Short Contribution

High proliferation rate characterizes the site of axis formation in the avian blastula-stage embryo

NETTA ZAHAVI, VANDA REICH and ODED KHANER*

Department of Cell and Animal Biology, The Institute of Life Sciences, The Hebrew University, Jerusalem, Israel

ABSTRACT Localized zones of high cell proliferation have been thought to be important in determining several phases of axis formation at early stages of chick development. It was suggested that a developmental center, a center of cellular activity such as proliferation and movement, is located in the posterior half of the area pellucida in the unincubated chick blastoderm. In the work reported here, we have used the bromodeoxyuridine (BrdU) incorporation procedure followed by immunoperoxidase detection to assess the rate of cell proliferation at particular sub-regions of pregastrulating chick blastoderms (stages X-XIII). Examination of whole-mount and histological sections of stages X through XII blastoderms, pulsed with BrdU, showed no distinguishable difference in labeled cells between particular regions of these blastoderms and also that there are no specific zones of high cellular proliferation in either the hypoblast or the epiblast layers of the area pellucida. However, our observations have shown a striking difference in the stage XIII blastoderm, in which a relatively high amount of labeled cells were detected all around the posterior region of the area opaca, the marginal zone, Koller's sickle and the epiblast. The relatively high proportion of cell divisions observed at the posterior end of a stage XIII blastoderm, the blastula stage of the avian embryo, may be associated with the major developmental ability of this region to initiate an embryonic axis. Directional axis formation, therefore, may be attributed to a region of proliferation in the posterior side of a stage XIII blastoderm.

KEY WORDS: *chick blastoderm, cell proliferation, axis formation, BrdU*

Localized zones of high cell proliferation have been thought to be important in determining several phases of axis formation at early stages of chick development. It was suggested that a developmental center is located in the posterior half of the *area pellucida* of unincubated blastoderm (Spratt and Haas, 1960,1961,1963; Spratt, 1966; Stern, 1979). The unique properties of the cells in the "developmental center" is their differentially greater mitotic and metabolic activities (Spratt and Haas, 1963).

Since Spratt (1966) has recognized the marginal zone and Koller's sickle as an area of crucial significance for embryonic development, experiments were made to study the capacity of different regions of the marginal zone to promote the formation of a primitive streak (Khaner and Eyal-Giladi, 1986). It was found that during normal development of the chick, only one primitive streak and consequently a single embryonic axis is initiated from the posterior marginal zone of the blastoderm and that lateral parts of the marginal zone region also have the potential to initiate an ectopic primitive streak. In addition, various experiments including homoplastic and heteroplastic transplantations have been done to examine whether a control mechanism, aimed to produce a single embryonic axis based on the presence of a developmental center, exists in the posterior marginal zone (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989; Khaner, 1993). These experiments revealed that the marginal zone expresses a gradient of potentialities to form a primitive streak. The highest potential of this developmental gradient was concentrated at the posterior marginal zone and Koller's sickle. However, the above experiments did not reveal whether the posterior marginal zone region in fact expresses a significantly greater mitotic activity, and if such activity may be associated with its high developmental potential.

The role of cell division during hypoblast development of early chick blastoderms (stages XI-XIII), was examined in colchicine treated blastoderms (Weinberger and Brick, 1982). Mitotic rate patterns, particularly accumulation of metaphase figures in colchicine treated embryos, were examined and have provided an indication to whether a proliferation center exists in the hypoblast layer. The reported data did not support the notion that a posterior

Abbreviations used in this paper: BrdU, Bromodeoxyuridine.

