CHEMICAL REVIEWS

Review

Molecular Aspects of Biomineralization of the Echinoderm Endoskeleton

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Molecular Aspects of Biomineralization of the Echinoderm Endoskeleton

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1. Introduction

During the latter half of the 19th century, there was an explosion of new discovery in the biological sciences, fueled mainly by better microscopes and the spirit of rational inquiry, which was dominant in intellectual circles in the United Kingdom and Europe. A marine station, the Stazione Zoologica, was founded in Naples in 1870. A cohort of the best zoologists from Germany and other European countries availed themselves of the opportunity to study unknown marine creatures. Echinoderms, especially sea urchins, became favorite material for investigations of cell biology and embryology, primarily because the material was abundant, the eggs were often optically clear, and fertilization and development could be followed using the improved microscopes. Some of the most important biological discoveries of the time were made using this material. Richard Fol and the Hertwig brothers demonstrated that sperm and egg united to form the zygote, and others showed the embryo formed by division of cells that arose from the egg. The larvae that arose from these fertilized eggs formed calcareous internal skeletons, called spicules, that were elaborate and whose forms were species specific. In a series of very famous experiments begun at Naples in 1889, Theodor Boveri showed that it is the nucleus and chromosomes that dictate development of the embryo. The main character used in that analysis was the morphology of the skeleton that formed in hybrids between two species. A sketch of interesting history of this early period of biology can be found in a recent article by Laubichler and Davidson.[1]

Adult echinoderms also possess calcareous skeletal elements, such as tests, plates, spines, pedicellariae, and teeth. These elements are abundant in the fossil record. Biologists, chemists, materials scientists, and paleontologists have all been fascinated by the extraordinary ability of echinoderms to carry out biomineralization, constructing beautiful, light, strong skeletal elements.

There is a large body of literature and recent reviews on various aspects of biomineralization in echinoderms, especially sea urchins, some of which will be cited in appropriate places. Wide ranging reviews of biomineralization in marine invertebrates can be found in the books by Lowenstam and Weiner,[2] Simkiss and Wilbur,[3] and Dove et al.[4] These sources consider some topics, such as biomechanics and materials properties of skeletal elements, that we are unable to cover here. In this review, we shall first present the basic outlines of spicule formation and biomineralization, and then proceed to emphasize (1) more recent discoveries on the structure and composition of the mineral and organic matrix of the spicule and the genes that encode them, (2) what is known of the function of matrix proteins in the deposition and properties of the spicule, (3) recent work on biomineralization in tissues of the adult, and (4) recent work on the genes involved in biomineralization and their evolution.

Echinoderms are the only nonchordate phylum that possesses an endoskeleton. Though many creatures possess hard skeletons or dentition and integument, these shells, carapaces, and spicules are secreted to form hard structures outside the body proper, that is, they are exoskeletons. Only vertebrates, invertebrate chordates, and echinoderms form their endoskeletons within the outer cellular covering of the organism. Echinoderms utilize primarily calcium carbonate in the form of calcite for skeleton building, while vertebrates utilize primarily carbonated apatite (calcium phosphate). Adults of all the echinoderm classes possess calcareous endoskeletal structures, but detailed study of the formation of the skeleton and its structure in larvae and adults has been carried out almost exclusively in the Echinoidea (sea urchins and sand dollars). Embryos of the brittle stars (Ophiuroidea) are the only other echinoderm class that possesses embryonic skeletons. The little that is known of the skeletons of other echinoderm classes has been recently reviewed.[5]

2. Embryonic Development of the Endoskeleton

Sea urchin embryos represent excellent material for the study of biomineralized structure formation. There is abundant material; a single female of some species can shed 10 mL of packed eggs, producing about 1.5×10^7 embryos. The embryos of many species are very clear, so observation of the process in living embryos is not difficult, and the processes can be observed in real time (reviewed by Wilt and Ettensohn[6]). Figure 1 shows some light micrographs of sea urchin embryos at various stages of larval development.

