controlled (Rogulska, 1968; Eyal-Giladi *et al.*, 1976; Ginsburg and Eyal-Giladi, 1989). In a parallel experiment, 5000 blastodermal cells were cultured without the feeder layers. In this case, the number of PGCs per embryo was significantly lower than that obtained from cultures initiated on feeder layers of STO cells but was the same as that obtained when whole embryos were dissociated and cultured on glass coverslips. Therefore, STO cells provide the factor or factors necessary to implement cellular/molecular events for the development of avian PGCs from dispersed embryo cells that do not have the capacity to spontaneously yield PGCs under *in vitro* conditions. This is evident from the differential results obtained with St. IX. Therefore, we are confident that culture on STO cells provides a model system to examine the factor(s) associated with the development and proliferation of avian PGCs. Hence, it would appear that the blastoderm does not harbor committed PGCs until Stage X and that additional development of the germ cells requires specific cellular contacts or a threshold level of organization.

Figure 3. Mean $\pm$ s.e. PGCs after culture on STO feeder cells. Embryos were isolated, cleaned and staged according to the scheme of Eyal-Giladi and Kochav (1976) in DMEM supplemented with antibiotics, L-glutamine and 10 % FBS. *Area pellucida* of the same stage embryos were pooled and dissociated by trypsinization. The number of blastodermal cells obtained per embryo was determined and 5000 cells/well were seeded onto feeder layers of mitotically arrested STO cells in 96-well plates. The cells were cultured for 48 hours at 37° C/ 5% CO₂. The cultures were fixed in 4% paraformaldehyde overnight. PGCs were identified on the basis of their morphology and reactivity to anti-SSEA-1. N=10-25.

The SSEA-1 positive cells found on the epiblast side of the hypoblast were assumed to be PGCs on the basis of expected number, morphological similarity to PGCs stained with anti-SSEA-1 in the blood and gonad, and finally their association with the hypoblast. Therefore, we tested the hypothesis that SSEA-1 positive cells of the hypoblast can give rise to the germ line *in vivo* and *in vitro* using the chick/quail chimera system. Since quail PGCs are PAS negative (Nakamura *et al.*, 1991 ; Nakamura *et al.*, 1992), it was possible to identify chicken PGCs by PAS staining in the chimeric gonad of recipient quail and also on a feeder layer of quail blastodermal cells. SSEA-1 positive cells gave rise to PGCs both *in vivo* and *in vitro*. No PGCs were detected in chimeras obtained from the injection nor after the culture of SSEA-1 negative cells on a quail blastodermal cell feeder layer. The number of PGCs derived from the SSEA-1 positive cells was

always lower than that expected suggesting that not all of the positive cells of the hypoblast are restricted to the germ cell lineage at St. XII - XIV. Alternatively, the low numbers could be due to viability effects of the vital labeling procedures. Nevertheless, to our best knowledge, the SSEA-1 positive cells of the hypoblast represent the earliest identifiable population of cells that have been demonstrated to give rise to the germline in the avian embryo.

The results of the above experiments clearly indicate that germline segregation in the chick embryo begins at Stage X and continues through to Stage XIV. Germ cells do not arise from a discrete region of the embryo, but emerge in a scattered fashion from the *area pellucida* and collect on the hypoblast. Finally, normal cell-to-cell contact among cells of the *area pellucida* are required for germ cell emergence and suggests the existence of specific intercellular signaling events that lead to germ cell segregation.

Objective 2: to determine the best location and stage of development to transfer donor cells for efficient germline chimerism (HUJ).

Given the results of objective 1 which indicate that avian germ cells do not arise from a particular localized areas in the pre-primitive streak embryo, the original strategy for constructing a germ-line chimera had to be altered. In order to have efficient germline chimerism, cells from a large portion of the *area pellucida* would have to be used.

