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Analysis of germ line development in the chick embryo using an anti-mouse EC cell antibody

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Summary

We have found that EMA-1, a monoclonal antibody originally raised against mouse embryonal carcinoma (Nulli SCC1) cells (Hahnel & Eddy, 1982), also labels chick primordial germ cells (PGCs). We have used this antibody in immunohistological studies to follow the development of PGCs in the chick embryo from the time of their initial appearance beneath the epiblast, through their migratory phase and subsequent colonization of the germinal epithelium. During hypoblast formation, individual EMA-1-labelled cells appeared to separate from the basal surface of the epiblast and enter the blastocoel, coincident with the appearance of morphologically identifiable PGCs in this same area. EMA-1 continued to label germ cells until the initiation of gametogenesis in each sex; specifically, labelling was absent by 7–8 days of incubation in females and started to decrease at 11 days of incubation in males. There was a recurrence of the epitope on oogonia at 15 days of incubation, but not on spermatogonia during the remainder of development through hatching. These observations are consistent with an epiblast origin for the avian germ line, and are strikingly similar to those reported for the early mouse embryo using the same antibody (Hahnel & Eddy, 1986).

Key words: primordial germ cells, EMA-1, monoclonal antibody, cell marker, chick embryo, germ line.

Introduction

The germ line, whether viewed as a continuum of totipotent cells or a newly formed cell line arising from somatic elements of each embryo, is, by definition, the only link between subsequent generations. Because of their unique role in the life cycle, the development of germ-line cells is particularly intriguing, especially with regard to understanding their determination, origin and relationship to somatic cells. Addressing these topics requires the ability to recognize and, ultimately, manipulate germ cells early in development.

Neither morphology nor the PAS marker can distinguish avian primordial germ cells (PGCs) prior to gastrulation, when they appear in the germinal crescent. Investigation of PGC origin has been limited largely to indirect *in vitro* studies in which chick–quail chimaeras are constructed, using epiblast and hypoblast from different species (Eyal-Giladi *et al.* 1981) or by culture of isolated hypoblasts and epiblasts until PGCs could be recognized by PAS staining (Sutasurya *et al.* 1983; Ginsburg & Eyal-Giladi, 1986). These studies all concluded that PGCs derive from the epiblast at stages prior to those at which they are identifiable by either morphological or histochemical criteria, and that the germinal crescent endoderm hypoblast is a secondary location in PGC development.

In order to investigate directly the origin of PGCs *in situ*, a germ cell marker is required that can distinguish PGCs at stages prior to the time when they become morphologically distinct. In this study, we use the antibody EMA-1 as a PGC marker in chick embryos. EMA-1, a monoclonal IgM antibody produced against a glycoprotein cell surface antigen of the embryonal carcinoma Nulli SCC1 (Hahnel & Eddy, 1982), recognizes fucosylated polylactosamine carbohydrate groups and has been previously shown to label murine PGCs (Hahnel & Eddy, 1986). We demonstrate here that EMA-1 labels chick germ cells at an earlier stage and for a longer period than PAS

staining does, and we provide additional evidence consistent with an epiblast origin of PGCs in the chick.

Materials and methods

Fertile White Leghorn eggs were purchased from a local commercial supplier (Donsing, Rio Linda, California). Eggs were incubated at 37·5°C and 55 % relative humidity.

Immunohistochemical or immunofluorescent studies using EMA-1 antibody (kindly provided by Dr E. M. Eddy) employed a total of 100 chick embryos and hatchlings ranging from preincubation embryos to newly hatched chicks. Both cryostat and paraffin sections were prepared. Each embryo was staged according to criteria established by Eyal-Giladi & Kochav (1976) or by Hamburger & Hamilton (1951).

