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Source: Zoological Science, 14(2) : 313-320

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.313

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ZOOLOGICAL SCIENCE **14**: 313–320 (1997) © 1997 Zoological Society of Japan

Individual Epiblast Cells Acquired Invasiveness Precedent to the Primitive Streak Formation in the Chick Embryo

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ABSTRACT—We developed a serum-free culture technique for chick epiblast explants in low density. In the condition free of unknown serum factors, we examined the invasiveness of chick epiblast cells by dissociating the cells into single cells and seeding them onto basement membrane analogues. At stage X-XI of Eyal-Giladi and Kochav's table (1976), 5-11% of the epiblast cells invaded laminin gel or the reconstituted basement membrane (EHS gel). The proportion of invasive cells increased quickly up to 50% at stage XII. Single prestreak epiblast cells on laminin gel still showed invasive behavior even in the simple medium without any growth factors or vitamins. They were also invasive on fibronectin gel, but not on type I collagen gel or a mixture of EHS gel and type I collagen gel. As for regionality, invasive epiblast cells were more densely distributed in the posterior region of *area pellucida* than in the anterior region. The early commitment of invasiveness in individual epiblast cells precedent to gastrulation was proved in our serum-free culture. In addition, we confirmed that prestreak epiblast cells are already heterogeneous in the invasive potency; they can be classified into two groups, invasive cells and non-invasive ones.

INTRODUCTION

In amniota, one of the most important cellular events during gastrulation is the ingression of a group of cells destined to form mesoderm and/or endoderm. By ingression, three germ layers are generated from the epiblast, a multipotent embryonic epithelium. In the avian embryo, a straight ridge of aggregated cells called initial streak is formed as a first axial structure at the posterior end of *area pellucida* (Vakaet, 1970; Takeuchi, 1984). Then, the basement membrane which once sealed the basal surface of the epiblast is lost along the primitive streak, and extracellular matrix molecules, such as fibronectin or laminin, are absent there (Sanders, 1979; Duband and Thiery, 1982; Sanders, 1982; Zagris and Chung, 1990). Therefore, proteolytic activity to dissolve the basement membrane has been assumed in the ingression of presumptive mesodermal and endodermal cells at the primitive streak (Mitrani, 1982; Vakaet, 1984; Harrisson *et al.*, 1991).

Sanders and Prasad (1989) revealed that the epiblast cells neighboring the initial streak just before ingression are already invasive (Sanders, 1991). Recently, we found that chick prestreak epiblast cells invaded laminin gel when they were cultured in the medium supplemented with chicken serum (Toyoizumi and Takeuchi, 1995). However, because we used serum in the culture, we could not eliminate out the possibility that unknown serum factor(s) artificially triggered or induced the invasive behavior of the prestreak epiblast in such a culture condition.

When and where do the epiblast cells acquire the invasive potency? Is it an autonomous process or an induced process? As a first step to answer these questions, we developed a culture technique for chick epiblast using a medium containing completely chemically defined compounds. We succeeded in culturing chick epiblast cells for more than a week in a serum-free condition. Using the medium, we confirmed that half of the epiblast cells acquired the invasiveness at stage XII, several hours before initial streak formation (Eyal-Giladi and Kochav, 1976). With our assay system, it was revealed that prestreak epiblast is composed of invasive cells and non-invasive cells. The substratum-dependency of their invasiveness was also found. The invasive cells were distributed densely in the posterior half of the *area pellucida*.

The implications of these findings for the mechanism of cellular ingression as one step of avian gastrulation will be discussed.

MATERIALS AND METHODS

Fertilized White Leghorn chicken eggs were incubated at 37°C for 0-6 hr until embryos reached stage X-XIII of Eyal-Giladi and Kochav's normal table (1976). For comparison, embryos at stage 2-3 of Hamburger and Hamilton's table (1951; H&H stage in abbreviation) obtained by 7-12 hr of incubation were also used.

Epiblast cells dissected from the following spots of the embryos at the following stages were used for our invasion assay:

- stage X central epiblast cells (in H&H stage 1, prestreak stage)

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- slightly caudal region of *area pellucida* epiblast cells from stage XI-XIII embryos (in H&H stage 1)
- stage XII epiblast cells from the most anterior region of the *area pellucida*
- H&H stage 2-3 epiblast cells from the most anterior region of the *area pellucida*
- H&H stage 2 epiblast cells facing the initial streak, but not in the initial streak
- H&H stage 2-3 initial streak cells

