Fibronectin-rich fibrillar extracellular matrix controls cell migration during amphibian gastrulation

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ABSTRACT We have reviewed the evidence supporting the notion that the fibrillar extracellular matrix on the basal surface of the blastocoel roof in amphibian embryos directs and guides mesodermal cell migration during gastrulation. Based on extensive experimental evidence in several different systems, we conclude the following: (i) the fibrillar extracellular matrix contains fibronectin (FN) and laminin. (ii) The fibrils are oriented in such a way as to promote directional migration of mesodermal cells during migration. (iii) We have used several different probes to disrupt the interaction between migrating mesodermal cells and the fibrillar extracellular matrix. These probes include: (a) nucleocytoplasmic and interspecific hybridization. Such embryos have defects in FN synthesis and gastrulation. (b) Fab' fragments of anti-FN and anti-integrin VLA-5 IgGs prohibit mesodermal cell adhesion both *in vitro* and *in vivo* and gastrulation is arrested. (c) Peptides containing the RGDS sequence specifically inhibit interactions between migrating mesodermal cells and the FN-fibrillar matrix. (d) Tenascin blocks cell adhesion to FN *in vitro* and gastrulation *in vivo*. (e) Antibodies against the cytoplasmic domain of ß1 integrin, when injected into blastomeres, prevent FN-fibrillogenesis in progeny of injected blastomeres and delay mesodermal cell migration selectively in the progeny of injected blastomeres but not in the uninjected blastomere progeny.

KEY WORDS: *amphibian, gastrulation, extracellular matrix, fibronectin*

Introduction

All metazoan organisms undergo an early developmental period where changes in cell shape, cell number, and cell-cell associations produce fundamental changes in embryonic morphology. Groups of cells are designated to perform particular assemblages of cell movements. At present, the molecular mechanisms involved in this designation and subsequent morphogenetic selectivity are poorly understood – typically, morphogenetic cell movements are controlled in a temporal and spatial pattern that is more or less the same in each embryo. Cells move along specific pathways within the embryo, moving from one location to another along a *particular* pathway. It is difficult enough to understand how cells move from one location to another inside the embryo but even more mysterious why they choose one particular pathway for this locomotion from among the large number of pathways theoretically available to them. Cell movements occur over a single path among the many possible paths as cells start in one location and end up in another. We will examine some of the factors that guide morphogenetic cell movements along specific pathways during amphibian gastrulation.

In all urodele amphibian embryos, the morphogenetic cell movements of gastrulation follow a common pattern and give rise to a blastopore. At the beginning of gastrulation, *bottle cells* form near the blastopore. This initial step in gastrulation leads to the formation of an invagination site in the marginal zone which quickly grows into a small curved slit (Fig. 1A) and then a crescent-shaped structure. In addition, cells above the dorsal lip of the blastopore begin to migrate towards and then over the dorsal lip in a movement known as *involution*. A broad sheet of cells, representing the primordium of the chordamesoderm and located above the dorsal lip of the blastopore, converges on the blastopore and moves toward it.

As chordamesodermal cells involute, their space on the surface of embryo is occupied by spreading presumptive ectodermal cells

Abbreviations used in this paper: DMZ, dorsal marginal zone; ECM, extracellular matrix; ESC, *rana esculenta*; FN, fibronectin; PIP, *rana pipiens*; TN, tenascin; VLA, very late antigen.

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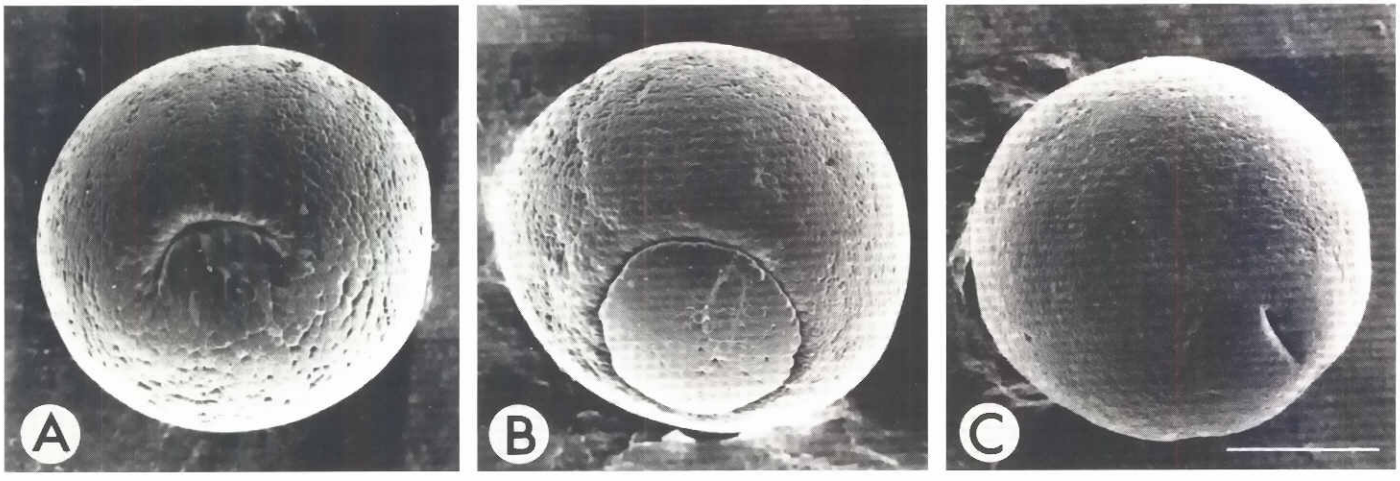

Fig. 1. Scanning electron micrographs. *Gastrulation of* Pleurodeles waltl *embryos from the vegetal aspect.* **(A)** *Blastopore is a pigmented area just beginning to invaginate.* **(B)** *Blastopore nearly a complete circle.* **(C)** *Yolk plug disappears from surface and blastopore closes. Bar: 500 μm.*

during *epiboly*, an event that is closely coordinated with involution. The blastopore continues to grow from a crescent into a circle and then the diameter of the circular blastopore shrinks by constriction (Fig. 1B). By the end of gastrulation, the presumptive endoderm is drawn completely inside the embryo (Fig. 1C).

