Original Article

Guidance of filopodial extension by fibronectin-rich extracellular matrix fibrils during avian gastrulation. A study using confocal microscopy

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ABSTRACT We have used double-label confocal microscopy to examine the relationships between the orientation of filopodial extension in mesoderm cells and the orientation of fibronectin-rich extracellular matrix fibrils during chick embryo gastrulation. We fluorescently labeled mesoderm tissue dissected from donor embryos by immersion in carboxyfluorescein and then grafted it into unlabeled host embryos at the same stage of gastrulation. After further incubation, the host embryos were fixed, the endoderm removed, and the extracellular matrix was immunostained with antibodies to fibronectin conjugated to Texas Red. We found that both the general shape of the mesoderm cells and the orientation of filopodial extension were influenced by the surrounding matrix fibrils. Elongated shape was associated with individual fibrils which impinge on only one side of the cell. Similarly, filopodial extension followed a single fibronectin-rich fibril, although filopodia were also observed to be channeled between pairs of parallel fibrils. Cells attached to non-aligned regions of substratum showed no polarity. The mesoderm cells themselves apparently synthesize their own fibronectin, and deposit this on the cell surface not attached to the substratum. We conclude that individual fibronectin-rich substratum fibrils, in the size range 0.7-2.8 μm, are able to exert contact guidance on the mesoderm cells, despite the production of endogenous fibronectin by the cells themselves. These results support the contention that contact guidance is a physiological mechanism influencing the orientation and directionality of cells during the morphogenetic movements of embryogenesis.

KEY WORDS: *chick embryo, gastrulation, mesoderm, contact guidance, confocal microscopy*

Introduction

Embryogenesis is characterized by extensive cellular re-arrangement and cell migration. For example, during early vertebrate development, active cell locomotion is involved in the morphogenesis of the mesodermal cells following the ingression movements of gastrulation (Sanders, 1986); the elaborate migrations of the neural crest cells (Newgreen and Erickson, 1986); and the long-distance migrations of primordial germ cells (Wylie *et al.*, 1986). In each of these cases, the mechanisms controlling the migrations must provide cues for the initiation, the directionality and the cessation of the movements. Directionality is thought to be imposed on the moving cells by a combination of phenomena including: contact inhibition of locomotion, chemotaxis, adhesion gradients (haptotaxis) and contact guidance by means of topographic heterogeneities in the substratum, such as fibrils or grooves (reviewed by Trinkaus, 1984; Sanders, 1989). Not all of these factors may be operating equally in any given circumstance, indeed some, such as chemotaxis, are extremely difficult to demonstrate *in vivo* and have no firm experimental support in these situations.

Contact guidance, by contrast, is a more tractable phenomenon, which has gained support as a possible means of directing cell movements in a variety of embryonic events (Trinkaus, 1982, 1984), and which has been widely studied *in vitro* (Dunn, 1982). The elongation of cells *in vitro* and their directional movement in response to substratum anisotropy, either on fibrillar or grooved surfaces, has long been appreciated (Weiss, 1958; Dunn, 1982), and there are reasons to believe that the phenomenon is operational *in vivo*. There are several *in vivo* examples of fibrillar substrata which are used by migrating cells during development. In these cases the fibrils are composed of extracellular components such as fibronectin, which is an adhesive glycoprotein for cells (Hynes, 1990). Because of this adhesive property, there are, in theory, at least two ways in which these fibrils could guide filopodial extension and cell movement (Trinkaus, 1982): either by virtue of the elongated shape of the fibrils, or by the restricted adhesive pathway that they offer. In either case, this guidance needs to act in conjunction with other influences, such as a gradient of adhesiveness, population pressure, or contact inhibition of locomotion in order to dictate a forward rather than a backward direction of movement.
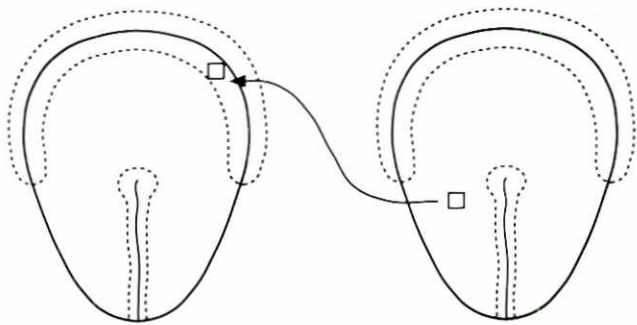
Fig. 1. Diagrammatic representation of the origin of the mesoderm graft and the position in the host embryo to which it was transplanted.

