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Skeletogenesis in the sea urchin embryo

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Introduction

Skeletogenesis, a key morphogenetic event in the embryonic development of vertebrates, is also a very important, albeit a transient, milestone in the development of a marine invertebrate, the sea urchin. In recent years this biological system, in which the biomineralization process can be investigated in a well-defined, single cell type (the primary mesenchyme cell), has received considerable study. The objective of this review is to summarize our current knowledge about skeletogenesis in the sea urchin embryo and highlight the outstanding questions that remain to be answered. It will become apparent that there are two broad sets of questions: one set pertains to cell differentiation and morphogenesis; the other is concerned with basic cellular processes that are common to most cell types in a developing organism, such as cell–cell interactions, membrane fusion, ion transport and exocytosis. The former set of questions has been reviewed on numerous occasions, most recently by Morrill & Santos, 1985; Davidson, 1986; Solursh, 1986; Wilt, 1987 and McClay & Ettensohn, 1987. Therefore, coverage of these issues will be restricted to points that pertain to the basic cellular processes involved in spiculogenesis.

Spicule formation in relation to cell differentiation and morphogenesis

Early work on the structure and ontogeny of the skeleton

One reason the skeleton of the sea urchin embryo has attracted attention is its remarkable structure, which is readily apparent upon microscopic examination of the embryo. This is illustrated in Fig. 1 which shows the embryo of *Strongylocentrotus purpuratus* at the pluteus stage, viewed by polarized light (Nomarski optics). The skeleton grows from two triradiate spicules that increase in complexity as the embryo progresses from the late gastrula to the pluteus stage. The inorganic component of the spicules is the mineral calcite, $CaCO_3$, plus a small amount (5 %) of $MgCO_3$.

The most distinctive feature of the skeleton is its apparently crystalline nature. Thus, although the skeleton is a complex, branched structure, the individual spicules behave 'optically as though chiseled out of a single crystal of calcite' (Okazaki & Inoue, 1976; Inoue & Okazaki, 1977). In fact, each spicule can be considered to have a polycrystalline structure, made of stacks of contiguous microcrystals. However, as pointed out most clearly by Okazaki & Inoue (1976), such microcrystals do not define the overall shape of the spicule. Rather, the morphology of the spicule is dictated by the syncytial structure produced by the primary mesenchyme cells.

Both optical birefringence and X-ray diffraction (Donnay & Pawson, 1969) indicate that the spicules are crystalline; however, no crystalline structure is apparent upon scanning electron microscopic examination of the intact or fractured spicules (Millonig, 1970). Even in the absence of obvious crystalline faces, the isolated spicule can serve as a template for growth of calcite crystals upon addition of $CaCl_2$ and $NaHCO_3$, and the crystals that grow on the spicule under these conditions are aligned parallel to each other and follow the form of the spicule (Okazaki & Inoue, 1976; Inoue & Okazaki, 1977). Thus, despite the absence of some of the expected features of a polycrystalline structure, there is little doubt that this extraordinary structure is composed of calcite crystals.

A second reason for interest in the spicule relates to its ontogeny. About a century ago three investigators, Selenka, Semar and Theel, independently carried out investigations on the origin of the spicule. In 1882, Theel wrote

"The manner in which the calcareous deposits originate in the Echinoderms, and the subsequent mode of increase of the skeleton are by no means clear and offer a great deal of

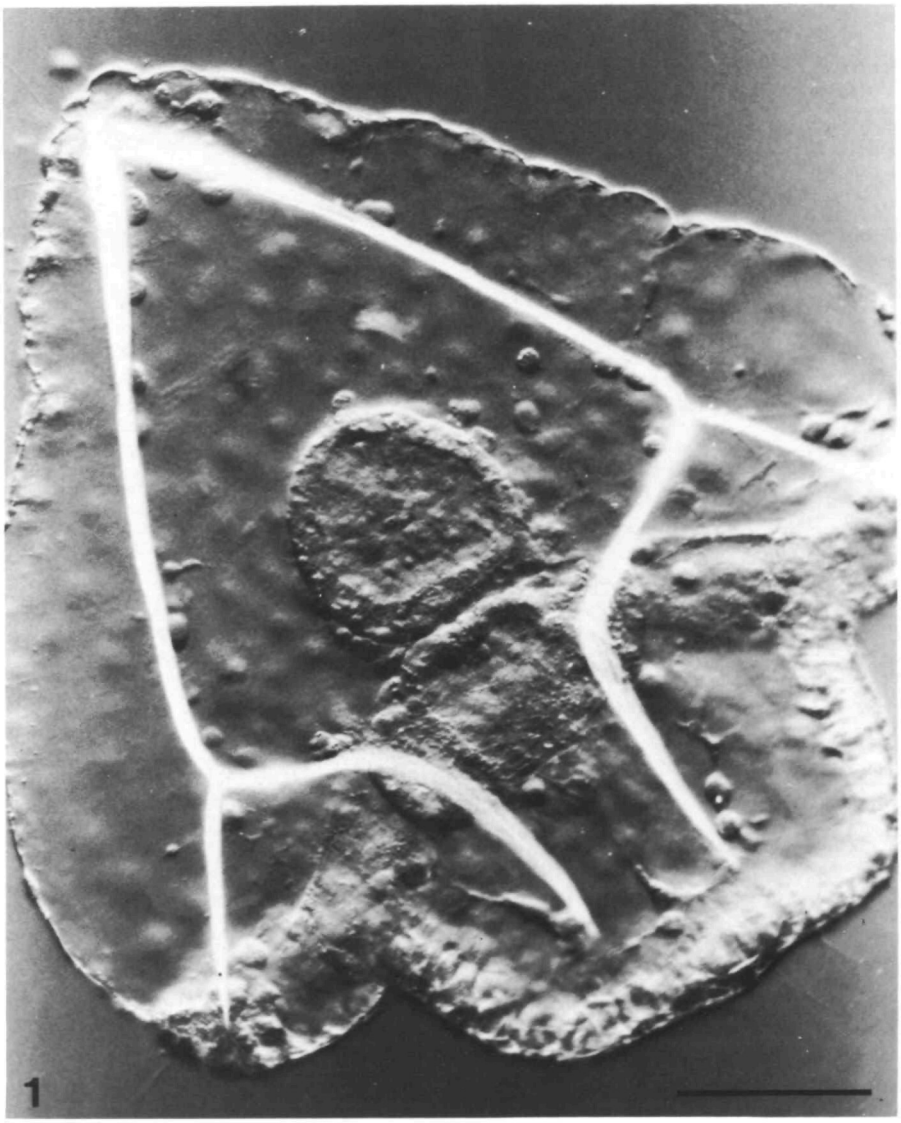

Fig. 1. Pluteus larval skeleton and associated primary mesenchyme cells. The *S. purpuratus* embryo has been flattened for viewing the entire skeleton. The body rods of the skeleton meet at the dorsal side (upper left). The stomatodaeum (mouth) opening is slightly out of the plane of focus on the opposite (ventral pole) end of the dorsoventral axis. Bar, 50 μm.

interest. When studying these processes, the investigator soon perceives that the calciferous cells, though free in the blastocoel, act by no means without method, and that they cooperate for a common goal as if they were conscious."

One hundred years later this 'cooperative' behaviour of the 'conscious', 'calciferous cells' is no less remarkable, and only slightly better understood. What is clear now is that the cells responsible for assembly of the skeleton are primary mesenchyme cells and that they originate from a subset of the micromeres formed in the 16-cell embryo (Fig. 2). At early blastula stage, after numerous rounds of cell division, the cells whose progenitors were the micromeres are indistinguishable from the other cells comprising the blastodermal layer of the embryo. Only upon formation of the vegetal plate of the blastula-stage embryo do these 30–50 cells emerge as a distinguishable cell type. At this point in the developmental program, the primary mesenchyme cells leave the blastoderm, enter the blastocoel and form a cell cluster at the vegetal pole. Subsequently, migratory behaviour is initiated and the primary mesenchyme cells extend filopodia and move along the blastocoelic wall.