During gastrulation, a new cavity, called the *archenteron*, grows from the blastopore as the invagination site at the blastopore deepens and expands toward the animal pole inside the embryo. As the archenteron expands, it grows at the expense of the shrinking blastocoel. The leading edge of the archenteron consists of groups of cells that adhere to and spread across the inner surface of the roof of the blastocoel. The cells at the leading edge of the growing archenteron form broad lamellipodial attachments on the basal surfaces of cells making up the roof of the blastocoel. These cells attach preferentially to this inner surface and move away from the blastopore. What guides these movements? What is the molecular basis for these specific interactions?

Recent work has revealed that there are significant differences in mesodermal cell formation in different amphibian species. Smith and Malacinski (1983) confirmed Keller's (1975, 1976) earlier observations in the anuran *Xenopus laevis* to show that mesodermal cells arise from deep cells in the dorsal marginal zone (DMZ). In addition, Smith and Malacinski (1983) found that mesodermal cells arise in the superficial DMZ in the urodele *Ambystoma mexicanum*. Furthermore, Lundmark (1986) has provided evidence that in *A. mexicanum* mesodermal cell migration may play a significant role in convergent extension. Recently, Shi *et al.* (1987) have investigated the capacity for extension of the DMZ in the urodele *Pleurodeles waltl* gastrulae. They showed that intercalation plays a role in convergent extension but also showed that when rotated 90° or 180°, grafted DMZ explants still involuted normally and extended in accordance with the appropriate animal pole-blastopore axis of host embryos. Furthermore, if the roof of the blastocoel was removed at the blastula stage, i.e. when mesodermal cells had not yet undergone migration on the blastocoel roof, involution and extension of the DMZ remained extremely limited. These results suggest that mesodermal cell migration may play an important role in DMZ extension in *Pleurodeles*. In contrast, Keller (1984) has shown that in the anuran *Xenopus laevis*, DMZs rotated 90° and then grafted into hosts failed to involute and, instead, showed extension in the appropriate direction with respect to the graft animal pole-blastopore axis rather than with respect to the same axis in the host. Clearly then, there are significant differences in the detailed mechanisms of gastrulation in anuran and urodele embryos.

Interactions of mesodermal cells with conditioned substratum

Ubiquitous nature of fibrillar extracellular matrix in amphibian gastrulae

Nakatsuji *et al.* (1982) and Boucaut and Darribère (1983a, b) discovered a dense network of extracellular fibrils lining the basal surface of epithelial cells comprising the roof of the blastocoel. These fibrils are sparse prior to gastrulation, appear at the beginning of gastrulation, and continue to accumulate during gastrulation. These fibrils have been observed in all 9 species of amphibian embryos examined to date including the urodeles *Ambystoma maculatum, A. mexicanum, Pleurodeles waltl* and *Cynops pyrrhogaster* and the anurans *Xenopus laevis, Rana pipiens, Rana sylvatica, Bufo bufo* and *B. calamita* and are known to contain FN (Boucaut and Darribère, 1983a; Nakatsuji and Johnson, 1983b; Johnson, 1984; Lee *et al.*,1984; Nakatsuji, 1984; Delarue *et al.*, 1985; Nakatsuji *et al.*, 1985b; Johnson *et al.*, 1987). These fibrils also contain laminin (Nakatsuji *et al.*, 1985a; Darribère *et al.*, 1986). FN synthesis occurs at low levels prior to gastrulation and is made in increasing amounts during gastrulation (Darribère *et al.*, 1984; Lee *et al.*, 1984). FN-containing fibrils accumulate preferentially on the inner surface of the roof of the blastocoel (Boucaut and Darribère, 1983a, b) presumably due to the differential distribution and function of integrin receptors for FN (Lee *et al.*, 1984; Darribère *et al.*, 1988, 1990).

Guidance of migrating mesodermal cells

Migrating mesodermal cells in amphibian gastrulae use an anastomosing network of extracellular matrix fibrils on the inner