There are a number of examples of *in vivo* situations in which contact guidance appears to occur during early development. Probably the best understood and substantiated involves the movement of mesoderm cells during gastrulation in amphibians, in which contact guidance by oriented fibronectin-rich fibrils on the roof of the blastocoel appears to have a role in the determination of cell directionality (Nakatsuji, 1984; Nakatsuji and Johnson, 1984; Winklbauer and Nagel, 1991). In this instance, in which closely comparable *in vitro* and *in vivo* studies have been possible, contact guidance seems to work together with contact inhibition of locomotion and with the inherent polarity of the mesoderm cell clusters. Other examples in which a case has been made for contact guidance are: neural crest cell migration, in both the amphibian embryo (Lofberg and Ahlfors, 1978) and the chick (Newgreen, 1989); and sclerotome cell movement (Ebendal, 1977). In these circumstances the situation is more complicated than in amphibian gastrulation because of the complexity of the migration spaces involved in neural crest and sclerotome morphogenesis, so the case is not as well substantiated in these instances.

Gastrulation in the chick embryo is particularly interesting in this regard, because after ingression through the primitive streak the mesoderm cells begin to move in the space between the overlying epiblast and the underlying endoderm (Bellairs, 1986; Sanders, 1986). A proportion of the mesoderm cells move on the basement membrane of the epiblast, which forms a planar substratum for locomotion. This basement membrane presents fibronectin-rich fibrils to the moving mesoderm cells, and these fibrils form patterns which vary in different regions of the embryo (Critchley *et al.*, 1979; Wakely and England, 1979). In some regions of the basement membrane, this extracellular matrix forms tracks of parallel fibrils, while in others the fibronectin is present as isolated fibrils or in a punctate pattern. The latter may correspond to the fibronectin-rich "interstitial bodies" observed on this basement membrane by electron microscopy (Sanders, 1982). That the fibronectin is important for the locomotion of the mesoderm cells may be inferred not only from detailed temporo-spatial studies of its occurrence (Duband and Thiery, 1982; Harrisson *et al.*, 1984; Hynes, 1990), but also from experimental studies, *in vitro* and *in vivo*, which show that perturbation of the fibronectin substratum, using antibodies or peptides from the cell-binding region of fibronectin, inhibits mesoderm cell movement (Sanders, 1980; Brown and Sanders, 1991; Harrisson *et al.*, 1993). By contrast, recent work with a fibronectin-deficient mouse mutant *(FN.null)* indicates that gastrulation and mesodermal movement in this species are apparently not solely

dependent on the presence of fibronectin (George *et al.*, 1993).

The original description of the fibrous band of parallel fibronectin-rich fibrils at the rostral limit of the area pellucida was accompanied by speculation that the tracks could be used by cells as a contact guidance mechanism for mesoderm cells or primordial germ cells (PGCs; Critchley *et al.*, 1979; Wakely and England, 1979), and this conjecture has subsequently been supported by *in vivo* and *in vitro* morphometric studies (Toyoizumi *et al.*, 1991; Toyoizumi and Takeuchi, 1992). However, this notion has been challenged on the grounds that mesoderm cells on the fibrous band cannot be seen, by scanning electron microscopy, to form lamellipodia (Andries *et al.*, 1985), and the band was therefore viewed by these authors as a barrier to cell movement. In an extension of this work, Harrisson *et al.* (1992) examined the morphology of cells grafted onto the fibrous band and similarly concluded that the band acts as a barrier to migration. Regardless of whether cells on the fibrous band form lamellipodia, it appears, by inspection of the scanning electron micrographs, that both mesoderm cells (Andries *et al.*, 1985; Toyoizumi and Takeuchi, 1992) and PGCs (England, 1983) possess filopodia which are aligned with extracellular fibrils, which are presumably fibronectin-rich, and that the cells as a whole orientate according to the axis of the band. Chronologically, the PGCs reach the region of the band of fibrils, from their place of origin, before the mesoderm cells do (Clawson and Domm, 1969; Harrisson *et al.*, 1985; England and Matsumura, 1993), and while the former are individual cells, the mesoderm cells, although occasionally single, are usually present in small clusters or as part of a cell sheet.