Materials and Methods:

Approach I (HUJ)

The first step undertaken was to try to construct a quail/chick germline chimera by transplanting a central area at Stage X *in ovo*. This attempt failed since the blastoderm is too weak to endure the pressure exerted by the yolk without mechanical protection by the vitelline membrane. Therefore, the slightest tearing of the vitelline membrane above the embryo immediately leads to a complete rupture. Alternatively, quail/chicken germline chimeras at Stage X were constructed *in vitro*. The chimeras were made by isolating the central area consisting of about 1/3 the diameter from one species and replacing it with an homologous area removed from the other species (Figure 4). After 48h of incubation using New's system the chimeras were fixed in Rossman's solution and were stained as whole mounts with PAS. After counting the chicken PGCs, the whole mounts were processed for histology, serially sectioned and the numbers of the chicken and quail PGCs were assessed. Thirty chimeras using quail as a recipient and a chicken central area were constructed.

Approach II (HUJ)

This series of experiments served two purposes, 1) to extend the period of development of an *in vitro* cultured embryo beyond the second day of incubation and 2) to rescue an embryo grown *in vitro* for 48 hours by transfer to an *in ovo* recipient. (See Figure 5)

This was done in 4 ways:

- 1) A yolk sac chimera was made by using a composite embryo from which its cranial part was taken from a chimera made at Stage X-XII using 1/3 of the central disk of a quail transplanting into a chick recipient, and incubating it for a further two days.
- 2) A yolk sac chimera was made by using a composite embryo from which the anterior (cranial) half came from a chick embryo cultured for two days and the posterior part was donated by a normal chicken embryo from a egg incubated for two days.
- 3) A yolk sac chimeras was made by using a composite embryo made from the anterior and posterior segments of a normal two-day incubated egg.
- 4) A yolk sac chimera made of a normal chicken embryo isolated from an egg after two days of incubation and transplanted onto the yolk sac of a similar embryo.

Groups 3 and 4 were used mainly as controls. The technique was mastered within a short time, relatively, due to the kind advice of Dr. Claude Martin. Groups 1 and 2 contained yolk sac chimeras of which the anterior part of the composite embryo was previously developed in vitro for two days.

Figure 4. A schematic representation of the development of a composite embryo made from the transplantation of the inner 1/3 of the central disk of a quail embryo which replaced an equivalent fragment in a chicken embryo. These composite embryos were then cultured on vitelline membranes for 48 h, and PGCs were examined in the germinal crescent.

Figure 5. A schematic representation of the production of a yolk sac chimera to extend development. The composite embryo cultured for 2 days as described in Figure 4 was too small to use for a yolk sac chimera. Instead, the anterior half of the 2 day in vitro cultured embryo was matched to the posterior half of a normal embryo obtained from an incubated egg. This composite embryo was then transplanted in ovo into a yolk sac to form the yolk sac chimera which was incubated for an additional two days. This procedure allowed the further development of the original in vitro cultured embryo.

Figure 4.

Figure 5.

The final approach to this objective utilized microinjections of lysinated-rhodamine dextran into individual cells at various areas of the Stage X chick blastoderm. This approach had been modified to use the carbocyanine dye Dil as a label and was restricted to in vitro culture. Dil was applied extracellularly on one or a few clusters of cells that ingress from the ventral surface of the epiblast. This labeling was followed using the procedure described by Selleck and Stern (1991). Blastoderms at Stages X and XIII were prepared according to New (1955) with the ventral surface facing up while the dorsal surface of the embryo is attached to the vitellin membrane. Stock solutions of Dil were made at a concentration of 0.5 in ethanol. The working solution was made 1:9 in 0.3M sucrose. The dye was applied through a pulled glass electrode connected to an injection apparatus attached to a Leitz micromanipulator. At Stage X the ingressed clusters of cells were readily accessible. For Stage XIII embryos the hypoblast had to be peeled back to label ingressing cells and was put back in place after injection. The embryos were incubated for two days and then fixed in Rossam's fluid and washed in ethanol. Fluorescence was checked under a fluorescent microscope. To allow histological processing, the Dil was photoconverted to an insoluble product using 3, 3'-diaminobenzidine. This step was achieved by UV irradiation of the embryos in DAB until evidence of fluorescence disappeared. The embryos were then stained with PAS and processed for paraffin sections. Sections were counter stained with hematoxylin. Five embryos at Stage X and 15 at Stage XIII were examined.