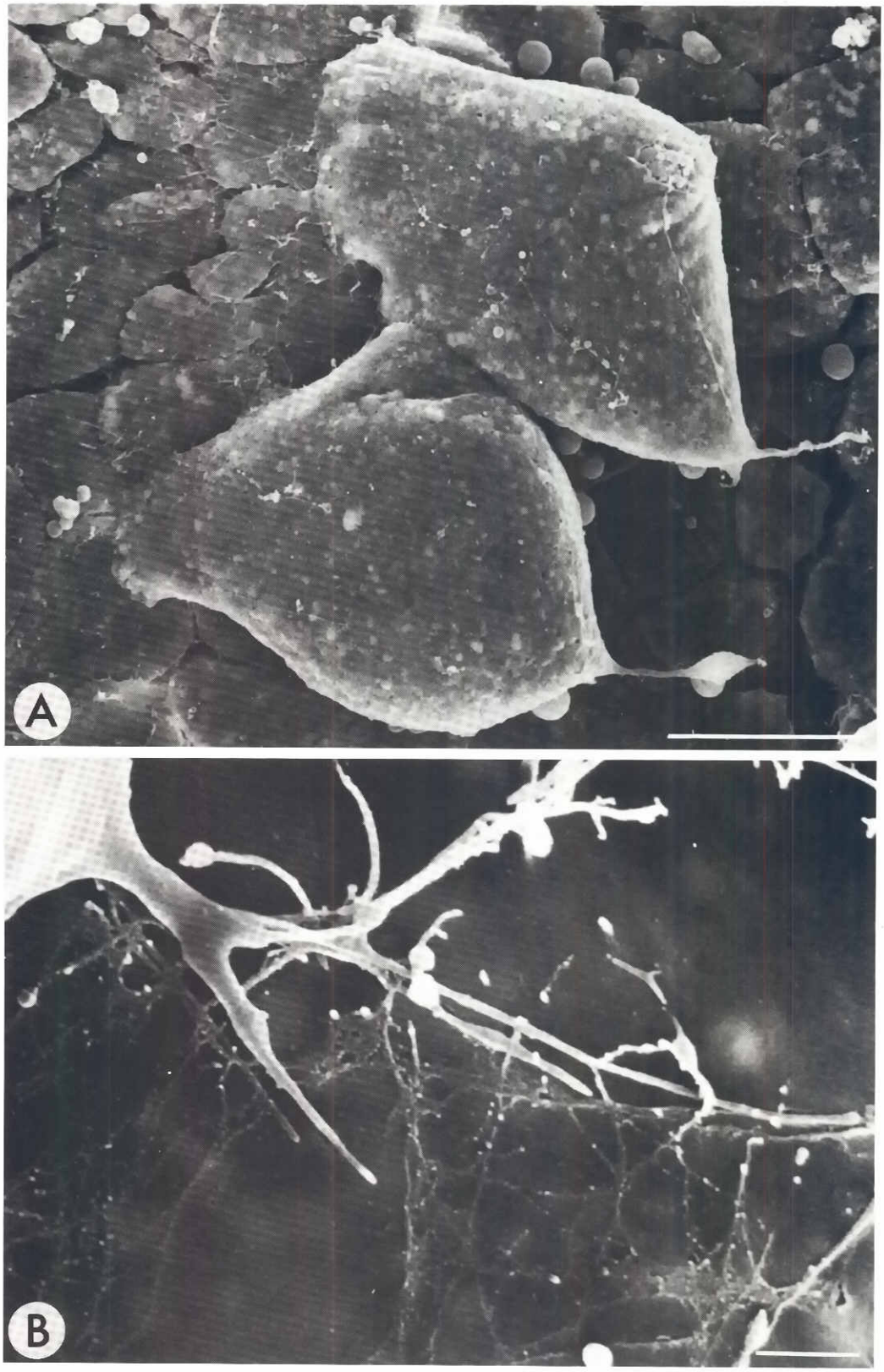

Fig. 2. Scanning electron micrographs of migrating mesodermal cells on the inner surface of the blastocoel roof in *Ambystoma maculatum* gastrulae. (A) *Two migrating mesodermal cells. The animal pole is to the left and the dorsal lip of the blastopore is to the right. Notice that the cells have large lamellipodia on the leading edge of the cells directed toward the animal pole and a uropod-like attachment on the trailing edge of the cells. Bar: 50 μm.* **(B)** *Filopodia on the leading edge of a lamellipodium associated with the fibrillar ECM on the basal surface of the blastocoel roof. Bar: 1 μm.*