In the present study, we have extended this work by detailed examination of the relationship between filopodial extension and fibrils on the basement membrane which are known to be fibronectin-rich. We have used carboxyfluorescein-labeled mesoderm cells from gastrulating donor embryos and grafted the cells into host embryos of the same stage. After allowing the grafted cells to attach and begin moving, we have fixed the embryos and immunostained for fibronectin. By means of double-label confocal microscopy, we have shown the manner in which individual filopodia interact with individual fibronectin fibrils.

Results

CFSE-labeled grafted mesoderm cells retained an intense fluorescence for several days, and during this time were readily observable within the embryo by means of conventional fluorescence microscopy (Fig. 2). The 6 h incubation period was found to be long enough to allow the grafted cells to attach to the underlying epiblast cell layer, and to begin moving on it. Fine cellular processes were visible with the conventional technique. Similarly, the Texas red-labeled fibronectin on the basal lamina of the epiblast was discernable with normal fluorescence microscopy, and displayed patterns previously described by Critchley *et al.* (1979) and Wakely and England (1979). In particular, the band of fibronectin-rich fibres was apparent (Fig. 3) in the rostral region of the area pellucida corresponding approximately to the position of the germinal crescent. The use of confocal microscopy to image the fibrous band and surrounding regions enhanced appreciation for this pattern by eliminating background and out-of-focus fluorescence, allowing a clear distinction to be made between the region of the fibrous band (Fig. 4, upper part), and the punctate patterns closely adjacent (Fig. 4, lower part). This method also allowed high definition imaging of individual fibronectin-rich fibrils and their

relationship to fine cellular processes. Individual fibrils were calculated to be 0.7-2.8 μm in diameter.

When the cells and the matrix were imaged simultaneously with the confocal microscope, it became clear that both general cell shape and the extension of individual filopodia were influenced by the surrounding matrix in several ways. Elongated cell shape was associated with alignment along a fibronectin fibril which impinged on only one side of the cell (Figs. 5,6,7,8). However, the direction of extension of filopodia seemed also frequently to be influenced by pairs of parallel fibrils which appeared to channel filopodia in a particular direction (Figs. 9,10). Clearly, from such images it is not possible to assume that filopodial extension is occurring, in contrast to filopodial retraction, but since fibronectin is known to be an adhesive protein for these cells, it seems more likely that retraction would occur on less ordered arrays of extracellular matrix. Such an occurrence appears to have been imaged in Fig. 11, in which a cell is attached to non-filamentous matrix, and correspondingly shows no polarity.

The mesoderm cells were sometimes present as an aggregate or cell sheet, rather than as individuals. In that case, shown in Fig. 12, cells fixed apparently in the process of separating from the sheet, or advancing in a new direction, were seen to follow the predominant orientation of matrix fibrils.

Fibronectin could also be detected associated with the surfaces of the mesoderm cells themselves (Fig. 13). This was distinguished from the substratum fibronectin by its plane of focus relative to the cells, and also by its patterning which showed a characteristic network appearance.