Cryostat sections

Embryos were prepared for cryosectioning by fixing for 15 min in 4 % paraformaldehyde, infiltrating at room temperature for 2 h each in 15 % and 30 % sucrose and freezing in OCT compound (Miles Laboratories) under liquid nitrogen. 12 μm sections were cut on a Bright Cryostat and postfixed by dipping the slides in 0·4 % paraformaldehyde. A 1:10000 dilution of EMA-1 in phosphate-buffered saline (PBS) was applied to the sections for ½ h, followed by a PBS rinse. Sections were then incubated in fluorescein- or rhodamine-conjugated goat anti-mouse immunoglobulin secondary antibody (1:100 and 1:200 dilutions, respectively; Cooper Biomedical). Sections were mounted in 2 % *n*-propyl gallate (Giloh & Sedat, 1982) and examined with a Leitz Dialux 20 microscope equipped for epifluorescence.

Paraffin sections

Embryos were fixed in Bouin's fixative, with care taken to remove and fix the entire area vasculosa in embryos younger than stage 18. They were then dehydrated, embedded in Paraplast Plus (Monoject Scientific), serially sectioned at 10 μm, deparaffinized and stained as described above. Alternatively, paraffin sections were secondarily labelled with the avidin/biotin-conjugated-alkaline phosphatase system (Vectastain ABC-AP kit, Vector Laboratories) according to manufacturer's suggestions. Sections were counterstained with the cytoplasmic stain Fast Green. Avidin–biotin system-stained slides were mounted in Pro-Texx (Lerner Laboratories). Controls for nonspecific antibody binding substituted monoclonal mouse IgM anti-human IgG (1:500 dilution) for EMA-1 on sections taken at intervals from representative stages of chick. These were consistently negative with the exception of blood cells and occasional yolky endoderm cells, which showed nonspecific staining under the conditions described above.

PAS staining

Sections from 23 chick embryos ranging in age from unincubated embryos to 13 days of incubation were stained with PAS, providing a demonstration of PGC location independent of EMA-1 labelling. Whole embryos or gonads were fixed in Rossman's fluid, double-embedded in celloidin and paraffin (Humason, 1972), and cut at 10 μm. Sections were deparaffinized and hydrated, oxidized for 20 min in 1 % periodic acid (Sigma), rinsed with water, stained with Schiff's reagent (Sigma) counterstained with haematoxylin, cleared and mounted in Pro-Texx. In order to determine directly whether individual germ cells stained with *both* PAS and EMA-1, two chick embryos at stage 20 (3½ days of incubation) were fixed in high alcohol fixatives (Rossman's in one case and Gendre's in the other), embedded in paraffin and sectioned at 5 and 7 μm, respectively. Alternate sections were stained with PAS or labelled with EMA-1. All embryos stained with ABC-AP or PAS were studied and photographed using a Zeiss Photomicroscope III. Cell size was measured with an ocular micrometer.

Results

EMA-1 labels primordial germ cells

EMA-1 labelling of chick germ cells was initially tested on stage-20 embryos because PGC morphology, distribution and location at this stage provide additional and independent criteria for their identification. In all eight embryos examined, EMA-1 labelled large (10 μm in diameter or more), round to oval cells in the germinal ridge area, posterior to the exit of the vitelline artery from the dorsal aorta. The labelled cells were distributed in mesenchyme of the body wall ventral to the dorsal aorta, in mesenchyme of the mesentery, and in the epithelium at the coelomic angle. In separate preparations, PAS-stained germ cells were identical to the EMA-1-positive cells according to all the above criteria, indicating that EMA-1 does label chick PGCs at this stage.