Cell preparation

The whole blastoderm was excised together with the covering vitelline membrane in Ca^{2+}, Mg^{2+}-free phosphate buffed saline (PBS), and was fixed on a paraffin bed by putting a stainless ring (about 3-8 mm in diameter) on its ventral surface. The non-epiblast cells located on the basal surface of the epiblast (mainly hypoblast) were then wiped out or pealed off with a pair of glass needles on the bed. A small piece of the prestreak epiblast sheet (0.4 mm × 0.4 mm) was cut out from the above region of *area pellucida* with a pair of glass needles. The fragments were further dissected into smaller cell clusters with the needles. They were collected with a pipetman (20 μl), and transferred into a culture well of 96-well test plates (Falcon 3072) filled with 200 μl of Spinner's minimal essential medium (S-MEM). S-MEM contains no calcium ions for floating culture. These cell clusters were incubated for 15-20 min at room temperature with flashing gently several times with a pipetman at every 5 min. With this procedure, most of the epiblast cells were dissociated into individual cells while conserving their affinity to the extracellular matrix proteins. For initial streak cells, very gentle flashing after a few minutes of S-MEM treatment was enough to obtain fully separated single cells.

Substrata

To examine a serum-free culture condition, type I collagen-coated dishes (35 mm in diameter; Iwaki Co., Japan) were used. Cell clusters of stage XII-XIII epiblast or those of H&H stage 2-3 epiblast containing 20-100 cells were cultured on the dishes.

For the invasion assay, 30 μl of laminin solution (1 mg/ml; Biomedical Technologies Inc. (USA), BT-276) was mounted on the central area (ca. 2 cm^2) of Falcon 3001 petri dishes. According to the equipped data sheet, this product forms a gel at room temperature. The dishes were incubated at 37°C for more than 4 hr in a humid chamber to form thick layer of laminin gel. Otherwise the laminin solution was coated and air-dried at 37°C for half a day and then reconstituted to be gel form by washing it with the medium.

Basement membrane-like substratum in gel form derived from mouse Engelbreth-Holm-Swarm tumor (Iwaki Co. (Japan), EHS gel in abbreviation) was also used as reconstituted basement membrane. This product contains laminin, fibronectin, entactin, heparan-sulfate proteoglycan, and reduced amount of type-IV collagen. In the case of the EHS gel, air-drying seemed to prevent the invasive process, so all the EHS dishes were prepared by incubating for 2-4 hr at 37°C in a humid chamber.

For comparison, we prepared and used type I collagen gel and fibronectin gel. Type I collagen gel derived from porcine tendons was prepared with a kit manufactured by Nitta Gelatin Co. (Japan). Twenty-five μl of collagen gel was mounted at the center of a dish, washed with the medium after gelation and used as a substratum. To make fibronectin gel, 25 μl of bovine fibronectin (3.1 mg/ml of phosphate-buffered 2 M urea solution (pH 7.2); Canadian Bioclinical Co. (Canada), BP-6) was coated at the center of a plastic petri dish (Falcon Co., "3001"), and air dried thoroughly at 37°C for a few days. Then the solvent was washed away with the medium by gentle stirring. Fibronectin deposits in threadlike gel form were left at the bottom of the culture dish. Preliminary experiments revealed that glycerol which is often contained in the manufactured fibronectin products as a preservative inhibits the gelation of fibronectin, so we purchased and used the glycerol-free fibronectin product.

Culture medium

We cultured chicken epiblast cells with RPMI1640 medium (Gibco Co.) supplemented with 0.2% Mito+ (Becton Dickinson Co.). We adopted RPMI1640 medium, because Mitrani and Shimoni (1990) reported that central epiblast disc alone formed an axial structure without the help of hypoblast tissue or marginal zone tissue when they were cultured on agar using RPMI1640 medium supplemented with 10% XTC-MIF supernatant containing activin molecules (Asashima *et al.*, 1990; Smith, 1990). Mito+™ is an additive developed for serum-free hybridoma culture that contains 11 compounds including so-called 3 indispensable factors for serum-free culture, that is, insulin, transferrin and selenious acid. The other compounds are epidermal growth factor, endothelial cell growth supplement, triiodothyronine, hydrocortisone, progesterone, testosterone, estradiol-17, and O-phosphoryl-ethanolamine (see Burdsal *et al.*, 1993).

RPMI 1640 medium supplemented with 0.2% ITS premix™ (Becton Dickinson Co.; containing 10 μg/ml insulin, 10 μg/ml transferrin and 10 ng/ml selenious acid in our final concentration) was also used in part. As a "minimum medium", Earle's balanced salt solution supplemented with 0.2% ITS premix™ and MEM amino acids' cocktail (Gibco Co.) was also used in our invasion assay.

Cell culture and observation

Single epiblast cells were cultured at 37°C in 5% CO_2 -95% air throughout our experiments. In the long term explant culture, medium exchange was conducted in every 2-3 days. Single epiblast cells were seeded onto the above substrata and incubated for 2 hr. Dead cells through mechanical dissection were then washed away thoroughly by medium exchange. The behavior of the epiblast cells on the substratum was observed and recorded after 18-24 hr of culture, because chick primitive streak formation occurs after 18-24 hr of incubation in normal intact embryos. Phase-contrast micrographs were taken with Neopan F film or Fujicolor film (Fuji film Co.). Some samples cultured on the laminin gel or on EHS gel were fixed with 0.2% glutarardehyde in PBS for one day. They were dehydrated, air-dried or dried with a critical point dryer, coated with Au using an ion coater, and observed with a scanning electron microscope (JEOL Co., TS-20).