Results and Discussion:

Quail/Chick Chimeras -

Series A:

Chimeras (30) were constructed using 1/3 of the diameter of the *area pellucida* of chicken embryos and was transplanted into a quail recipient. Of these, 18 developed into an embryo with a normal axis. Seven of these were stained with PAS as whole mounts and were examine for the distribution of PAS positive PGCs. The whole mount assessments were later confirmed by detecting both the chicken and quail PGCs in histological sections. The chicken PGCs were located within the germinal crescent either in a concentrated region or evenly distributed (Plate 12).

Table 1. Embryos from Series A in which PGCs were evenly distributed:

Embryo #	Quail	Chick	cPGCs	qPGCs	%qPGCs
9R	XII+	X+	200	12	5.7
11R	XI	XII	155	65	29
14R	XII	XII	186	28	13

Table 2. Embryos from series A in which PGCs were collected in one region of the embryo.

Embryo #	Quail	Chick	cPGCs	qPGCs	%qPGCs
7R	XII	XI+	122*		
8R	XII	X	46	141	33%
10R	XII	XI+	44*		
24R	XII	XII	32	139	50%

*cPGCs in whole mounts

Table 3. Embryos from series B

Embryo #	Quail	Chick	cPGCs	qPGCs	%qPGCs
34R	XII+	XII	51*		
36R	XII	XI	82	153	
39R	XII	XI	35	117	

The PGCs were always detected in the area of the germinal crescent of the chimeric embryo, after it had reached Stage 10. As demonstrated above, the relative contribution of the central area versus the peripheral area to the PGC population was variable. It would appear that about 2/3 of the PGC population was derived from the transplanted central area. At the same time, histological analysis of the embryos which developed in the chimeras were derived mainly from the recipient component. No specific staining was done to differentiate the quail somatic

cells from the chicken cells. However, the general subjective impression has been that in most chimeras made with the 1/3 of the central disk of a chick embryo the anterior regions of the nervous system, usually the prosencephalon, were derived from the donor chick cells while the remaining portions were from the recipient quail cells. However, in the reciprocal experiments, the somatic tissues were mainly of donor quail cells. It was necessary to extend the time of culture beyond 48h to study the fate of PGCs in the recipient. Several techniques were tried: 1. transfer of the chimeric embryo onto a fresh vitelline membrane after 48h. 2. transfer of the embryo after the first 48h onto an enriched agarose substrate. 3. transfer of the embryo onto the chorio-allantoic membrane of an 8-day chicken embryo. None of the above systems supported development of the embryo. Finally, it became obvious that the only means to allow further development of the *in vitro* cultured chimera was to introduce it back into an egg. A technique for constructing yolk-sac chimeras was devised by Claud Martin (1972). The process is rather complicated and difficult to master. It involves removing the embryo proper from the recipient egg and leaving only its yolk sac and implanting in its place a donor embryo. The edges of the transplanted embryo and the recipient yolk-sac are seamed together by resectioning their margins simultaneously with Pashew scissors. The egg is then sealed and reincubated. This technique could be carried out on embryos ranging from 8 to 22 pairs of somites. Transplantation of *in vitro* cultured embryos has never been attempted until now.

Attempts to transplant *in vitro* cultured quail/chicken chimeras developed from Stages X-XII were not successful since even after 48h of culture the chimera was too small to replace a normal embryo of the same developmental stage (Figure 5). Rather than grafting the whole embryo, only the anterior portion of the chimera, which would contain the PGCs, was transplanted to recipient

eggs. This operation involved constructing a whole embryo from the anterior region of the quail/chicken chimera and the posterior region from the recipient embryo. A normal chicken embryo at Stage 10 was isolated and placed back to back and head to tail in relation to the quail/chick chimera. The two embryos were cut simultaneously, perpendicular to the long axis. Cutting ensured adherence of the complementary anterior end of the *in vitro* embryo and posterior end of the normal embryo. In a second step, the composite hybrid embryo was grafted onto a recipient yolk sac (Figure 5). Once the procedure was mastered, the transplanted whole embryos would have circulation develop across the seam allowing development of the embryo. Some hybrids, constructed according to the above plan continued to develop for a few days, but were not yet normal.

