Int. J. Dev. Biol. 36: 123-137 (1992)

The arrest of cell migration in the chicken blastoderm: experimental evidence for the involvement of a band of extracellular fibrils associated with the basal lamina

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ABSTRACT **This article overviews our current knowledge of the occurrence and distribution of oriented extracellular fibrils associated with the basal lamina, and their presumptive role in contact guidance of cells in early embryos. To investigate the role of the band of extracellular fibrils situated at the basal side of the epiblast at the cranial edge of the area pellucida of the chicken blastoderm, we determined the precise location and morphology of the fibrils using TEM and SEM, described the relationship between migrating mesoblast cells and the fibrillar band, and, finally, tested experimentally the behavior of homologous and heterologous tissues in the vicinity of the fibrillar band. The descriptive analysis demonstrated that a horseshoe-shaped, 170 μm-wide band of fibrils occurs at the cranial and lateral edges of the area pellucida and area opaca, the highest density being found in the area pellucida. Migrating mesoblast cells presented a surface morphology that was different from the morphology of cells reaching the fibrils at the lateral edge of the area pellucida. Mesoblast cells never crossed the fibrils, an observation that may explain why during gastrulation, mesoblast cells invade the area opaca only in the caudal region, where no fibrillar band is present. The experimental analysis, which involved transplantation and healing experiments, demonstrated that the arrest of cell migration, that occurred in all cases in the vicinity of the fibrillar band, was correlated with changes in surface morphology suggesting a decreased cell adhesion to the fibrils. From these observations emerged the view that the horseshoe-shaped fibrillar band functions as a barrier inhibiting migration of individual mesoblast cells and expansion of tissue sheets, rather than as an extracellular substrate mediating the oriented guidance of cells. In addition to its inhibitory role in cell migration, the extracellular band may also be regarded as a factor that stabilizes the polarity of the early embryo by determining the cranial and lateral limits between embryonic and extraembryonic tissues.**

KEY WORDS: *chicken blastoderm, cell migration, extracellular fibrils, basal lamina, gastrulation, contact guidance*

Introduction

Cell migration during early development involves a complex series of coordinated mechanisms that, in time, may be defined as the initiation of cell migration, the directional cell movement, and the inhibition and final arrest of migration. During chicken gastrulation, the formation of mesoblast is initiated at the level of the primitive streak (for review see Bellairs, 1986), by de-epithelialization of upper-layer cells and subsequent cell detachment as a consequence of the synthesis of hyaluronate (Vanroelen *et al.*, 1980; Van Hoof *et al.*, 1986; for review see Harrisson *et al.*, 1988). The directional movement of single mesoblast cells occurs using the basal lamina and the neighboring mesoblast cells as an appropriate, fibronectin-rich substrate for adhesion (Sanders, 1982; Harrisson *et al.*, 1984, 1985). The mechanisms determining directional movement of mesoblast cells (for general reviews see Katz and Lasek, 1980 and Oster *et al.*, 1983) probably include haptotaxis on a fibronectin gradient in the basal lamina (Harrisson, 1989), along with contact inhibition of movement and population pressure arising from a high cell-packing density that decreases from the primitive streak towards the lateral edge of the area pellucida. In

Abbreviations used in this paper: AO, area opaca; AP, area pellucida; BL, basal lamina; DL, deep layer; HN, Hensen's node; ML, middle layer; PS, primitive streak; SEM, scanning electron microscopy; TEM, transmission electron microscopy; UL, upper layer; VM, vitelline membrane.

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0214-6282/92/$03.00

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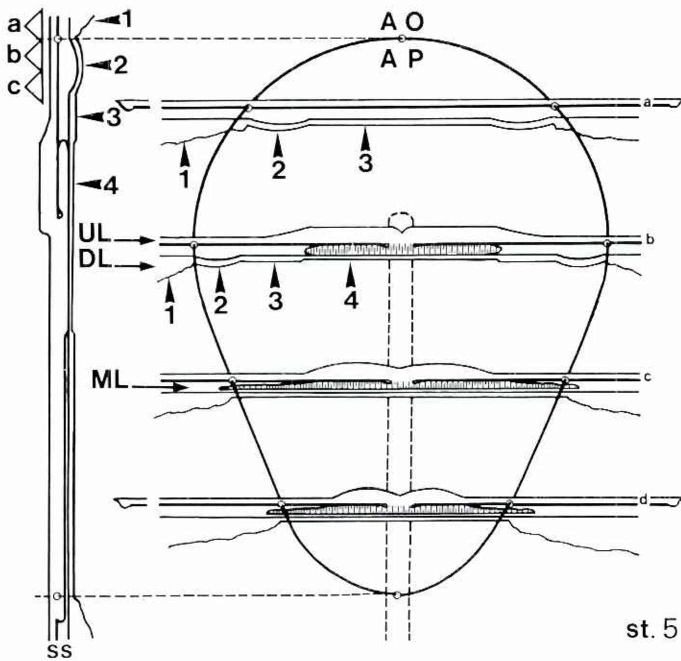


Fig. 1. Schematic representation of a stage-5 blastoderm, to show the disposition of the different germ layers in a sagittal section (SS) and in transverse sections (a-d). *The Arabic numerals indicate the different regions of the deep layer: 1, yolk endoderm; 2, endophyll; 3, hypoblast; 4, definitive endoblast. a, b, c, insets in Fig. 2.*

addition to these mechanisms providing directionality to the movement, physical contact guidance along oriented extracellular fibrils may contribute to the orderly movement of cells. Indeed, Ebendal (1977) hypothesized that extracellular matrix fibrils may play a role in orientation of cell migration and axon extension, and, at early stages of chicken development, Critchley *et al.* (1979) and Wakely and England (1979) demonstrated the presence of fibronectin-rich fibrils associated with the basal lamina at the basis of the epiblast, mainly along the cranial border of the area pellucida, and sparsely in relation to mesoblast cells. These authors concluded that fibronectin-rich fibrils may serve as a contact-guidance system utilized by primordial germ cells and by mesoblast cells during their migration. This is corroborated by Kucera and Monnet-Tschudi (1987), who observed that the presence of fibronectin arranged in radially oriented fibrils in the area opaca corresponds to the direction of migration of mesoderm cells. During chicken heart organogenesis, individual cells also migrate out from the endothelium, along uniformly oriented bundles of extracellular fibers, and populate the acellular cardiac jelly (Markwald *et al.*, 1979). Since the time of appearance of these reports, similar extracellular matrix fibrils aligned in the direction of migration of cells have been described in several embryonic systems. Most information originates from the study of amphibian gastrulation. Indeed, in amphibian gastrulae, presumptive mesoderm cells migrate from the blastopore towards the animal pole along the inner surface of the ectoderm. The substrate for migration is a network of anastomosing extracellular matrix fibrils underlying the roof of the blastocoel (Nakatsuji *et al.*, 1982; Boucaut and Darribère, 1983b; Nakatsuji and Johnson, 1983a,b, 1984a,b; Nakatsuji, 1984, 1986; Komazaki, 1985, 1986). Moreover, these fibrils contain fibronectin (Boucaut and Darribère, 1983a,b; Lee *et al.*, 1984; Darribère *et al.*, 1985; Nakatsuji *et al.*, 1985b; Winklbauer, 1988; Johnson *et al.*, 1990) and laminin (Nakatsuji *et al.*, 1985a; Darribère *et al.*, 1986; Riou *et al.*, 1987). These observations have made it very likely that the extracellular fibrillar network not only provides an adequate substrate for migration of amphibian mesoderm cells, which are themselves devoid of fibronectin, but also orients the movement by contact guidance. This is even more evident since microinjection of a synthetic decapeptide containing the cell attachment sequence of fibronectin (Boucaut *et al.*, 1984b) or of anti-fibronectin antibody (Boucaut *et al.*, 1984a, 1985) inhibits gastrulation. After gastrulation, contact guidance along extracellular fibrillar matrices also seems to be implicated in various other morphogenetic movements such as migration of neural crest cells and of sclerotome cells (Löfberg *et al.*, 1980) and migration of primordial germ cells (Heasman *et al.*, 1981). In teleosts, similar fibrils termed «actinotrichia» provide the guidance cues to the migration of mesenchymal cells within the developing fin (Wood and Thorogood, 1987). In the sea urchin embryo (Katow and Solursh, 1979; Kawabe *et al.*, 1981; Amemiya, 1986, 1989) and in the starfish (Crawford and Chia, 1982), a fibrillar matrix composed of sulfated glycosaminoglycans is closely associated with the basal lamina of ectodermal cells, and is believed to mediate migration of primary mesenchymal cells.