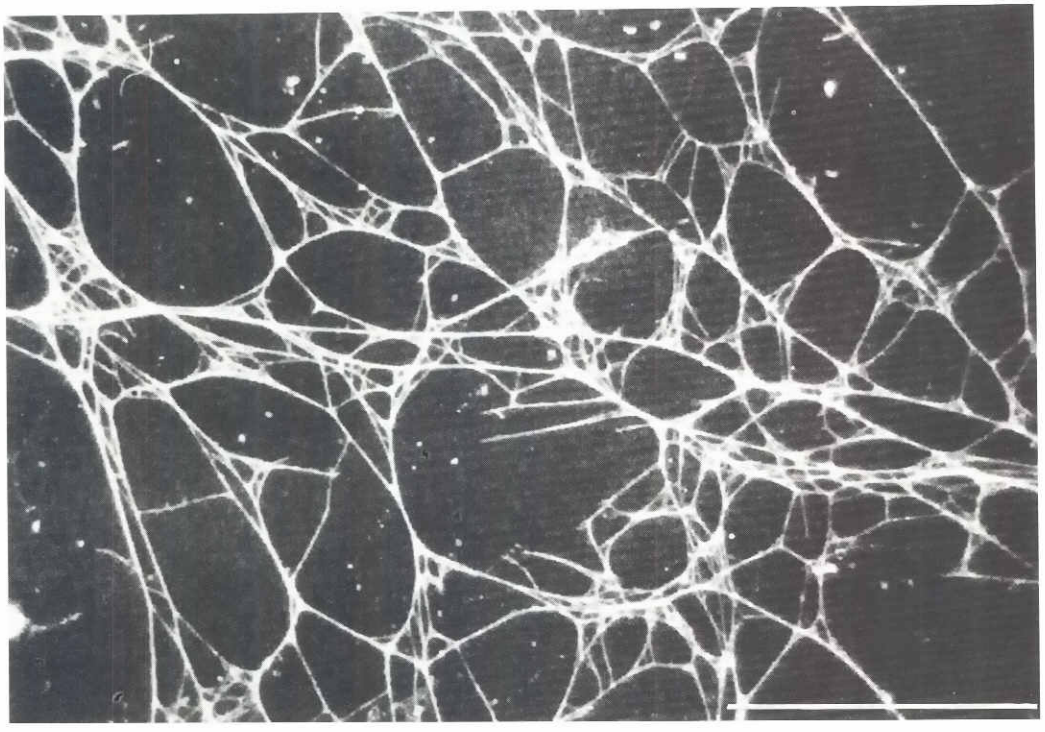

Fig. 3. Fluorescent micrograph of ECM fibrils transferred from the basal surface of the blastocoel roof of an *Ambystoma maculatum* gastrula to a plastic dish. *Deposited fibrils were stained with a polyclonal antibody directed against* A. mexicanum *plasma fibronectin. The dorsal lip region of the conditioning explant was on the left and the animal pole region was on the right. Notice that the fibrils are highly aligned parallel to the blastopore-animal pole axis (parallel to the long axis of the photograph). Migrating mesodermal cells or explants of the DMZ adhere to such substrata and migrate preferentially toward the animal pole region of such conditioned areas. Bar: 50 μm.*

surface of the ectoderm layer as their substratum for cell migration (Nakatsuji *et al.*, 1982; Nakatsuji and Johnson, 1983a, 1984). Boucaut *et al.*, (1984a) have shown that when the animal cap is inverted 180° so that the surface facing the perivitelline space now faces into the blastocoel, grafted explants do not provide a suitable substratum for mesodermal cell migration because extracellular matrix fibrils are not available. Migrating mesodermal cells often have large lamellipodia on their leading edges, projecting toward the animal pole (Fig. 2A) and have numerous filopodia on the leading edges of lamellipodia of migrating mesodermal cells, which often show close association with the fibrils (Fig. 2B), suggesting that the fibrils serve as a preferential adhesion site for filopodia and lamellipodia (Boucaut *et al.*, 1984a). Furthermore, the presence of the statistically significant alignement of this fibril network along the

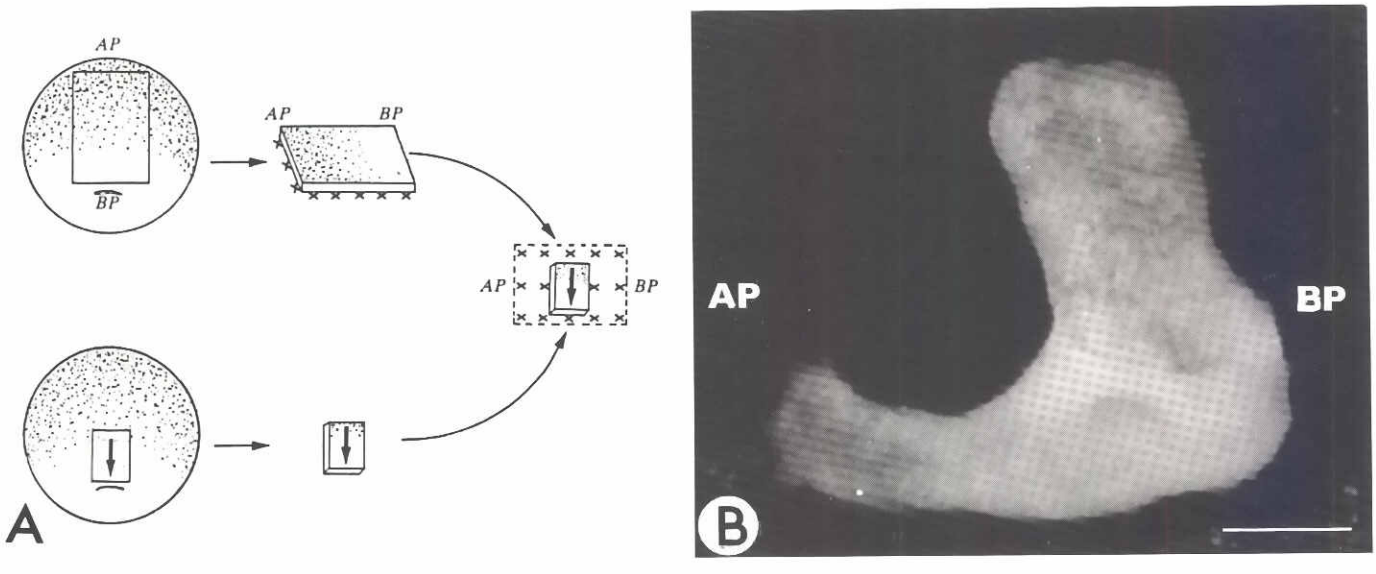

Fig. 4. Orientation of DMZ outgrowth toward the animal pole (AP) on ECM-conditioned substratum. (A) *Diagram of explants containing DMZ and adjacent ectoderm was deposited in the center of a substratum conditioned by ECM deposition from the basal surface of the blastocoel roof. The original animal pole to blastopore axis (thick arrow) was perpendicular to that of the fragment used to condition the substratum.* **(B)** *After 24 h of culture. DMZ outgrowth has fully spread toward the animal pole, a cohesive cell sheet developed on this side. The front of migration reached the edge of animal pole and stopped there because ECM-conditioned substratum was no longer available. AP, animal pole ; BP, blastopore. Bar: 500 μm.*