***Address for reprints:** Department of Cell and Animal Biology, The Institute of Life Sciences, The Hebrew University, Jerusalem, Israel 91904. FAX: 972 2 5617-918. e-mail: ODEDK@VMS.HUJI.AC.IL

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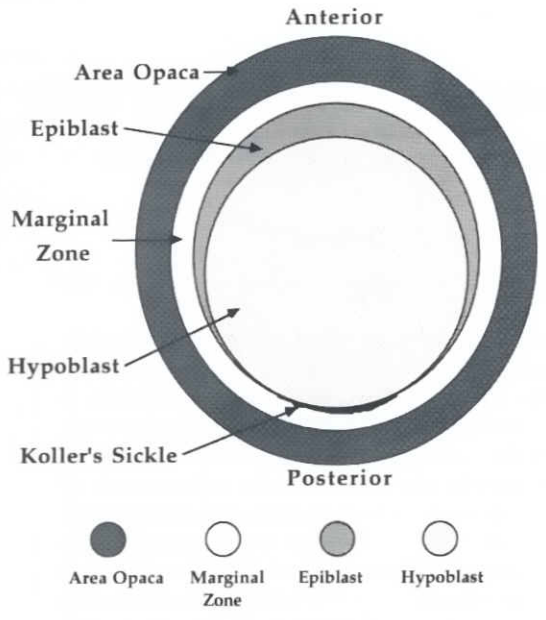

Fig. 1. Scheme of a stage XIII blastoderm, showing its particular regions: *the epiblast, the hypoblast, the marginal zone, Koller's sickle and the area opaca.*

proliferative center, which could provide the motive force for growth and expansion, is located in the posterior half of the hypoblast in unincubated chick blastoderm. In another report, the role of mitosis in the formation and function of the hypoblast was examined in irradiated unincubated blastoderms (Mitrani, 1984). It was demonstrated that mitosis inhibition by irradiation does not prevent formation of a functional lower hypoblast layer.

As mentioned above, Spratt and Haas suggested that a developmental center is located in the posterior half of the *area pellucida* of unincubated blastoderm. However, the stage they referred to as unincubated blastoderm comprises four particular stages (X, XI, XII and XIII) by the new normal table of chick development of Eyal-Giladi and Kochav (1976). These staging series did not yet exist when Spratt and Haas conducted their research. In the work reported here, the blastoderms were staged using the new table of chick development (stages X, XI, XII and XIII of EG and K), and the temporal and spatial variations in proliferation during these stages of development were examined. Particular regions examined included the epiblast, the hypoblast, the marginal zone and Koller's sickle and the area opaca. Detection of proliferation in these regions was achieved using nuclear incorporation of BrdU, a modern technique that marks cells in S phase of the mitotic cycle, followed by an immuno-cyto-chemical detection (Sanders *et al.*, 1993; Bellomo *et al.*, 1996). This protocol was designed to reveal particular areas of cell proliferation in whole-mount and serial sections of the examined blastoderms.

The results reported here were obtained from 13 blastoderms at stage X, 16 blastoderms at stage XI, 17 blastoderms at stage XII, and 26 blastoderms at stage XIII. To distinguish between stained cells in each particular layer, each blastoderm was viewed from both sides, dorsal side (epiblast layer) and ventral side (hypoblast layer). The number of BrdU incorporating cells, and the total number of cells was counted in each region of the blastoderm (area opaca, marginal zone, Koller's sickle, epiblast and hypoblast) -identified by their morphological characteristics, through these regions (Fig. 1).

Stages X-XI: At these stages, the hypoblast starts to form at the posterior side of the blastoderm. Labeled cells at these stages of development could be observed in particularly at the posterior side of the epiblast (29-30%), anterior and lateral parts of the epiblast layer (32-33%) and the posterior side of the hypoblast (5%) (Fig. 2).

Stage XII: At this stage of development, the hypoblast is almost completed at the posterior region of the blastoderm, but it continues to develop in anterior and lateral directions. About the same number of labeled cells were observed at the posterior side of the epiblast layer (31%) as in its anterior and lateral parts (33%). Only about 4% of labeled cells were found in the hypoblast layer at this stage. There was not a significant visible difference in labeled cells between stages X-XII, in the area opaca region (31-32%), nor was there a difference in labeled cells at the marginal zone (25-26%) and Koller's sickle (30-31%) (Fig. 2).