Table 4: Survival of Yolk Sac Chimeras after 2-3 days of Incubation:

Group	Number Made	No. Survived	% Survival
1	58	19	33
2	28	3	11
3	40	19	48
4	14	10	71

Groups 1 and 2 contain embryos in which the anterior portion has been previously developed in New's culture. These groups had a survival rate of 25.6%. The construction of a composite embryo was the main obstacle to this complex system, whether using *in vitro* cultured material or embryos developed *in ovo*. The neural system must be correctly joined between the two parts so that the future development of the heart is relatively normal (50) the embryo will survive the required time. Most of the embryos from group 4 appeared normal and healthy after 2-3 days of incubation (Plate 16). Most of the yolk sac chimeras in the other three groups had severe distortions of the long axis, i.e. bent and twisted (Plate 17). Since a yolk sac chimera cannot be made in a quail egg, we had to construct the *in vitro* chimeras in such a way that the transplanted 1/3 of the central disc would be of quail origin. In histological sections, chick & quail PGCs were found (Plate 18).

Analysis of Dil Labeled Cells

The ingressed clusters that were labeled with Dil in the center of the embryo were detected in anterior regions of the embryo later in incubation. At the end of incubation fluorescence was

found at the germinal crescent. DAB labeling was found in sections of the blastoderms marked at Stage X or Stage XIII in cells exclusively in the middle layer of the extraembryonic mesoderm.

Objective 3: to transfect donor cells and produce transgenic/germline, chimeric embryos.

Materials and Methods

This was approached with the cooperation of both laboratories. As the results of objectives 1 and 2 appeared, the primary ideas of the project had to be adapted to the facts which

became established. Specifically, the germline begins to segregate very early at Stage X and that the segregation process is gradual. Most importantly, PGCs are formed from the ventral surface of the *area pellucida* in a scattered pattern and not from one specific area as in other vertebrates. In an attempt to transfer foreign DNA into PGCs, the liposome N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium sulfate (DOTAP) was used. The reporter construct used was pmiwZ. This plasmid contains the bacterial beta-galactosidase from *E. coli* expressed with chicken beta-actin and Rous' Sarcoma Virus promoters in tandem. DOTAP (45 μ l) was diluted in 5 μ l of Hank's balanced salts (HBS) and was mixed with 50 μ l of plasmid DNA diluted in HBS at a concentration of 0.225 μ g/ μ l. The lipid to DNA ratio was 4:1.

Eggs were positioned horizontally 24 hrs prior to injection. A window was opened in the equatorial region of the eggshell. Several drops of Tyrode's solutions assisted viewing and positioning of the blastoderm. About 5 μ l of the DOTAP:DNA mixture was injected using a micropipet. After injection, the air cell was punctured and the embryo was lowered back to its original position. The window in the shell was sealed and the egg was maintained at 37 C for 2 days.

In another experiment, unincubated blastoderms were isolated from eggs and were incubated in the liposome:DNA complex diluted in Tyrode's solution for 90 minutes at 37 C. Several dilutions of the DNA complex were tested: 1) undiluted, 2) 1:1, 3) 1:20, 4) 1:100. After the treatment, the blastoderms were transferred onto a vitelline membrane and were further incubated in vitro using New's culture for 2 days. Subsequently, the embryos were washed with

PBS and stained for bacterial beta-galactosidase activity according to Brazolot *et al.* (1991). After viewing the embryos and photodocumentation, those that contained stained cells were dehydrated in an ethanol series, embedded in paraffin and serially sectioned. Sections were counter stained with hematoxylin.

Results and Discussion:

From the embryos that were injected *in ovo*, only 2 out of 60 stained cells were detected at the boundary of the *area pellucida* and the *area opaca* (Plate 19). No cells appeared to exhibit clonal growth. In sections, the stained cells appeared only as part of the extraembryonic endoderm.

Efficient transfection of target cells is a major hurdle required for the routine production of transgenic animals of any species. In the present study, this issue was approached with the idea of limiting the transfection procedures to the source of the germ cell lineage in the developing early embryo so that incorporation events could be amplified as the germ cells naturally proliferate. However, as can be seen from the results of objectives 1 and 2, the development of the germ cell lineage does not lend itself to this approach since germ cells do not arise from one particular cluster of cells in the embryo. Nevertheless, several attempts were made to overcome this obstacle using liposome-mediated transfection. Admittedly, such an approach does not overcome the second issue in regards to transfection efficiency, that of stable incorporation of the exogenous DNA into the genome. During this study, however, stable incorporation was a secondary issue dependent upon efficient transfection of the embryo cells themselves. Recently, other groups have reported on liposome mediated transfection of the early avian embryo. For the

most part, the expression of DNA appears to be highly transient. Our work is in agreement with these findings.