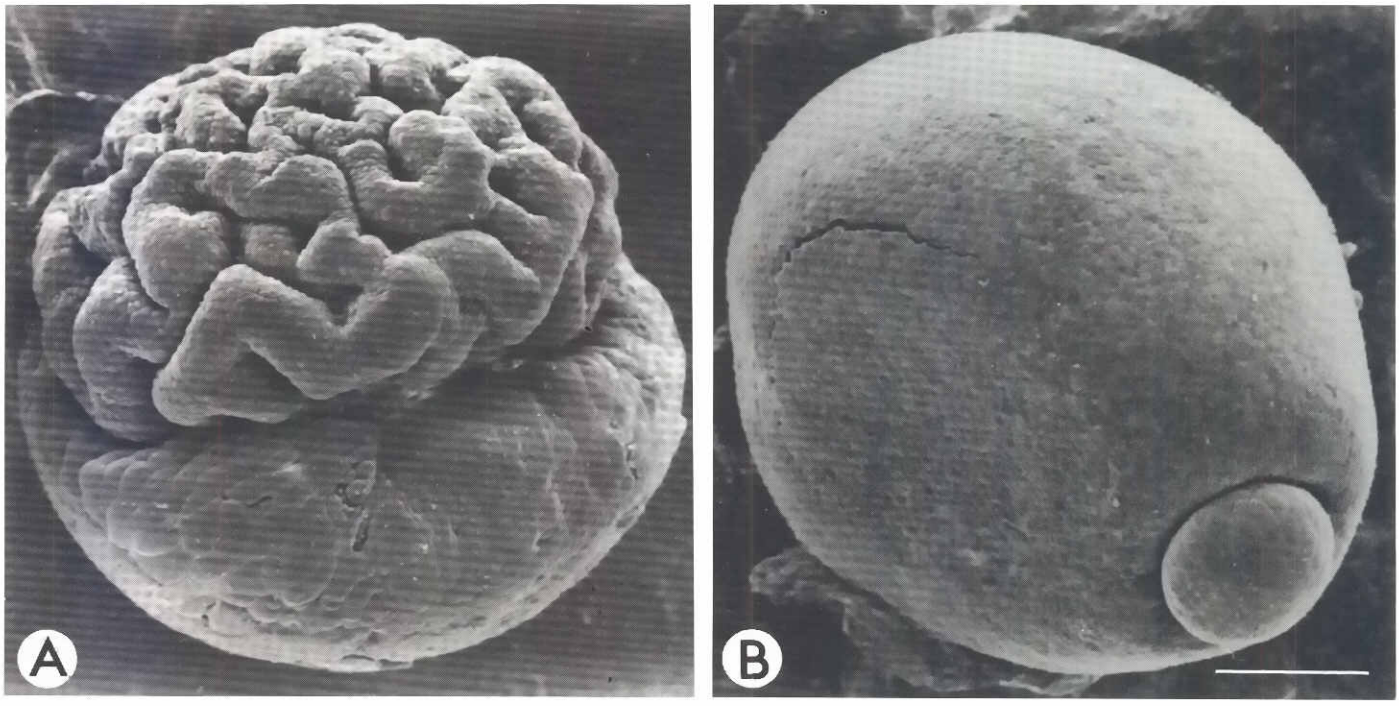

Fig. 5. Scanning electron micrograph of the *in vivo* effect of Fab' fragments of anti-FN during gastrulation. Pleurodeles waltl *embryos were microinjected at the early gastrula stage (Stage 8b) and observed 24 h later.* **(A)** *Injection of Fab' fragments of anti-FN. This result is typical of the more severely arrested embryos. A complete inhibition of gastrulation was observed. Note the highly convoluted roof of the blastocoel, circular blastopore, and smooth exposed endodermal mass.* **(B)** *Control embryo injected with Fab' preimmune IgG. The embryo undergoes normal gastrulation. An early neural plate has formed in this control embryo indicating that primary embryonic induction has taken place. Bar: 250 μm.*

blastopore-animal pole axis in *Ambystoma* (Nakatsuji *et al.*, 1982), indicates an interesting possibility for an actual role *in vivo* for a long-postulated contact guidance system (Weiss, 1945) by an aligned fibril network (Nakatsuji *et al.*, 1982 ; Nakatsuji, 1984).

To test this hypothesis, Nakatsuji and Johnson (1983a) and Shi *et al.*, (1989) transferred the extracellular fibril network onto a cover slip surface. A rectangular piece of the presumptive ectoderm was dissected from the dorsal part of early gastrulae of *Ambystoma maculatum* or *Pleurodeles waltl* and explanted onto a plastic cover slip with the inner surface facing down on the cover slip surface. After suitable culture, the explant outline and an arrow marking the direction toward the animal pole of the original embryo were scratched on the cover slip. After the explant was mechanically removed from the cover slip, dissociated mesoderm cells isolated from gastrulae or DMZ explants were placed on the conditioned surface. A dense matrix of fibrils was transferred from the conditioning explant to the substratum (Fig. 3). Cells and explants adhered rapidly to such conditioned surfaces. They extended lamellipodia and filopodia, and migrated actively on the conditioned surface. Isolated cells moved preferentially toward the animal pole region of the conditioned substratum. Similarly, the outgrowth of mesodermal cells from DMZ explants was distorted toward the animal pole region of conditioned substrata (Fig. 4).