Summarizing, a significant spatial and temporal correlation between the presence of extracellular fibrils, in which fibronectin, laminin and glycosaminoglycans have been localized by several authors, and the positioning of the mesenchyme during gastrulation has been firmly established in echinoderms, anurans and urodeles, and suggested in the chicken. However, when studying the relationship between the band of extracellular fibrils situated at the cranial and lateral edges of the chicken area pellucida and the migration of mesoblast cells, Andries *et al.* (1985) had some doubts about the implication of this fibrillar band in the contact-guided migration of mesoblast cells. Indeed, they observed that migratory mesoblast cells adhering to a smooth basal lamina were flattened and possessed lamellae, whereas cells in the vicinity of the fibrillar band were rounded and devoid of lamellae. Consequently, they assumed that the fibrils may represent a barrier that inhibits mesoblast-cell motility during gastrulation, rather than a contact-guidance system.

The present study is intended to test the hypothesis that the fibrillar band mentioned above is capable of inhibiting cell movement. The ultrastructural morphology and precise location of extracellular fibrils was reinvestigated first, before several experiments were designed *in vitro*. In a first series of experiments, pieces of primitive streak were explanted in the immediate vicinity of the fibrillar zone or on the fibrillar band of blastoderms deprived of their hypoblast. These blastoderms were briefly fixed before transplantation and culture, in order to avoid induction of a secondary primitive streak or of a neural plate by the graft. In a second series, pieces of heterologous hypoblast were transplanted into living blastoderms also deprived of their own deep layer. Finally, in a third series of experiments, the repair of blastoderms deprived of part of their hypoblast (Vanroelen *et al.*, 1982) was followed in embryo culture. The results indicate that the band of extracellular fibrils described along the cranial and lateral edges of the area pellucida constitutes a barrier for migration of single cells and for expansion of tissue sheets.

Fig. 2. Mediosagittal section of a stage-5 chicken blastoderm at the level of the endophyllic crescent and at the edge of the area opaca (AO)/ area pellucida (AP). *The insets indicate the areas chosen for ultrathin sectioning. DL, deep layer (endophyll); UL, upper layer or epiblast. Magnification: x450.*

Results

Localization and ultrastructure of fibrils

The location of the band of extracellular fibrils at the cranial and lateral edges of the area pellucida has been mapped in stage-5 blastoderms. In order to localize this band as precisely as possible, a condition that had to be fulfilled before transplanting pieces of tissue on it, semi-thin mediosagittal sections were cut in epoxy-embedded blastoderms (Fig. 2), and three adjacent areas in the region of the fibrillar band were chosen for ultrathin sectioning (Fig. 1 and insets a, b, c in Fig. 2). These areas were entirely photographed from caudal to cranial, and mounted in a line. The results showed that the basal lamina of the upper layer is associated with a variable quantity of diffuse extracellular materials and a variable number of interstitial bodies (Low, 1970) and cross-sections of fibrils, which were not distinguishable from one another. The number of cross-sections through interstitial bodies and fibrils per 10 μm was plotted. It was found that the highest number of basal lamina-associated structures was present in a 170- μm-wide band situated partly in the area opaca, over a width of about 50 μm, and partly in the area pellucida, over a width of about 120 μm. The relative density and location of basal lamina-associated structures is shown in Fig. 3.

Figs. 4 through 7 illustrate the morphology of the basal lamina and associated structures in this region. The most cranial part that

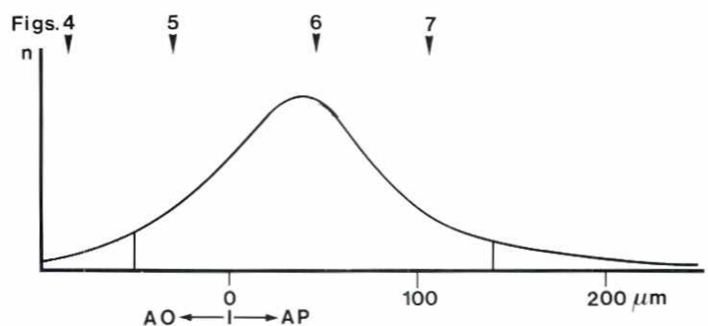
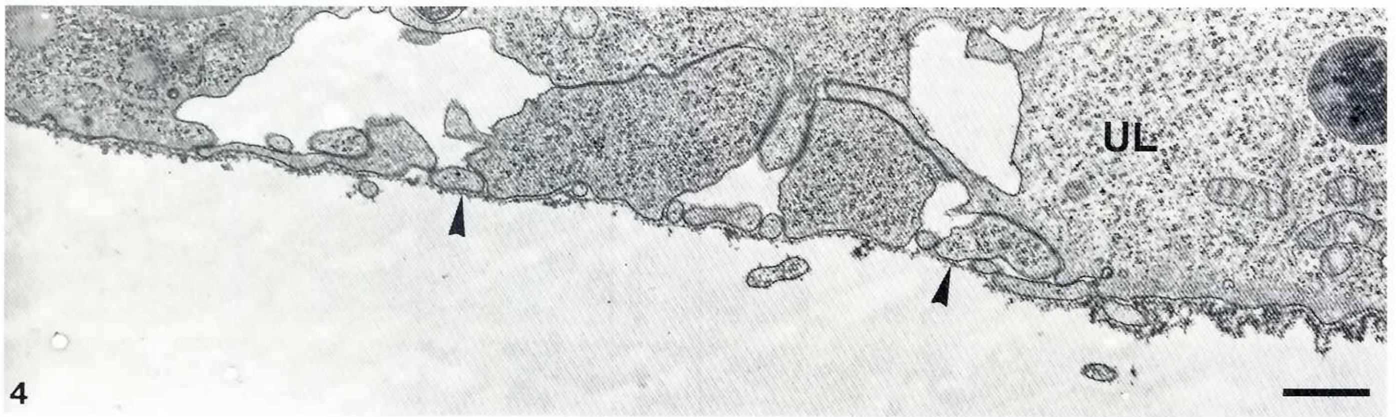