Description of Cooperation:

Throughout the project period, both laboratories have been in constant communication. Initially, this was through the transmission of letters using a fax-machine; however, during the project, both laboratories acquired electronic mail. This resulted in extensive, cost effective communication between the two laboratories. Over 250 communications were exchanged electronically during the project. This yielded not only an exchange of results but of procedures and reagents useful in various phases of the project. NCSU provided co-investigators with plasmids, several antibodies (QH-1, EMA-1, SSEA-1), and detailed procedures on using these reagents in their work. Several independent experiments using different approaches were carried out that verified the results of each laboratory. For example, NCSU used immunohistochemistry and cell culture to indicate that germ cell development began at Stage X, while HUJ used a series of *in vitro* and *in ovo* transplantation experiments that complemented these results. The conclusions from both of these approaches were basically the same thereby strengthening the overall results. Exchange visit to each laboratory were particularly fruitful. Investigators at the HUJ were able to examine first-hand the results of the immunohistochemistry slides that were done at NCSU. Likewise, the results and problems associated with the technical aspects of the chimera work at HUJ were addressed during the same visit. In addition, the NCSU visiting

scientist was able to provide a seminar for the host institution on various aspects of transgenesis in birds. The visit by Dr. Mike Ginsburg to NCSU was an intensive two weeks for the host laboratory. All of the remaining issues and interpretations of the results were debated with several laboratory staff. Dr. Ginsburg's visit to NCSU culminated in a presentation at the symposium "Current Advances in Avian Embryology and Incubation" at the annual meeting of the Poultry Science Association. This provided a unique opportunity for exchange beyond the two laboratories.

Evaluation of the Research Achievement:

In surveying the research directions in attempts to develop transgenic poultry, the use of chimeric intermediates is at the forefront of technology at this time. The two approaches to producing germ line chimeras are the transfer of blastodermal cells from the uncubated embryo and the transfer of committed primordial germ cells. The former method is really a form of primordial germ cell transfer in that it is hoped that precursors of primordial germ cells will be contained in the mixture of cells injected into donor embryos. One implicit assumption in the use of blastodermal cell transfer has been that the Stage X embryo does not harbor committed germ cells. Until now, this assumption was based upon the identification of committed PGCs only at germinal crescent stages of development. However, the present study clearly shows that cells which give rise to the avian germ line begin to segregate at Stage X, 2/3 of the germ cell population arises from the inner 1/3 of the *area pellucida*, that the *area pellucida* has the potential to give rise to germ cells as it is formed at Stages VII-IX but loses this capacity if disorganized, and that a population of cells on the hypoblast of the Stage XIII embryo, which is immunologically

distinct, can give rise to germ cells. In addition, cell lineage experiments suggests that immunologically identifiable germ cells arise from the polyingressing cells beneath the epiblast between Stages X-XIII in association with cells that will give rise to extraembryonic mesoderm. Thus, the process of germ cell segregation in the early avian embryo is much more complex than previously observed. All of these features have implications for strategies designed to efficiently introduce novel genes into the germline of the chicken. Attempts to transect early germ cells using liposome-mediated transfection were problematic, specifically because of the biology of germ cell origins. Efficient transfection and stable integration of DNA into the avian genome will require the ability to culture germ cells or their precursors. In this regard, this project has begun the first systematic efforts towards this goal through the development of an *in vitro* assay for PGC emergence. This assay represents the first time that avian PGCs could be cultured in the absence of organized embryonic tissue. This assay is significant in that it is now possible to quantitate the effect of peptide growth factors on the emergence of PGCs *in vitro*. This could very well lead to culture conditions that would allow the transfection of PGCs and amplification of PGCs that have integrated the exogenous DNA for the production of transgenic/chimeric intermediates. Therefore, we have successfully completed objectives 1 and 2, and the results of the latter have given new directions for further research on objective 3. Manuscripts detailing this work are currently being prepared for publication.