In order to confirm that EMA-1 labels PGCs and to determine the efficiency of the EMA-1 label relative to the PAS marker, alternate sections were stained with EMA-1 or PAS, and counts per section were made of germ cells labelled by each method. EMA-1-positive cells were consistently PAS positive, although not all PAS-positive cells were EMA-1 labelled (Fig. 1). Quantitatively, EMA-1 labelled one-third to two-fifths as many cells as were stained with PAS (Table 1). The same ratio was seen when comparing germ cell number in all sections of separate stage-20 embryos, each prepared optimally for the separate staining regimens: fixation in Bouin's and paraffin embedment for EMA-1 labelling, and fixation in Rossman's and celloidin/paraffin embedment for PAS staining. In addition, the intensity of EMA-1 labelling varied from germ cell to germ cell. Sufficient numbers of PGCs were clearly labelled with EMA-1, however, to follow directly their migration and development, from their initial appearance be-

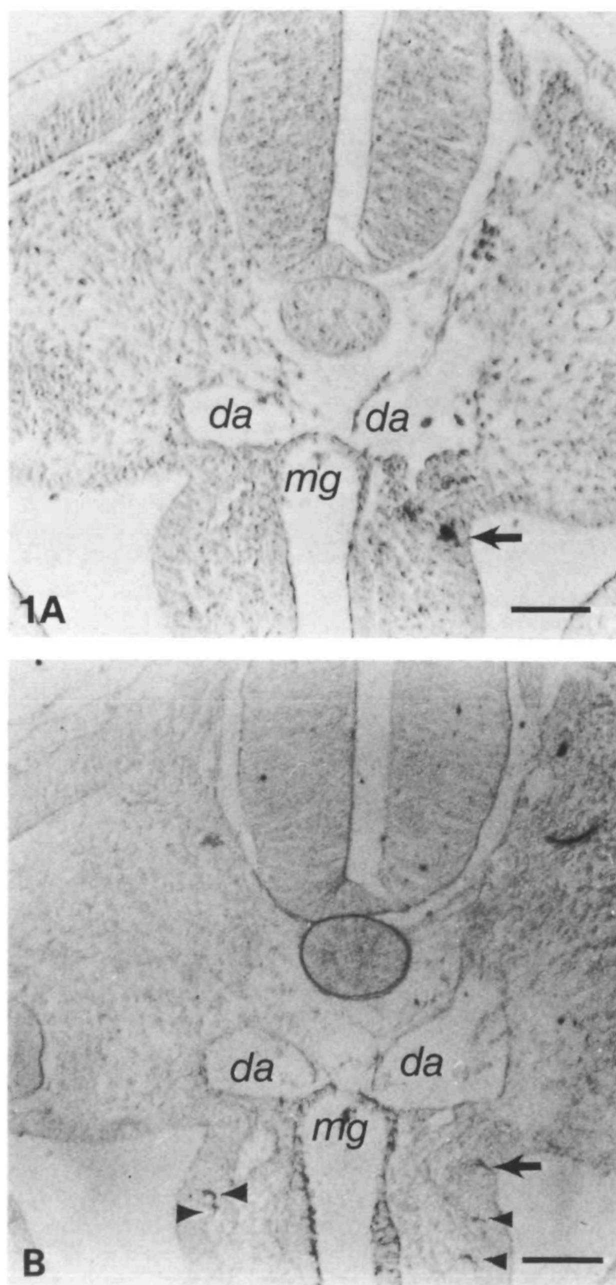

Fig. 1. Confirmation of PGC labelling with EMA-1. In a Gendre-fixed, paraffin-embedded stage 20 (3·5 days of incubation) chick embryo, EMA-1 labelled a subset of chick primordial germ cells in the germinal ridge area. These alternate sections show a PGC simultaneously labelled with EMA-1 (A) and with PAS (arrows) (B), as well as PAS-positive, EMA-1-negative PGCs (arrowheads). *mg*, midgut; *da*, dorsal aorta. Bars, 50 μm.

neath the epiblast through their migration to the germinal epithelium and their subsequent entry into gametogenesis.