Skeletal elements of embryos can be obtained in large quantities, and vestiges of adherent soft tissue are removed

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Christopher Killian was born in New York City. He attended Franklin and Marshall College and received an A.B. degree in 1981. He then attended Georgetown University and received a Ph.D. degree from the Department of Biology in 1985. His graduate studies in the laboratory of David Nishioka focused on biochemical and cellular changes during sea urchin fertilization and early development. Christopher joined Fred Wilt's laboratory at the University of California, Berkeley, in 1985 as a postdoctoral researcher. He has remained as a research associate in Fred Wilt's laboratory at Berkeley. His studies in the Wilt laboratory were centered initially on the molecular details of tissue differentiation in the sea urchin embryo. His research interests have refocused on the cellular and molecular mechanisms underlying the process of biomineralization during sea urchin spicule formation. Using biochemical, cellular, molecular, and genomic approaches, he and others in Fred Wilt's laboratory have identified and characterized a number of the integral matrix proteins and the cognate genes that are involved in sea urchin biomineralization.

Fred H. Wilt was born in the small Northern Indiana town, Nappanee. He received a B.A. in Zoology from Indiana University (1956) and the Ph.D. in Biology from Johns Hopkins University (1959). After postdoctoral work in biochemistry at University of Liverpool and in embryology at the College de France, he joined the Biology Department at Purdue University from 1960 to 1964 and then joined the Zoology Department at the University of California, Berkeley, where he is now Professor Emeritus in the Department of Molecular and Cellular Biology. His main research interests have focused on understanding cell differentiation in embryos using the tools of biochemistry and molecular biology, and he has studied development of visual pigments in amphibians, the formation of erythrocytes and hemoglobin in chicks, and early development of the sea urchin embryo, especially its endoskeleton. During the past 20 years, the focus of the research has gradually shifted to understanding the cellular and molecular basis of biomineralization of the endoskeletal spicule of sea urchin embryos. His research group has identified, isolated, and characterized some of the genes and proteins that are involved in the formation of these skeletal elements and that confer unusual material properties on these protein−calcite composites.

by treatment with NaClO. The cells that secrete the skeleton, micromeres and their descendants, which are called primary mesenchyme cells (PMCs), can be cultured *in vitro*, where they recapitulate the processes they carry out in the intact embryo.[7] The genome of the purple sea urchin, *Strongylocentrotus purpuratus*, has been completely sequenced and partially annotated,[8,9] and the network of genes that direct the establishment and development of the PMCs has been elucidated.[10,11]

The embryonic development leading to spicule formation has been intensively studied and recently reviewed.[6,12,13] In brief, development of the spicule can be divided into five phases: micromere formation, PMC formation, syncytium formation, skeleton deposition, and skeleton remodeling and elaboration [see Figure 2]. Micromeres are four small cells that arise during the fourth cell division at the vegetal pole of the cleaving zygote. This quartet divides once more to give rise to four large and four small daughter cells. The larger daughters will continue to divide three or four (depending on the species) more times to form a torus of cells at the vegetal pole of the hollow blastula.

During the next phase, which usually occurs between 8 and 24 h postfertilization, depending on the temperature and the species, the micromere descendants, that is PMCs, detach themselves from the wall and ingress into the hollow blastula, forming a migratory population of 32−64 PMCs. The PMCs adopt stereotypical locations within the blastula as other complex gastrulation movements are occurring to produce a primitive gut. Adjacent PMCs fuse with one another forming a continuous multicellular syncytium [see Figure 1C]. Within a short time, usually only a few hours, small, rhombohedral, birefringent granules can be detected in the syncytium located in the future ventrolateral position of the larva. Spicules form by continued deposition of calcareous material in three radii emanating from this initial granule, elongating in the crystallographic *a*-axes, then bending at nearly right angles and continuing to elongate in the *c*-axis. During late embryonic and early larval development the endoskeleton will become many times larger and more complex. During this time, the skeletal elements will grow by deposition of mineral at the tips of pre-existent spicules and also by initiation of new skeletal rods, de novo, presumably from mesenchyme cells separated from the syncytium. When embryonic development has ceased and a feeding larva has formed, the larva will grow as part of a planktonic community. The scenario we have just described applies to indirectly developing euechinoid species, which constitutes the majority of sea urchin species. The only other echinoderm group that forms endoskeletal spicules in the embryo are the Ophiuroidea, the brittle stars. All the other classes do, however, form mineralized structures in the adult, which we shall describe later.