RESULTS

To test the suitability of our serum-free medium, we tried to culture chick prestreak epiblast clusters with the medium for several days, since the cells in clusters were handled more easily than the completely dissociated single cells. Epiblast explants containing approximately 20-100 cells were cut out from the center of *area pellucida*, and seeded onto the type I collagen-coated dishes. On type I collagen, stage XII-XIII prestreak epiblast explants spread as coherent cell sheets within a day (n=30; Fig. 1a), while on fibronectin, they spread to be monolayer within half a day (n=12; data not shown). After 3-4 days of culture, the explants dissected from the posterior region of *area pellucida*, scattered fibroblastic single cells around their margin or beneath the sheet (n=12; Fig. 1b), while anterior *area pellucida* epiblast cells continued to be coherent continuous monolayer, and only a few cells wandering out of the explant (n=7; Fig. 1c).

After several days of culture, the explants often came close to one another gradually and at last combined together into one large sheet. Sometimes, tubular structures (Fig. 1d) differentiated in a week. After 8-13 days of culture, highly elongated bipolar cells (probably neuroblasts) were recognized

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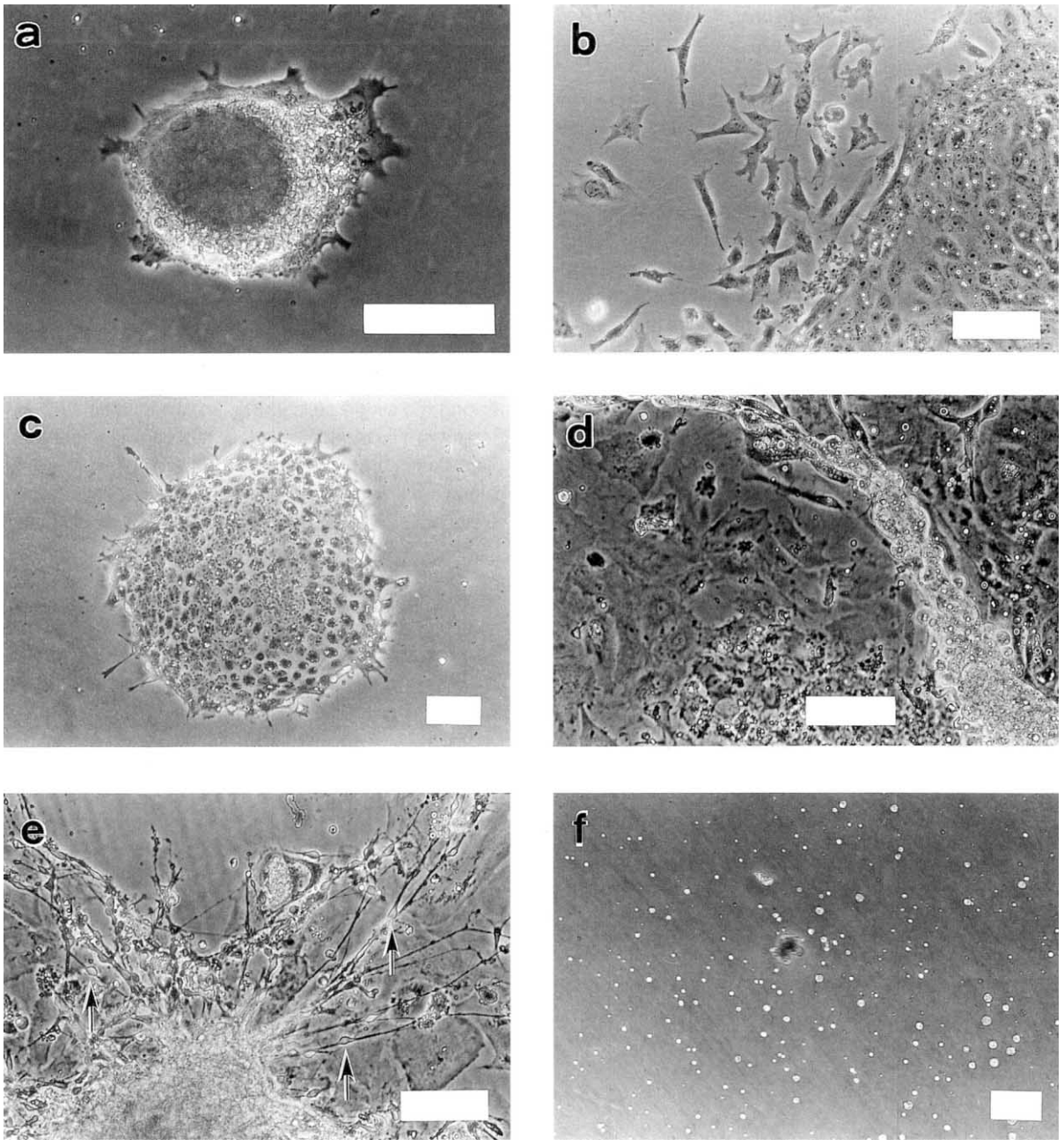