Distribution of EMA-1 epitope

(1) *Somatic tissue labelling*

Unincubated embryos often had only a few labelled cells in the epiblast. Progressively older embryos had increasing numbers of epiblast cells labelled with EMA-1. By gastrulation, all of the epiblast cells in the centre of the area pellucida were strongly labelled with EMA-1, whereas labelled cells became gradually less common peripherally until none were seen in the area opaca. Mesenchymal cells moving through the primitive streak showed reduced intensity of label compared to the epiblast cells from which they had just detached. The head process stained slightly, and the notochord, paraxial, intermediate and lateral plate mesoderm all showed scattered antibody labelling immediately anterior to the regressing primitive streak. Labelling of the ectoderm derived from the epiblast ceased by the end of gastrulation, with the exception of some localized staining in the diencephalon and spinal cord. Between stages 13 and 19, portions of the medial midgut began to label with EMA-1. Progressively more foregut and midgut endoderm labelled in older embryos. Many of the mesonephric tubules stained after stage 25 (5 days of incubation).

(2) *Early development (0–24 h incubation)*

In two of nine unincubated (stage X–XI) embryos, no EMA-1-positive cells were found. In the other seven unincubated embryos, columnar cells of the epiblast had begun to label in a scattered fashion. By stage XII (6–12 h of incubation), some of the labelled and unlabelled epiblast cells appeared to be in the process of, or just completing, ingression into the blastocoel, either individually or in small clusters (Fig. 2A). In pre-primitive streak preparations that showed epiblast labelling, one to fourteen morphologically identifiable EMA-1-positive PGCs were located in the hypoblast (Fig. 2A) and in the blastocoel of each embryo, including some closely associated with the undersurface of the epiblast.

It was impossible to use PAS to identify PGCs definitively in stage X–XI (unincubated) and stage XII (midhypoblast formation) embryos because all cells of the embryo, as well as the underlying yolk, stained deeply (Fig. 2B). By stage XIV (pre-primitive streak), the PAS stain was more specific for germ cells and, in this and all older embryos, PAS-positive PGC morphology and distribution paralleled that described for the EMA-1 preparations.

(3) *PGC migration (1–5 days of incubation)*

EMA-1-labelled cells detaching from the epiblast were apparent as late as stage 7 (one somite). EMA-1-positive PGCs were identifiable throughout their migration route (reviewed in McCarrey & Abbott, 1979): in the germinal crescent during gastrulation, in the circulatory system during their transit through the blood vascular system, and in the germinal ridges and

Table 1.

	Gendre fix		Rosman fix		Rossman	Bouin
	PAS	EMA-1	PAS	EMA-1	PAS	EMA-1
Section width (μm)	7	7	5	5	10	10
Alternate sections	64	64	122	122	–	–
PGCs	136	46	433	175	238	99
PAS(+)/EMA-1(+)	2·96		2·47		2·40	