Stage XIII: At this stage, the hypoblast layer is completed, and the blastoderm is constituted from two unique layers of cells, the epiblast and the hypoblast. A relatively variable distribution of labeled cells were observed in particular regions of the blastoderm when compared with earlier stage blastoderms (X-XII). The hypoblast layer was already formed, and only 1.7% of labeled cells could be detected in this layer. At this stage, 35% of labeled cells were observed at the anterior and lateral parts of the epiblast layer, however, 41% of labeled cells were observed at the posterior side of the epiblast layer. Also, a significant increase in numbers of labeled cells was observed in the posterior regions of the area opaca (40-47%), the marginal zone (46%) and Koller's sickle (41%) (Figs. 2 and 3A,C).

Control experiments: Blastoderms at appropriate stages (X, XI, XII and XIII), were exposed to Ringer solution instead of BrdU, and therefore, served as control experiments. Examination of whole-mount and serial sections, stages X through XIII control blastoderms, showed no labeled cells in all areas of the blastoderm (Fig. 3B and D).

Examination of whole-mount and histological sections of stages X-XII showed no distinguishable difference in labeled cells between particular regions of these blastoderms and that there are not specific zones of high cellular proliferation. However, our observations have shown a relatively high amount of labeled cells which were detected all around the posterior region of the area opaca, the marginal zone, Koller's sickle and the epiblast in a stage XIII blastoderm.

What then is the developmental significance of high cellular proliferation in this region at a stage XIII blastoderm? Recent experiments have demonstrated that at stage XIII, the posterior marginal zone and Koller's sickle cells, as well as the posterior side of the epiblast, have a remarkable ability to initiate an ectopic primitive streak (Khaner, in press). Izpisúa-Belmonte *et al.*, (1993), have shown that the homeobox gene goos*ecoid*, a marker of organizer cells, is first expressed in a small cell population in the middle layer of the prestreak stage embryo, associated with the posterior marginal zone, Koller's sickle and in the posterior side

of the epiblast layer. These early *goosecoid*- expressing cells were found to have the ability to induce other cells to express the same gene. Moreover, *cVg1* (a powerful inducer of organized axial tissue amphibia) is also expressed in the posterior marginal zone and Koller's sickle and in the posterior side of the epiblast layer at early stages of development (Seleiro *et al.*, 1996). The pattern of expression of both genes (*goosecoid*, and *cVg1*) seems to converge with this particular posterior region of a stage XIII blastoderm, which also expresses high cellular proliferation. These observations may indicate the possibility that a posterior proliferative region, which expresses inducer molecules of organized axial tissue, is required as a driving force for directional axis formation in the chick embryo. The cells formed all around the posterior side of the blastoderm might migrate in a specific direction such as the fountain-like pattern of cell movement described previously (Spratt, 1966), and possibly, induce gene expression in selected groups of cells. Later reports using fate-mapping of the posterior marginal zone and the posterior side of the epiblast layer of early chick embryos, also demonstrate the crucial role of this particular region in the process of axis formation (Hatada and Stern, 1994).

The hypoblast is known to develop from two sources of cells. Cells that move centrally from the posterior marginal zone and Koller's sickle and populate it's posterior part. The other source are cells which polyingress from the epiblast (Stages X-XIII) (EG and K). Our examinations have shown that a relatively low labeling of cells were detected in the hypoblast layer. This might be because of polyngression and migration during the process of hypoblast formation, but not because of cell division. Similar kinds of findings were also reported by Weinberger and Brick, (1982), who found that the rate of cell division during hypoblast formation is limited.

The relatively high labeling of cells observed in the *area opaca*, especially at stage XIII, may be associated primarily with blastoderm radial expansion which follows immediately after. This interpretation finds support in the report of New (1959), who found that when chick blastoderms were kept from expanding by culturing them on the vitelline membrane, the outer surface become thicker at the periphery. In addition, Weinberger and Brick (1982), found that the mitotic index within the *area opaca* region is higher, and they too correlated it with the blastoderm radial expansion.