Fig. 1. Long term serum-free culture of the epiblast explant. They were cultured with RPMI 1640 medium supplemented mainly with insulin, transferrin and selenious acid. Bars, 100 μm. (**a**) A stage XIII central epiblast explant at the beginning of spreading after overnight culture on type I collagen-coated dishes. The small fragments once aggregated on the substratum, then spread within a day. (**b**) A stage XIII prestreak epiblast dissected from the central area pellucida scattering many fibroblastic cells around the sheet after 3 days of culture. In 2-3 days of culture, many epiblast explants from the central or posterior *area pellucida* scattered fibroblastic cells around the margin or underneath the sheet. (**c**) A stage XIV most anterior *area pellucida* explant cultured for 3 days. Anterior *area pellucida* explants continued to be coherent epithelial sheets for 3-4 days of culture. (**d**) A stage XIII central epiblast explant cultured on type I collagen for seven days. The monolayer was disordered by the cells forming a cord-like structure on the apical surface of the sheet. (**e**) An explant dissected from the anterior *area pellucida* epiblast after 13 days of culture. Bipolar neuroblasts are recognized (arrows). (**f**) A stage XII epiblast explant collapsed after three days of culture in RPMI1640 medium without any additives. This shows that the culture additives we used in substitution for serum were valid for the survival of the epiblast cells.

in the epiblast clusters from anterior *area pellucida* (Fig. 1e). These data show that epiblast cells survived in our serum-free medium at least for a week. We cultured the epiblast explants for 2 weeks at maximum (n=7). There was no recognizable difference between the effect of Mito+ and that of ITS on the growth of epiblast explants. When the explants

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were cultured with RPMI1640 medium only, they once attached to the substratum to be a spread sheet, but fragmented into single dead cells within 2 days (Fig. 1f).

These results show the suitability of our serum-free medium for epiblast culture. Thus, we used the above serum-free medium in the following invasion assay.

Invasion of single epiblast cells into EHS gel/laminin gel

Most of the single epiblast cells attached to the EHS gel or laminin gel within 2 hr and kept the state for 2 days. Some of the cells dug holes after overnight culture (Fig. 2a). Most of the invading cells attached to the wall of the holes at the same time, suggesting that they not only dissolve the substratum, but simultaneously advance into the holes. The diameters of the holes after overnight culture were nearly the same size as those of the single epiblast cells, suggesting that locomotive activity in parallel with the surface of the gel was very low, though their invading process was active. Sometimes, at the gel margin where the thickness of the gel was thin, the ridge-like structures surrounding the holes were remarkable (Fig. 2b).

Through the low power view of phase-contrast microscopy, it was recognized that some cells apparently showed invasive behavior, while the others simply attached to the substratum, which was clearly revealed in 16 hr (Fig. 2c). Observation with SEM showed that the characteristic marks around the single cells in the phase-contrast micrographs were surely the holes made by the invasion (Fig. 2d).

Stage-dependency and regionality of the ratio of invasive epiblast cells

To examine further the heterogeneity of invasiveness among the single cells in Fig. 2c, we investigated the stage-dependent incidence of invasive cells on laminin gel (Fig. 3, black line graph). The ratio of invasive cells quickly increased during the short period from stage XI (8%, 78 cells out of 992) to stage XII (50%, 930 cells out of 1872). The increase leveled