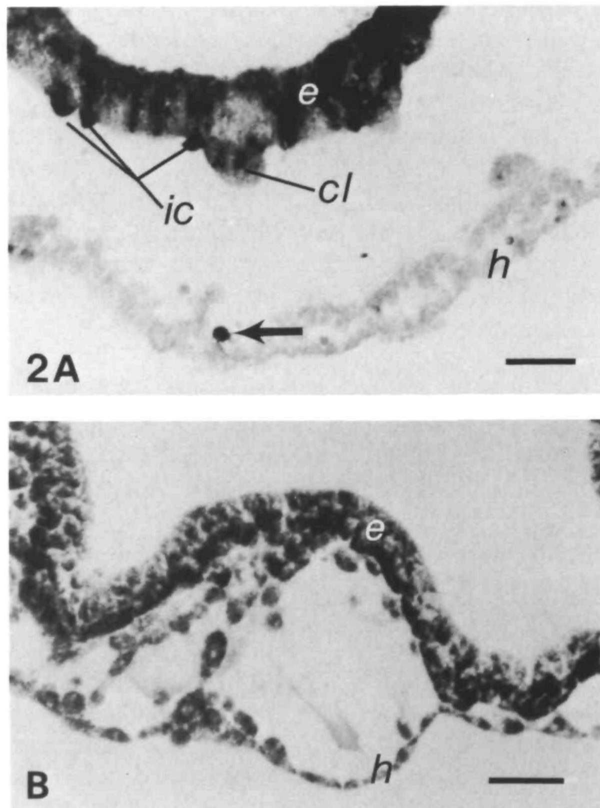

Fig. 2. EMA-1 labelling in embryos after approximately 12 h of incubation. (A) A Bouin's-fixed, paraffin-embedded stage-XII, embryo shows EMA-1 staining some cells of the epiblast (*e*) in cross section, including individual cells apparently in the process of ingression (*ic*) and some cells in a cluster (*cl*). A PGC (arrow) is located in the hypoblast (*h*). (B) PAS stains cells nonspecifically in stage XII embryos. This embryo was prepared by fixation in Rossman's fluid and celloidin/paraffin embedment. Bars, 50 μm.

adjacent mesenchyme during the period of germinal ridge invasion.

(4) *Gonadal development and differentiation (5–18 days incubation)*

PGCs continued to be labelled by EMA-1 throughout the period of growth of the germinal ridge and formation of the sexually indifferent gonad. In later stages, EMA-1 reactivity showed a marked sexual dimorphism. In females, germ cells lost the antigen at the time that overt gonadal sexual differentiation began at 7–8 days of incubation. Few labelled germ cells were seen in the germ cell clusters of the ovarian cortex between days 7 and 14 days of incubation (stages 32 and 40), although some germ cells in the stroma were labelled (Fig. 3A). The EMA-1 epitope reappeared on cortical oogonia by 15 days of incubation, with nearly 100 % labelling efficiency of cells in germ cell clusters through the time of hatching and with few, if any, labelled germ cells still present in the stroma (Fig. 3B). Unlike germ cells in the female, germ cells in the male retained EMA-1 antigenicity until after gonadal sexual differentiation, with a gradual decrease in numbers of labelled cells becoming apparent at 11 days of incubation (stage 37) (Fig. 4A) and continuing until essentially all spermatogonia were unreactive with EMA-1 at the time of hatching (Fig. 4B).

Discussion

EMA-1, a monoclonal antibody raised against mouse embryonal carcinoma (Nulli SCC1) cell surface antigens (Hahnel & Eddy, 1986), identifies germ cells in chick embryos throughout the development of the germ line. EMA-1 is the only monoclonal antibody thus far reported in the literature that can serve to identify chick PGCs. In addition, EMA-1 represents a direct marker that effectively extends the temporal limits of specific germ cell labelling in the chick embryo both back into the early stages of hypoblast formation, and ahead, beyond the period of gonadal colonization. Like PAS, EMA-1 allows easy identification of PGCs from the germinal crescent stages through the migratory phase. However, EMA-1 represents the first chick germ cell marker reported to label subsequent to the beginning of primary sex differentiation.

The EMA-1 epitope appears in both somatic and germ line tissues in the chick embryo in a pattern strikingly similar to that reported for the mouse embryo (Hahnel & Eddy, 1986), and preliminary