The appearance of the primary mesenchyme cells in the vegetal half of the early-gastrula-stage embryo is shown by scanning electron microscopy in Fig. 3. At this stage of development the primary mesenchyme cells cease their migration in the blastocoel and become positioned as two cell clusters that are formed bilaterally with respect to the site where the endoderm invaginates. Subsequently, as shown in Fig. 4, an annular pattern of cells is formed and two loci become readily evident by light microscopy. The cells at each locus have fused with each other and, within the syncytial structures that are formed, the birefringent, polycrystalline spicules grow. Upon reaching pluteus stage, an abundance of extracellular matrix is also found associated with the syncytia and blastocoelic wall (Fig. 5). Throughout development

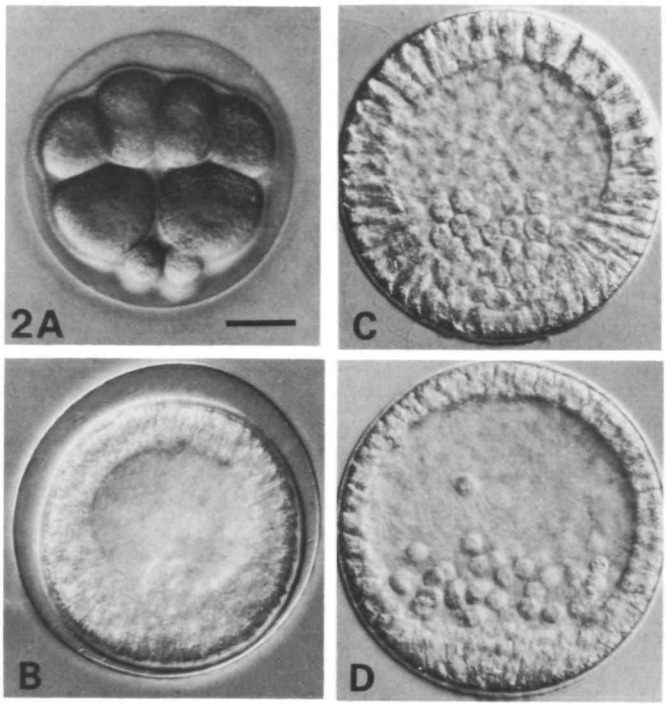

Fig. 2. Lineage determination, ingression and migration of the primary mesenchyme cells within the blastocoel of an *S. purpuratus* embryo. (A) 16-cell-stage embryo showing four progenitor micromeres at vegetal pole that mark the site on the animal–vegetal axis where the primary mesenchyme cells will emerge at blastula stage. (B) Primary mesenchyme cells emerging from the blastoderm at the vegetal plate, just prior to hatching. (C) Primary mesenchyme cells accumulating in a cluster at the vegetal pole, and later (D) migrating towards the animal pole and then returning to form centres for spicule formation (see text). Bar, 25 μm.

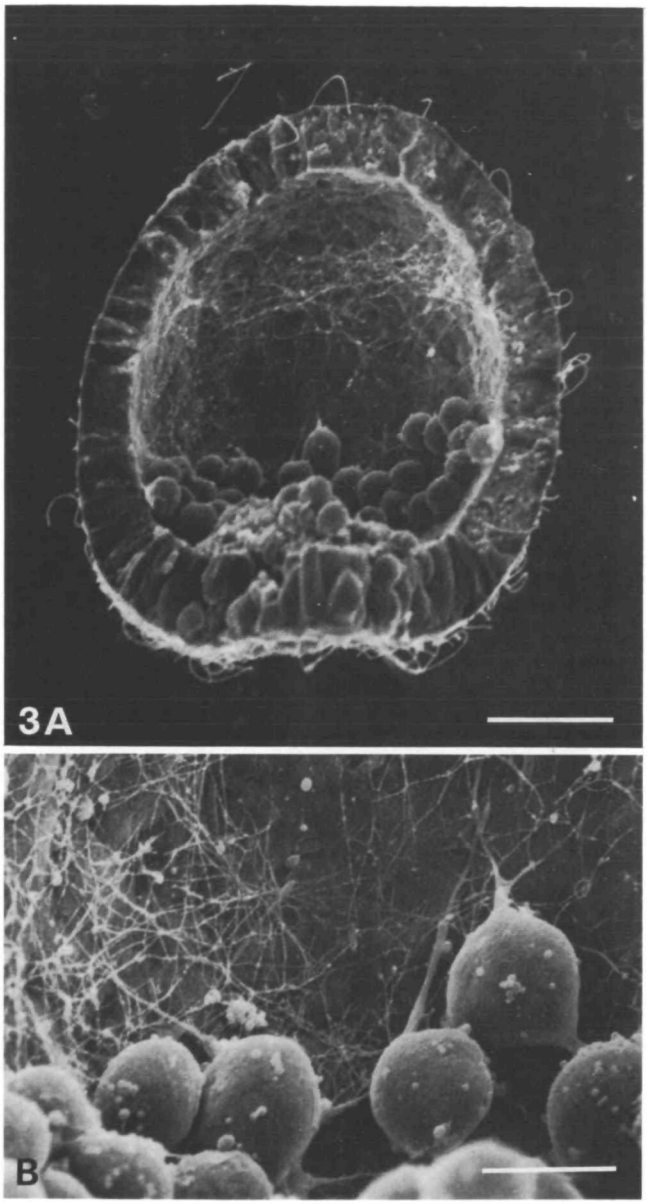

Fig. 3. Scanning electron micrograph of early gastrula stage *Lytechinus variegatus* embryos. (A) Clusters of primary mesenchyme cells position bilaterally with respect to the advancing endoderm at the vegetal pole. (B) Higher magnification of region of lower left portion of the blastocoel showing cell bodies and filopodial extensions that fuse to form a syncytium. Bars (A) 24 μm; (B) 5 μm. Prepared as described by J. B. Morrill (1986).

from gastrula to pluteus stages the skeleton grows in both size and complexity. Its existence, however, is only transient in the overall life cycle of the sea urchin because it is "lost" when the pluteus larva undergoes metamorphosis to form the juvenile sea urchin. For more comprehensive coverage of issues pertaining to the ontogeny of the spicule-forming cells, the reader is referred to the important studies of Horstadius (1973), the recent review by Wilt (1987) and the monograph by Davidson (1986).

What factors are required for expression of the differentiated phenotype of primary mesenchyme cells?

One important finding was that isolated micromeres (but not meso- or macromeres) cultivated in sea water containing serum could differentiate *in vitro* into spicule-forming primary mesenchyme cells (Okazaki, 1975*a*). This observation suggested that isolated micromeres are already programmed for the differentiation process and that external cues provided by other blastomeres are not necessary. This idea was supported by the findings that primary mesenchyme cells isolated from mesenchyme-blastula-stage embryos by virtue of their selective adhesiveness to the surface of tissue culture dishes (Venkatasubramanian & Solursh, 1984; Carson *et al.* 1985) or to the immobilized wheat germ agglutinin (Ettensohn & McClay, 1987) produce spicules in the apparent absence of other cell types. This is also observed in

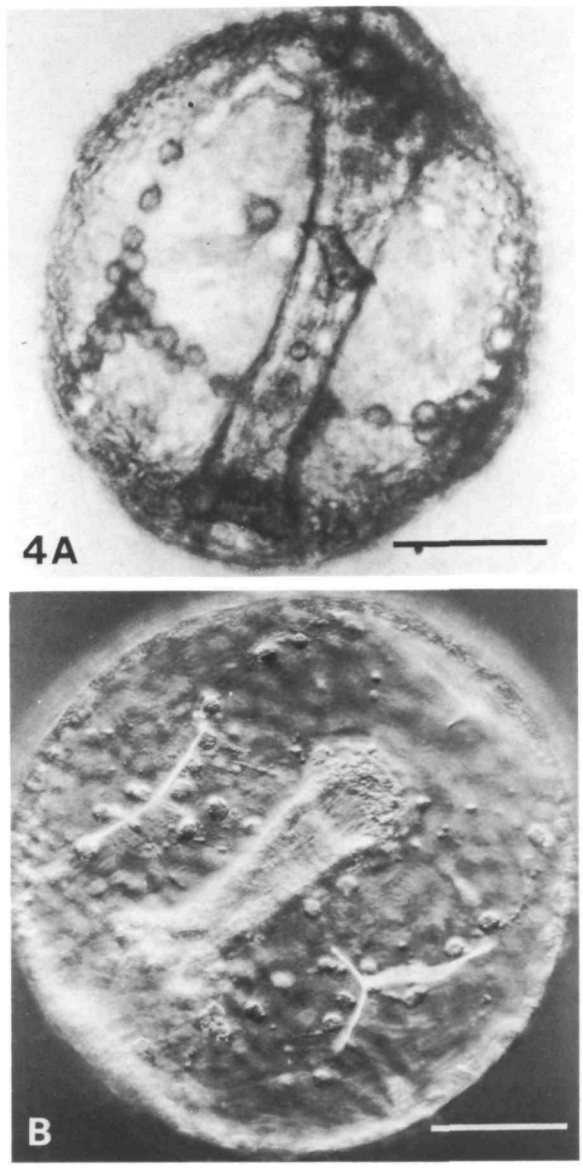

Fig. 4. Light micrographs of spicule-forming centres of primary mesenchyme cells at gastrulation stage. (A) Annular pattern of primary mesenchyme cells of *Lytechinus pictus* extending around the wall of the blastocoel (bright field mode); (B) *S. purpuratus* triradial spicules produced by the primary mesenchyme cells by late gastrula stage (Nomarski mode). Bars, 50 μm.

another, somewhat more complex, *in vitro* system consisting of primary mesenchyme cells enclosed by the basal lamina of the mesenchyme-blastula-stage embryo that has been stripped of ectodermal cells (blastula 'bags') (Harkey & Whiteley, 1980). In all three systems (cultured micromeres, primary mesenchyme cells or primary mesenchyme cells within basal laminar bags) the majority of the spicules formed are linear, i.e. they lack the complex form found in the intact embryo. Similarly, in all three systems, the sea water normally does not support spicule formation unless it is supplemented with horse serum, although in the case of blastula 'bags' a low level of spicule formation can be observed in the absence of serum.