The results reported here have thus demonstrated that the gradual morphogenetic processes that establish the embryonic axis in the early chick blastoderm (stages X-XII) include cell proliferation evenly distributed throughout blastoderm. However, at stage XIII, the blastula stage of the avian embryo, the relatively high proportion of cell division observed around the posterior side of the epiblast, the marginal zone and Koller's sickle, may be associated with its major developmental potential to initiate the embryonic axis.

Experimental Procedures

Bromodeoxyuridine incorporation

Blastoderms at appropriate stages (X, XI, XII and XIII), were exposed to 5-bromo-2-deoxy-uridine (BrdU, Sigma) at a concentration of 50 μg/ml for 30 min, at 37°C, by applying 0.25 ml BrdU solution on top of the blastoderm and the same volume underneath. Incubations were carried out in a humid chamber, making sure at each step that every blastoderm was exposed to the reagent. Since no difference in labeling was noted between incubation from 30 min to 2 h, a 30 minute period was chosen as the standard incubation time. After the 30 min of incubation at 37° in BrdU, the blastoderms were fixed with 4% paraformaldehyde overnight at 4°C. The blastoderms were washed three times in phosphate-buffered saline (PBS) for ten minutes, incubated with 2N hydrochloride acid (HCL) for 30 min at room temperature, washed 3 times in PBS for ten minutes, and then incubated with 1% bovine serum albumen (BSA) containing 0.1% Triton and 0.13% Sodium Azide, for 60 min at room temperature to block non-specific antibody binding.

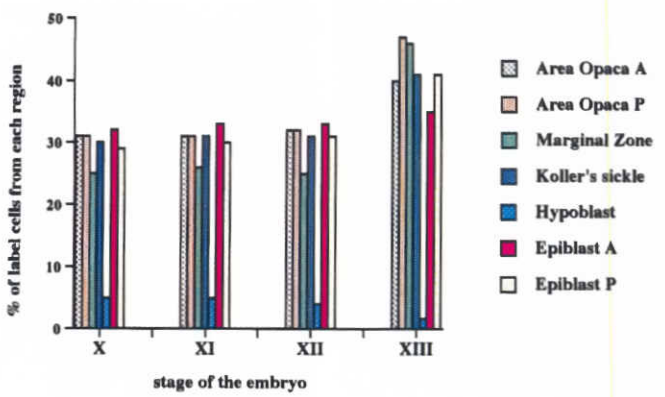

Fig. 2. Comparison of cell proliferation in different regions of early chick blastoderms (stages X-XIII), as measured by % BrdU labeled cells counts of sagittal serial sections. *P, posterior; A, anterior.*

Three blastoderms at each appropriate stage (X, XI, XII and XIII), were utilized for control experiments. These blastoderms were not exposed to BrdU, but instead they were incubated for 30 min at 37°C by applying 0.25 ml Ringer solution on top of the blastoderm and the same volume underneath. After the incubation in Ringer solution, the control blastoderms were treated the same as the experimental blastoderms.

The primary antibody used to detect incorporated BrdU was a monoclonal mouse anti Bromodeoxyuridine, obtained from Zymed Laboratories inc. The antibody, used at a dilution of 1:10 in PBS plus 1% BSA with 0.1% Triton and 0.13% sodium azide, was applied overnight at 4°C, followed by washing 3 times for 10 min each with PBS. The secondary antibody was peroxidase-goat anti-mouse IgG (H+L) obtained from Zymed Laboratories inc., applied at a dilution of 1:100 in PBS plus 1% BSA with 0.1% Triton for 4 h at room temperature, followed by washing 3 times, 10 min each, with PBS. Binding was detected with diaminobenzidine (DAB) Peroxidase substrate tablet set (Sigma fast). The blastoderms were then washed 3 times for 10 min with PBS, which stopped the DAB reaction.