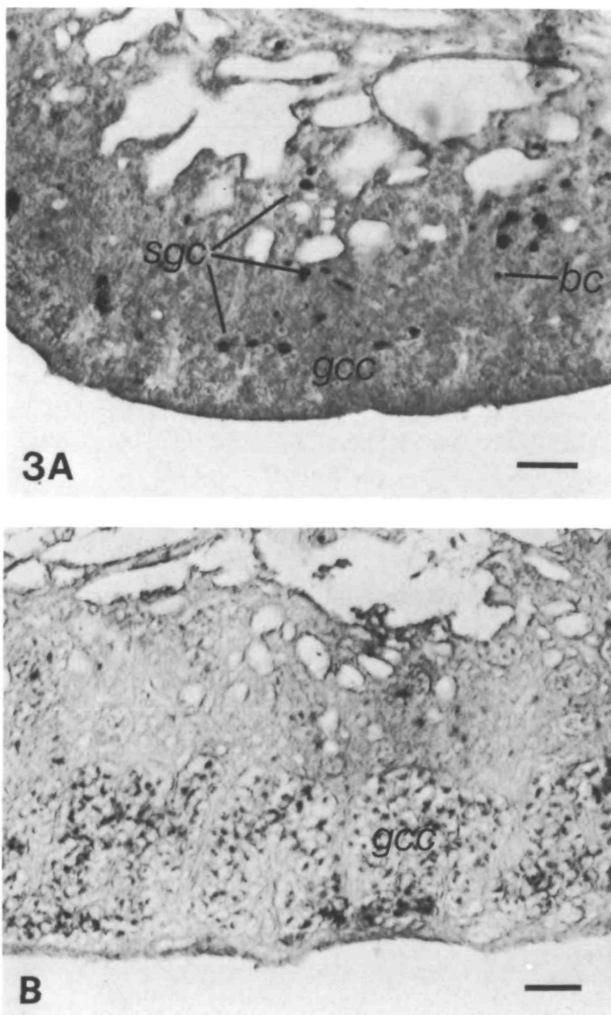

Fig. 3. EMA-1 labelling of sexually differentiated left female gonads, prepared by Bouin's fixation and paraffin embedment. (A) An ovary at 10 days of incubation (stage 36) shows large EMA-1-positive germ cells remaining in the stroma (*sgc*). Some blood elements (*bc*) also show nonspecific binding of the antibody–enzyme complex in these later stages and are distinguished from germ cells by their small size. Germ cell clusters (*gcc*) in the cortex are not labelled. (B) An ovary from a newly hatched chick shows label on nearly all oogonia in the germ cell clusters (*gcc*). Bars, 50 μm.