Fig. 3. Diagram showing the relative number (n) of fibrils and, eventually, interstitial bodies associated with the basal lamina vs the distance in μm from the edge of the area opaca/area pellucida.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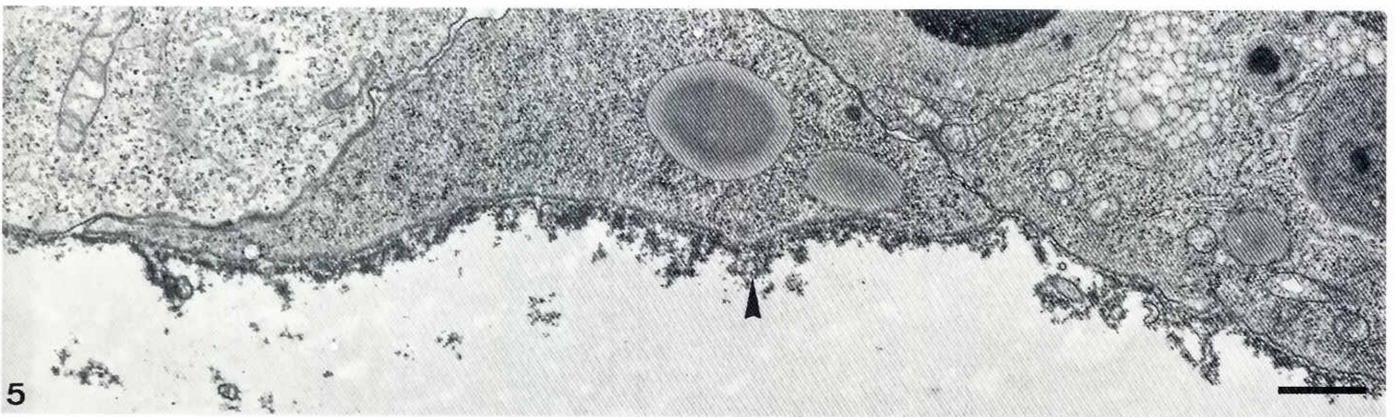
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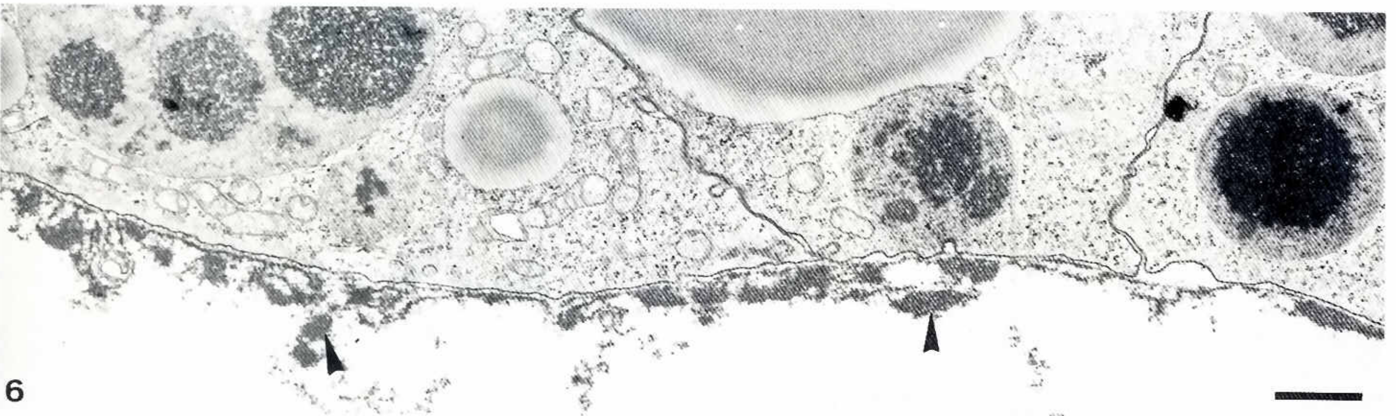
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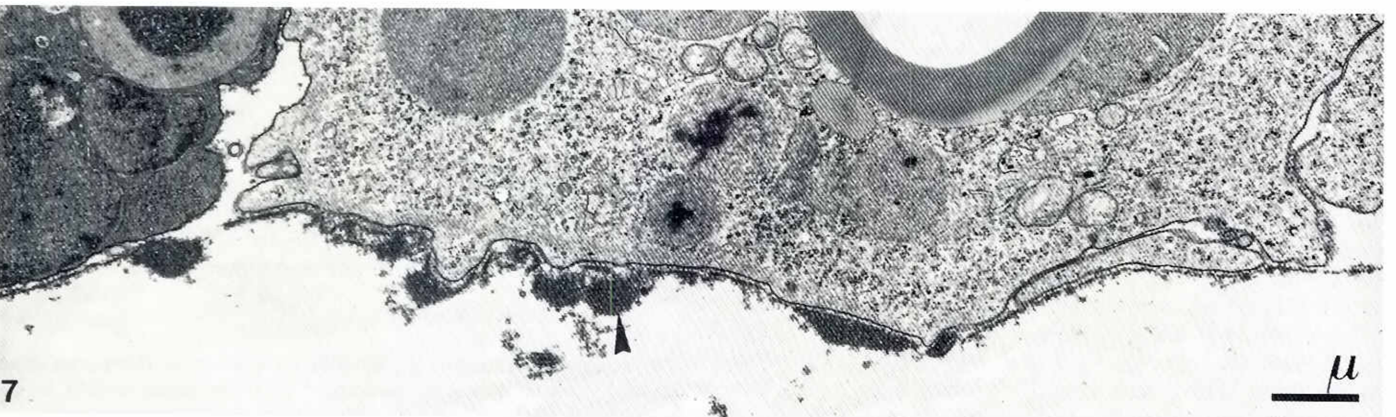
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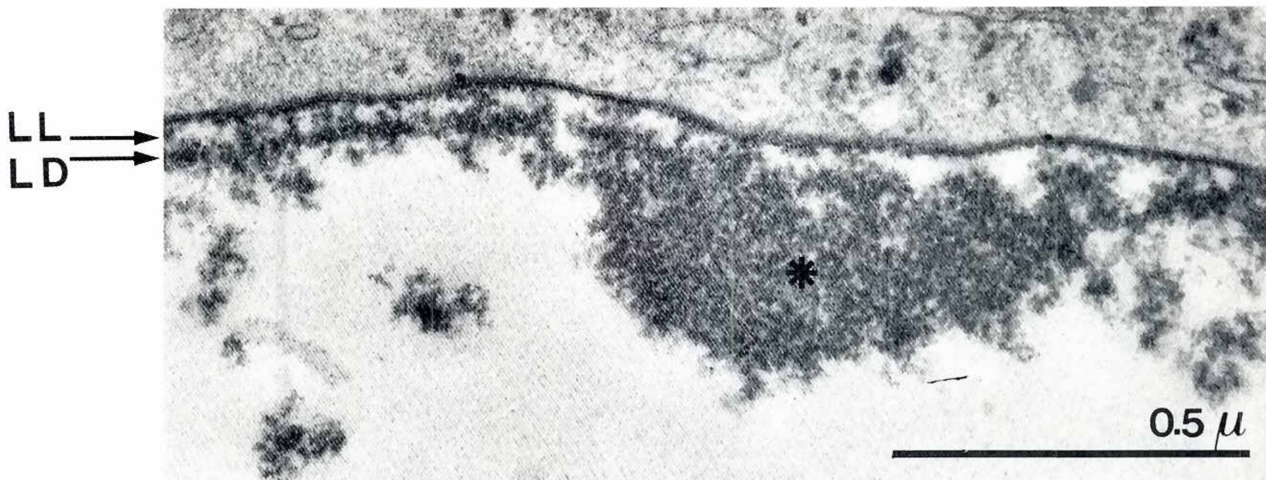


Fig. 8. High magnification of a basal lamina-associated fibril (asterisk) of 0.3 μm x 0.7 μm in size. *The basal lamina, clearly resolved into a lamina lucida (LL) and a lamina densa (LD), is closely apposed to the plasma membrane. Magnification: x94000.*

has been investigated in the area opaca (Fig. 4) was characterized by the presence of a continuous and smooth basal lamina, with little associated extracellular material. When looking more caudally, but still in the area opaca, the basal lamina was progressively more and more associated with a diffuse extracellular matrix, which was sometimes assembled into small aggregates (Fig. 5). The number and size of these aggregates, which obviously represented cross-sections of fibrils and interstitial bodies, was highest at the cranial edge of the area pellucida (Fig. 6). More caudally in the area pellucida, the basal lamina-associated structures appeared as well-defined electron-dense aggregates with little diffuse extracellular material in their vicinity (Fig. 7). At higher magnification (Fig. 8), these structures appeared to be 1.05±0.3 μm wide and 0.39±0.12 μm high.

The location and morphology of the fibrillar band were investigated with SEM in stages-5 to -7 blastoderms. Therefore, the blastoderms were explanted on a glass ring, according to the method of New (1955), and their deep layer was completely removed, except in the region of the primitive streak. The photomicrographs demonstrated the presence of a horseshoe-shaped fibrillar band situated at the edge of the cranial and lateral borders of the area pellucida. This band was widest in the cranial region, and progressively decreased in thickness towards the lateral and more caudal edges (Fig. 5). The basal lamina of the area pellucida enclosed within the horseshoe-shaped fibrillar band was rather smooth: only interstitial bodies and short fibrils without preferential direction were noted (Figs. 10-12). So far, it can not be ruled out that the short fibrillar strands are retraction fibers or filopodia pulled off from migrating cells or from the deep-layer cells that have been removed experimentally.

The preparations made for the SEM study of the basal lamina and its associated structures also allowed us to study the shape and surface morphology of cells adhering to a smooth basal lamina or to fibrils. Migrating mesoblast cells leaving the primitive streak were observed cranial to Hensen's node and lateral to the primitive streak, i.e., inside the fibrillar band (Figs. 10-12). These cells formed a loosely connected cell sheet in which many cells showed interdigitating and overlapping cell processes. Moreover, these cells, which adhered to a rather smooth basal lamina, were flattened and showed small lamellae and filopodia. At high magnification, it was observed that the fibrils of the band may be associated with knob-like protuberances (Figs. 13-14). Only a few cells (primordial germ cells?) appeared to adhere to the band in the cranial region. These cells were rounded and showed little protrusive activity (Fig. 14).

Summarizing, this descriptive study has unequivocally localized a band of extracellular fibrils associated with the basal lamina to a thin cranial and lateral zone bridging the edge of the area pellucida/ area opaca. The surface morphology of cells adhering to these fibrils is different from the cells adhering to the smooth basal lamina inside the fibrillar band. Taking advantage of the greater opacity of the area opaca, due to the presence of yolk beneath the extra-embryonic upper layer, it is now possible to define *in toto* the precise location of the fibrillar band in a blastoderm explanted in New culture, to transplant cells on it, either inside or outside the fibrillar band, and to observe their behavior and surface morphology using SEM.

