Role of Fibronectin in Primary Mesenchyme Cell Migration in the Sea Urchin

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ABSTRACT We studied the effect of fibronectin (FN) on the behavior of primary mesenchyme cells isolated from sea urchin mesenchyme blastulae in vitro using a time-lapse technique. The migration of isolated primary mesenchyme cells reconstituted in seawater and horse serum is dependent on the presence or absence of exogenous FN in the culture media. The cells in FN, 4 and 40 μg/ml, show a high percentage of migration and migrate long distances, whereas a higher concentration of FN at 400 μg/ml tends to inhibit migration.

In sea urchin embryos, the primary mesenchyme cells (PMCs),[1] the first migratory cells, are formed at the blastula stage from the blastocoel wall at the vegetal plate (e.g., references 11, 18, 31, and 38). These cells then migrate using their cell processes (11, 20, 23) that extend toward the basal lamina (20). The basal lamina is composed of 25–30-nm-diam granules and thinner fibers (17, 23, 37, 40). These cells form spicules after cell fusion (10, 31). This spicule formation also occurs in vitro with an adequate amount of horse serum in the medium (24, 30). During the period of PMC migration, a number of changes occur. The extracellular matrix in the blastocoel, which contains sulfated proteoglycans (16, 34) rich in heparan sulfate, chondroitin-6-sulfate (37), and dermatan sulfate (37, 44) undergoes some ultrastructurally recognizable changes (17, 19, 23), as well as changes in the spatial distribution of concanavalin A-binding materials (19, 21, 41). Collagen has been detected from the hatched blastula stage (4, 41). The interaction between PMCs and the components of the extracellular matrix used for cell locomotion requires further definition.

Among the numerous extracellular matrix components in vertebrate tissues, fibronectin (FN) has received considerable attention for its role in cell–matrix interactions. This glycoprotein binds to cell surfaces (12, 42, 43) and contains domains that can bind to extracellular sulfated glycosaminoglycans, such as heparin (6, 12, 33, 35, 42), heparan sulfate (12, 25, 26, 36), and dermatan sulfate (25, 26), in addition to other matrix components such as collagen (12, 24, 25, 42, 45). FN is involved in such cell behavior as adhesion (2, 12), motility (e.g., references 2, 3, 7, 12, 27, 32, and 44), and proliferation (2, 12). FN-like proteins appear to be widely distributed among different species. FN has been reported in some invertebrates including echinoids. In the sea urchin it has been isolated from the basement membrane of the ovary (13, 14) and detected immunohistologically between ectodermal cells of the embryo (12), on the surface of PMCs (12, 41), and in the blastocoel (15, 39, 41).

The present study indicates that PMC migration is dependent on the presence and concentration of exogenous FN in vitro.

MATERIALS AND METHODS

The eggs of the sea urchin *Pseudocentrotus depressus*, which were collected near the Misaki Marine Biological Station and the Shimoda Marine Research Center, Japan, were released by intracoelomic injection of 0.5 M KCl. The embryos were incubated in filtered seawater or artificial seawater JAMARINE U (Jamarine Laboratory, Osaka) at 14–17°C until the hatched or mesenchyme blastula stage.

Time-lapse Study of Cell Behavior In Situ: Embryos at the early mesenchyme blastula stage, in which the PMCs had ingressed and piled up on the vegetal plate, were attached to the bottom of poly-L-lysine (Sigma Chemical Co., St. Louis, MO) coated 35-mm-diam Falcon plastic Petri dishes (Falcon Labware, Oxnard, CA). The embryos were incubated in the dishes at 25°C during observation. 35-mm photographs of 41 different PMCs in 10 different embryos were taken for 100 min at 5- or 10-min intervals using a Nikon inverted light microscope. The photographs were enlarged 340 times and the path of each PMC was traced and examined on each print. Under the present culture condition, the embryos reached the pluteus stage and formed spicules.

PMCs that migrated along the animal–vegetal axis of the embryo within a fixed focusing zone of the light microscope were examined. Those PMCs that migrated on the basal surface of the ectoderm along the equator of the embryo were excluded from the examination since during observation they tended to move out of the focusing zone of the light microscope. With such precautions the risk of miscalculating the actual distance of cell migration was minimized.