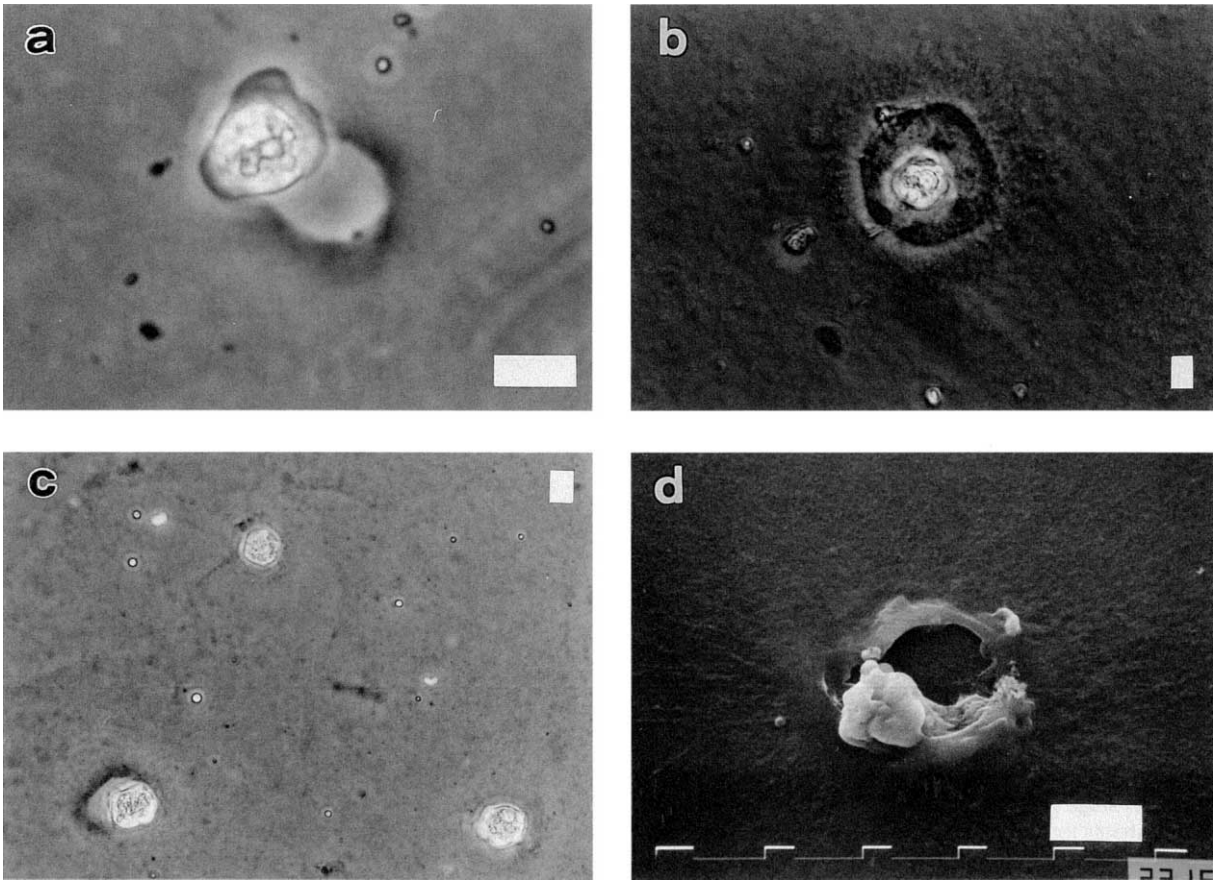

Fig. 2. Invasive behavior of the single prestreak epiblast cells on laminin gel or EHS gel (basement membrane-like gel) in serum-free culture. Samples were photographed after fixation with 4% formaldehyde solution. Bars, 10 μm. (**a**) Phase-contrast micrograph of a stage XII epiblast cell on EHS gel after 18 hr of culture. Note that a hole is made beneath the cell, while the surface of the gel is very smooth elsewhere except the hole. (**b**) Phase-contrast micrograph of a stage XIII epiblast cell on EHS gel after 18 hr of culture. Note that a circular ridge is made around the invading cell, which was often observed at the peripheral margin of the gel where the gel was relatively thin. (**c**) Phase-contrast micrograph of stage XII single epiblast cells on laminin gel cultured for 18 hr. Note that the left cell is observed to have invaded the laminin gel, while on the contrary, the other two cells simply attach to the substratum and there are no signs of invasion. (**d**) SEM photograph of a stage XIII epiblast cell on EHS gel after 20 hr of culture. Note that this cell invades the substratum and simultaneously firmly attaches to the proteolytic hole. Elsewhere, the gel surface is smooth except the ridge around the hole.

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off at stage XII–XIII (43%, 621 cells out of 1450). The proportion of invasive cells on EHS gel also increased during stage XI–stage XII, showing a similar stage-dependent pattern (Fig. 3, gray line graph). The increase of the incidence of invasive cells during stage XI-XII was judged to be significant by the chi-square test for 2×2 contingency table at 0.1% significance level (chi-value=497(laminin), 39.4(EHS)).