Whole-mount and serially sectioned preparation

A treated blastoderm was put into a drop of a glycerol on a glass slide and then covered with a coverslip. The edges of the coverslip around the blastoderms were then sealed with a transparent nail varnish. At this stage the blastoderms were viewed with a microscope, analyzed and photographed. The coverslip upon the blastoderm was removed, the blastoderm was transferred through increasing concentrations of ethanol and then embedded in paraffin. The blastoderm was sagittally serially sectioned at 10 μm thickness and mounted on slides. After deparaffinization, the sections were stained with methyl green to visualize the borders of the cell. A few drops of Entellan and a coverslip were applied. The stained sections were viewed with a microscope, analyzed and photographed.

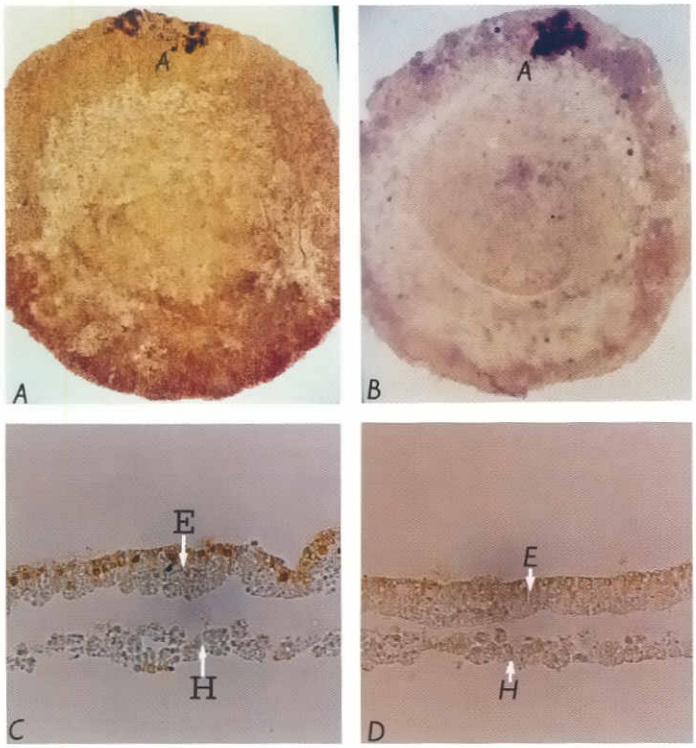

Fig. 3. Whole-mount and histological sections of a stage XIII blastoderm. (A) *A stage XIII blastoderm viewed from its ventral side after detection of incorporated BrdU (purple-brown). Relatively high labeling of cells is observed in the posterior region which includes: the area opaca, the marginal zone and Koller's sickle and the epiblast layer. A, anterior. Magnification, X75.* **(B)** *A control of a stage XIII blastoderm viewed from its ventral side. A, anterior. Magnification: X75.* **(C)** *A sagittal section of a stage XIII blastoderm, focusing on the area pellucida region which includes the epiblast and the hypoblast layers. Note that in this specific section labeled cells (brown) are shown only in the epiblast layer. E, epiblast; H, hypoblast. Magnification: X1200.* **(D)** *Control sagittal section of a stage XIII blastoderm, focusing on the area pellucida region which includes the epiblast and the hypoblast layers. No labeled cells are detected in the epiblast or in the hypoblast. E, epiblast; H, hypoblast. Magnification: X1200.*

Cell number counts

The percentage of BrdU incorporating cells in sectioned blastoderms at stages X-XIII was counted in sagittal sections of BrdU experimental blastoderms. The number of BrdU incorporating cells, and the total number of cells was counted in each region of the blastoderm (area opaca, marginal zone, Koller's sickle, epiblast and hypoblast-identified by their morphological characteristics) in all consecutive serial sections through these regions. BrdU labeled cells could be observed because of the intense brown stained nuclei. The average percentage of cells incorporating BrdU in each region was calculated from approximately 10-15 sections in each of the experimental blastoderms.

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