Experiments during the halcyon days of experimental embryology (cf. Wilt and Ettensohn[6]) showed that the detailed morphology of the spicules, for example, whether plain or fenestrated, is a property of the PMCs. However, the overall form of the spicule, indeed whether it is formed at all, depends upon signals that emanate from the adjacent epithelial wall of the blastula. It has recently been shown that the growth factor VEGF (vascular endothelial growth factor) is secreted by a small number of cells in the ectodermal wall and that the PMCs express the receptor for VEGF.[14] Initiation of biomineralization depends upon this signaling.

Formation of mineralized tissues of the adult begins during the planktonic phase of larval existence and is quite complex. All the major organ systems form initially in a juvenile

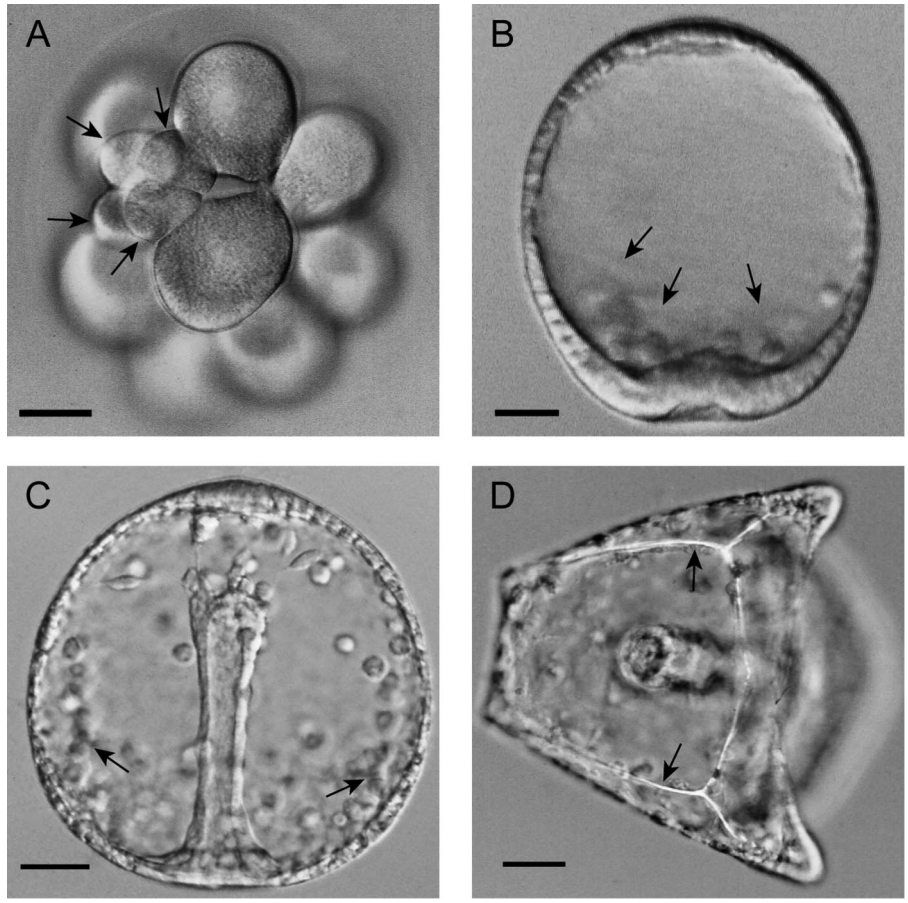

Figure 1. Photomicrographs of sea urchin embryos: (A) a 16-cell-stage embryo viewed from the vegetal pole (arrows indicate the forming micromeres; bar = 20 μm); (B) a late blastula stage (~22 h post fertilization) with arrows indicating ingressing PMCs (bar = 30 μm); (C) an embryo nearing the completion of