PMC Separation: 1 vol mesenchyme blastulae was rinsed twice at 5-min intervals in 20 vol calcium-magnesium-deficient artificial seawater containing 100 μM EDTA. The embryos were briefly incubated in 20 vol calcium-deficient artificial seawater and then gently pipetted. The embryos promptly dissociated. The cell suspension was briefly centrifuged with a hand centrifuge to remove tissue debris. The result was a supernatant of cell suspension

[1] *Abbreviations used in this paper:* FN, fibronectin; PMC, primary mesenchyme cell; SEM, scanning electron microscope.

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composed mostly of single cells. The cell suspension was layered into plastic Petri dishes for 5 min at 10°C. Cells that did not attach to the plastic dishes were discarded. The Petri dishes were rinsed twice with calcium-deficient artificial seawater to remove loosely attached ciliated cells. The Petri dishes were then vigorously rinsed with calcium-deficient artificial seawater to remove any cells that were still attached to the plastic dishes. These cells were collected and centrifuged at 1,000 rpm for 3 min. Most of these cells that had been firmly attached to the Petri dishes had no cilium (Fig. 1) and formed spicules when they were cultured in artificial seawater that contained 2% horse serum (22), thus indicating that these are PMCs. Incubation of the embryonic cell suspension for >5 min in the Petri dishes caused some contamination with ciliated cells. That these cells formed swimming blastuloid cell aggregates when incubated over a long period indicates that these ciliated cells are ectodermal cells.

Scanning Electron Microscopy (SEM): PMCs separated as above were plated in plastic Petri dishes prefixed in 2.5% glutaraldehyde, and postfixed in 1% OsO_4 in a 0.20 M phosphate buffer (pH 7.4). The specimens were dehydrated in ethanol and critical point dried (1). After the specimens were coated with gold-palladium they were examined under a Hitachi-S430 SEM.

Time-lapse Study of PMCs In Vitro: Drops of cell suspension, 0.3 ml of 1.5×10^5 cells/ml, in calcium-deficient artificial seawater were put into 35-mm-diam plastic Petri dishes and left for 5 min at 10°C. 2 ml of one of the experimental media was added (Table I). The amount of FN in the 2% horse serum was estimated to be 4.4 ± 0.8 μg/ml, as discussed later. The 0.4, 4, 40, and 400 μg/ml of FN concentrations were ~0.1, 1, 10, and 100 times the concentration of that in the 2% horse serum, respectively.

Time-lapse photographs were taken with a phase-contrast microscope every 5 min during the 100 min observation period. The micrographs were magnified 300 times and the initial position of each cell was marked on transparent plastic sheets. The migratory behavior of the PMCs was analyzed by two different criteria: (*a*) A percent migration that represents the proportion of cells that migrated more than one cell diameter (~8 μm). Such a measurement was chosen because the diameter distance is substantial. (*b*) A cell migration pattern that represents the proportional distribution of the population of PMCs that migrated a certain distance. This latter parameter is divided into 18 groups ranging from those in which the cells migrated from 0 to 2 μm, 2.1 to 4.0 μm, and so on to a group of cells that migrated >36.1 μm. The population of cells in each group is expressed as a percentage of the total number of cells examined. The numbers of cells and experiments in each culture condition are summarized in Table I.

Preparation of FN and FN-depleted Horse Serum: FN and FN-depleted horse serum were prepared at room temperature by gelatin column chromatography (8). Horse serum (Nippon Bio-Supply Center, Tokyo) was passed through a precolumn of Sepharose 4B and then applied to a gelatin-Sepharose column. The flow-through fraction from the gelatin column was collected and used as FN-depleted horse serum. The column was washed with 0.13 M NaCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.0), and then eluted with 4 M urea and 10 mM Tris-HCl (pH 7.0). The eluted fraction, pure FN, showing a doublet at M_r 230,000 upon SDS PAGE, was precipitated by 40% saturated ammonium sulfate, and dialyzed against artificial seawater at 4°C. The concentration of FN in the horse serum was estimated from the amount of FN bound to the gelatin column (28) to be 0.22 ± 0.04 mg/ml.

RESULTS

PMC Behavior In Situ

The PMCs in the blastocoel migrated in a zig-zag path on the basal surface of the ectoderm (Fig. 2). During migration these cells retained a round cell contour with a smooth cell surface, particularly on the blastocoel side. The cells migrated various distances during the 100-min observation, mainly from 10 to 38 μm. Some cells migrated >44 μm. Thus, in situ the PMCs migrated at various migration velocities. The cell migration pattern, therefore, indicated a rather even distribution of the PMC population. The percent migration was 55 (Fig. 5).