The requirement for horse serum is presumably indicative of the need for molecular components that have been lost in the process of dissociating the embryos (Okazaki, 1975*a*,*b*). However, such components have yet to be identified. Studies on this apparent requirement suggest that a factor present in serum induces the fusion of filopodial networks that form prior to spiculogenesis (McCarthy & Spiegel, 1983). Presumably the component(s) of horse serum is present in the blastocoel, as are fibronectin and acid polysaccharides and/or proteoglycans (Iwata & Nakano, 1985*a*,*b*). Progress in elucidating the role of these molecules in the various stages leading to formation of the spicule is discussed below.

What defines the complex shape of the spicule synthesized in the embryo?

The three-dimensional arrangement of the spicules is genetically determined, since the primitive skeleton for each species of sea urchin has a unique structure (Horstadius, 1973). Studies with cells *in vitro* indicate that conditions favouring cell aggregates promote formation of multiradiate spicules (Kinoshita & Okazaki, 1984). However, it is clear that the highly reproducible, genetically determined, structures formed *in vivo* are defined by more complex factors. At the early stage of spicule formation, the overall structure of the spicule is determined by the three-dimensional arrangement of the primary mesenchyme cells in the blastocoel. But what defines the arrangement of these cells? What spatially organized cues instruct the primary mesenchyme cells to cease migration at a particular site in the blastocoel?

One possibility is that the basal lamina is not compositionally uniform and that domains of specialized molecules serve as 'stop' signals for the migratory primary mesenchyme cells. This would implicate similar regional specialization of the blastodermal cells, since these cells are attached to the opposite side of the basal lamina. Although this idea is not unreasonable, no direct evidence for regional specialization of the basal lamina exists. However, Wessel *et al.* (1984), studying the ontogeny of the basal lamina, reported that individual extracellular matrix components are incorporated into this complex structure at different stages of development.

Another line of evidence indicates that blastodermal cells, perhaps directly, play an instructional role in the placement of primary mesenchyme cells in locations that subsequently define the shape of the spicule. Harkey & Whiteley (1980), studying spicule formation by primary mesenchyme cells contained within basal laminar bags, found that in the presence

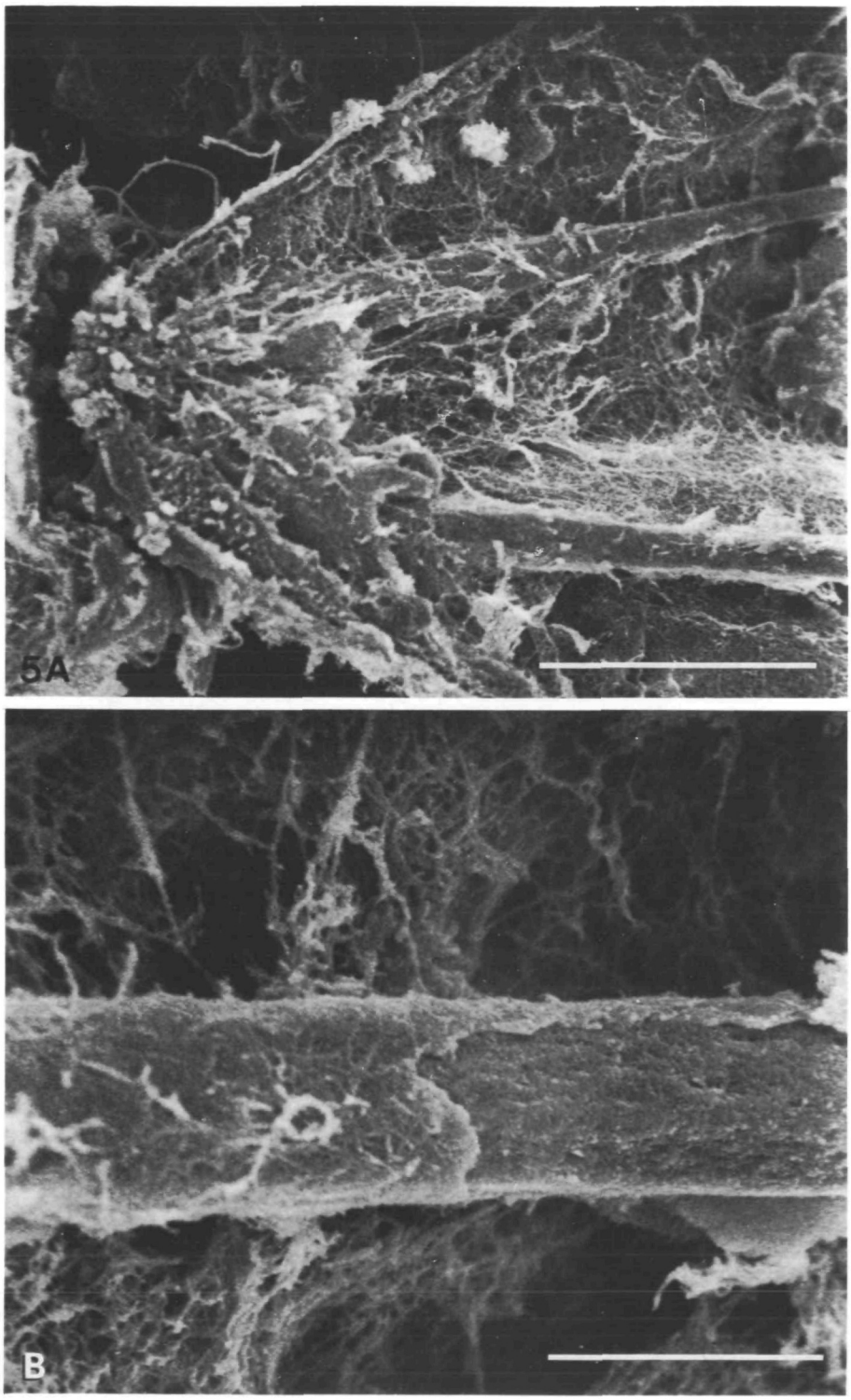

Fig. 5. Scanning electron micrograph of the skeleton in the early-pluteus-stage embryo of *S. purpuratus*. (A) Body rods of skeleton at dorsal side of dissected dry-fractured embryo showing extensive extracellular matrix within the blastocoel; (B) Region of A, upper right, showing exposed spicule partially surrounded by a filopodia-derived sheath. Bars (A) 20 μm; (B) 3 μm.

of a low level of exogenously added blastodermal cells the spicules formed by primary mesenchyme cells were linear. However, when the concentration of added blastodermal cells was high, partial reformation of the blastoderm occurred and the morphology of the spicules formed approached that of spicules made in the normal embryo. On the basis of these observations, and in accordance with the earlier proposals of Gustafson & Wolpert (1963) and Okazaki (1975*a*,*b*), Harkey & Whiteley (1980) proposed that filopodia of the primary mesenchyme cells can actually interact with cells that comprise the blastoderm. In any case, if such interaction leads to specific positioning of the primary mesenchyme cells, then

one still must postulate that all blastodermal cells are not equal and that some must express the 'stop' signals discussed above.

How is the crystallinity of the spicule controlled?

Despite the fact that biomineralization is a fundamental cellular process that occurs in the development of both the vertebrate and invertebrate, there is still a great deal of uncertainty regarding basic aspects of this process.

With respect to the skeleton of the sea urchin, it is not clear what factors define the mineral type that forms; the carbonate mineral in the sea urchin spicule could in principle be either aragonite, vaterite or monohydrocalcite, rather than the calcite that is found. In addition, as noted earlier, there are difficulties in describing the mineral phase in the sense that it is unclear if the calcite in a single spicule is a single crystal or a collection of crystal fragments, i.e. a polycrystalline aggregate of preferred orientation (Okazaki & Inoue, 1976). Thus, as reviewed by Okazaki & Inoue (1976), the results of polarizing light microscopy and X-ray diffraction analysis are consistent with a single crystal structure, whereas analysis by electron microscopy suggests a polycrystalline array.

Second, the concept of an organic matrix, first developed by workers studying mammalian bone, has become an important component of theories of invertebrate biomineralization (Weiner, 1984). Two general models for the role of these molecules in the biomineralization process, including growth of the crystal, have evolved (Towe, 1972). In one, the epitaxy model, the organic molecules serve as a template to define both the overall shape and the crystal form of the structure. However, there are basic mechanistic reasons (see Towe, 1972) why the crystal properties cannot be completely explained by epitaxy. In the second model, the compartment model, the organic matrix does not serve as a template *per se*, but defines a compartment within which the biomineral grows. In this model, the overall shape of the biomineral is defined by the compartment, but the issue of how the crystallinity is controlled is not addressed.

With respect to the organic matrix, it is important to note that defining its location with respect to the spicule is not operationally easy. As will be discussed below, the spicule is wreathed in a membrane. As shown in Fig. 6, when this membrane is removed by treatment with urea and sodium dodecyl sulphate and the mineral is subsequently dissolved by addition of acid or chelator, an organic 'matrix' in the shape of the original spicule remains (Benson *et al.* 1983). Is this all of the 'matrix' or was part of it associated with the inner face of the membrane and lost at the initial step of urea–sodium dodecyl sulphate treatment? Recent studies (see below) make it clear that proteins are present in the mineral *per se*, but how they are related to any molecules that might have been present on the surface of the mineral remains to be established.