gastrulation, showing a primitive gut stretched across the embryo (arrows indicate fused PMCs initiating triradiate spicules; bar = 30 μm); (D) a prism stage embryo (~48 h postfertilization) with arrows indicating calcareous spicules (bar = 30 μm).

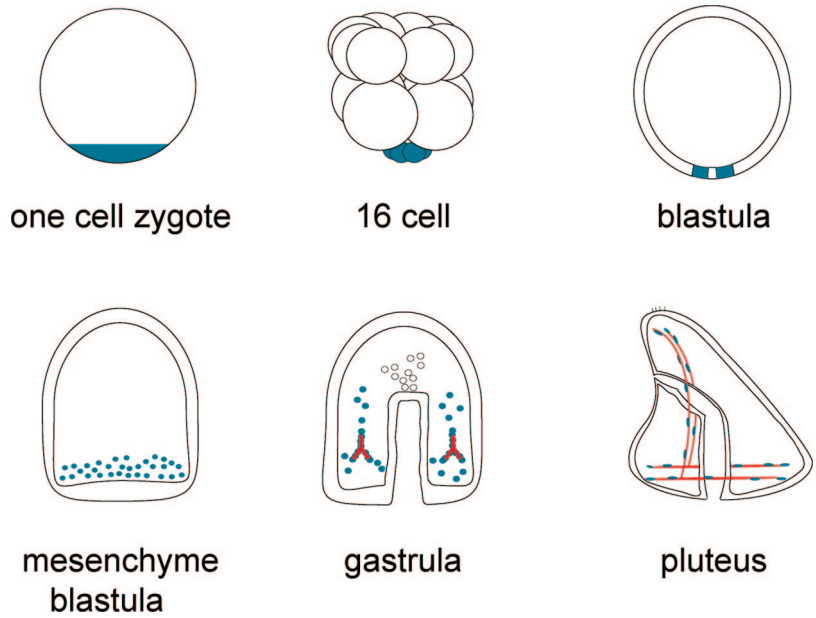

Figure 2. Diagrams of spicule formation. The six diagrams show successive stages of the development of endoskeletal spicules. The one cell zygote at the top left indicates a special cytoplasmic region at the vegetal pole, indicated by blue shading. The diameter of the egg is approximately 100 μm. By the fourth cell division, this cytoplasmic region has been segregated to four small micromeres, which continue to divide and form a torus of prospective PMCs in the wall of the blastula. The PMCs ingress (lower left), then form a syncytium and deposit the spicule (red). This figure is adapted from ref 119 by permission. Copyright 1999 Elsevier, Ltd.

rudiment that develops primarily from cells in a coelomic sac adjacent to the larval gut; there are also some contributions of larval ectoderm to the juvenile. Formation of biomineralized spines and test plates can be observed in the