Discussion

The results presented here show that both mesodermal cell elongation and the direction of filopodial extension may be influenced by the orientation of individual fibronectin-rich extracellular matrix fibrils. In some cases, the cells were apparently able to follow single fibrils, while in other examples the filopodia seemed to be channeled in a particular direction by being sandwiched between two parallel fibrils. Although earlier morphometric studies have statistically confirmed that such fibrils guide the locomotion of the mesoderm cells (Toyoizumi and Takeuchi, 1992), as originally suggested (Critchley *et al.*, 1979; Wakely and England, 1979), the present work shows the detail of the filopodial/fibril interaction that is responsible for this contact guidance, at greater resolution than previously possible. Presumably, it can be inferred that the interactions described here also apply to the other established examples of fibril-mediated contact guidance of cells, at least those occurring during early embryogenesis (Newgreen, 1989; Winklbauer and Nagel, 1991).

The distinction has been drawn between two possible modes of action of extracellular matrix fibrils in the promotion of contact guidance (Trinkaus, 1982; Newgreen, 1989). The first is a steric form of guidance, in which cells are influenced by inhomogeneity in the shape of the substratum; and the second is an adhesive phenomenon in which cells follow fibrils because they are more adhesive than the surrounding substratum. The present observations suggest that these two phenomena operate simultaneously and in concert with one another. The fact that filopodia may be sandwiched between parallel fibrils suggests that steric guidance may be operating, at least once the filopodia have found the parallel tracks; while the observation that filopodia can follow single fibrils would suggest the influence of adhesive guidance. On the other hand, *in vitro* studies have shown that substratum inhomogeneities as narrow as 0.25 μm are able to elicit contact
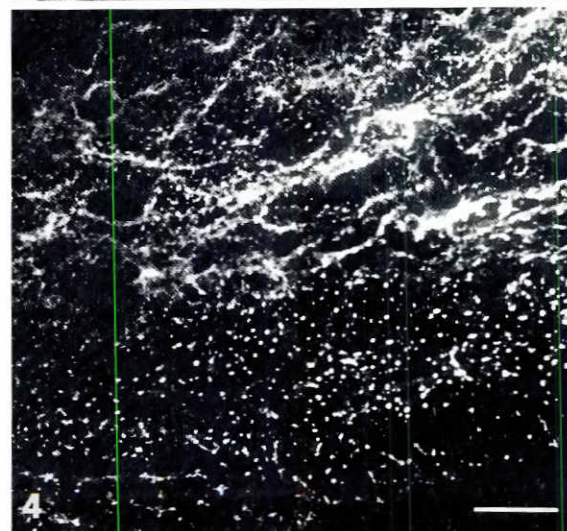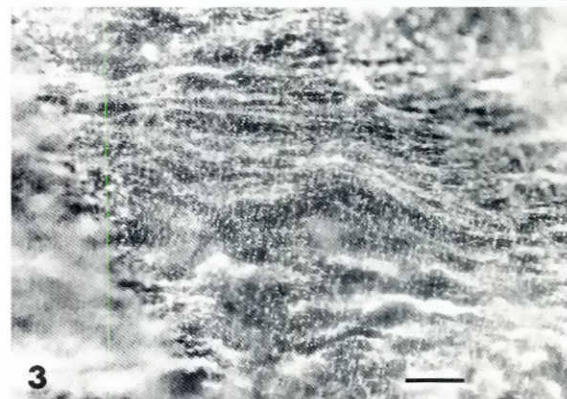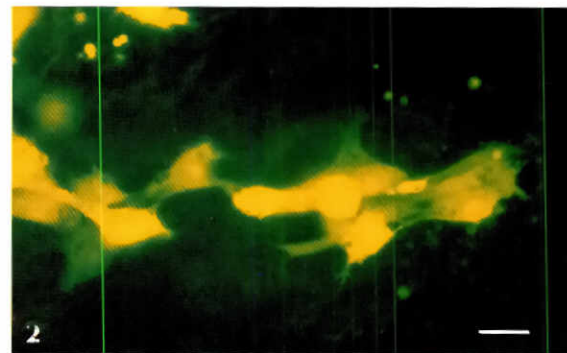

Fig. 2. CFSE-stained mesoderm cells spreading in a host embryo, 6 h after grafting. *Imaged using a conventional fluorescence microscope. x550. Bar, 20 μm.*