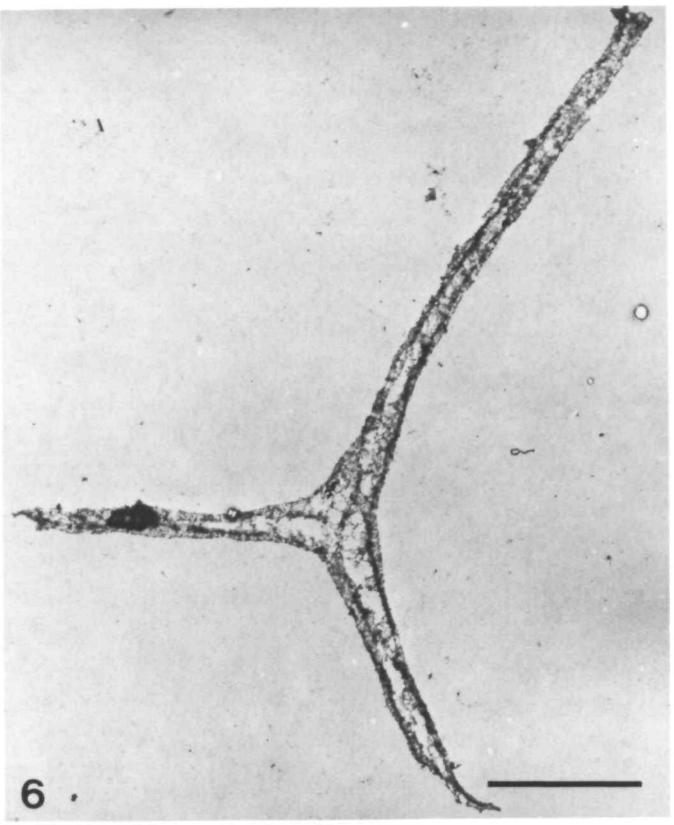

Fig. 6. The morphology of the organic matrix of a demineralized triradiate spicule exhibiting a morphology comparable to that of the intact spicule at gastrula stage (from Benson *et al.* 1983). Bar, 4 μm.

Finally, whatever the role of the proteinaceous matrix in biogenesis of the spicule, the fact is that because the spicule is a composite of calcite and proteins it exhibits less stiffness and more strength than it would if made of pure calcite (Emlet, 1982). Hence, the macromolecules may function not only in directing synthesis of the spicule, but also in conferring properties that provide a survival advantage. Clearly, more information on the overall organization of the matrix and an understanding of the properties of the molecules in it will be useful in unravelling these complex issues pertaining to the nature of the biomineral.

Recently, some progress has been made in this respect: earlier work on the so-called matrix proteins of mineralized tissue of a wide variety of invertebrates suggested that in some cases collagen-like proteins are present, whereas in others silk fibroin-

like proteins rich in Asp, Gly and Ser are the major components (Travis *et al.* 1967). More recently, studies by Kingsley & Watabe (1983) have confirmed the presence of proteins high in aspartic acid content in both the soluble and insoluble spicule matrix contents of the gorgonian *Leptogorgia virgulata*. Subsequently, the kinetics of synthesis of these molecules by pulse-chase labelling techniques with [^{3}H]aspartic acid was studied (Kingsley & Watabe, 1984). Although this approach was limited by a lack of probes for specific, individual proteins, the authors conclude that matrix protein synthesis occurs in the endomembrane system of the rough endoplasmic reticulum and Golgi complex and takes two hours from appearance in the rough endoplasmic reticulum to deposition in the spicule.

Studies by Wilt and Benson and their co-workers have recently provided the first detailed information on individual proteins of the matrix of the sea urchin spicule. As described earlier, Benson *et al.* (1983) demonstrated that after demineralization of isolated spicules, an organic matrix remained that had an overall structure similar to the spicule's. The organic material in this matrix did not have the characteristics of collagen, but apparently was organized in concentric layers within the spicule.

Subsequent SDS–PAGE analysis of the protein components of this matrix (Benson *et al.* 1986) revealed the presence of ten acidic glycoproteins. This mixture of protein was shown to bind Ca^{2+}, albeit with relatively low affinity (10^{-4} M) (Benson *et al.* 1986). Similar findings on the occurrence of matrix proteins were reported by Venkatesan & Simpson (1986). Later, Benson *et al.* (1987) prepared a polyclonal antibody to these spicule matrix proteins and used it to screen a cDNA expression library. A cDNA probe isolated by this approach was used to identify a cognate RNA transcript that encoded a $50\times10^3 M_r$ (50K) protein identical to the major protein in the mixture of proteins isolated earlier from the matrix (Benson *et al.* 1986). Hybridization studies revealed that the 2·2 kb mRNA encoding this protein first appeared at late cleavage stages; from the blastula stage onward its concentration increased in an essentially linear manner. *In situ* hybridization experiments first detected the mRNA in primary mesenchyme cells ingressing into the blastocoel of the embryo.

These studies, which clearly established that a 50K matrix protein is synthesized in primary mesenchyme cells, were extended in order to define the structure of the gene and the protein (Sucov *et al.* 1987). The polypeptide sequence derived from the structure of the gene indicates the presence of a signal peptide and an *N*-linked glycosylation site, as expected for a secretory glycoprotein. The most novel feature of the protein is that almost half of the sequence is composed of a 13-residue repeat element of the structure:

$$\text{Try–Val–Gly–Asp–Asn–Gln–Ala–}\begin{matrix}\text{Leu}\\\text{Trp}\end{matrix}\text{–Val–}\begin{matrix}\text{Ile}\\\text{Asp}\end{matrix}\text{–Asn–}\begin{matrix}\text{Gln}\\\text{Pro}\end{matrix}\text{–}\begin{matrix}\text{Val}\\\text{Gln}\end{matrix}$$

No homology between this sequence and known sequences was detected. A second feature of interest is the high abundance (one out of three residues) of proline in an amino terminal domain of 55 residues. A weak homology between this domain and a group of human salivary proteins was detected. Clearly, however, the functional significance of these structural features with respect to the biomineralization process remains to be established.

Basic cellular processes involved in spicule formation

Several rather complex questions about the process of spiculogenesis were raised but not answered in the preceding section. To simplify the questions, a number of investigators have in recent years focused on individual steps in the overall process of spiculogenesis, using either intact embryos or a variety of *in vitro* systems consisting of cultured cells. All of the latter systems are based on the pioneering observations of Okazaki (1975*a*,*b*) who established that micromeres cultivated *in vitro* in the presence of horse serum underwent apparent differentiation to primary mesenchyme cells and produced spicules.

Cell ingression

Biochemical studies (Schneider *et al.* 1978) revealed that although gastrulation was inhibited by the *N*-linked glycoprotein inhibitor tunicamycin, the earlier invasion of incipient primary mesenchyme cells into the blastocoel was not blocked. Similarly, neither a block in the addition of glycosaminoglycan chains to polypeptide chains by cultivation of embryos in the presence of β-xylosides (Solursh *et al.* 1986) nor a deficiency in sulphate in the medium (Karp & Solursh, 1974) resulted in a block in ingression. Thus, ingression seems to exhibit relatively little dependence on *de novo* synthesis of glycoproteins or proteoglycans.

Ultrastructural studies by Katow & Solursh (1980) revealed that the first step in the overall process of ingression involves the loss of a well-defined basal lamina in the vegetal plate region and the loss of cilia from these cells. These authors observed shape changes in which the blastodermal cells protruded into the blastocoel, lost their apical junctions, rounded up and then entered the blastocoel. McClay and his co-workers (McClay & Fink, 1982; Fink & McClay, 1985; McClay & Ettensohn, 1987) very

elegantly showed that, consistent with these observed ultrastructural changes, the presumptive primary mesenchyme cells lost their ability to bind to hyalin and to other cells and acquired the ability to migrate through the basal lamina into the blastocoel.

Cell migration

Shortly after entering the blastocoel, the primary mesenchyme cells migrate. Several studies have focused on the nature of the molecules either on the surface of the primary mesenchyme cells or in the blastocoel that may be involved in the migratory process. One of the best described mediators of cell–cell and cell–substratum adhesion events is fibronectin, which is known to have binding domains for a cell surface receptor, collagen and glycosaminoglycans (Ruoslahti & Pierschbacher, 1987). In fact, fibronectin has been found to be specifically associated with the primary mesenchyme cells during the stage in which they exhibit migratory behaviour (Katow *et al.* 1982). It seems likely but is not established that the binding of fibronectin to the cells is mediated by a cell surface receptor of the type shown in other systems to recognize the sequence Arg–Gly–Asp in fibronectin. For example, such a system has been shown to be functional in mouse blastocyst attachment and outgrowth (Armant *et al.* 1986). For the fibronectin to participate in the migration process it might be expected that, in addition to interaction with the primary mesenchyme cells, it would interact with either collagen or certain of the glycosaminoglycan chains of proteoglycans in the blastocoel. The available evidence suggests that, although collagen is not involved in cell migration (Wessel & McClay, 1987 and references cited therein), proteoglycans are. In contrast to ingression, migration of primary mesenchyme cells is inhibited by either the presence of β-xylosides (Solursh *et al.* 1986; Kinoshita & Saiga, 1979; Akasaka *et al.* 1980) or the absence of sulphate in the sea water (Karp & Solursh, 1974). Initially, Katow & Solursh (1981) reported that primary mesenchyme cells in sulphate-depleted embryos exhibited defects in the behaviour of the long and short cellular extensions that participate in the migration of these cells. Subsequent *in vitro* studies with isolated primary mesenchyme cells confirmed these observations (Venkatasubramanian & Solursh, 1984).