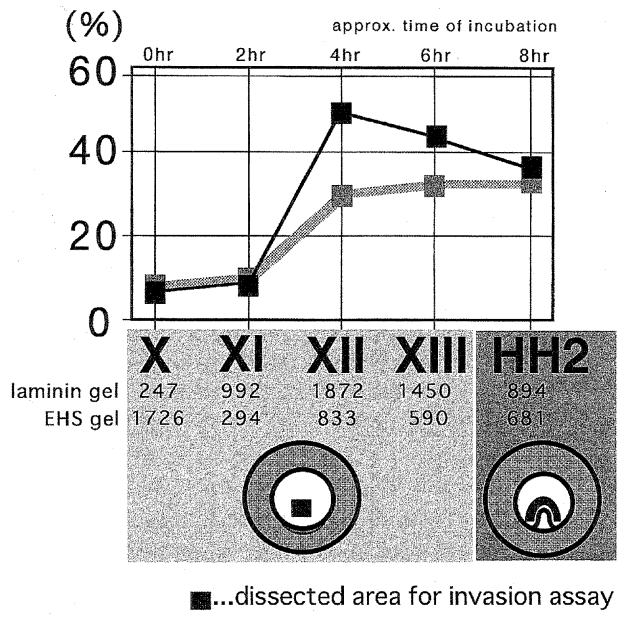

Fig. 3. Stage-dependency of the incidence of invasive cells among the cells attached to the basement membrane analogues. The black line graph represents the transition of the proportion of the invasive cells on laminin gel. The gray line graph represents the same on EHS gel. Numbers below the x-axis represent the total cells counted for each spot (n=5 embryos in average for each spot). Note that in stage X-XI, the proportion of invasive cells is less than 10%. Then, immediately at stage XII, the proportion of invasive cells increases toward 50%. The incidence is saturated in stage XII-XIII.

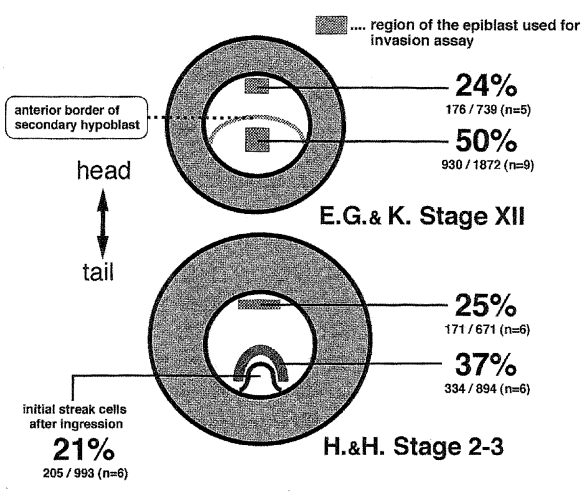

Fig. 4. Regional differences in the incidence of invasive cells on the laminin gel. Note that invasive cells were widely distributed at the prestreak stage, and at the same time, they were more densely distributed in the posterior region than the anterior.

The density of invasive cells at the prestreak stage was found to vary according to the region of the *area pellucida* where the cells were collected (Fig. 4). In stage XII embryos, invasive cells on laminin gel were less in number in the anterior *area pellucida* compared with the posterior area, but some of the anterior epiblast cells were still invasive. Nine hundred and thirty posterior cells out of 1872 (50%, n=9) were invasive on laminin gel, while only 176 anterior cells out of 739 (24%, n=5) were invasive on it (Fig. 4). This means that invasive cells were more densely distributed in the posterior half of the prestreak blastoderm than the anterior. This distribution gradient of invasive cells was warranted by the chi-square test for 2×2 contingency table at 0.1% significance level (chi-value=145). Also in initial streak stage (H&H stage 2-3) embryos, there were few invasive cells in the anterior periphery of the *area pellucida* (171/671=25% on laminin gel, n=6; Fig. 4). A considerable number of initial streak cells was observed to invade the gels, but it was difficult to culture single initial streak cells more than one day in our serum-free medium.

Invasiveness in the culture medium with minimal compounds

RPMI 1640 medium contains various compounds such as vitamins and inositol phosphates etc., some of which are small in amount but quite effective. Thus we checked the necessity of such compounds for invasion. With a medium containing no vitamins or growth factors, we conducted the single cell invasion assay. Invasiveness was still recognized after overnight culture even when we used the simplest medium, which was composed of the Earle's balanced salts solution supplemented only with 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml selenious acid, and with a group of amino acids contained in Earle's minimal essential medium. Figure 5 shows a prestreak stage XII epiblast cell on laminin gel undergoing invasive movement in this medium. Forty-seven

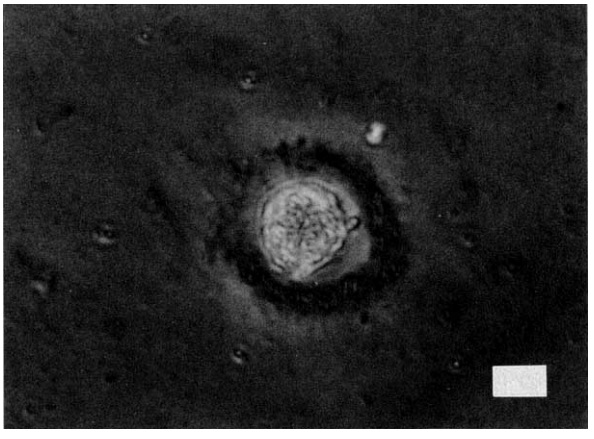

Fig. 5. Invasiveness of a stage XII epiblast cell cultured on laminin gel in Earle's balanced salt solution supplemented only with amino acids, insulin, transferrin, and selenious acid. This phase-contrast micrograph was taken after 21 hr of culture. Bar, 10 μm.