Conclusions:

The work of the present project shows that the development of the avian germ line is a complex process. Cells with the potential to give rise to the germ line are acquired with the formation of the *area pellucida* at St. VII-IX, but that potential is lost without normal cell-to-cell contacts. The process of germ cell emergence begins in earnest at St. X, about the time of oviposition of the egg, and continues to the formation of the primitive streak. About 2/3 of the germ cell lineage is contributed by the inner 1/3 of the *area pellucida*. Immunologically distinct cells accumulate on the hypoblast between Stages XII-XIV and are the earliest identifiable population known to give rise to the germ line. These cells first appear associated with the polyingressing cells of the epiblast and may be linked to the development of the extraembryonic mesoderm. This developmental history of PGCs explains the difficulties involved in introducing exogenous DNA into the avian germ line. However, the *in vitro* assay of germ cell emergence developed during this work may lead to the identification of culture conditions that allow efficient growth of PGCs for stable integration of exogenous DNA.

Publications:

Publications of this work are currently in preparation.

References:

- Bosselman, R.A., H. Rou-Yin, T. Boggs, S. Hu, J. Bruszewski, *et al.* (1989) Germline transmission of exogenous genes in the chicken. *Science* 243:533-535.
- Brazolot, C.L., J.N. Petite, R.J. Etches, and A.M. Verrinder Gibbins (1991) Efficient transfection of chicken cells by lipofection and introduction of transfected blastodermal cells into the embryo. *Molecular Reproduction and Development* 30:304-312.
- Carscience, R.S., M.E. Clark, A.M. Verrinder Gibbins, and R.J. Etches (1993) Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. *Development* 117:669-675.
- Canning D.R. and C.D. Stern (1988) Changes in the expression of the carbohydrate epitope HNK-1 associated with mesoderm induction in the chick embryo. *Development* 104:643-655.
- Donovan, P.J. (1994) Growth factor regulation of mouse primordial germ cell development. Pages 189-218. In: *Current Topics in Developmental Biology*, R. Pederson ed., Academic Press, New York.
- Eyal-Giladi, H. and S. Kochav (1976) A complementary normal table and a new look at the first stages of the development of the chick. 1. General morphology. *Developmental Biology* 49:321-337.

- Eyal-Giladi, H., S. Kochav, and M.K. Menashi (1976) On the origin of the epimordial germ cells in the chick embryo. *Differentiation* 6:13-16.
- Ginsburg, M. And H. Eyal-Giladi (1986) Primordial germ cells of the young chick blastoderm originate from the central zone of the A. pellucida irrespective of the embryo-forming process. *Development* 101:209-211.
- Ginsburg, M. and H. Eyal-Giladi. (1989) Primordial germ cell development in cultures of dispersed central disks of Stage X chick blastoderms. *Gamete Research* 23:421-427.
- Ginsburg, M., J. Hochman, and H. Eyal-Giladi (1989) Immunohistochemical analysis of the segregation process of the quail germ cell lineage. *International Journal of Developmental Biology* 33:389-395.
- Gooi, H.C., T. Feizi, A.Kapadia, B.B. Knowles, D. Solter, and M.J. Evans (1981) Stage-specific embryonic antigen 1 involves $\alpha 1 \rightarrow 3$ fucosylated type 2 blood group chains. *Nature* 292:156-158.
- Gordon, J.W., J.A. Scangos, D.J. Plotkin, J.A. Barbosa, and F.H. Ruddle (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. *Proceedings of the National Academy of Science USA* 77:7380-7384.

- Harper, E.H. (1904) The fertilisation and early development of the pigeon's egg. *American Journal of Anatomy* 3:349-386.
- Love, J., C.Gribbin, C. Mather, and H. Sang (1994) Transgenic birds by DNA microinjection. *Biotechnology* 12:60-63.
- Naito, M., K. Nirasawa, and T. Oishi (1990) Development in culture of the chick embryo from fertilized ovum to hatching. *Journal of Experimental Zoology* 254:322-326.
- Naito, M., M. Watanabe, M. Kinutani, K. Nirasawa, and T. Oshi (1991) Production of quail-chick chimeras by blastoderm cell transfer. *British Poultry Science* 32:79-86.
- Nakamura, M., H. Maeda, and T. Fujimoto (1991) Behaviour of chick promordial germ cells injected into the blood stream of quail embryos. *Ikakimas Folia Anatomy Japan* 67:473-478.
- Nakamura, M., K. Yoshinaga, and T. Fujimoto (1992) Histochemical identification and behaviour of quail promordial germ cell injected into chick embryos by the intravascular route. *The Journal of Experimental Zoology* 261:479-483.