Adhesion and movement of mesodermal cells on substrata coated with fibronectin or laminin

Culture dish surfaces coated with fibronectin or laminin were found to be good substrata for attachment and migration by *Xenopus* and *Pleurodeles* gastrula mesodermal cells (Nakatsuji, 1986; Darribère *et al.*, 1988). For example, about 80% of *Xenopus* mesodermal cells attached to FN-coated substrata within 1 h and moved actively at a mean rate of 2.8 μm/min. Johnson (1985) and Johnson and Silver (1989) also observed adhesion and migration of mesodermal cells attached to Sepharose beads coupled with FN. Furthermore, Darribère *et al.*, (1988) showed that Fab' fragments of anti-VLA5 ($\alpha_5\beta_1$) integrin receptor caused detachment of *Pleurodeles* cells previously attached to FN-coated substrata. Nakatsuji (1986) also showed that type IV collagen and heparan sulfate supported neither adhesion nor movement of *Xenopus* gastrula mesodermal cells. Another important finding was that a heterogeneous distribution of fibronectin or laminin molecules *in vitro* caused guiding effects on the adhesion and migration of the mesodermal cells. For example, *Bufo bufo japonicus* gastrula mesodermal cells accumulate inside an area coated with fibronectin. *Rana pipiens* cells adhered preferentially and moved along FN strands. Furthermore, under certain conditions, laminin molecules make fibrillar aggregates *in vitro*. When a network of such fibrils was aligned accidentally, mesodermal cells elongated and moved along the axis of fibril alignment, illustrating a clear case of the contact guidance *in vitro*. These observations show that it is possible to guide the mesodermal cells by heterogenous distributions of ECM *in vivo* and *in vitro*. If these extracellular fibrils serve as a contact guidance system for migrating mesodermal cells, one would expect that probes to disrupt cell-fibronectin interaction would have a

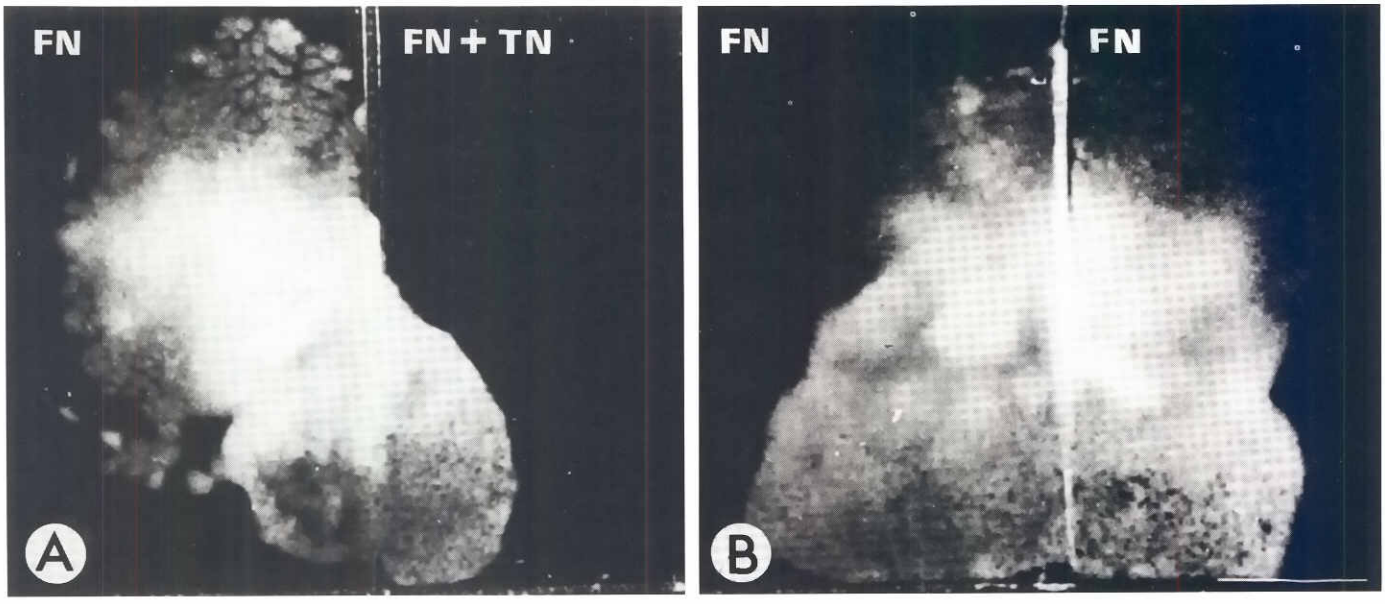

Fig. 6. Migratory behavior of mesodermal cells *in vitro* from *Pleurodeles waltl* gastrulae on a substrate conditioned by FN and TN. (A) *An explant containing the DMZ and adjacent ectoderm was placed at the edge of one track conditioned by FN (left) and another conditioned by FN and TN (right). The migrating mesodermal sheet fails to spread on FN- and TN-coated substratum. The vertical lines through the micrographs represent the boundaries between each type of substratum.* **(B)** *Control where both tracks were conditioned by FN alone. The cell sheet spread equally in both tracks. Bar: 500μm.*

profound effect on cell migration. Boucaut *et al.*, (1984a, b) have provided evidence that this prediction was correct.

Studies with probes to disrupt function of ECM molecules *in vivo*

Studies with Fab' fragments of anti-FN IgG, anti-VLA5 IgG, and peptides containing the RGDS sequence

First, when living embryos were injected with Fab' fragments of anti-FN IgG at the early gastrula stage, migration of mesodermal cells was inhibited and gastrulation was blocked (Fig. 5). The antibodies interacted with FN-containing fibrils *in vivo* in such a way as to prevent mesodermal cell adhesion to the fibrils. Similar injections at the late gastrula stage had no noticeable effect on neurulation (Boucaut *et al.*, 1984a). Second, when early gastrula were probed with synthetic peptides representing the cell-binding domain of FN, again migration of mesodermal cells was inhibited and gastrulation was blocked completely. A peptide representing the collagen-binding domain of FN had no effect on gastrulation (Boucaut *et al.*, 1984b, 1985). Third, when Darribère *et al.*, (1988) injected Fab' fragments of anti-VLA5 IgG into living early gastrulae, once again, mesodermal cell migration and gastrulation were completely inhibited.