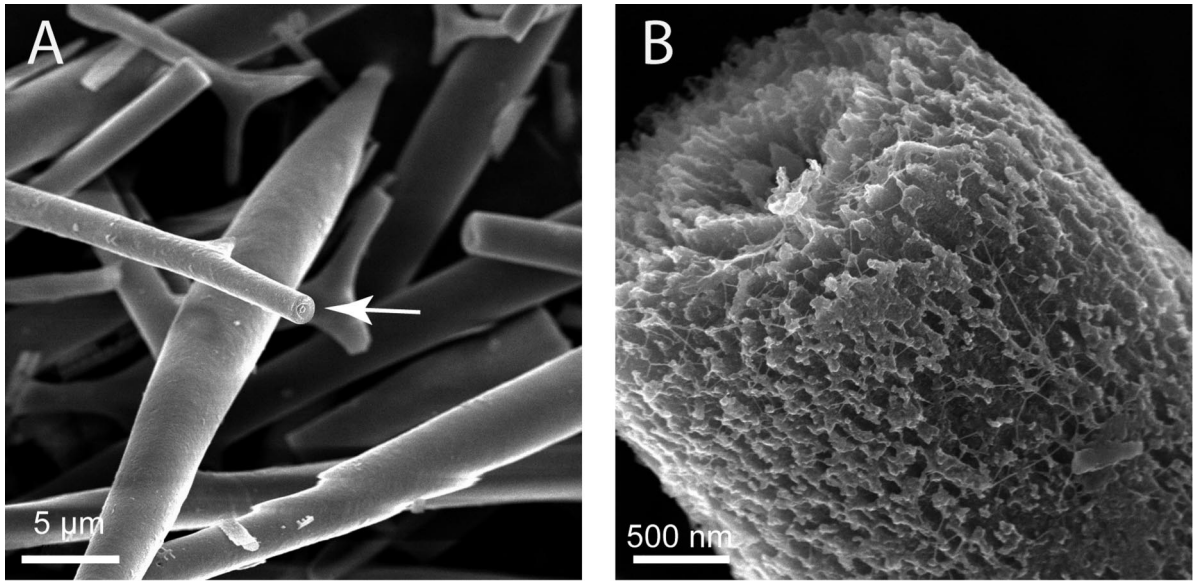

Figure 3. Scanning electron micrograph of purified spicules: (A) Spicules were purified from pluteus stage embryos; vestiges of extracellular material were removed by treatment with NaOCl. Arrow indicates broken end of a spicule. (B) Scanning electron micrograph of an etched spicule. Purified spicules were etched by exposure to EDTA in the presence of 1% glutaraldehyde. The end of a broken spicule viewed at high magnification by SEM shows deep etching of the calcite, which appears as concentric laminae. Small fibers (~15−20 nm) can be observed coursing in all directions through the mineral.

rudiment, usually forming in conjunction with or close to larval spicules.

During settling of the larva and metamorphosis, the larval tissues undergo autolysis and the juvenile urchin begins further growth and development attached to a substrate on the floor of the ocean or intertidal zone.

3. The Larval Spicules

3.1. Mineral and the Endoskeleton

The sea urchin embryonic and adult endoskeleton is comprised of magnesian calcite (usually about 5% $MgCO_3$ or sometimes more) and occluded matrix proteins. Figure 3A shows a tangle of clean isolated spicules with an arrow indicating a broken end. This end reveals the concentric organization of layering within the larval spicule.[15] Figure 3B shows a partially demineralized spicule at a broken end at a higher magnification. This treatment reveals more strikingly the concentric layers of mineral in the spicule as well as a meshwork, presumably organic, that run throughout the spicule. Under polarized light, the embryonic spicule and adult mineralized tissue appears to be a single crystal of calcite. In all adult sea urchins, the *c*-axis of the calcite in the spines is parallel to the length of the spine.[16] The crystal axis orientation of the adult test varies with the species of sea urchin. *Strongylocentrotus purpuratus* orients the calcite *c*-axis perpendicular to the surface of the plates making up the test.[16−18] In the spicule of the sea urchin embryo, the calcite crystal *c*-axis is parallel to the long axis of the body rod and perpendicular to the axis of the initially synthesized triradiate.[19] Yet the spicules have a smooth surface and after fracture display conchoidal (smooth and slightly concave) fracture planes, both of which are physical characteristics different from pure calcite. In addition, the embryonic spicules have greater flexural strength than pure calcite.[20] These physical properties of the spicules are believed to be mediated by the integral matrix proteins.