In view of the apparent importance of sulphated proteoglycans in the blastocoel in the migration process, these molecules were studied in both *S. purpuratus* and *L. pictus* embryos at the mesenchyme blastula stage of development (Solursh & Katow, 1982). These studies revealed the presence of the following glycosaminoglycan chains in the proteoglycans of *S. purpuratus*: dermatan sulphate (37%), chondroitin 6-sulphate (16%), and heparan sulphate (13%). Detailed studies on the effect of β-xylosides revealed that sulphated glycosaminoglycan chains not attached to proteins accumulated in the presence of this agent (Solursh *et al.* 1986). This result strongly suggests that the functional molecules in cell migration are intact proteoglycans. In this context, it is of interest that Karp & Solursh (1985*a*) have shown that when the filopodia of isolated primary mesenchyme cells make contact with an aggregate of extracellular matrix material, the cell body moves towards this material.

It is clear that, although considerable progress has been made in understanding the migration of these cells, the intact relevant molecules have not been directly identified. Moreover, a mechanism that is merely based on binding of molecules on the surface of these cells to molecules on the inner surface of the blastocoel would not explain the fact that although initially these cells go through a phase of *continuous* migration, they subsequently stop at specific sites.

It is clear from the above studies that the environment of the blastocoel plays a crucial role in the overall program of primary mesenchyme cell activities, from ingression to spicule formation. Further documentation of this conclusion and support for the idea that the environment must be changing over the course of development is provided by the previously cited work of Wessel *et al.* (1984) and the important recent studies of McClay and co-workers (Ettensohn & McClay, 1986; McClay & Ettensohn, 1987). These authors carried out microscopic observations of vitally stained primary mesenchyme cells that had been injected into the blastocoel of an embryo. They found that the behaviour of the primary mesenchyme cells is dependent on the stage of the host embryo, that is, primary mesenchyme cells injected into the blastocoel of a blastula embryo prior to hatching migrate to the vegetal pole but then remain static until the 'correct' time for migration. At that time they undergo the same migrations as the normal 'resident' primary mesenchyme cells. In contrast, primary mesenchyme cells injected into later-stage embryos no longer can recognize and migrate to the vegetal pole. Thus, the host blastodermal surface seems to provide the programme of information necessary for correct migration of primary mesenchyme cells.

Interaction and fusion of primary mesenchyme cells

As described earlier, the migration of the primary mesenchyme cells within the blastocoel culminates with their interaction *via* filopodia, followed by fusion to form a syncytial network that defines the geometry of the soon-to-form spicule. Several aspects of the fusion event have been investigated. In early studies,

Hagstrom & Lonning (1969) reported that both micromeres and their descendants, the primary mesenchyme cells, have a strong propensity to undergo fusion. Moreover, Karp & Solursh (1985*b*), studying isolated primary mesenchyme cells in culture, established by time-lapse videomicroscopy not only that these cells fuse with each other, but that the syncytial entity formed can undergo 'division' into mononucleated or anucleated cells. These cells are thus remarkable in their plasticity: they fuse with each other like myoblasts during terminal differentiation and divide like all cells during mitosis. A scanning electron micrograph showing several primary mesenchyme cells that apparently have undergone fusion *via* a common syncytium is shown in Fig. 7.

In this context, it should be noted that evidence recently obtained implicates metalloendoproteases in membrane fusion events in myotubule formation from myoblasts (Couch & Strittmatter, 1983, 1984), secretion in mast cells and in adrenal chromaffin cells (Mundy & Strittmatter, 1985), the acrosome reaction in sea urchin sperm (H. A. Farach *et al.* 1987), and sea urchin gamete fusion (Roe *et al.* 1988). To this list can now be added primary mesenchyme cell fusion because very recent studies indicate that metalloendoprotease inhibitors block spicule formation by primary mesenchyme cells in culture (J. L. Roe, H. Park, W. Strittmatter and W. J. Lennarz, unpublished observations). Because attachment of the cells to the culture dish is not affected by these inhibitors, the most likely site of action is the fusion event that is a prerequisite to spicule formation.

The biomineralization process

Biomineralization and, more specifically, calcification are fundamental biological processes that are of great importance. With respect to molecular studies, the sea urchin embryo offers several advantages. First, because the overall process of formation of a $CaCO_3$-containing spicule involves a massive accumulation of Ca^{2+}, studies on the events that precede deposition, such as entry and 'packaging', should be facilitated. Second, the lineage of the primary mesenchyme cells that carry out the spiculogenic process is well defined. Third, the calcification process can be studied with a relatively simple *in vitro* system consisting of primary mesenchyme cells in sea water containing horse serum.

We began efforts to define individual steps in the calcification process using cells in culture. Because the system of Okazaki (1975*a*), involving isolation of micromeres that differentiate *in vitro* to spicule-forming primary mesenchyme cells, did not in our hands reproducibly yield sufficient cells for biochemi-

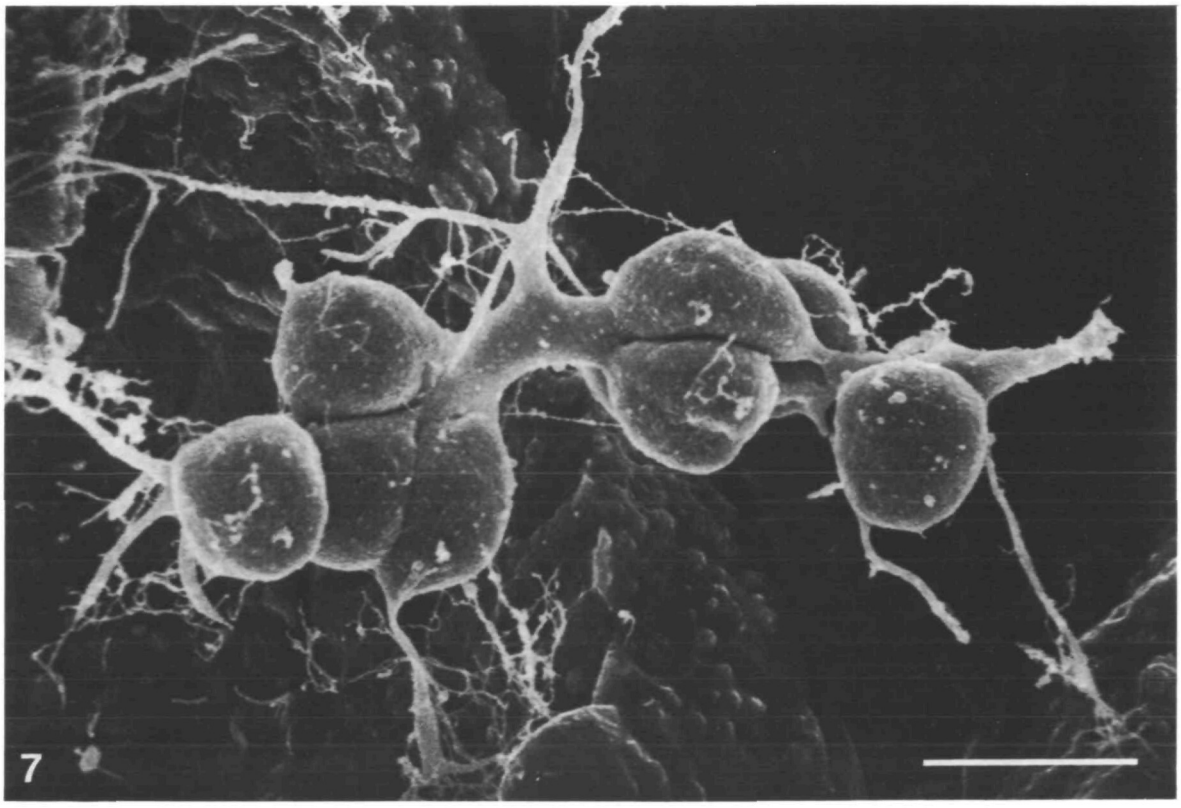

Fig. 7. Early-gastrula-stage embryo of *Lytechinus variegatus* showing syncytial nature of primary mesenchyme cells that were dislodged from the blastocoel during preparation for scanning electron microscopy. Bar, 10 μm.

cal studies, we first used a mixed cell system consisting of primary mesenchyme cells and blastodermal cells obtained by dissociating embryos at the mesenchyme blastula stage (Mintz *et al.* 1981). After 10 h, a significant portion of the cells formed aggregates and after 48 h linear spicules were formed in many of these aggregates. Inhibitors of DNA synthesis had no effect on spicule formation or on the number of cells in the culture. Both of these observations are consistent with the idea that terminal differentiation occurred and that cell division ceased.