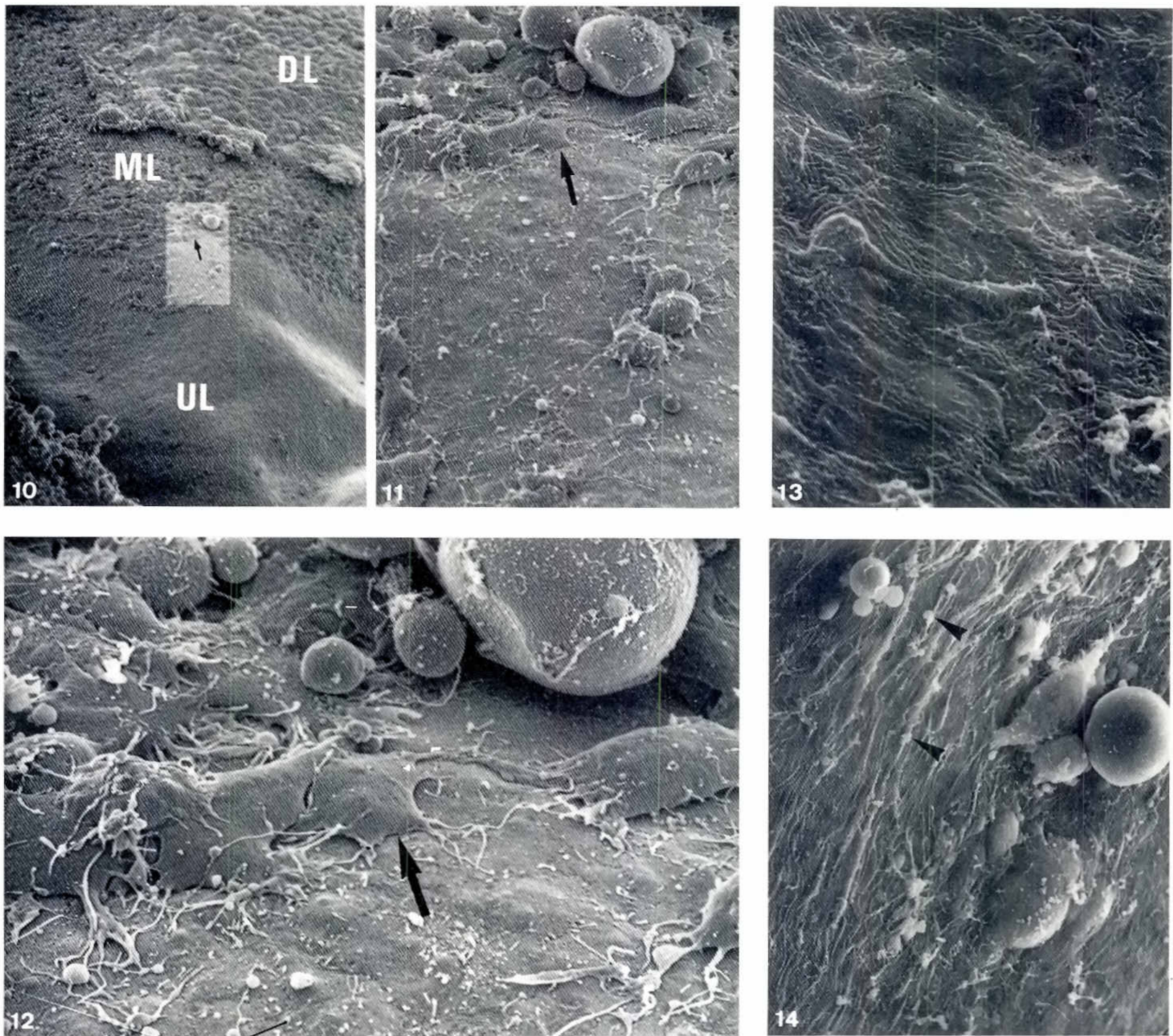
Transplantation of primitive streak grafts

The behavior of primitive streak grafts (Hensen's node, midstreak, and caudal streak) transplanted either onto the fibrillar band or on the smooth basal lamina of the area opaca or of the area pellucida

Figs. 4-7. TEM photomicrographs taken in the areas depicted in Fig. 3, at the basal side of the epiblast. Magnifications: x11100. *From cranial to caudal:* **(4)** *shows a smooth basal lamina (arrowheads) in the area opaca, outside the fibrillar band;* **(5)** *shows a basal lamina associated with diffuse extracellular materials (arrowhead) and small aggregates in the area opaca, close to the edge area opaca/area pellucida;* **(6)** *shows a high density of basal lamina-associated structures (arrowheads) in the area pellucida, close to the edge area opaca/area pellucida;* **(7)** *shows large, but few in number, cross-sections of fibrils (arrowhead) and interstitial bodies in a region situated somewhat more caudally than in Fig. 6. UL, upper layer or epiblast.*

Fig. 9. SEM photomicrograph at low magnification of the ventral side of a stage-6 blastoderm after removal of its deep layer. *The asterisks indicate the location of a horseshoe-shaped fibrillar band. Inside this band, the basal lamina is smooth, and only interstitial bodies are associated with it (white, dot-like structures). AO, area opaca; AP, area pellucida; HN, Hensen's node; PS, primitive streak. Magnification: x120.*

was investigated using time-lapse photomicrography and SEM. The observations were similar with all grafts used, whatever their original location in the primitive streak. In contrast, the nature of the substrate on which they were transplanted profoundly influenced their surface morphology and behavior. When the grafts were placed in the immediate vicinity of the fibrillar band, either outside the band, in the area opaca (Figs. 16-18), or inside the band, in the area pellucida (Figs. 19-21), an eccentric outgrowth of the grafted tissue always occurred in the direction opposite to the fibrillar band, i.e., in a centrifugal direction for grafts in the area opaca, and in a



Figs. 10-12. Progressive higher magnifications of the basal side of the epiblast, showing flattened mesoblast cells with small lamellae, migrating on a smooth basal lamina. *The arrows indicate the same cell in all figures. Magnifications: x330, x1900, and x5000, respectively.*

Figs. 13-14. SEM photomicrographs of the fibrillar band at the cranial edge of the area pellucida. *The arrowheads indicate knobs associated with the fibrils. The cells adhering to these fibrils are rounded, with little protrusive activity (14). Magnifications: x1500.*

centripetal direction for grafts in the area pellucida. After 3 h of culture, the grafts were perfectly circular in shape (Figs. 17 and 20), and then they progressively flattened out and became more transparent (Figs. 18 and 21). Using SEM, it was clear that during the time of culture, single cells of Hensen's node left the graft and moved individually (Fig. 22). It may be speculated that these cells represent mesoblast cells having kept their mesenchymal phenotype and their migratory behavior. Indeed, these cells extended a high number of long, thin filopodia towards the basal lamina, which was associated with interstitial bodies only (Fig. 23). A number of grafted cells presented, however, a quite different surface morphology, which was characterized by the presence of broad lamellipodia covering the basal lamina, in addition to several filopodia (Fig. 24). When the graft was placed so that it partially covered the fibrillar zone, the cells neighboring the fibrils displayed a completely different surface morphology and shape. They had a more rounded

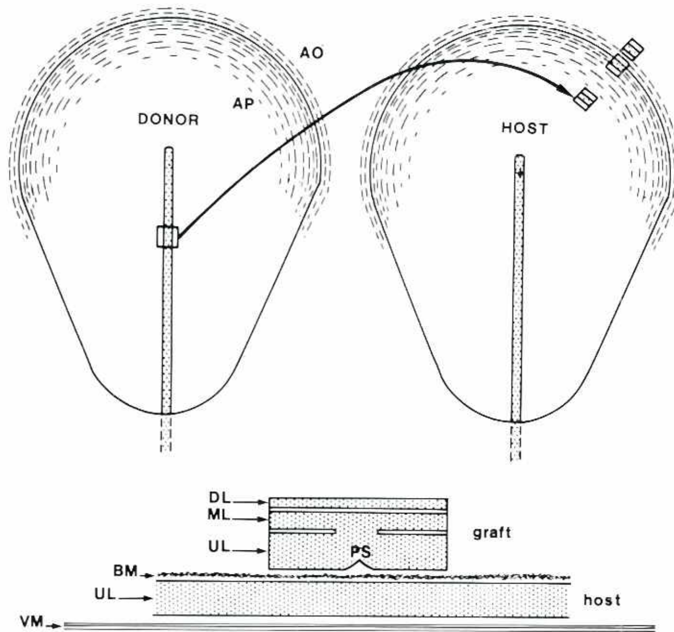


Fig. 15. Schematic representation of the experimental procedure used for transplantation of a piece of primitive streak into a host blastoderm. *The graft was transplanted so that the apical surface of its upper layer faces the basal side of the host upper layer.*

shape and retracted their cell protrusions (Fig. 25). Spreading of a graft across the band of fibrils was never observed, neither in a centrifugal nor in a centripetal direction.