Fig. 3. The band of extracellular matrix fibrils, stained with Texas Red-labeled anti-fibronectin antiserum, and imaged with a conventional fluorescence microscope. *x490. Bar, 20 μm.*

Fig. 4. As Fig. 3, but imaged with the confocal microscope. *The band of fibrils is present at the top of the illustration, and non-oriented matrix is towards the bottom. x720. Bar on all confocal images, 20 μm.*
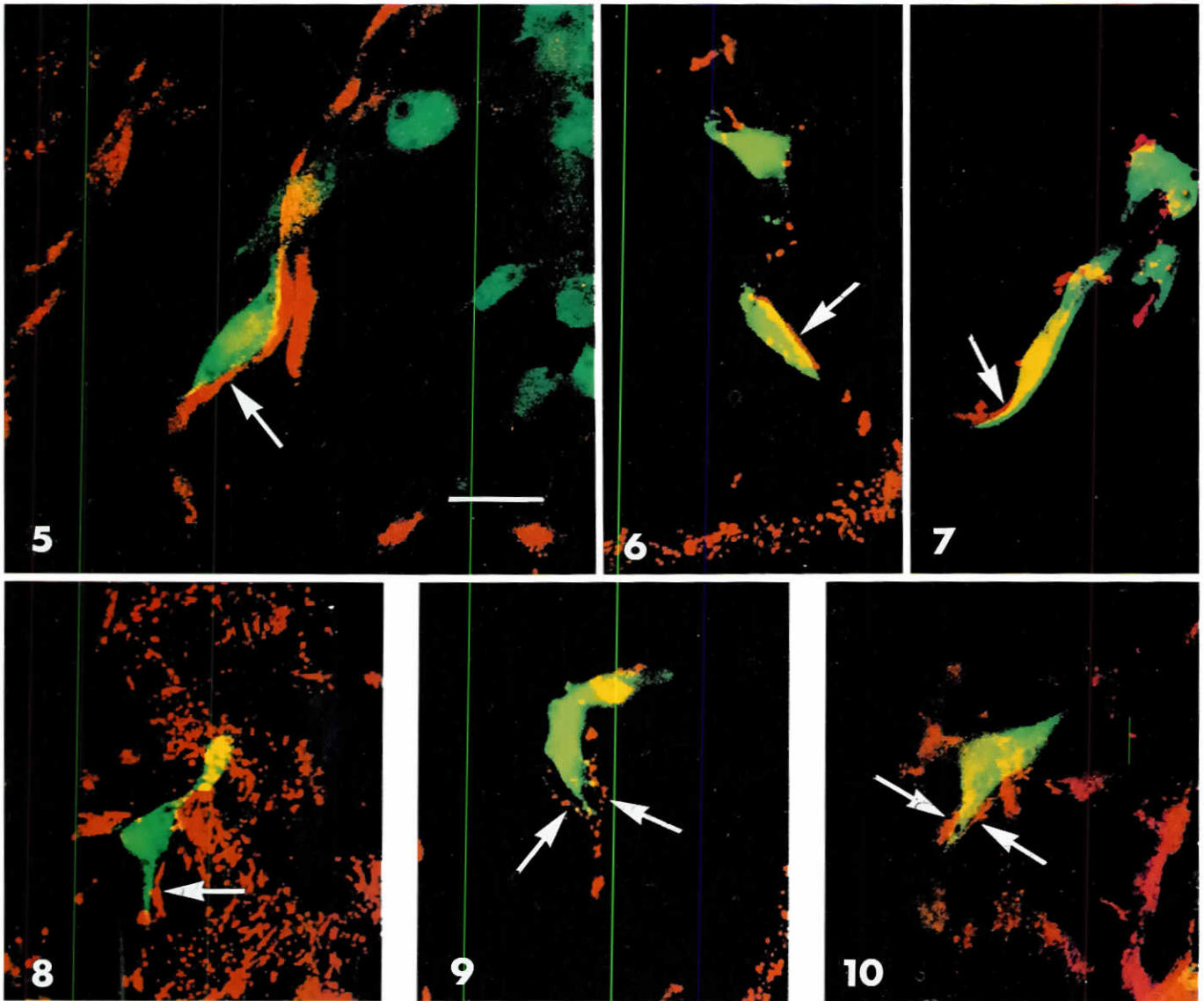
Figs. 5,6,7,8. Confocal double images showing mesoderm cells (green) elongated against a single fibronectin-rich red-labeled extracellular matrix fibril (arrows).