A marked increase in incorporation of [^{3}H]glucosamine and $^{45}Ca^{2+}$ into the cells was detected at the same time as spicule formation. Incorporation of [^{3}H]glucosamine into glycoconjugates and $^{45}Ca^{2+}$ into spicules was blocked by tunicamycin, a specific inhibitor of *N*-glycosylation of proteins. This observation suggested a possible role for *N*-linked glycoproteins in spicule development. In addition, one or more novel hydroxyproline-containing proteins appeared to be synthesized by the primary mesenchyme cells during formation of skeletal spicules. This protein did not appear to have the properties typical of collagen since it did not contain pepsin-resistant domains and it was not degraded by collagenase. However, its synthesis and processing seemed to be essential for spicule formation since inhibitors of prolyl hydroxylase, lysyl hydroxylase and lysyl oxidase, three enzymes involved in post-translational modification of amino acid residues in collagen-like proteins, blocked spicule formation.

These initial studies in an *in vitro* system consisting of mixed cell types provide evidence supporting earlier observations with intact embryos (Schneider *et al.* 1978; Heifetz & Lennarz, 1979) that the biosynthesis of *N*-linked glycoproteins is a prerequisite for skeleton formation. Subsequent studies using this system and inhibitors of phosphodiesterase also suggested that a decrease in cAMP may be a prerequisite for terminal differentiation to spicule-forming cells (Mintz & Lennarz, 1982).

In studies using cultured micromeres, Blankenship & Benson (1984) showed that these cells did not undergo the final stages of differentiation and form spicules in the presence of inhibitors of collagen processing, but did complete the process if exogenous collagen was added. This finding strongly suggests a requirement for collagen in spicule formation, probably as a component of an extracellular matrix. The relationship between this observation and a peak at the blastula stage in an mRNA encoding a type IV collagen and its later marked decline remains to be established (Venkatesan *et al.* 1986).

A facile technique for isolating a highly enriched population of *S. purpuratus* primary mesenchyme cells was developed by taking advantage of the fact that primary mesenchyme cells attach to culture dishes but blastodermal cells do not (Venkatasubramanian & Solursh, 1984; Carson *et al.* 1985). The appearance of the primary mesenchyme cells and associated spicules as visualized by light or scanning microscopy is shown in Fig. 8. This system has been used in a series of studies directed at gaining an understanding of the molecular aspects of the Ca^{2+} deposition process. Studies with *S. purpuratus* embryos revealed a 130×10^3 M_r (130K) stage-specific antigen that became concentrated on primary mesenchyme cells at the mesenchyme blastula stage (Carson *et al.* 1985). Of particular interest was that the binding of a monoclonal antibody (1223) to the 130K protein of primary mesenchyme cells in culture arrested spicule elongation, measured by either light microscopy or incorporation of $^{45}Ca^{2+}$. A Fab fragment of the 1223 antibody had the same inhibitory effect. Both findings are illustrated in Fig. 9 (Grant *et al.* 1985).

Because of the rapidity of the effect, it was suggested that the binding of the antibody to the 130K surface antigen blocked Ca^{2+} entry. Subsequently, it was shown (Grant *et al.* 1985) that, in cells detached from the culture dish, conversion of Ca^{2+} to $CaCO_3$ was minimal. Yet under these conditions the antibody still inhibited $^{45}Ca^{2+}$ uptake markedly, a finding consistent with the idea that the antigen is somehow involved in the Ca^{2+} uptake process, rather than in its subsequent conversion to $CaCO_3$.

The developmental expression of the 130K protein was also studied in both *S. purpuratus* and *L. pictus* embryos and in cultured primary mesenchyme cells isolated from *S. purpuratus* embryos (M. C. Farach *et al.* 1987). In the latter *in vitro* system it was found that the expression of the 130K protein correlated temporally with Ca^{2+} accumulation in spicules. Using antibody and indirect immunofluorescence, as well as immunoblotting techniques, it was found that the 1223 epitope detected on the 130K protein was present in the *S. purpuratus* egg and in low levels in all cell types of the embryo up to the gastrula stage. After this stage, synthesis and expression of the 130K protein was found to be localized to the skeleton-forming primary mesenchyme cells (Fig. 10). In contrast, in *L. pictus* the 130K protein was undetectable in eggs or in embryos until the primary mesenchyme cells were apparent. Hybrid embryos prepared from *S. purpuratus* crossed with *L. pictus* exhibited a pattern of expression indistinguishable from that of the species contributing the maternal genome, suggesting that early expression of the protein in *S. purpuratus* embryos was due to maternal transcripts. Perhaps later expression of this protein in primary mesenchyme cells of both species is the result of cell-type-specific synthesis, encoded by embryonic tran-

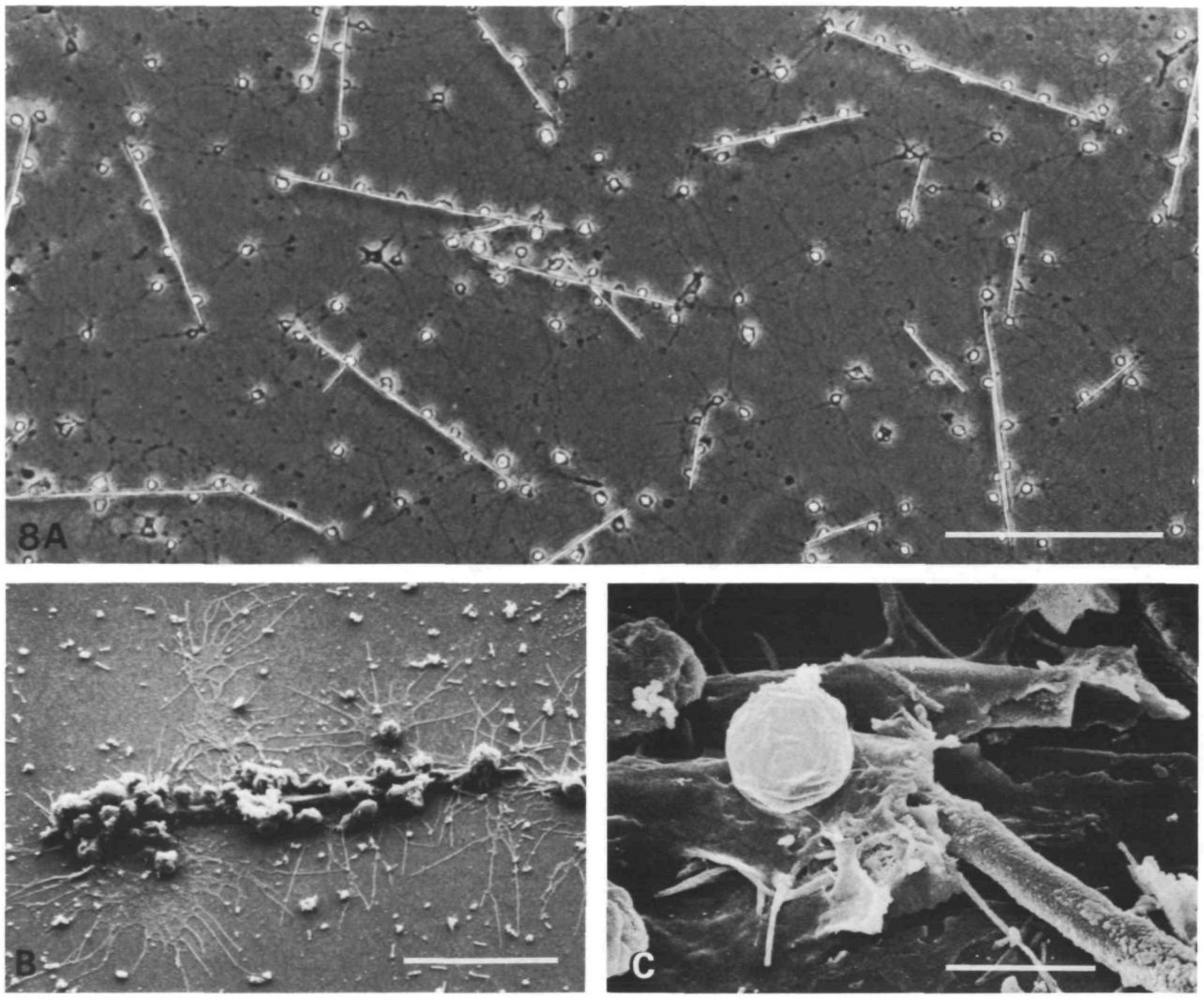

Fig. 8. Primary mesenchyme cells cultured *in vitro*. (A) Light micrograph (phase mode) of spicules and associated cells cultured *in vitro* for 72 h in sea water containing horse serum; (B,C) Scanning electron micrographs of syncytium before (B) and after fracturing (C) to reveal spicule enclosed in the filopodia-derived sheath. Note granularity of spicule surface. Prepared as described earlier by Decker *et al.* 1987. Bars (A) 150 μm; (B) 40 μm; (C) 5 μm.

scripts.