Transplantation of grafts of heterologous deep layer

Pieces of quail deep layer of different sizes have been transplanted onto the basal lamina of the area pellucida of chicken blastoderms previously deprived of their own deep layer, both at the levels of the area pellucida and of the area opaca (Fig. 26). The grafted tissue was placed so that its dorso-ventral polarity was maintained. Time-lapse photomicrography showed that the grafted deep layer rapidly spread. After a period of 5-7 h of culture, the chicken area pellucida was completely covered by a deep layer, which had reached the fibrillar zone (Fig. 27). When looking at the surface morphology of the cells spreading on the smooth chicken basal lamina, it was observed that the grafted tissue layer was closely apposed to the host basal lamina and that its edge cells extended a high number of thin filopodia towards the basal lamina. As in normal blastoderms, a high number of interstitial bodies appeared to be associated with the basal lamina. Even after culturing the blastoderms for periods over 7 h, the chicken deep layer of the area opaca never healed, either by centrifugal spreading of the quail deep layer across the fibrillar zone, or by centripetal movement of chicken material of the area opaca. Instead, when the edge cells of the grafted deep layer reached the fibrillar zone, they tended to roll up, losing their flattened shape, and retracting their protrusions (Fig. 29), suggesting that the fibrillar band may function as a barrier that inhibits the spreading of the graft.

Healing of wounded deep layer

In this series of experiments, the deep layer was partially removed at the levels of the area pellucida and of the area opaca (Fig. 30), and healing of the wounded deep layer was followed using time-lapse photomicrography, and analyzed using SEM. In all blastoderms studied, it appeared that the deep layer of the area pellucida was completely healed within the first 3 h of culture, by a centripetal movement of the wounded deep layer of the area pellucida (Fig. 31). Longer periods of culture did not allow the healing of the deep layer at the level of the area opaca, either by centrifugal spreading of the deep layer across the fibrillar band, or by ingrowth of deep-layer material of the area opaca. As in the series of experiments in which a heterologous deep layer had been grafted, the edge cells of the wounded tissue, which spread on a smooth basal lamina associated with interstitial bodies only, extended filopodia adhering to the basal lamina (Fig. 32). At the level of the band of fibrils, where wound healing was arrested, the edge cells presented little protrusive activity (Fig. 33).

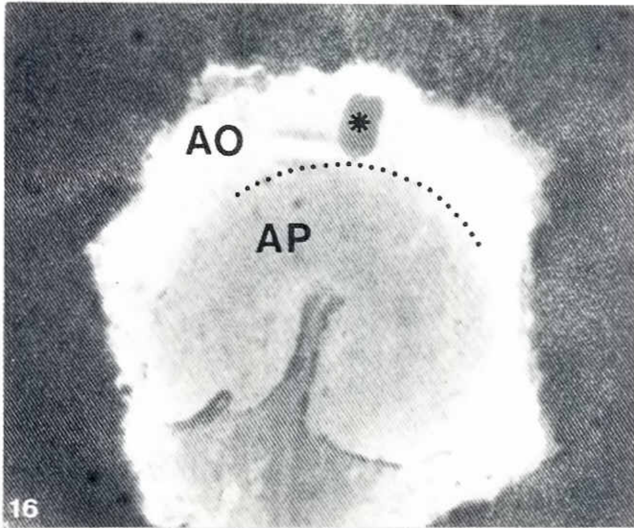
Discussion

Embryonic cells migrate in a highly directional manner, and evidence has been provided that the environment through which these cells pass plays a crucial role in this directionality (for review see Thiery *et al.*, 1985). Löfberg (1976) and Ebendal (1977) were probably the first to suggest that within this environment, extracellular fibrillar matrices may influence directional movement by a kind of contact guidance, as defined earlier by Weiss (1961). During chicken gastrulation, the presence of aligned extracellular fibrils at the basal side of the epiblast was first reported by Low (1968). Critchley *et al.* (1979), Wakely and England (1979) and Zagris *et al.* (1989) observed cells attached along these fibronectin-rich fibrils, and hypothesized that they may specify the direction of primordial germ cells and mesoblast cells by contact guidance. Experimental evidence supporting this view has, however, never been provided in the chicken blastoderm.

In the present study, we intended to determine the precise location of these fibrils, and to test experimentally the behavior of cells brought in contact with the fibrillar band. The behavior of cells on a smooth basal lamina and in the vicinity of fibrils were compared. The descriptive part of this study has unequivocally localized a 170-μm-wide band of fibrils at the cranial and lateral borders between the area opaca and the area pellucida. A systematic counting of basal lamina-associated structures in TEM photomicrographs showed that the highest density of fibrils is found

Figs. 16-18. Time-lapse photomicrographs showing the behavior of a caudal piece of a stage-6 primitive streak (asterisk) transplanted outside the fibrillar band (dotted line) of a stage-6 blastoderm. *An eccentric outgrowth in a centrifugal direction, away from the fibrillar band, is noted. AO, area opaca; AP, area pellucida: PS, primitive streak.*