- New, D.A.T. (1955) A new technique for the cultivation of the chick embryo *in vitro*. *Journal of Embryology and Experimental Morphology* 3:320-331.
- Palmiter, R.D. and R.L. Brinster (1985) Transgenic mice. *Cell* 41:343-345.
- Perry, M.M. (1987) Nuclear events from fertilisation to the early cleavage stages in the domestic fowl (*Gallus domesticus*). *Journal of Anatomy* 150:99-109.
- Perry, M.M. (1988) A complete culture system for the chick embryo. *Nature (London)* 331:70-72.
- Petitte, J.N., C.L. Brazolot, M.E. Clark, G. Liu, A.M. Verrinder Gibbins, and R.J. Etches (1993) Accessing the genome of the chicken using germline chimeras. Pages 81-101. In: *Manipulation of the Avian Genome*, R.J. Etches and A.M. Verrinder Gibbins eds., CRC Press, London.
- Petitte, J.N., M.E. Clark and R.J. Etches (1991) Assessment of functional gametes in chickens after transfer of primordial germ cells. *Journal of Reproduction and Fertility* 92:225-229 .
- Petitte, J.N. M.E. Clark, G. Liu, A.M. Verrinder Gibbins, and R.J. Etches (1990) Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. *Development* 108:185-189.

- Piedrahita, J.A., L. Guillespie, and N. Maida (1990) Establishing an embryonic (ES) stem cell line utilizing hamster embryos. *Biology of Reproduction* 42(Suppl. 1):175.
- Riese, J., R. Zeller, and R. Dono (1995) Nucleo-cytoplasmic translocation and secretion of fibroblast growth factor-2 during avian gastrulation. *Mechanisms of Development* 49:39-22.
- Rogulska, T. (1968) Primordial germ cells in normal and transected duck blastoderms. *Journal of Embryological Experimental Morphology* 20:247-260.
- Rowlett, K. and K. Simkiss (1987) Explanted embryo culture: *in vitro* and *in ovo* techniques for the domestic fowl. *British Poultry Science* 28:91-101.
- Salter, D.W., E.J. Smith, S.H. Hughes, S.E. Wright, and L.B. Crittenden (1987) Transgenic chickens: insertion of retroviral genes into the chicken germline. *Virology* 157:236-240.
- Salter, D.W., E.J. Smith, S.H. Hughes, S.E. Wright, A.M. Fadly, R.L. Witter, and L.B. Crittenden (1986) Gene insertion into the chicken germ line by retroviruses. *Poultry Science* 65:1445-1458.
- Sang, H. and M.M. Perry (1989) Episomal replication of cloned DNA injected into the fertilized ovum of the hen, *Gallus domesticus*. *Molecular Reproduction and Development* 1:89-106.

- Selleck, M.A.J. and C.D. Stern (1991) Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* 112:615-626.
- Shuman, R.M. and R.N. Shoffner (1986) Gene transfer by avian retroviruses. *Poultry Science* 65:1437-1444.
- Simkiss, K., K. Rowlett, N. Bumstead, B.M. Freeman (1989) Transfer of primordial germ cell DNA between embryos. *Protoplasma* 151: 164-166.
- Simkiss, K., L. Vick, G. Luke, N. Page, and D. Savva (1990) Infection of primordial germ cells with defective retroviruses and their transfer to the developing embryo. *Proceedings 4th World Congress on Genetics Applied to Livestock Production, Edinburgh, XVI*, 111-114.
- Souza, L.M., T.C. Boone, D. Murdick, K. Langley, J. Wypych, *et al.* (1984) Application of recombinant DNA technologies to studies on chicken growth hormone. *Journal of Experimental Zoology* 232:465-473.
- Stern, C.D. and D.R. Canning (1990) Origin of cells giving rise to mesoderm and endoderm in chick embryo. *Nature* 343:273-275.
- Temin, H.M. (1989) Retrovirus vectors: promise and reality. *Science* 246:983.