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percent of the stage XII cells (78/167) were invasive in this medium. Thus it was proved that minor components of the RPMI1640 medium or Mito+ were unnecessary for the invasive behavior of prestreak epiblast cells.

Substratum-dependency of invasiveness

To know whether the proteolytic process of the epiblast cells was affected by the kind of extracellular matrix molecules, fibronectin gel and type I collagen gel were used as culture substrata for our invasion test. Stage XII-XIII central epiblast cells hardly invaded the type I collagen gel after overnight culture. Most cells simply attached to the substratum, showing no invasive activities (2025/2038=99%, n=12). No invasive holes were recognized beneath the single cells on type I collagen gel in our observation with SEM, too (Fig. 6a). Thirteen stage XII-XIII prestreak epiblast cells from 4 embryos on type I collagen gel were photographed with SEM, but no invasive holes were recognized beneath the cells. Next we prepared a mixture made by the same quantity of type I collagen gel and EHS gel. Nevertheless, no cells dig holes, either (1813 cells were observed, n=13).

On the other hand, 180 cells out of 851 stage XII-XIII epiblast cells were recognized to invade the fibronectin gel (n=9; Fig. 6b). Also, on fibronectin gel, the heterogeneity of invasiveness among the epiblast cells existed. The other cells simply attached to the fibronectin gel.

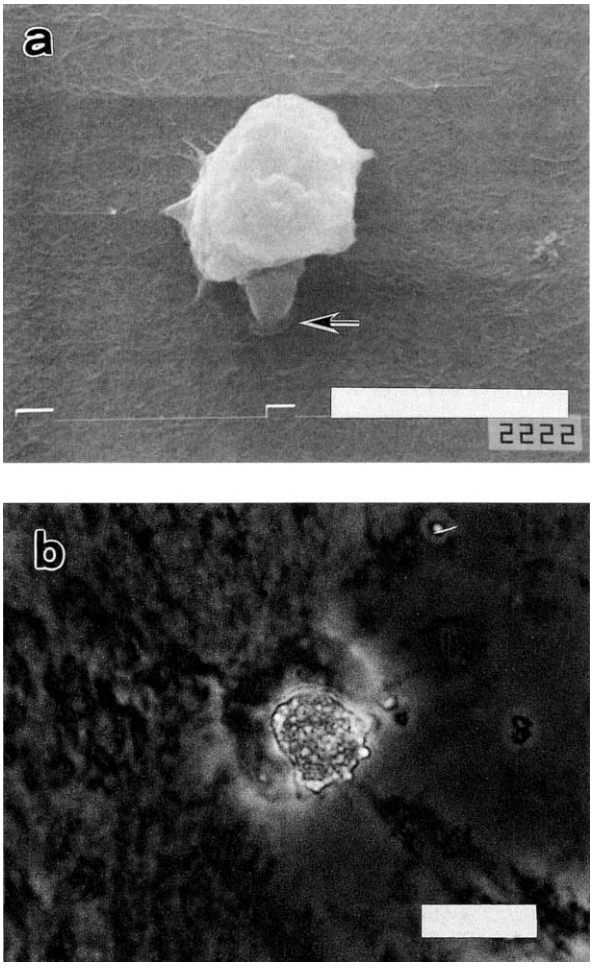

Fig. 6. Single prestreak epiblast cells on type I collagen gel (**a**) and fibronectin gel (**b**). The prestreak epiblast cells invaded the fibronectin gel, but not the type I collagen gel. Bars, 10 μm. (**a**) SEM photograph of a single stage XII epiblast cell on type I collagen gel after 20 hr of culture. Note that this cell firmly attaches to the substratum with extending filopodia and a lobopodium, which appears to push the substratum (arrow head), but it does not undergo the proteolytic process. (**b**) Phase-contrast micrograph of a stage XII epiblast cell on fibronectin gel. Note that this cell dug a hole similar to the invasive cells on laminin gel or on EHS gel.

DISCUSSION

It was clearly shown that part of the epiblast cells at the prestreak stages had acquired the ability to invade laminin gel or EHS gel through serum-free culture. Our results proved that the invasiveness was not induced by unknown factor(s) in serum. Even without many kinds of components contained in RPMI1640 or Mito+, a considerable number of epiblast cells could invade the laminin gel (Fig. 5). Thus we conclude that culture medium or the culture additives in it did not cause the cells to invade the gels, but simply offered an environment enabling the cells to invade the gels. Using the assay system reported here, it was demonstrated that individual epiblast cells acquired their invasiveness several hours before initial streak formation.