TABLE I. *Number of Artificial Extracellular Matrices Used for Observation of PMC Behavior*

Matrix component	No. (*n*)*
Plain seawater	246 (3)
2% Horse serum	394 (2)
2% FN-depleted horse serum	219 (2)
0.4 μg/ml FN	212 (2)
4 μg/ml FN	543 (2)
40 μg/ml FN	173 (2)
400 μg/ml FN	366 (2)
2% FN-depleted horse serum + 4 μg/ml FN	233 (2)
2% FN-depleted horse serum + 40 μg/ml FN	155 (2)

* Number of cells (No.) and experiments (*n*) analyzed.

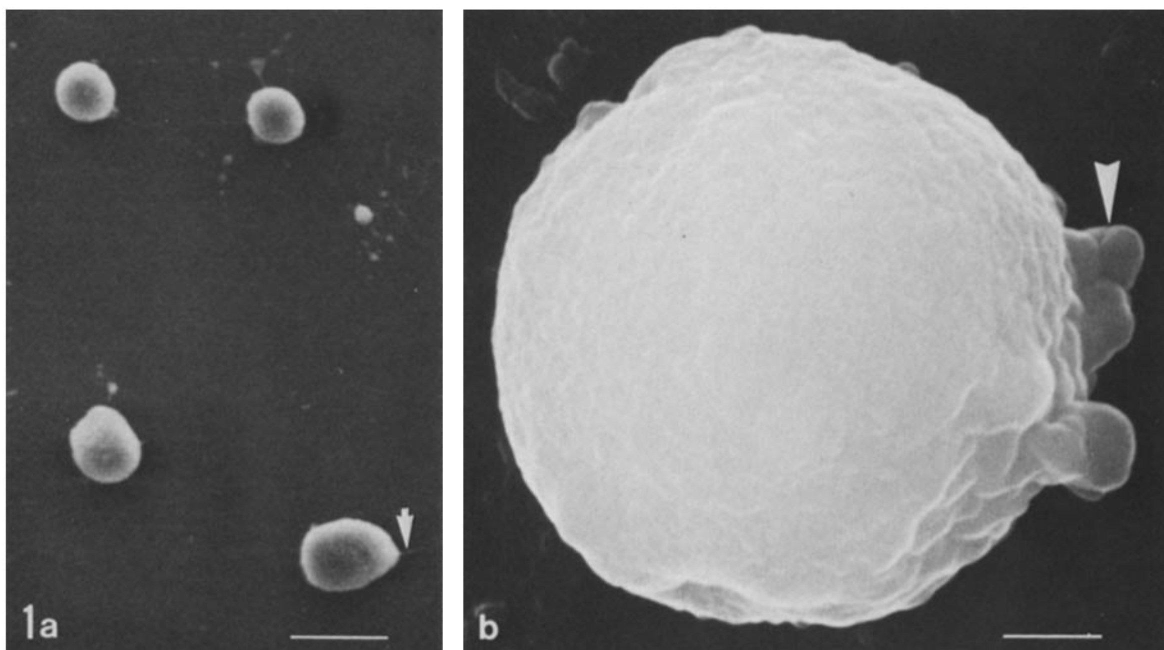

FIGURE 1 (*a*) SEM of PMCs isolated from mesenchyme blastulae. PMCs have no cilium and occasionally extend small cell process (arrow). × 1,300. (*b*) Highly magnified SEM of a PMC on the plastic substrate. The cell surface is smooth and very short cell processes are extended (arrowhead). × 13,000. (*a* and *b*) Bars, 10 and 1 μm, respectively.

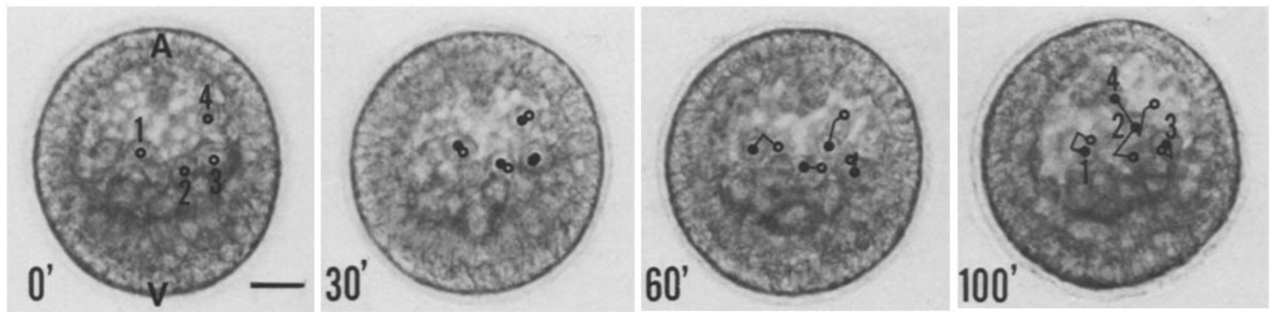

FIGURE 2 Time-lapse micrographs of a mesenchyme blastula showing PMCs behavior in situ. Solid lines indicate paths of migration of cells that started from numbered open circles. Solid circles indicate the location of cells at the time indicated at the lower left-hand corner of each frame. Bar, 20 μm. × 360.