While the embryonic spicules of sea urchins appear from their outward appearance to be composed of only calcite, the initially deposited spicule is composed largely of amorphous calcium carbonate. Beniash et al.[21] and Politi et al.[22] observed that more than half the mineral of the newly synthesized spicule is comprised of amorphous calcium carbonate (ACC). Using X-ray absorption and infrared spectroscopy, they reported that between the prism and pluteus stage, the spicule ACC converts to calcite. Numerous observations of other mineralized tissues of vertebrates and invertebrates have shown that when mineralized tissue is first formed, the mineral portion often has very short-range order and does not coherently diffract X-rays. This amorphous mineral is transformed as the tissue develops into a crystalline form with much longer-range order that gives good diffraction patterns.[2,23−26]

Since ACC is thermodynamically unstable, the sea urchin embryo must have a mechanism of stabilizing ACC. Integral matrix proteins are intimately associated with the mineral phase of the spicule, thus making them likely candidates to play a role in stabilizing ACC. To address this possibility, Raz et al.[27] inquired whether the spicule integral matrix proteins could stabilize ACC *in vitro*. They found that spicule matrix proteins isolated from earlier stage spicules that have high levels of ACC (from late gastrula/prism stage embryos) will stabilize amorphous calcium carbonate *in vitro* in the presence of Mg^{2+}, while spicule matrix proteins isolated from older spicules that have little ACC (from pluteus stage) will not. These results point to the matrix proteins playing a role in stabilizing ACC during spicule formation.

3.2. Integral Matrix Proteins

The integral matrix proteins comprise approximately 0.1% of the spicule.[28] Benson et al.[29] first observed the morphology of the integral matrix by electron microscopy of fixed partially demineralized spicules. They found that the spicule matrix is arranged in concentric sleeves of fibrillar material with connections between the sleeves of matrix. Figure 3A,B shows this concentric layer organization in the spicules. Seto et al. [15] looked at the localization of specific spicule matrix proteins, and they found that the proteins they analyzed were