Figs. 19-21. Time-lapse photomicrographs showing the behavior of a piece of a stage-6 midstreak (asterisk) transplanted inside the fibrillar band (dotted line) of a stage-6 blastoderm. *An eccentric outgrowth in a centripetal direction, away from the fibrillar band, is noted.*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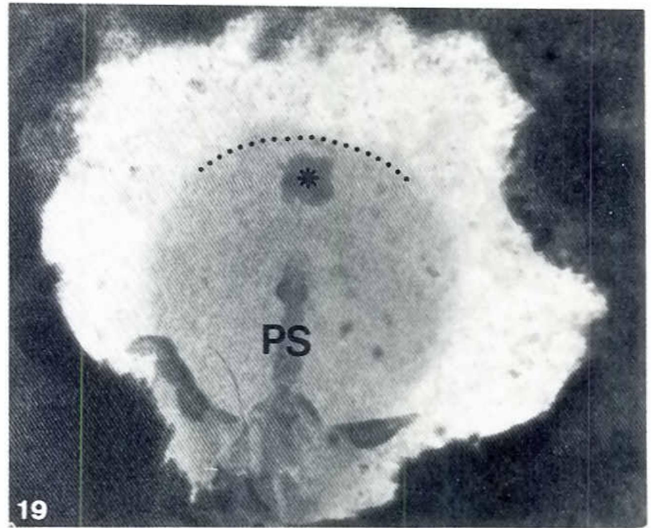
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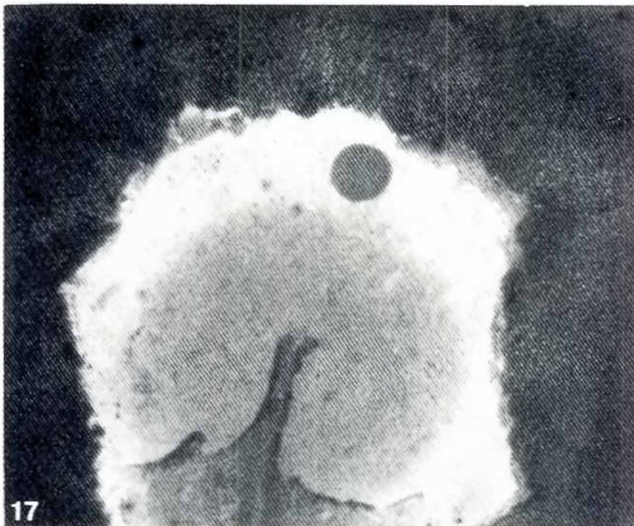
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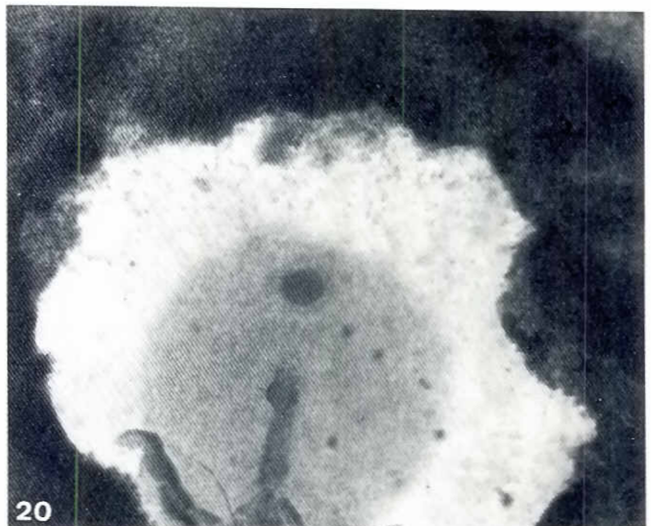
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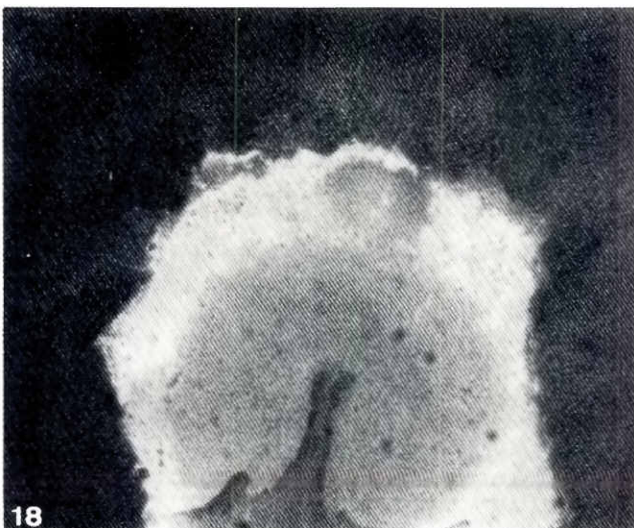
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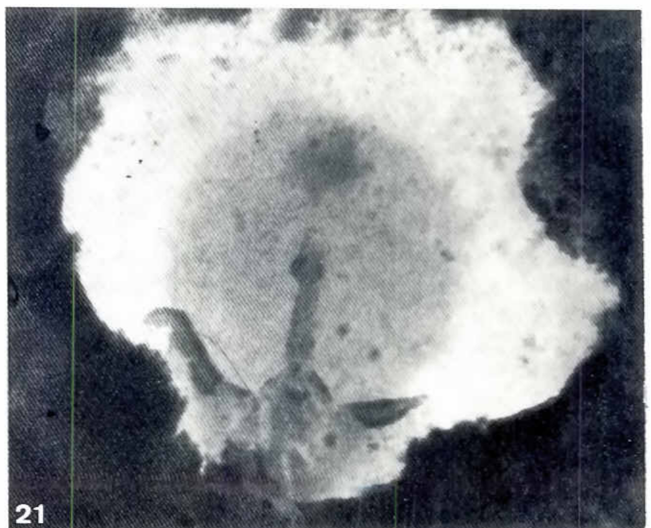
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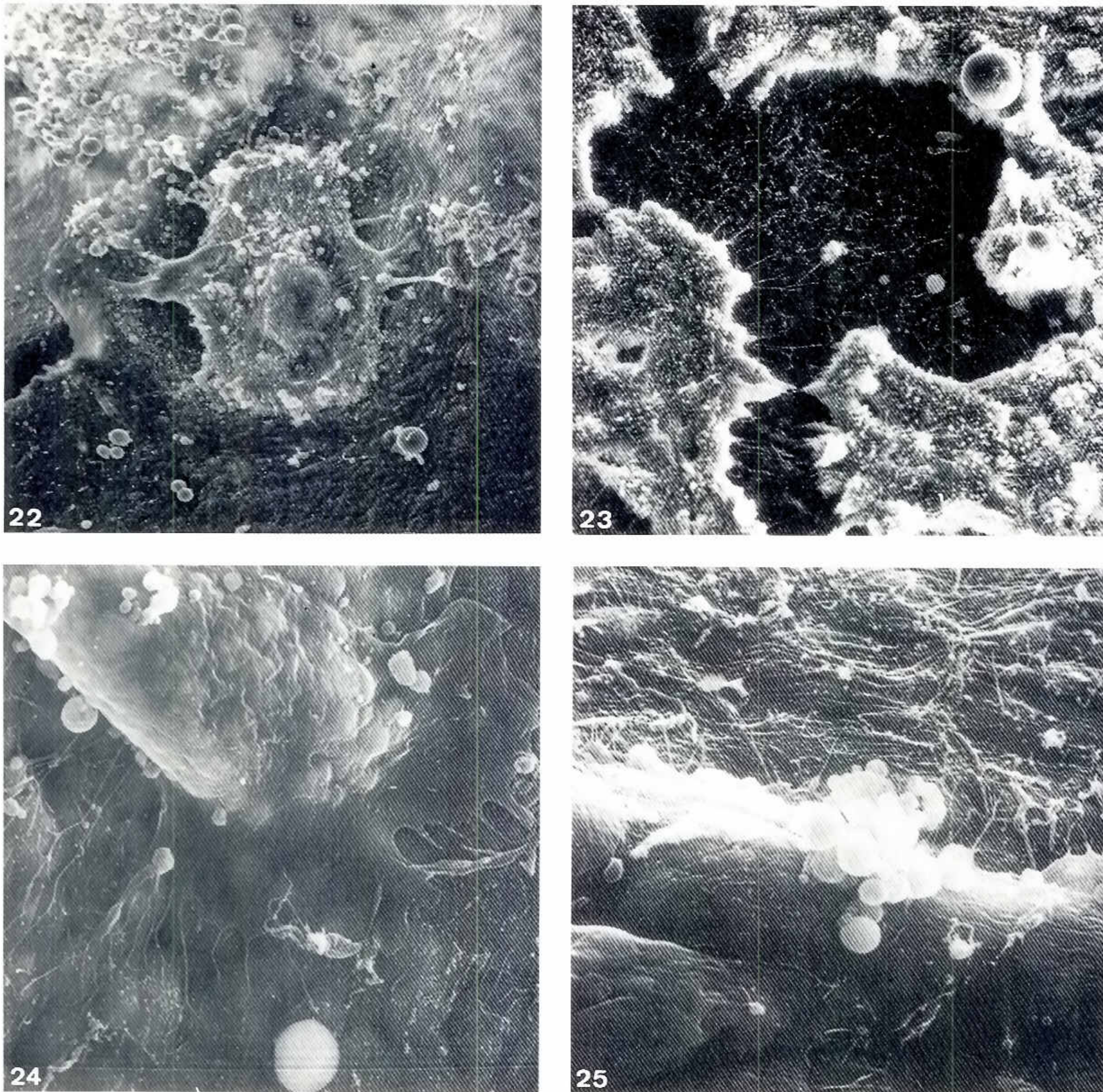
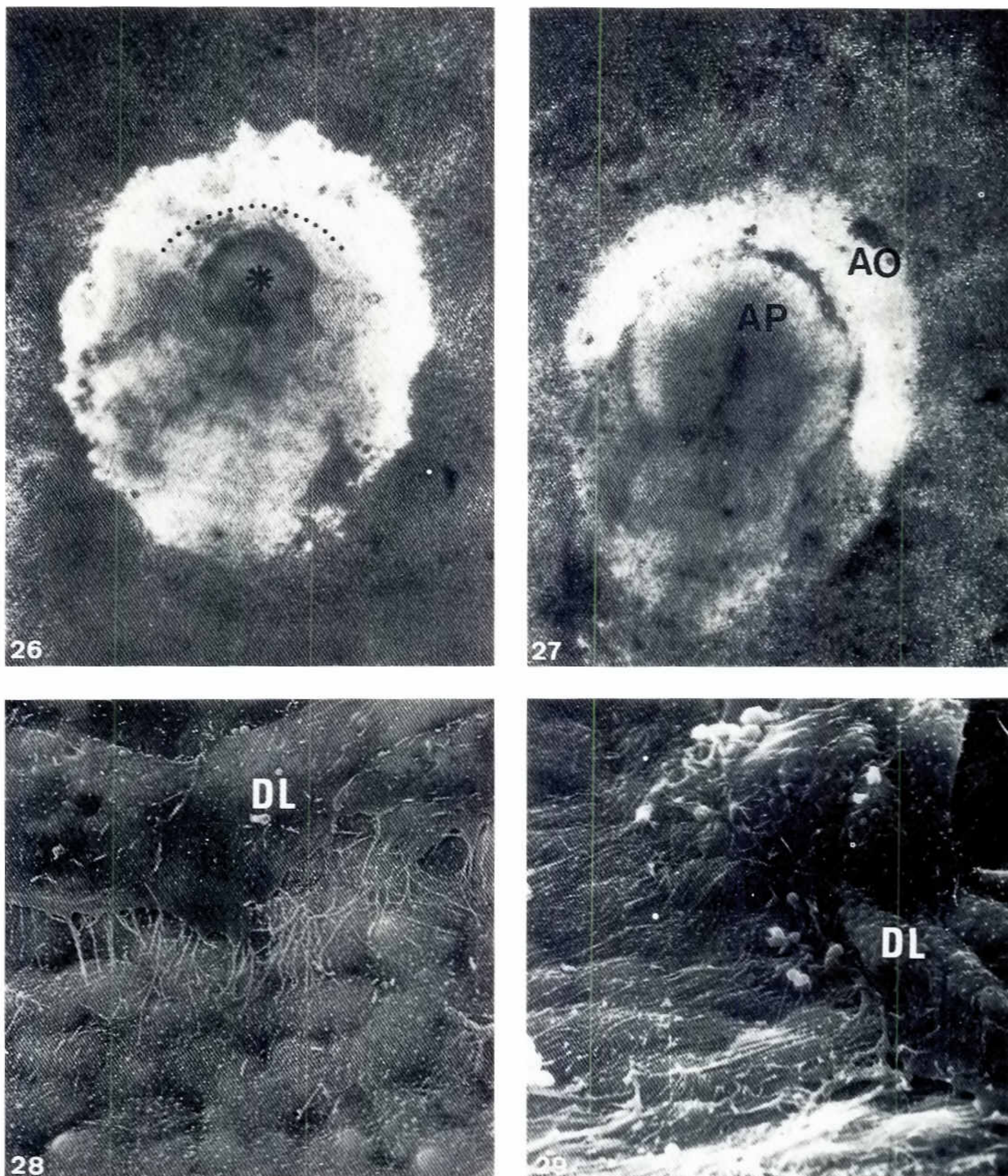