FN-dependent Migration In Vitro

The PMCs separated from the mesenchyme blastulae, shortly after ingression yet before active migration from the vegetal plate, retained an almost round cell contour on the plastic surface (Fig. 1). The cells that migrated extensively on the plastic surface formed very short cell processes (Figs. 1 and 4) which were occasionally difficult to identify under the light microscope.

MIGRATION IN MEDIA WITH OR WITHOUT FN: In plain seawater the percent migration was 22.2 ± 1 (Fig. 5). The migration pattern was characterized by an extremely high proportion of cells that migrated <2 μm (Fig. 3). These cells formed short cell processes and survived in this condition for at least 6 h, the longest culture period used in this study.

In seawater containing 2% horse serum the contour of the PMCs was identical to that in plain seawater, but PMCs in seawater that contained 2% horse serum migrated extensively with a fairly complex path (Fig. 4). The percent migration was 65.3 ± 5.9 (Fig. 5).

The PMCs in seawater containing 2% FN-depleted horse serum migrated very little. The percent migration was only 39.2 ± 1.5 (Fig. 5). The cell migration pattern was similar to that of cells in plain seawater (Fig. 3). Therefore, there was a decline of cell motility by depletion of FN from the horse serum. Furthermore, since no morphological alteration of PMCs, such as flattening or extensive formation of cell processes, was observed in the presence or absence of FN in the culture media, this indicated that only migration behavior was affected.

MIGRATION DEPENDS ON FN CONCENTRATION IN THE MEDIA: These results suggest that FN contained in the horse serum plays a crucial role in PMC migration. To determine whether there is an optimal concentration of FN for PMC migration, PMCs were incubated in seawater containing various amounts of FN.

In seawater containing 0.4 μg/ml of FN, the PMCs migrated very little. The percent migration was 15.0 ± 8 (Fig. 6). The cell migration pattern was similar to that of cells in plain seawater (Fig. 7*a*). In seawater containing the amount of FN in 2% horse serum, 4 μg/ml, however, the percent migration rose to 38.2 ± 4.5 (Fig. 6). Still, this was not a full recovery as compared with PMCs in the 2% horse serum, despite the presence of the same amount of FN in the medium. The migration pattern differed considerably from that of PMCs in the previous two media (Fig. 7*b*). The PMCs in seawater containing a 10-times-higher concentration, 40 μg/ml, of FN than that in the 2% horse serum migrated most extensively

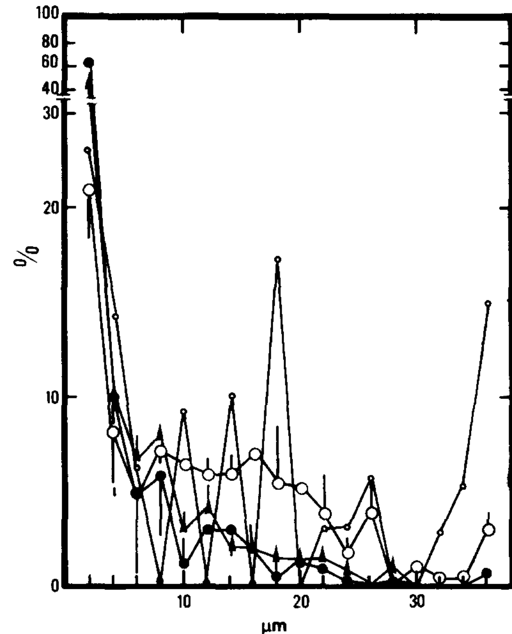

FIGURE 3 Cell migration pattern of PMCs in seawater containing 2% horse serum (○), 2% FN-depleted horse serum (▲), in plain seawater (●), and in situ (o). Abscissa indicates distance (micrometers) the cells migrated in 100 min. The ordinate indicates percentage of the cell population that migrated, classified by distance, as explained in Materials and Methods.

in terms of percent migration and cell migration pattern. The percent migration was 60.5 ± 2.2 (Fig. 6). The migration pattern was quite different from any of the previous three experiments (Fig. 7*c*). In seawater that contained a 100-times-higher concentration, 400 μg/ml, of FN than that in 2% horse serum, the PMCs migrated extensively (Fig. 7*d*), but the percent migration declined a little from that of PMCs in 40 μg/ml of FN (Fig. 6). These findings indicated that a concentration of FN that is 100 times higher is excessive and results in the inhibition of cell migration.