In an independent study, Leaf *et al.* (1987) reported the identification of a primary mesenchyme cell-specific cDNA that encoded a 130K protein. Antibody was prepared against a β-galactosidase fusion protein containing the last 88 amino acid residues of the 130K protein. This antibody detected a 130K protein on the surface of primary mesenchyme cells. RNA gel blots failed to reveal the transcript encoding this protein in eggs, but detected a marked increase in

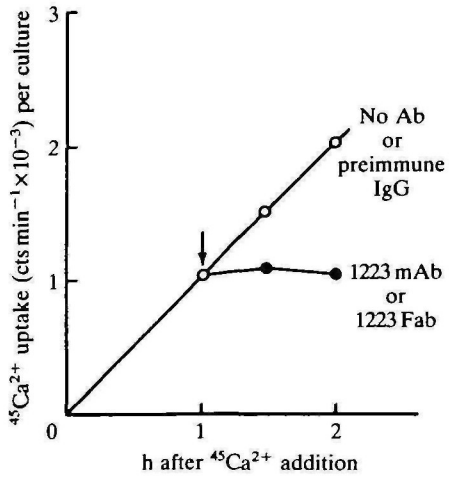

Fig. 9. Inhibition of $^{45}Ca^{2+}$ uptake in primary mesenchyme cell cultures exposed to a monoclonal antibody (1223) that binds to the cell surface of these cells. Ordinate represents $^{45}Ca^{2+}$ incorporated with or without addition of 1223 antibody or its Fab fragment; time after addition of $^{45}Ca^{2+}$ is shown on the abscissa (Modified from Grant *et al.* 1985).

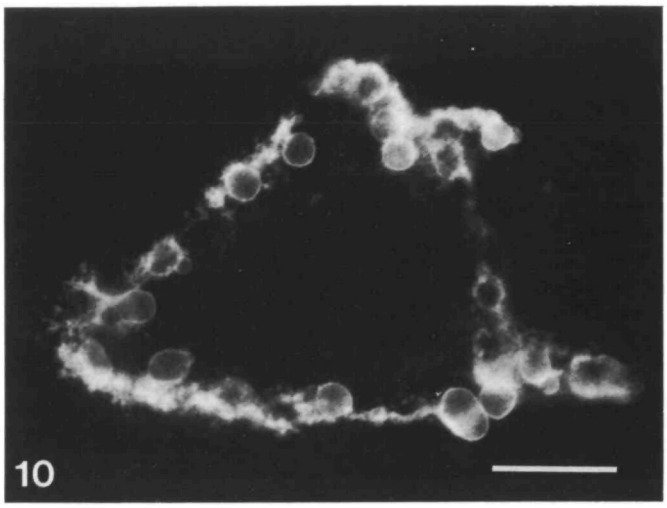

Fig. 10. Immunofluorescent localization of MAb 1223 bound specifically to the cell surfaces of primary mesenchyme syncytia showing form of demineralized syncytia to be similar to shape of skeleton. Prepared as described in M. C. Farach *et al.* 1987. Bar, 30 μm.

the level of specific message at and subsequent to primary mesenchyme cell ingression within the blastocoel. Subsequently, the 130K antigen reported by this group (Anstrom *et al.* 1987) was studied using both a monoclonal antibody (B2C2) directed toward it and the polyclonal antibody to the fusion protein mentioned above. The authors presented evidence that the 130K protein was glycosylated and that the monoclonal antibody only recognized the native form of the polypeptide, and not the fusion protein synthesized by *Escherichia coli*. This observation is consistent with the idea that the epitope recognized by B2C2 is the carbohydrate chain, which is proposed to be sulphated. In a recent extension of these studies (Anstrom *et al.* 1988), it was reported that this epitope is present in eggs and is localized to a subset (20%) of the cortical granules. In agreement with these observations, Decker *et al.* (1988) using the immunogold technique found that the 1223 antigen in the egg is localized to the cortical granules; however, in this case the antigen was found in all of the cortical vesicles. Decker *et al.* (1988) found that upon fertilization the antigen is exocytosed with the other components of the cortical granules; subsequently, only low levels of it are found in the embryos. However, upon appearance of the primary mesenchyme cells in the blastocoel, the antigen is greatly increased in level and is found concentrated in the Golgi complex and at the surface of the cells and spicule compartment. These results provide strong evidence for the earlier hypothesis (M. C. Farach *et al.* 1987) that the expression of the 1223 antigen is biphasic, with maximal localized expression in the eggs and the primary mesenchyme cells of the embryo. Very recently, M. C. Farach & Lennarz (unpublished observations) have obtained direct evidence that the 130K protein is a glycoprotein and have shown that the epitope towards which the monoclonal antibody is directed is the carbohydrate chain. This chain, which is anionic, has an apparent molecular mass of 3200. It binds Ca^{2+}, which results in its aggregation. Whether such binding plays a functional role *in vivo* remains to be established. Clearly, these results from two different laboratories establish that the primary mesenchyme cell contains a unique cell surface glycoprotein epitope. Precisely how this molecule and/or its carbohydrate chain is involved in the calcification process remains to be established.

Three other proteins that appear to be localized to primary mesenchyme cells have been reported. One of these, a 380K protein (meso 1) that is first detected at the onset of primary mesenchyme cell ingression in *L. variegatus*, is subsequently found associated with the membranous spicule sheath (Wessel & McClay, 1985). Using micromeres from *Hemicentrotus pulcherrimus* cultured *in vitro*, it was shown that a block in the synthesis of a 32K protein correlates with conditions that block synthesis of the spicules (Kitajima, 1986). Yet another protein (180K), shown to be present in embryos of several species of sea urchins including *H. pulcherrimus* and *S. purpuratus* under conditions of spicule formation, was found to bind Ca^{2+} (Iwata & Nakano, 1986).

Another important overall feature of the spicule formation process that must be clarified before individual steps can be delineated is the site of initiation of calcification and the mechanism of growth of the spicule. Over a century ago (Selenka, 1879), it was reported that a 'mini-spicule' became evident within one primary mesenchyme cell in each of the two clusters of primary mesenchyme cells in the blastocoel. How this entity is related to the spicules that grow in association with the syncytial cells found in the embryo and in primary mesenchyme cell cultures remains unclear; however, it appears that initiation of spicule growth occurs at these sites within the embryo.

To clarify some of these issues, electron microscopic techniques were used to study the primary mesenchyme cells and spicules formed in culture (Decker *et al.* 1987). This study revealed that the cell bodies and the filopodia and lamellipodia formed spatial associations similar to those seen in the blastocoel of the intact embryo (Fig. 11). It was proposed that the spicule was enclosed by a membrane-limited sheath generated by fusion of filopodia. However, apparently this sheath does not completely separate the spicules from the external milieu because the mineral could be dissolved in living or fixed cells by a chelator of divalent cations or

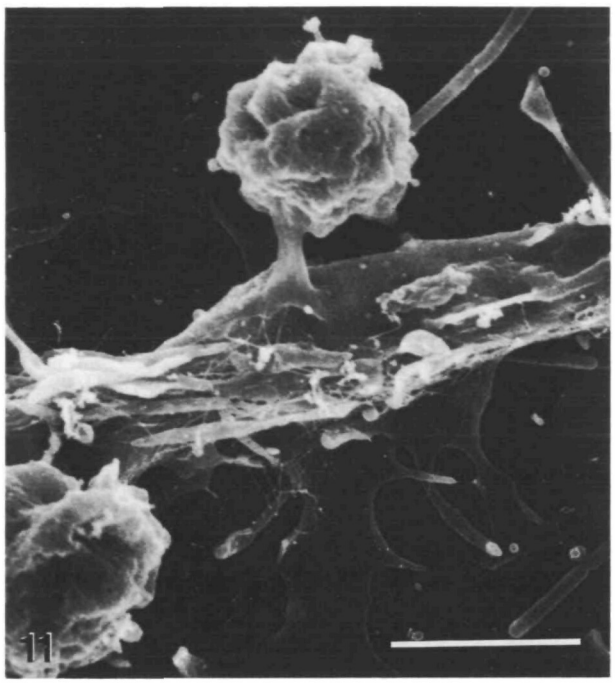

Fig. 11. Scanning electron micrograph of *in vitro* culture of primary mesenchyme cells (72 h). Note the thin stalk that interconnects the cell body and the sheath forming the spicule cavity which apparently has openings between the filopodial processes. Prepared as described in Decker *et al.* 1987. Bar, 5 μm.

by lowering the pH of the medium. In contrast, granular deposits in the cell bodies appeared relatively refractory to such treatments, indicating that they were inaccessible to agents that dissolved the spicules.