All three probes prohibit the interaction between migrating mesodermal cells and the FN-rich ECM on the inner surface of the roof of the blastocoel, albeit by different mechanims. The anti-FN antibodies coat FN-fibrils (Darribère *et al.*, 1985), preventing access by migrating mesodermal cell receptor. The anti-VLA5 antibodies bind to the major cell surface receptor for FN and thereby prevent mesodermal cell adhesion to FN-containing fibrils. The synthetic peptides bind to cell surface VLA5 integrin receptor and thus prohibit cells from adhering to the FN-rich ECM. Each probe has the net effect of preventing the crucial interaction between migrating mesodermal cells and FN-fibrils deposited on the inner surface of the roof of the blastocoel.

Studies with normal and hybrid frog embryos

Delarue *et al.* (1985) showed that while both *Bufo bufo* and *B. calamita* gastrulae have extensive FN-rich ECM, gastrula arrested nucleocytoplasmic hybrids lack this matrix. Johnson (1985) and Johnson and Silver (1989) showed that normal *Rana pipiens* show a progressive increase in cell adhesion to FN-Sepharose beads while cells from several different arrested hybrid embryos show defects in adhesion to FN-Sepharose beads. *Rana pipiens* eggs fertilized by *Rana esculenta* sperm (ESC) hybrid embryos develop until gastrulation in control *Rana pipiens* embryos (PIP) and then show morphogenetic arrest (Johnson *et al.*, 1990). After arrest, ESC do not gastrulate but live for 5 days as a blastula-like embryos. We studied the distribution of FN-fibrils and integrin in PIP and ESC. There are many FN-fibrils in PIP organized in anastomosing networks radiating away from the center of individual cells and across intercellular boundaries. ESC have fewer fibrils compared to PIP. After arrest in ESC, when PIP are neurulae, many more FN-fibrils appear. We found that ESC cells are partially defective in their attachment to and translocation on FN-substrata and native ECM conditioned substrata. We made reciprocal grafts of the blastocoel roof between PIP and ESC early gastrulae. We found that PIP cells would adhere to ESC blastocoel roof, even though partially depleted of FN. These results may mean that even a sparse FN network may support cell locomotion. In contrast, they may suggest that the FN-rich ECM on the blastocoel roof in anura may not play as important a role as that found in urodeles.