In a mixture of 2% FN-depleted horse serum and 4 μg/ml of FN (a culture similar to 2% horse serum) the percent migration was 65.8 ± 1.8 (Fig. 5). The value indicates a higher recovery of cell motility than that of PMCs in 4 μg/ml of FN alone. In a mixture of 2% FN-depleted horse serum and 40 μg/ml of FN the percent migration declined to 36.2 ± 15.3 (Fig. 5). The migration pattern of PMCs in this medium was not significantly different from that of PMCs in plain seawater. We may conclude, therefore, that in seawater containing

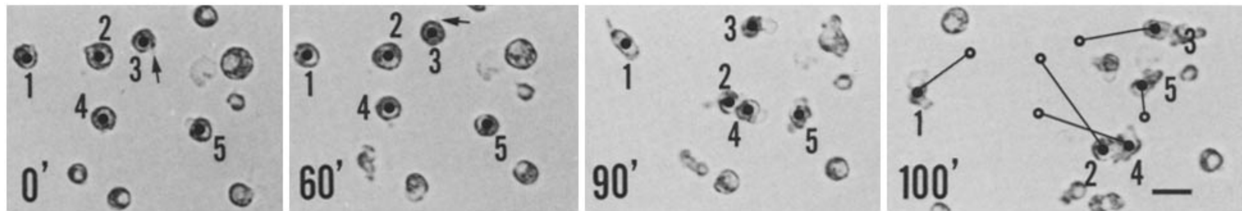

FIGURE 4 Time-lapse micrographs of a typical PMC migration in seawater containing 2% horse serum. Solid lines indicate the direction of cells migrating from original locations (open circles) to final ones during 100 min of observation. Closed circles indicate the location of cells at the time shown at the lower left-hand of each frame. Five cells are numbered with closed circles as a sample. Arrows indicate short cell processes. Bar, 20 μm. × 260.

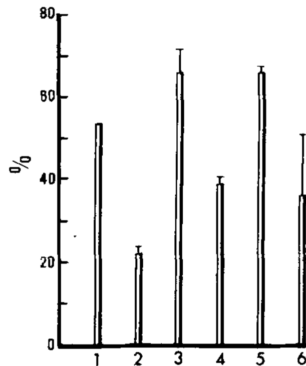

FIGURE 5 Percent migration of PMCs in situ (*1*), and in plain seawater (*2*), seawater containing 2% horse serum (*3*), seawater containing 2% FN-depleted horse serum (*4*), a mixture of 2% FN-depleted horse serum and 4 μg/ml of FN (*5*), and a mixture of 2% FN-depleted horse serum and 40 μg/ml of FN (*6*). Vertical bars show standard deviation.

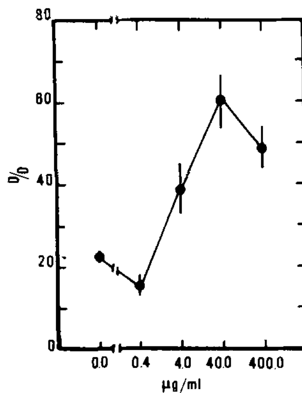

FIGURE 6 Percent migration of the PMCs in seawater containing various amounts of FN, indicated along the abscissa. Vertical bars show standard deviation.