Figs. 9,10. Grafted mesoderm cells extending filopodia between two fibrils of extracellular matrix (arrows).

guidance (Ohara and Buck, 1979; but see Dunn, 1982), so that individual fibrils shown here, with a diameter of 0.7-2.8 µm, could possibly provide the topographic cues required for steric guidance alone.

The conclusion reached here is not in accordance with that of Andries *et al.* (1985) and Harrisson *et al.* (1992), who contend that the fibrous band is a barrier to cell migration rather than a contact guidance system. The ultimate test would be time-lapse microscopy of cells on the fibrils, but in the absence of this data it is probably fair to say that the present work supports the view that contact guidance by these fibrils is *possible*, but that the actual *in vivo* situation is still not strictly known.

The present results are of relevance not only to situations in which contact guidance occurs, but also to those in which it does not. For example, in the early chick embryo the directional move-

ment of the precardiac mesoderm is correlated with an adhesive gradient of fibronectin in the matrix (i.e. haptotaxis), but not with the orientation of the fibronectin-rich extracellular meshwork (Linask and Lash, 1986). Presumably, the type of filopodial/fibril interaction described here also occurs in such a situation, except that adhesive guidance is a stronger influence than steric guidance. So, the alignment of filopodia can apparently serve either a "strategic" contact guidance function in guiding the alignment and directionality of the entire cell, or a "tactical" function in which individual filopodia move along an adhesive gradient on individual fibrils and towards populations of fibrils of greater adhesiveness in a randomly oriented fibrillar substratum.

Notwithstanding the recent results of George *et al.* (1993) showing, with the *FN.null* mutant, that fibronectin may not be essential for gastrulation in the mouse, one may ask whether the
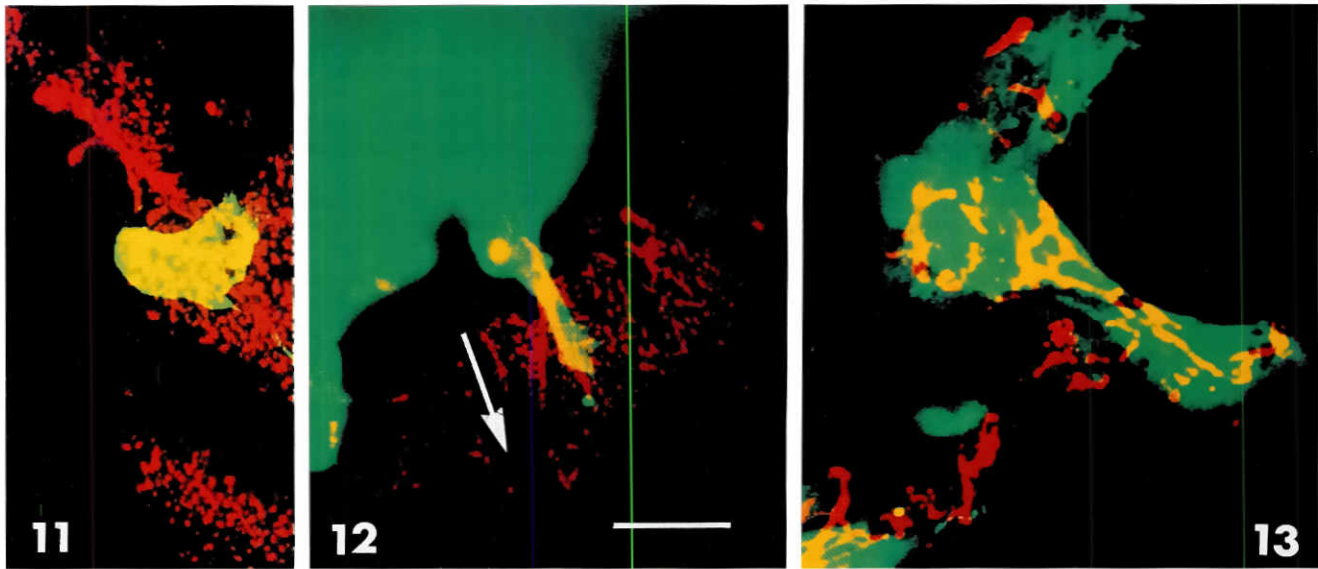
Fig. 11. A mesoderm cell attached to non-oriented fibronectin-rich extracellular matrix. *The cell shows no polarity, and there are no filopodia extending from the cell.*