Urven, L.E., C.A. Erickson, U.K. Abbott, and J.R. McCarrey (1988) Analysis of germ line development in the chick embryo using mouse EC cell antibody. *Development* 103:299-304.

Wentworth, B.C., H. Tsai, J.H. Hallett, D.S. Gonzales, and G. Rajcic-Spasojevic (1989) Manipulation of avian primordial germ cells and gonadal differentiation. *Poultry Science* 68:999-1010.

Plate Legends:

Plate 1. Blood smear taken from a Stage 15 embryo stained with anti-SSEA-1. Note that the large primordial germ cells are distinctively stained (arrow).

Plate 2. Dispersed gonadal cells from a 6 day embryo. Cells were stained with anti-SSEA-1 followed by periodic acid Schiff. All germ cells were stained with PAS and SSEA-1 (arrows).

Plate 3. Low magnification of a whole Stage IX embryo after immunohistochemical staining for SSEA-1. No part of the embryo is stained.

Plate 4. High magnification of a whole Stage IX embryo after immunohistochemical staining for SSEA-1. None of the cells show any staining.

Plate 5. Low magnification of a whole Stage X embryo after immunohistochemical staining for SSEA-1. At this stage, stained cells are located in the *area pellucida* most often associated with the polyingressing cells on the ventral surface of the hypoblast.

Plate 6. High magnification of the same embryo in Plate 5 showing the staining of the polyingressing cells on the ventral surface of the epiblast in the region of the *area pellucida*.

Plate 7. A high magnification of a polyingressing cell stained for SSEA-1 in association with unstained polyingressing cells.

Plate 8. Low magnification of a whole Stage XIII embryo with the hypoblast removed. Numerous SSEA-1 positive cells can be seen outlining the *area pellucida*.

Plate 9. High magnification of the same embryo in Plate 8 at the border between the *area pellucida* and the *area opaca*. Note that SSEA-1 expression is limited to the *area pellucida* and the varying intensities of staining.

Plate 10. Low magnification of a Stage XIII hypoblast stained with anti-SSEA-1. Even at this low magnification, distinctive staining of cells can be seen.

Plate 11. High magnification of the SSEA-1 staining of cells on the Stage XIII hypoblast. Stained cells occur singly or in clusters and can only be found on the side adjacent to the epiblast.

Plate 12. Low magnification of SSEA-1 staining of 5,000 dispersed Stage X blastodermal cells cultured on an STO feeder layer. Note the patches of stained cells.

Plate 13. Higher magnification of a region in Plate 12 showing SSEA-1 stained PGCs.

Plate 14. High magnification of PGCs in Plate 13 showing the morphological detail of the PGCs stained using anti-SSEA-1.

Plate 15. (A) Whole mount of a 2 day *in vitro* cultured quail embryo whose 1/3 central disk (CD) has been previously replaced by a CD from a chick embryo of the same stage. The arrows indicate chicken PGCs localized within a defined area lateral to the head. (B) Whole mount of a 2 day chicken embryo cultured *in vitro* in which the inner 1/3 of the CD was replaced with the same area taken from a quail embryo. Chicken PGCs revealed by PAS staining were detected only at the area marked by the arrow. (C) Higher magnification of the area indicated in B.

Plate 16. (A) A two day yolk sac chimera made of a quail/chick chimera which was cultured *in vitro* and then transplanted *in ovo* as a composite embryo. (B) A two day yolk sac chimera made of a 2 day old *in ovo* embryo.

Plate 17. A yolk sac chimera made of a 2 day embryo that has been transplanted into the yolk sac of another embryo and was cultured for two more days.

Plate 18. Quail PGCs are detected adjacent to chicken PGCs (PAS stained) in sections of a yolk sac chimera in which the anterior part was contributed by an *in vitro* chimera whose 1/3 central disk was transplanted from a quail embryo.

Plate 19. Ventral view of an embryo that was transfected *in vitro* with pmiwZ using DOPTAP (1:2) in Tyrode solution for 90 min and cultured *in vitro* for 2 days. Dark spots indicate clonal staining for bacterial beta-galactosidase activity which was limited to the extraembryonic areas.

6

8

5

7

10

12

9

11

13

14

16

A

B