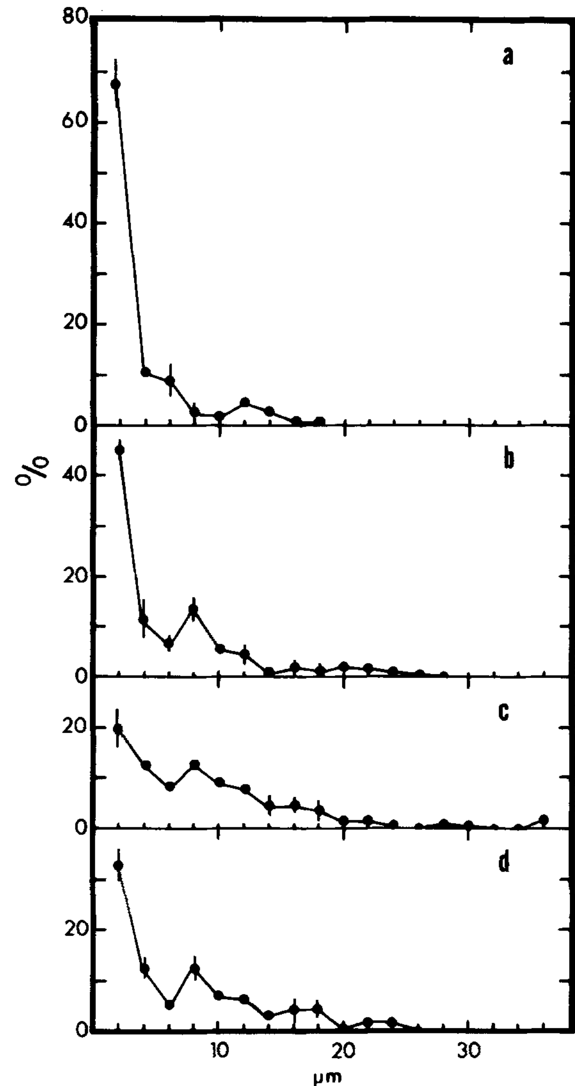

FIGURE 7 Cell migration pattern of the PMC in (*a*) 0.4, (*b*) 4, (*c*) 40 and (*d*) 400 μg/ml of FN. Vertical bars show standard deviation.

2% FN-depleted horse serum and 40 μg/ml of FN the migration of PMCs is inhibited.

DISCUSSION

There have been few measures with which to express cell behavior quantitatively. The velocity of cell migration and the proportion of cells that migrate for a certain distance during a certain period are the two principal measures used in the past. In some instances, one or the other has been successful in quantifying cell behavior. Unfortunately, such measures were often insufficient for an adequate understanding of cell behavior. In the present study, we attempted to express cell migration behavior more accurately by employing a cell migration pattern in additon to percent migration. The percent migration provides an overview of the responses made by many cells in a particular culture condition so that one can compare responses at large. It does not, however, provide information as to how many cells migrated how far, which is important for understanding the migration behavior of cells. These cells are potentially heterogenous in some instances. The importance of the cell migration pattern is better appreciated when one analyzes random cell behavior as we have done.

The in situ behavior of PMCs in this species consistently confirmed previous observations that PMCs migrate with a round cell shape, forming short cell processes (20, 23, 38), and that the cells migrate along a zig-zag course (23). This migration course in the sea urchin embryo suggests a lack of directionality, in contrast to that found in presumptive mesodermal cells of the *Ambystoma maculatum* (29). The oriented extracellular fibers found in *Ambystoma* have never

been found in sea urchins. They possess no obvious directionality (17). Furthermore, they are heavily coated with 25-30-nm-diam granules (17, 19, 20, 23). The migration pattern of the PMCs in situ indicates a difference in motility from cell to cell, which suggests that the PMCs are not a homogenous cell population in terms of motility. Whether this variation in cell motility is related to a difference in the cell's role in the formation of spicule is not yet fully understood. However, PMCs that migrate slowly may initiate spicule formation in situ (23).

The present results indicate that plasma FN in horse serum promotes sea urchin PMC migration and that cell migration is inhibited by a decrease of FN concentration in the medium. Such findings support the idea that PMC behavior is regulated by quantitative changes of exogenous FN. In addition, these results support previous immunohistological studies that found that the PMCs have FN on their surface during the migratory period (12, 41), as seen in amphibian embryonic cells (3) and mice primordial germ cells (9). Although in avian neural crest cell migration, FN in the extracellular space rather than on the cell surface appears to regulate the cell migration (5, 12), FN both on the cell surface and in the extracellular matrix may be contributing to the PMC migration mechanism. For sea urchins, there are two optimal concentrations of FN that are different depending on whether FN is applied alone or in a mixture. The requirement for a higher optimal concentration of FN in the absence of horse serum could have at least two explanations: serum provides some other factor that substitutes in part for FN, or serum protects FN in some way. In both incubation conditions, with or without 2% FN-depleted horse serum, too much or too little FN inhibits cell locomotion. Since the PMC has FN on the cell surface in situ (12, 41), even after separation in vitro (40), enough FN in the extracellular space in vitro might mediate an attachment between the substrate and cell.

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