Fig. 12. A sheet of mesoderm cells (green), with one cell extending out over extracellular matrix. *The alignment of the extending cell matches the predominant alignment of the fibrils in the matrix (arrow).*

Fig. 13. A mesoderm cell with its cell surface fibronectin-rich matrix (yellow). *The matrix image was made at the plane of the upper surface of the mesoderm cell, showing the fibronectin deposited as a network on this surface of the cell.*

type of guidance demonstrated here is of physiological significance in the avian embryo. The current observations support the original view of Critchley *et al.* (1979) and Wakely and England (1979), that the "fibrous band" on the basement membrane of the epiblast functions to guide either primordial germ cells or mesoderm cells during the period of gastrulation. Although this rostral region is not heavily populated by mesoderm cells at this time, it is possible that the parallel fibrils serve to orient "pioneer" mesoderm cells that are observed to arrive at the fibrous band ahead of the main mesodermal sheet, and that these cells are able to influence the pattern of expansion of the following cells, including those not actually in contact with the basement membrane. The initiation of mesodermal cell movement, and movements more centrally in the area pellucida where there are no oriented fibrils, must clearly be mediated by other mechanisms. The situation was further complicated, however, by observations indicating that the basement membrane itself is moving medially with the overlying epiblast during the morphogenetic movements of gastrulation (Sanders, 1984).

Fibronectin is obviously not the only extracellular matrix molecule to which the mesoderm cells are exposed. Other constituents of the basement membrane include fibrillin (Gallagher *et al.*, 1993) and laminin (Bortier *et al.*, 1989). Although it is not known whether either of these substrata can impart any directionality to cells either *in vivo* or *in vitro*, it may be significant that the latter, at least, can serve as a substratum for mesoderm cell attachment and spreading (Brown and Sanders, 1991). Indeed, it is possible that the levels of such molecules are up-regulated in the homozygous *FN.null* mutants (George *et al.*, 1993) to account for the apparently normal gastrulation in the absence of fibronectin.

Mesoderm cells in three-dimensional collagen latices *in vitro* are

able to re-orient and align the matrix fibrils by virtue of the tension applied to them as a result the traction exerted by the moving cells (Sanders and Prasad, 1983). Whether or not such matrix reorganization occurs *in vivo* is not known, so that it is not clear that the alignment of the fibrils *in vivo* in the current situation is totally independent of the activity of the cells moving on them. However, we observed that cells also attached to non-fibrillar, punctate, fibronectin-rich bodies which probably correspond to the "interstitial bodies" seen by electron microscopy (Sanders, 1982), and that these structures were clearly not re-organized by the presence of moving cells.