Figs. 22-23. SEM photomicrographs of a graft (Hensen's node) *transplanted outside the fibrillar band (22). The cells at the edge of the graft extend long, thin filopodia towards the smooth basal lamina (23). Magnifications: x1500.*

Figs. 24-25. Higher magnifications of grafted cells adhering to a smooth basal lamina (24) and cells situated in the vicinity of the fibrillar band (25). *In the former case, the cell extends a broad lamellipodium and several filopodia towards the basal lamina; in the latter case, the rounded cells exhibit little protrusive activity. Magnifications: x1500.*

in the area pellucida. SEM confirmed that the structures that were counted are cross-sections of fibrils and, to a much lesser extent, interstitial bodies (Low, 1970). The latter, moreover, are associated with the whole basal lamina.

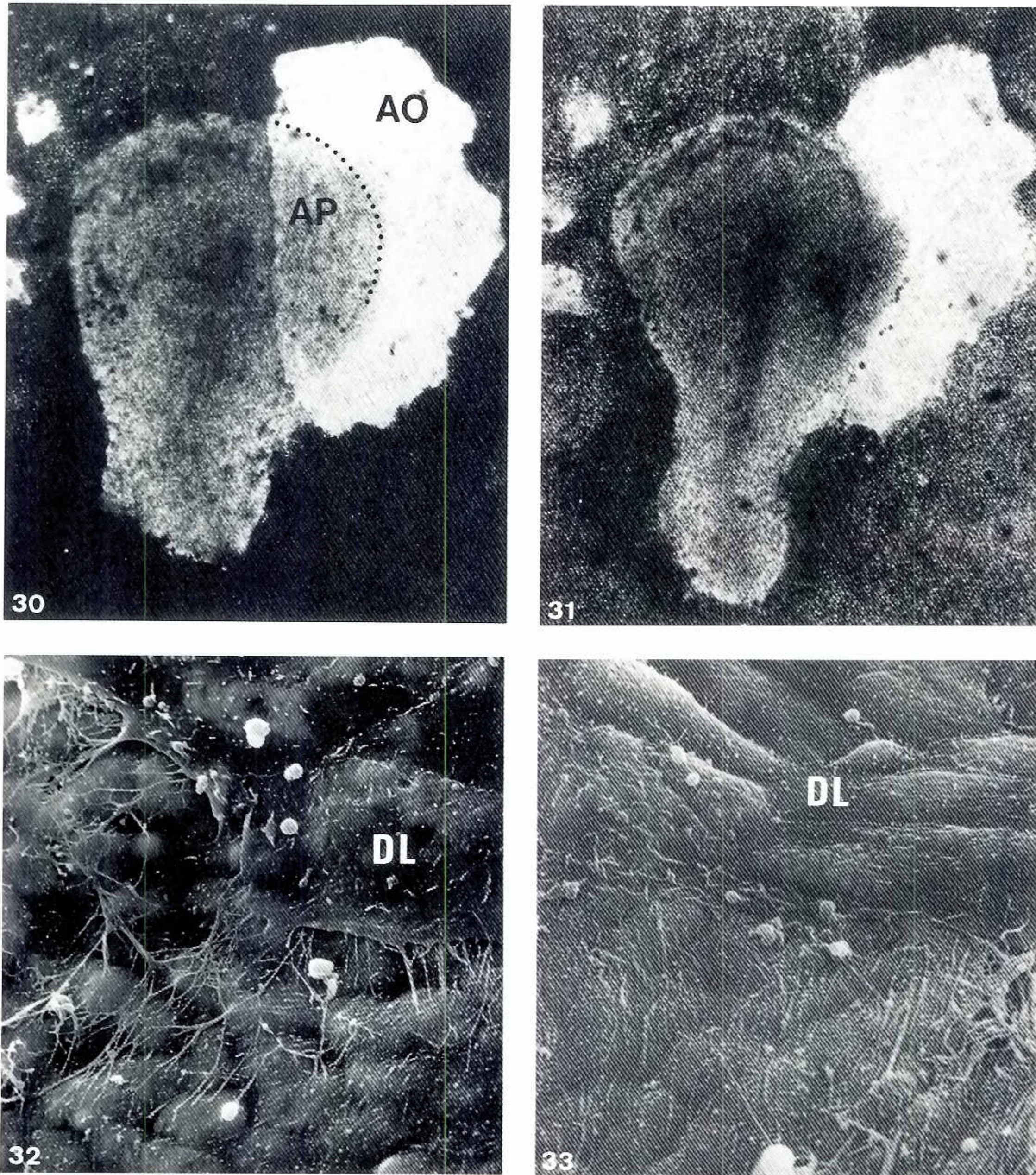
SEM observations of the surface morphology of migrating mesoblast cells confirmed observations made earlier by Andries *et al.* (1985) in chicken and quail embryos. The smooth basal lamina situated centrally in the area pellucida apparently permits the spreading of mesoblast cells, which adhere to the substratum. The cells often extend broad lamellae at the leading edge, and a high number of filopodia is a common feature. This polarized shape and morphology suggest cell locomotion and strongly recall observations made on cells spreading *in vitro* (Heaysman *et al.*, 1982). The surface morphology of cells reaching the band of fibrils at the lateral border of the area pellucida is different in several aspects. The spherical shape and the absence of cell protrusions suggest that this substratum is not favorable to cell spreading. This observation strongly contrasts with the conclusions drawn by other investigators (Critchley *et al.*, 1979; Wakely and England, 1979; Zagris *et al.*, 1989), who suggested that the fibrils may guide the migration of



Figs. 26-27. Time-lapse photomicrographs *showing the behavior of a piece of quail deep layer (asterisk) transplanted in a stage-6 blastoderm deprived of its own deep layer (26). After 7 h of culture (27), the deep layer is completely restored in the area pellucida. The grafted tissue never spreads across the fibrillar band (dotted line) and the area opaca is never healed.*

Figs. 28-29. SEM photomicrographs showing the surface morphology of a grafted deep layer (DL) on a smooth basal lamina (28) and on the fibrillar band (29). *In the former case, the cells of the migrating tissue are flat and extend numerous filopodia towards the basal lamina; in the latter case, the cells reaching the fibrils round up and form a roll at the edge of the tissue. Magnifications: x1500.*

mesoblast cells and primordial germ cells. In our opinion, the lateral part of the band of fibrils rather represents a barrier to spreading of mesoblast cells during gastrulation. Haptotactic migration on a gradient of fibronectin in the basal lamina (Harrisson, 1989), possibly in conjunction with other mechanisms, such as contact inhibition of movement and population pressures, may explain the directional spreading of mesoblast cells along a smooth basal lamina. We do not, however, exclude the possibility that physical guidance along the band of fibrils may contribute to the guidance of primordial germ cells (for review, see England, 1983), the movement of which corresponds to the orientation of the band of fibrils. However, as pointed out by Trinkaus (1976), alignment of a fibrillar



Figs. 30-31. Time-lapse photomicrographs showing a stage-7 blastoderm partially deprived of its deep layer (30) and the same blastoderm after 3 h of culture (31). *The deep layer of the area pellucida is completely healed, whereas the deep layer of the area opaca will never heal.*

Figs. 32-33. SEM photomicrographs illustrating the surface morphology of deep-layer cells moving on a smooth basal lamina (32) and deep-layer cells in the vicinity of the fibrillar hand (33). *In the latter region, where the centrifugal spreading of the deep layer is arrested, the edge cells retract their protrusions and adopt a more rounded shape. Magnifications: x1500.*

substratum can only give orientation to moving cells, not directionality, so that other factors must be involved in the directional migration of primordial germ cells.