After rapid freezing and anhydrous fixation to preserve the syncytia, electron microscopy and X-ray microprobe analysis indicated that the electron-dense deposits in the cell bodies contain elements (Ca and Mg) common to the spicule. Transmission electron microscopic examination of the spicule cavity (Fig. 12) after dissolution of the spicule mineral revealed openings in the filopodia-derived sheath, coated pits within the limiting membrane and a residual matrix that stained with ruthenium red. Exogenously introduced Concanavalin A–gold entered this spicule cavity and bound to matrix glycoproteins. Based on these observations, it was proposed that components of the spicule are sequestered intracellularly and that spicule elongation occurs in an extracellular cavity (Decker *et al.* 1987). It seems possible that Ca^{2+} and associated glycoconjugates (perhaps those matrix proteins discussed earlier) are routed to this cavity *via* a secretory pathway. However, at present there is no direct evidence for an intracellular pathway for the packaging and secretion of Ca^{2+}, alone or with glycoconjugates.

In another effort to unravel the events in the calcification process, the site of spicule growth was studied in the same primary mesenchyme cell culture system (Decker & Lennarz, 1988). The question posed was: where are the sites of growth of the spicule? To answer this question, we used pulse-chase labelling with $^{45}Ca^{2+}$ followed by radioautography; we found that in this cell culture system, in which most of the spicules are linear, there were two focal points of growth and the growth occurred by addition of approximately equal amounts of Ca^{2+} to both ends of each rod. Multiform spicules having more than two elongation sites (tips) showed a similar pattern of growth.

Several conclusions were derived from this study. First, because the $^{45}Ca^{2+}$ remained in the spicule during the chase period it must not be in a highly dynamic state and not readily exchangeable with unlabelled Ca^{2+} in the culture medium. Indeed, if such exchange had occurred all of the $^{45}Ca^{2+}$ label introduced during the pulse would have been lost during the chase (15 min–24 h). Second, that there are two growth loci in linear spicules means that one end of the spicule is not in a static state with respect to the other end. In addition, the fact that the length of the two unlabelled tips of these linear spicules are approximately equal in length indicates that growth proceeds at an approximately equal rate in opposite directions. Finally, the observation that the location of the $^{45}Ca^{2+}$ deposition with respect to the spicule tips was the same in linear and multiform spicules suggests that a common mechanism for Ca^{2+} deposition is operative in both forms.

With respect to the molecular aspects of the deposition process, it is important to point out that it is clear from studies with a variety of vertebrate systems that Ca^{2+} homeostasis is a crucial cellular process that is controlled by several systems (Carafoli, 1987). These include Ca^{2+} transporting systems in the mitochondria, the endoplasmic and sarcoplasmic reticulum and the plasma membrane. With regard to the latter, it is important to note that the Ca^{2+} concentration in sea water is 10 mM, and it seems highly likely that a Ca^{2+} pump in the plasma membrane of primary mesenchyme cells continually functions to lower the cytoplasmic Ca^{2+} concentration. If this is the case, the influx of Ca^{2+} at the time of spiculogenesis might be accomplished by attenuation of this pump. In this respect, it is noteworthy that several Ca^{2+} channel blockers have been reported to block Ca^{2+} incorporation into the spicule (Iwata & Nakano, 1985*a*). Clearly, more work, using the tools developed in vertebrate systems, must be done to elucidate the molecular aspects of calcification that

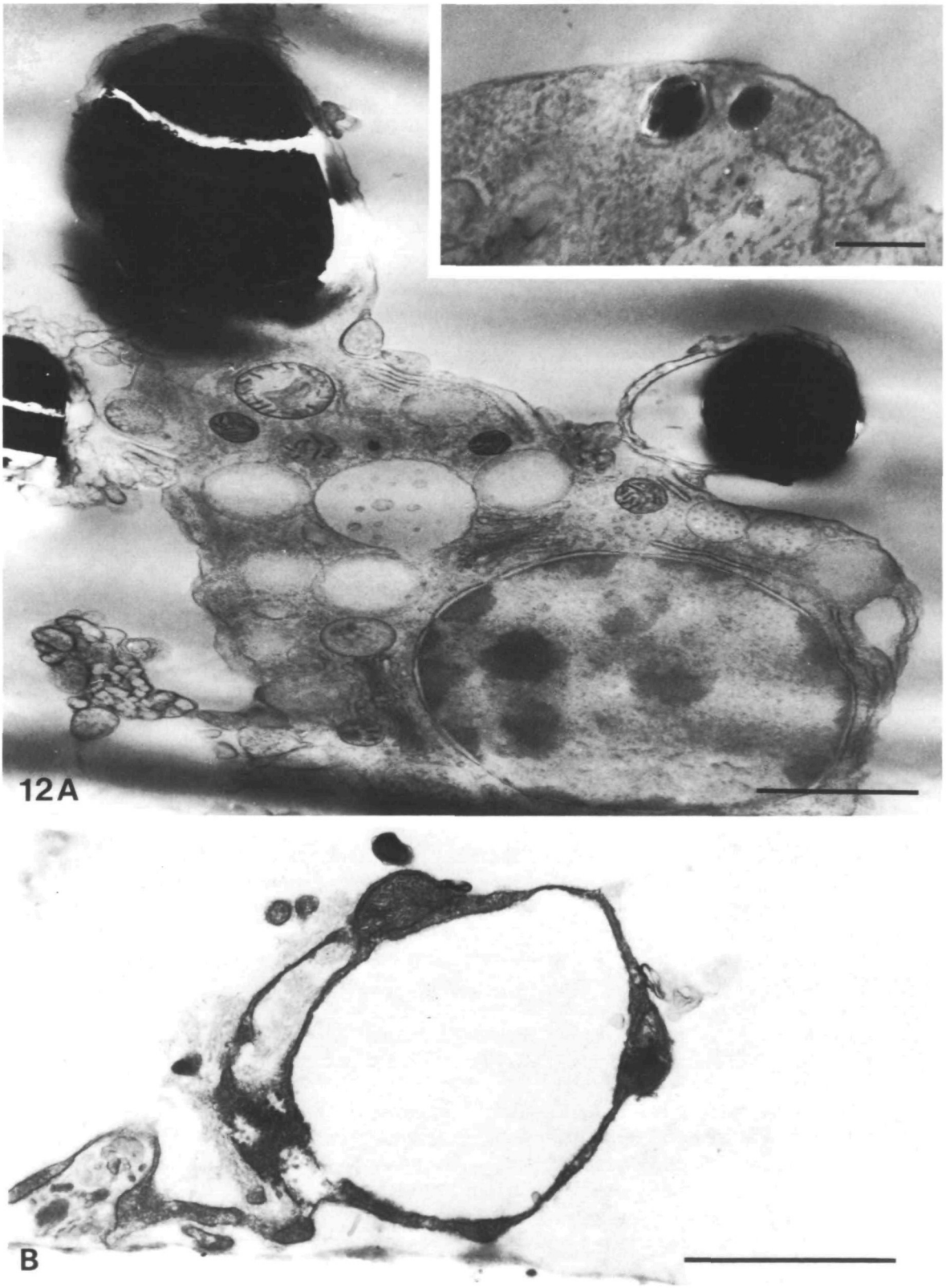

Fig. 12. Electron micrographs of isolated primary mesenchyme cells before and after demineralization. (A) Transverse section showing electron-dense spicules enclosed in a thin sheath which appears to form an extracellular cavity; inset shows intracellular deposits near the cell surface that may be routed to the spicule cavity. (B) Demineralized cavity of transversely sectioned syncytium. Note the presence of a lamellipodial process showing cytoplasmic continuity with the sheath that forms the spicule cavity. Prepared as described in Decker *et al.* 1987. Bars (A) 2 μm; inset 0·2 μm; (B) 1 μm.

occur in the primary mesenchyme cells during spiculogenesis.

Conclusions and prospects

Although the assembly of the skeleton in the sea urchin embryo has been studied for a century, only in the last decade have we seen considerable progress in understanding the cellular and molecular aspects of this intriguing process. This is the result of at least two factors: the application of cell culture techniques to study spiculogenesis with a single cell type and the utilization of state-of-the-art biochemical and molecular biological techniques.

The conclusions that can be tentatively drawn as a result of these approaches are as follows: differentiation into a spicule-forming cell results in the cessation of cell division and involves fusion of cells *via* filopodia to form a syncytial template for spicule growth. The spicules grow bidirectionally within this syncytium and the available evidence suggests the involvement of a secretory pathway. Spicule formation requires the synthesis of glycoproteins, one or more of which are cell surface molecules. Newly synthesized glycoproteins are also found in the spicule matrix. Synthesis of a collagen-like molecule, perhaps necessary for the substratum attachment process, is also required.

In the broader perspective, our understanding of the molecules that are expressed during spicule formation should shed light on a variety of basic cellular processes, i.e. cell migration, cell fusion, and Ca^{2+} uptake and deposition. Thus, for example, it is likely that as more information on cell adhesion molecules such as fibronectin, laminin and their receptors becomes available, a clearer picture will emerge that may explain basic molecular aspects of the migratory behaviour exhibited by primary mesenchyme cells inside the blastocoel. Hopefully the application of biochemical and molecular biological tools will enable one to expand relatively rapidly the list of molecules that function in the basic steps involved in spiculogenesis. The long-term challenge will then be to devise ways to test directly and to elucidate the functions of these molecules in each of these component biological steps. Clearly, this is a very considerable task, but one that has multiple long-term benefits because many of the cellular processes are certain to have analogues in a wide variety of biological systems.

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