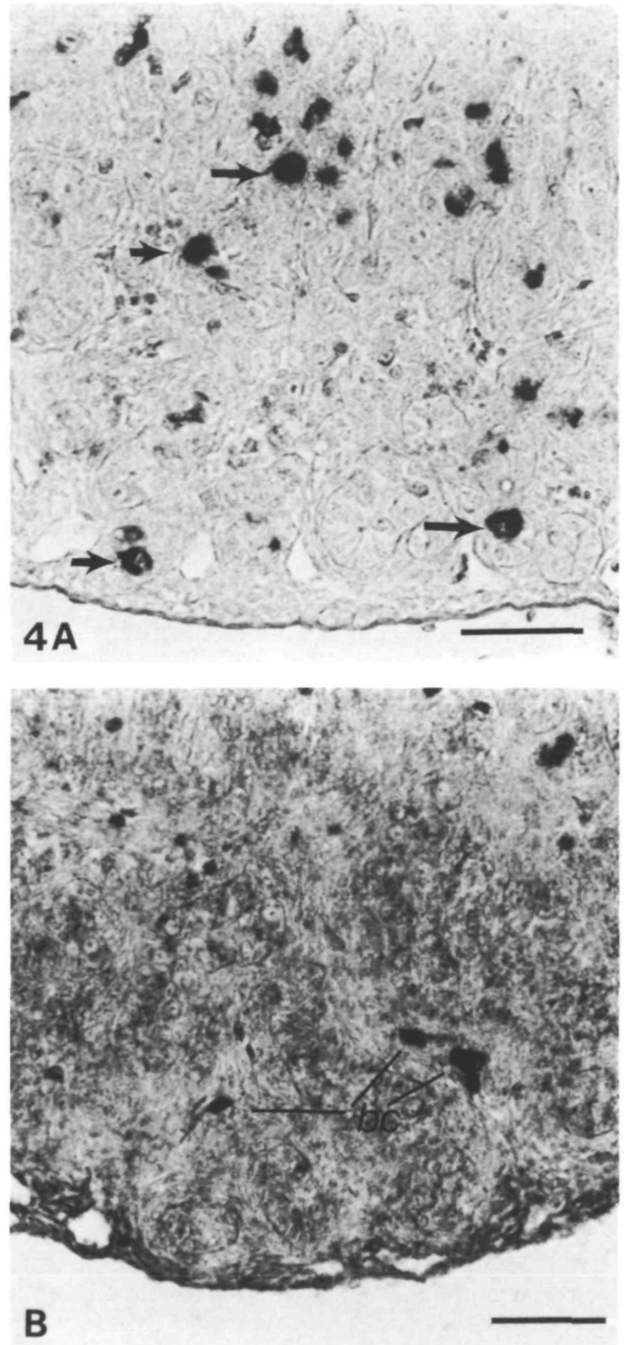

Fig. 4. EMA-1 labelling of sexually differentiated male gonads, prepared by Bouin's fixation and paraffin embedment. (A) Spermatogonia in the sex cords can still be identified at 11 days of incubation (stage 37) as the large EMA-1-positive cells (arrows). (B) A testis from a newly hatched chick does not show labelling of spermatogonia in sex cords. *bc*, nonspecifically stained blood cells. Bars, 50 μm.

results suggest that the same is true for quail. The resemblance between EMA-1-labelling patterns for mouse and chick PGCs as well as other cell types may reflect an unknown, but common and necessary, developmental function for the epitope.

EMA-1 labelled germ cells according to a sexually dimorphic temporal pattern. The temporary loss of labelling on PGCs in the ovarian cortex occurs at the time of overt gonadal sex differentiation and coincides with the time when these cells are reported to lose their migratory capacity (Dubois, 1968) and when they begin more rapid mitotic cell division (Goldsmith, 1928). The epitope reappeared on oogonia just before their reported entry into meiotic prophase (Narbaitz & Adler, 1966). Germ cells in males retained the epitope through day 11, roughly in

parallel with the reported maintenance of migratory ability by male PGCs through day 12 (Dubois, 1968), and loss of label is followed by the burst of germ cell mitotic activity in the testis after 15 days of incubation (Goldsmith, 1928). Thus, in each case, initial disappearance of EMA-1 label coincides roughly with differentiation of germ cells into either oogonia at 7–8 days or spermatogonia at 13 days (Hamilton, 1952).

Recent experimental evidence using chick–quail chimaeras (Eyal-Giladi *et al.* 1981) and embryo-fragment isolation (Sutasurya *et al.* 1983; Ginsburg & Eyal-Giladi, 1986) strongly suggests that avian PGCs originate in the epiblast, rather than in the hypoblast. Our findings of early chick PGC distribution, in agreement with studies of quail PGCs using either the QH1 monoclonal antibody, which labels PGCs in quail (but not chick) (Pardenaud *et al.* 1987), or an anti-quail PGC polyclonal antiserum (Ginsburg *et al.* 1987) provide direct observations consistent with an epiblast origin for germ cells in birds. Epiblast cells bound EMA-1 in increasing numbers, beginning at stages prior to incubation, simultaneous with the first appearance of EMA-1-positive PGCs in the blastocoel and among EMA-1-negative hypoblast cells. This suggests that a more common lineage exists between PGCs and cells of the epiblast than between PGCs and cells of the hypoblast. In addition, EMA-1 labels cells that appear to be in the process of ingressing from the epiblast into the blastocoel. The only other cells believed to be derived from the epiblast in this manner are primary hypoblast cells, based on gross morphology (Eyal-Giladi & Kochav, 1976), cinemicrography (Vakaet, 1970), and histology (Kochav *et al.* 1980). These somatic tissues are shown here to be EMA-1 negative. It seems likely on these bases that PGCs are indeed derived from these ingressing EMA-1-positive epiblast cells.

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