Knowing the precise location of the horseshoe-shaped band of fibrils at the cranial and lateral edges of the area pellucida, it was possible to transplant homologous or heterologous tissues in the vicinity of these fibrils, either in the area opaca or in the area pellucida of blastoderms deprived of their deep layer. Indeed, the greater opacity of the area opaca, due to the presence of yolk endoderm beneath the extraembryonic epiblast, made it possible to clearly observe the limits of the area pellucida in blastoderms explanted *in toto* according to New (1955). Common observations made in our three series of experiments were that cell spreading was always arrested at the level of the fibrils, and that the surface

morphology of cells situated on or outside the fibrillar band suggested differences of cell-substratum adhesiveness. The fibrillar band appeared as an area with decreased cell adhesion, which inhibited cell spreading and locomotion. Cells reaching the band of fibrils, whatever their nature, never crossed the fibrillar band, either in a centrifugal or in a centripetal direction. This explains why healing of the deep layer of the area opaca never occurs after wounding or after transplantation of a heterologous deep layer within the area pellucida. Ingrowth over the fibrillar band of extraembryonic deep layer or of tissue transplanted in the area opaca (not shown in this article) also never occurs. In this respect, it is worth mentioning that in the caudal part of the area pellucida, where a fibrillar band is not present, ingrowth of extraembryonic deep layer may occur (Vakaet, 1962), and migrating mesoblast cells partly invade the area opaca (Vakaet, 1970). We conclude that the fibrillar band induces the arrest of migration of individual mesoblast cells and expansion of tissue sheets. The molecular basis that differentiates the permissive basal lamina from the restrictive extracellular fibrils is still unknown, but it may be anticipated that the adhesive properties of particular extracellular macromolecules are altered by masking by other components, that some particular adhesive macromolecule is underexpressed in fibrils, or that a repellent molecule is expressed in fibrils, as compared to the basal lamina. The band of fibrils may also stabilize the polarity of the early blastoderm by determining the cranial and lateral limits between the embryonic and the extraembryonic areas of the blastoderm.

Materials and Methods

Tissue preparation for transmission electron microscopy (TEM)

Fertilized chicken eggs (Warren SSL strain) from a commercial stock were incubated for 12-18 h at 38°C to obtain stage-4 to stage-6 blastoderms (Vakaet, 1970). The embryos were explanted on a glass ring according to the technique of New (1955), fixed for 1 h at room temperature in a solution containing 1% (w/v) glutaraldehyde and 1% tannic acid (mol. wt. 1701) in 0.1 M cacodylate buffer at pH 7.4, and rinsed overnight in buffer after the blastoderms had been detached from their vitelline membrane. The blastoderms were postfixed for 1 h at room temperature in a 1% (w/v) solution of osmium tetroxide in the same buffer. The tissues were dehydrated through a series of graded concentrations of ethanol, transferred to propylene oxide, and embedded in LX-112 epoxy resin (Ladd Research Industries, Burlington, USA). Semi-thin, mediosagittal sections were cut for phase contrast microscopy (Fig. 2), and the areas indicated in Figs. 1 and 2 were chosen for ultrathin sectioning. Plastic sections of gray interference color were mounted on formvar-coated copper grids, stained for 7.5 min in 25% (w/v) uranyl acetate dissolved in methanol (Stempak and Ward, 1964), counterstained for 30 sec in aqueous lead citrate prepared according to Reynolds (1963), and examined in a JEOL 100 B electron microscope operated at 60 kV.

In order to localize the band of fibrils of the endophyllic crescent as precisely as possible, three adjacent ultrathin mediosagittal sections about 120 μm-wide each were made in the areas indicated in Fig. 2 (insets), entirely photographed from caudal to cranial at a magnification of x9300, and mounted in a line over a distance of 328 cm. Details of the basal lamina and its associated structures were photographed afterwards at a higher magnification. The number of basal lamina-associated structures was plotted over a distance of about 350 μm.

Tissue preparation for scanning electron microscopy (SEM)

The blastoderms were explanted with their vitelline membrane on a glass ring and fixed for 1 h at room temperature in a 1% (w/v) solution of glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The deep layer, which faces upwards in the glass ring, was removed using 0.05-mm-diameter watchmaker needles, in front of the endophyllic crescent (area opaca), at the level of the endophyllic crescent itself, and lateral to the primitive streak. This intervention made it possible to expose the basal surface of the epiblast in the region where a band of fibrils had previously been observed. Subsequently, the blastoderms were detached from their vitelline membrane, rinsed overnight in buffer, and postfixed for an additional hour in a 1% (w/v) solution of osmium tetroxide in buffer. The blastoderms were dehydrated through a series of graded concentrations of acetone, critical-point dried with carbon dioxide as transition fluid, mounted on stubs with Tempfix (Neubauer, Münster, Germany), sputter-coated with a 10 to 20-nm-thick layer of gold-palladium, and examined either in a Leitz AMR 1200 B scanning electron microscope operated at 15 kV or in a JEOL 100 B electron microscope with ASID high resolution scanning device.

Experimental procedure

Three series of experiments were designed to investigate the behavior of individual cells and tissue sheets on or in the vicinity of the band of fibrils:

Transplantation of grafts of primitive streak into fixed blastoderms

Stage-4 to stage-6 host blastoderms were explanted on a glass ring and briefly fixed for 10 min in a solution containing 1% (w/v) paraformaldehyde, 0.5% (w/v) glutaraldehyde and 1 mM calcium chloride in 0.1 M cacodylate buffer at pH 7.4 (Mayer *et al.*, 1981). The deep layer of these blastoderms was removed as indicated for SEM. Donor blastoderms at the same developmental stage were explanted on a glass ring, and a piece of their (unfixed) primitive streak (0.25 x 0.25 mm in size) was transplanted onto the band of fibrils of a host blastoderm, or in its immediate vicinity, either inside (area pellucida) or outside (area opaca) the band of fibrils (Fig 15). The grafts (n=25) were transplanted so that the apical side of the graft faced the basal lamina of the host (Fig. 15). The grafts were allowed to spread for 18 h onto the host basal lamina, and time-lapse photomicrographs were taken. The position of the graft was controlled using SEM.

Transplantation of grafts of quail deep layer into living chicken blastoderms

Stage-4 to stage-6 host blastoderms were explanted on a glass ring and deprived of their deep layer as indicated for SEM. Pieces of quail deep layer of various sizes were transplanted into these wounded chicken blastoderms, in such a way that the right polarity of the graft was maintained in the blastoderm. The chimeric blastoderms (n=9) were cultured for different time intervals, ranging from 3 to 7 h, and time-lapse photomicrographs were taken.

Healing of wounded deep layer in the living blastoderm

Stage-4 to stage-6 blastoderms (n=11) were explanted on a glass ring, deprived of part of their deep layer at the level of the endophyllic crescent and of the area opaca, and cultured for 5 h. Time-lapse photomicrographs were taken.

After culture, the blastoderms of all experimental series were fixed for 1 h in Mayer's fixative (Mayer *et al.*, 1981) and further processed for SEM.

Acknowledgments

The authors wish to express their gratitude to Dr. Ch. Vanroelen and Dr. J. Van Hoof, who initiated these investigations before leaving the Department of Anatomy and Embryology of the University of Antwerp. The authors also wish to thank Mr. F. De Bruyn, Mr. Ch. De Schepper, Mrs. E. Haest-Van Nueten, and Mr. G. Van den Broeck for skilful technical assistance.

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