It was clear from the present work that the mesoderm cells possessed their own cell surface fibronectin, which was deposited as a network on the free surface of the cells, i.e. the surface not in contact with the substratum (see also Harrisson *et al.*, 1985, 1993). Clearly, it is not possible by the present methods to determine the source of the fibronectin in the substratum, but since the fibrous band is present in advance of the mesoderm cells, it is presumably deposited in the basement membrane by the epiblast or endoblast cells. It has been argued (ffrench-Constant and Hynes, 1988) that endogenous fibronectin production by migrating embryonic mesenchyme cells must mask any generalized guidance effects of fibronectin in the substratum. However, we have shown that despite the synthesis of fibronectin by the mesoderm cells themselves, the exogenous substratum-bound fibronectin is still able to guide filopodia and orient the cells. We therefore suggest that these cells are susceptible to guidance by the fibronectin-rich fibrillar matrix, and that the latter is a significant physiological factor in the morphogenetic movements of the early mesoderm, despite the possibility that the presence of fibronectin may not be crucial for normal gastrulation in experimental situations (George *et al.*, 1993).

Materials and Methods

White Leghorn hen's eggs were incubated at 37°C in a humid chamber until they had reached stage 4 of development (Hamburger and Hamilton, 1951). Embryos destined to be hosts for grafted cells were then explanted on their vitelline membranes according to the method of New (1955) and replaced in the incubator. In this method, the vitelline membrane is stretched over a 25 mm internal diameter glass ring with the ventral surface of the embryo uppermost. Meanwhile, other embryos at a similar stage of development were removed from their yolk and vitelline membrane, and washed free of yolk in Pannett and Compton's saline. The latter embryos were used as a source of mesoderm cells. The mesoderm cells were dissected from the donor embryos using electrolytically sharpened tungsten needles and without the use of dissociating enzymes. After removal of the endoderm, the mesoderm cells could be teased away from the basal surface of the overlying epiblast in small groups.

The clusters of dissected mesoderm cells were then exposed to a solution of 5- (and -6)-carboxy-2',7'-dichlorofluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Inc.) at a concentration of 6 µg/ml in Tyrode's saline for 10 min at room temperature. The tissue pieces were then washed free of CFSE with Tyrode's saline.

The host embryos were prepared for grafting by removing excess fluid from the surface, and by making a small hole in the endoderm with a sharpened needle. The hole was usually located in or near the "germinal crescent" (see England and Matsumura, 1993), an arc of endoderm at the rostral tip of the area pellucida near the border with the area opaca (Fig. 1). A small piece of CFSE-labeled donor mesoderm was inserted into the hole in the endoderm, and the host was returned to the incubator for a further 6 h.

At the end of the incubation period, each embryo, still attached to its glass ring, was washed in warm phosphate buffered saline (PBS) and then fixed in 4% paraformaldehyde for 12-18 h. at 4°C. The embryos were then washed several times in PBS, and the endoderm overlying the general region of the grafted cells was removed with sharpened tungsten needles. All subsequent steps were performed at room temperature. The embryos were carefully removed from their vitelline membranes, washed again in PBS, and then incubated for 1 h in 10% goat serum in PBS. Specimens were then incubated in rabbit anti-human fibronectin antiserum (Collaborative Research Inc.), diluted 1:40 in PBS plus goat serum for 2 h, washed 3 times for 20 min each in PBS plus goat serum, and then incubated in goat anti-rabbit IgG conjugated to Texas Red (Calbiochem), diluted 1:100 in PBS plus goat serum, for 1 h. After 2 washes in PBS for 20 min each, the embryos were left in PBS overnight at 4°C. The following morning each embryo was dehydrated in methanol and mounted on a glass slide in methanol under a coverglass which had been weighted down in order to flatten the embryo. The coverglass was sealed with nail polish.

Confocal microscopy was carried out using a Leica confocal laser scanning microscope equipped with an argon/krypton laser. A short pass excitation filter was used (KP590) with the beam splitter in the neutral position. The barrier filter used in the Texas Red channel was OG530, and that in the fluorescein channel was OG550. Images from the 2 channels were collected sequentially as 16-scan averages, and were then digitally combined and artificially colored to give the final double-label image. The CFSE-stained cells were colored green, and the Texas Red-stained extracellular matrix was colored red. Overlap of these two colors produced a yellow image. In addition, some specimens were examined and photographed by conventional immunofluorescence microscopy using standard rhodamine and fluorescein filter sets.

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