On the increase of invasive epiblast cells during prestreak stages

Why does the ratio of invasive cells increase quickly at stage XII in prestreak-stage blastoderms? There are two possible explanations. One is that selective proliferation of the invasive epiblast cells increased their ratio to the non-invasive cells. Another is that previously non-invasive cells acquire invasiveness as a result of embryonic induction by surrounding blastodermal tissues.

It takes only a few hours for stage XI blastoderms to develop to stage XII. In such a short period, the ratio of invasive cells increased from 10% up to 50%; a fivefold increase existed there. Thus, explaining the increase of invasive cells at stage XII only by the selective proliferation of specific epiblast cells seemed difficult (Sanders, 1993), and so we support the latter explanation. In chick embryos, axial mesoderm cells are thought to be induced by the underlying hypoblast layer at stage XII (Khaner and Eyal-Giladi, 1989; Eyal-Giladi *et al.*, 1994). If the invasive cells are identical to the cells destined to be mesoderm and endoderm, this can explain the emergence of the invasive cells as a consequence of mesoderm induction. This idea is supported by the fate map study of prestreak blastoderms. Presumptive axial mesodermal and endodermal cells are distributed in the posterior part of the *area pellucida* (Hatada and Stern, 1994), where we found that invasive epiblast cells were densely distributed than in the anterior.

Stern and Canning (1990) proposed that presumptive

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initial streak cells selectively gather from all over the *area pellucida* to form the axial structure, while the rest cells differentiate into ectoderm (Canning and Stern, 1988). Coincidentally, Toyoizumi and Takeuchi (1995) showed that individual prestreak epiblast cells have migratory activity, which makes such selective directional migration in their hypothesis possible. If their idea is right, the concentration gradient of invasive epiblast cells is a reflection of the process of initial streak formation by the invasive cells.

Though the population is small, some of the epiblast cells were invasive before the stage XII. What kind of the cells were they? During stage X-XI, primary hypoblast cells actively undergo "polyingression" throughout the *area pellucida* (Kochav *et al.*, 1980; Harrisson *et al.*, 1991; Low and Mcclugage, 1993; Watt *et al.*, 1993); individual presumptive hypoblast cells ingress toward the subgerminal cavity throughout the *area pellucida*. Recently we seeded dissociated hypoblast cells onto the laminin gel and EHS gel. We found that those prestreak-stage hypoblast cells also make holes on these gels (Mogi *et al.*, 1996). Thus we speculate that minor invasive cells at stage X-XI were destined to be primary hypoblast cells which still existed in the early upper layer at the moment of tissue dissection. Hypoblast-specific molecular markers that are not yet known will be expected in the future.

On the substratum-dependency of the invasiveness

We revealed the substratum-dependency of invasiveness of the epiblast cells. The epiblast cells dug holes in simple laminin gel, fibronectin gel, and heterogeneous EHS gel. The EHS gel used in our experiment were mainly made of laminin and fibronectin. The basement membrane at the basal surface of epiblast in the early chick embryo was rich in fibronectin and laminin possibly secreted from both hypoblast cells and epiblast cells (Sanders, 1980; Mitrani and Farberov, 1982; Bortier *et al.*, 1989; Zagris and Chung, 1990), and so its major compounds were common to those of EHS gel. On these gel substrata, individual epiblast cells made proteolytic holes in the serum-free simple medium. This means that each cell secreted protease to dissolve the substratum, and this process of itself required no support from the surrounding cells.

Here, studies on cancer invasion hint to us the mechanism of epiblast cell invasion (Stetlerstevenson, 1993). It has been revealed that enzymes for basement membrane degradation called matrix metalloproteinases (MMPs) are secreted in the form of proenzymes by the invasive cancer cells themselves or by the surrounding connective tissues nearby. Then the proenzymes of MMPs are spliced to be active forms by the malignant cells proper. Concerning the single prestreak epiblast cells, those invading cells themselves may have individually secreted and activated MMP by itself, because those single epiblast cells invaded the gels in spite of very low density culture free of mutual interaction. We suppose that in chicken blastoderms, only the cells in the midst of ingression activated MMP very locally, in contrast to the uniform secretion of laminin and fibronectin by all the *area pellucida* cells. In EHS tumor cell line, active secretion of both basement membrane components and gelatinases(MMPs) occurred in the same cells (Mackay *et al.*, 1990). So we can suppose that invasive epiblast cells secrete both basement membrane components and MMPs at the same time. There are not yet any reports on the distribution of the MMPs in early avian blastoderms, but such reports will verify the above idea.

On type I collagen gel, no significant holes were made by the epiblast cells. We think this is because the epiblast cells could not synthesize a proteolytic enzyme such as MMP-1 to dissolve the type I collagen. The mixture of EHS gel and type I collagen gel inhibited the epiblast cells from invading. Thus we speculate that type I collagen in chick early blastoderms (Manasek, 1975) may act as a kind of stop signal for invasion.

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(Received September 12, 1996 / Accepted January 14, 1997)

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