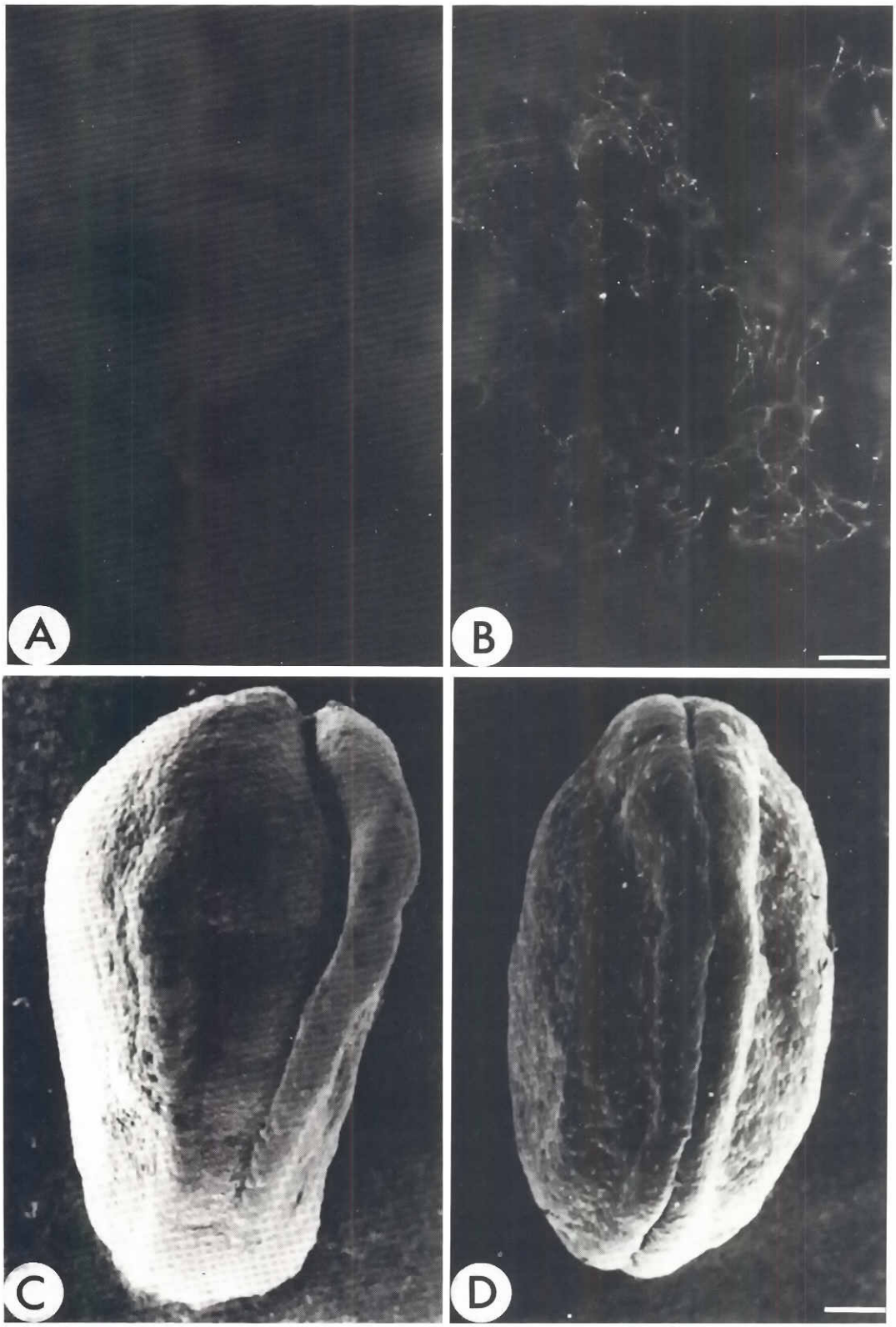

Fig. 7. Inhibition of FN-fibril formation in selected blastomeres by Fab' to cytoplasmic domain of integrin ß$_1$ subunit. (A-B) *Monovalent antibodies against the cytoplasmic domain of integrin ß$_1$ (50 ng/embryo) were injected into each uncleaved* Pleurodeles waltl *embryo. Embryos were maintained at 18°C, then dissected after the indicated times of incubation. Immunodetection of FN was done on whole mounts of the blastocoel roof.* **(A)** *Twenty-four hours after injection, the embryo has reached the late blastula stage (stage 7), but no staining for FN can be detected.* **(B)** *The first FN fibrils appear at the early gastrula stage (stage 8a). They are distributed around the periphery of cells. Their pattern is comparable to normal embryos observed at the early blastula stage (17 h earlier). Bar: 5 µm.* **(C-D)** *Embryos injected with antibodies to the cytoplasmic domain of integrin ß$_1$ subunit.* **(C)** *Embryo at the two-cell stage was injected into the left blastomere with 100 ng of anti-ß$_1$ COOH Fab'. At the time of observation 72 h later, the left neural fold is defective.* **(D)** *Control experiment. Anti-ß$_1$ COOH Fab' was preincubated with amphibian integrin ß$_1$ and injected into the left blastomere at the two-cell stage. Neurulation occurs normally. Bar: 0.3 mm.*

Studies with tenascin

Tenascin (TN) is a noncollagenous glycoprotein of the extracellular matrix with a spatially and temporally restricted tissue distribution. It was isolated by Chiquet and Fambrough (1984a, b) and was shown to be present at specific stages of development and may modulate integrin-mediated cell attachment to FN both *in vivo* and *in vitro* (Crossin *et al.*, 1986; Chiquet-Ehrismann *et al.*, 1988; Mackie *et al.*, 1988; Riou *et al.*, 1988). We have studied the effects of tenascin (TN) on mesodermal cell migration in *Pleurodeles waltl* gastrulae (Riou *et al.*, 1990). We found that TN added to culture medium inhibited attachment and spreading of DMZ cells on FN-coated substrata. DMZ outgrowth was also inhibited on substrata covered with mixtures of FN and TN (Fig.6). TN injected into the blastocoel of late blastulae bound to the fibrillar extracellular matrix and inhibited gastrulation by preventing mesodermal cell migration *in vivo*. Finally, we showed that mesodermal cell migration *in vivo* and *in vitro* was restored in the presence of TN and a monoclonal antibody known to mask the cell binding site of tenascin. These results provide additional evidence supporting the notion that cell binding to the fibrillar extracellular matrix is required for normal mesodermal cell migration and gastrulation.

Studies of fibrillogenesis

The interactions of cells with FN occur through specific cell surface receptors belonging to the large integrin familly (Hynes, 1987). The integrins consist of a heterodimeric complex of an α and a ß subunit noncovalently associated. The $ß_1$ integrins include the high-affinity VLA5 fibronectin receptors ($\alpha_5ß_1$) that interact with the RGDS sequence of the cell binding domain of FN (Akiyama *et al.*, 1989). Recently, we have made an extensive study of the organization of FN-fibrils on the inner blastocoel roof in *Pleurodeles waltl* embryos, and of the integrins involved in the assembly of FN into fibrils (Darribère *et al.*, 1990). Native FN begins to assemble at the early blastula stage and forms a complex extracellular matrix before gastrulation begins. When we injected FITC-labeled bovine plasma FN into the blastocoel of living embryos, we found that exogenous labeled FN was assembled into fibrils in the same spatio-temporal pattern as observed for endogenous FN. This suggests that the cell surface regulates fibrillogenesis. Fibrillogenesis of exogenous FITC-FN is inhibited in a dose-dependent manner by both the GRGDS peptide and monospecific antibodies to amphibian integrin. Injection of antibodies into the cytoplasmic domain of integrin $ß_1$ subunit produces a reversible delay in FN-fibril formation that follows early cell lineages (Fig. 7A-B) and causes delays in development (Fig. 7C-D). Together, these data indicate that *in vivo*, the integrin $ß_1$ subunit and the RGDs recognition signal are essential for the proper assembly of FN fibrils. Also, they suggest that normal gastrulation requires normal assembly of a FN-rich fibrillar extracellular matrix.

Conclusion

With our clearer understanding of the role of FN and integrins in gastrulation, we can begin to ask questions concerning the control of FN synthesis and distribution during gastrulation. In the future, we hope to learn more about the genetic control of the spatial and temporal pattern of FN synthesis and the factors that control the appearance and the function of integrins. We feel that genetically programmed synthetic events lead to the production of new extracellular matrix components. Also, the expression of appropriate cell surface receptors may both control the intraembryonic distribution of extracellular matrix components and also enable cells to respond specifically to changes in their pericellular environment. Finally, one set of morphogenetic movements such as epiboly may impose important epigenetic organizational order on the extracellular matrix, allowing specific contact guidance of other moving cells. Together, these genetic and epigenetic events lead to directing morphogenetic cell movements.

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