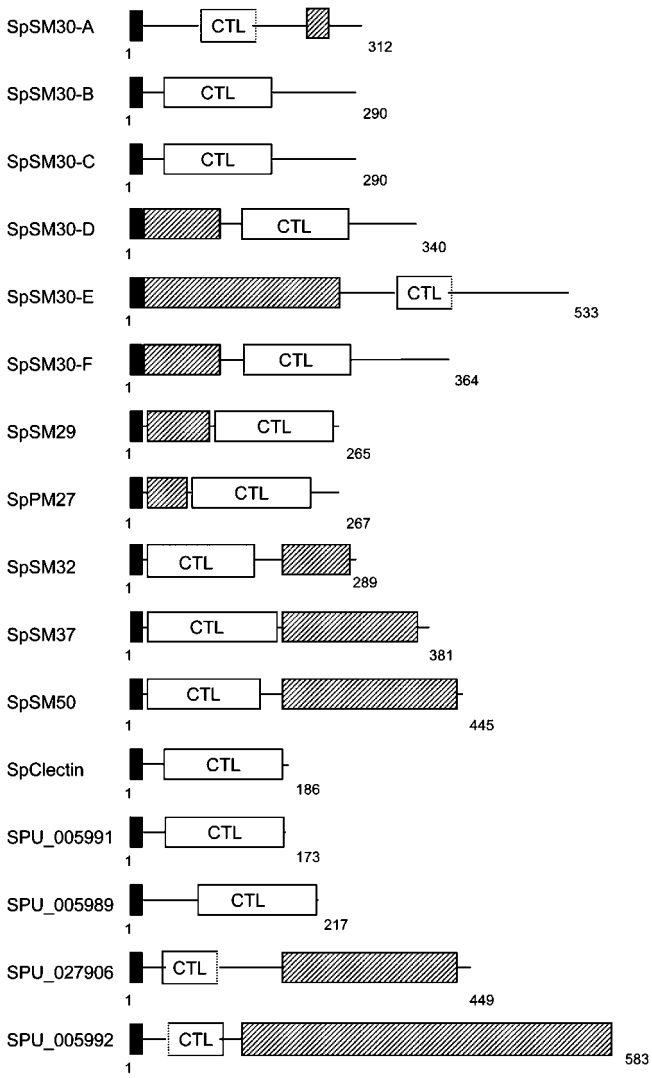

Figure 4. A diagram of integral spicule matrix proteins. The domain structure of known spicule matrix proteins is shown. Black box = signal sequence; hatched box = proline-rich repeat domain; CTL = c-type lectin domain. Reprinted with permission from ref 8. Copyright 2006 Elsevier, Ltd.

widely distributed throughout the mineral of the spicules. They suggested that the spicule matrix is woven finely around the microcrystalline domains of calcite.

Benson et al.[28] and Venkatesan and Simpson[30] using one-dimensional SDS-PAGE identified eight to ten proteins as comprising the integral spicule matrix. Higher resolution studies by Killian and Wilt[31] used ^{35}S-methionine-radiolabeled spicule matrix proteins and analyzed the integral matrix proteins with 2-D gel electrophoresis. These studies found that the spicule matrix was actually comprised of more than four dozen proteins. Most of the proteins are acidic, but there are also a number of proteins with alkaline isoelectric points.

Sixteen genes encoding proteins that are thought to be integral matrix proteins for the embryonic spicules or adult mineralized tissues of *Strongylocentrotus purpuratus* have been identified (see Figure 4). Prior to the release of the sea urchin genome (*S. purpuratus*) in November 2006, there were seven genes and a portion of an eighth that were identified as likely to be encoding integral matrix proteins. Six of these genes were initially isolated by cDNA or EST cloning from PMC-enriched libraries. These genes included *SM50, SM30, SM32, SM29, SpC-lectin*, and *PM27*.[32−35] One gene, *SM37*, and a portion of another gene, *SM30β*, were isolated initially from genomic clones.[36,37] All of these genes have a single c-type lectin domain.

Eight other genes were subsequently identified as likely to be encoding integral matrix genes.[8,9] The criteria for designating these genes as encoding putative integral matrix proteins are as follows: (1) the gene is closely linked to previously cloned integral matrix genes; (2) the derived amino acid sequence indicates that it is a secreted protein; (3) the gene encodes a protein that contains a c-type lectin domain[8] or is very similar to a previously identified spicule matrix gene; (4) it is found in PMC EST databases (libraries).

SM50 and *SM30* were the first genes identified as encoding *Strongylocentrotus purpuratus* spicule matrix proteins. These genes were initially isolated using polyclonal antibodies directed against all of the spicule matrix proteins.[32,33] These antibodies were then used to screen phage expression *S. purpuratus* cDNA libraries.

SM50 was found to be a single copy gene that encodes a secreted, nonglycosylated protein of approximately 48.5 kDa with an alkaline p*I*.[31,32,38,39] SM50 protein has a proline-rich repeat region as well as a region similar to a c-type lectin[34] (see Figure 4). The proline-containing repeats are similar to some found in elastin where they confer a β-spiral structure, but there is no direct evidence for this conformation in SM50. *SM50* mRNA expression sharply increases at the time of PMC ingression into the blastocoel in the sea urchin embryo and continues through spicule formation.[32,40] Specific antibodies raised against SM50 confirm that SM50 is an integral spicule matrix protein as well as an integral matrix protein of adult mineralized tissues.[31,41] Kitajima and Urakami[42] used monoclonal antibodies to localize SM50 epitopes and showed higher concentrations of SM50 in the triradiate (*a* axis) portion and the growing tip of the body rod. However, these data were obtained from staining whole embryos or isolated spicules cleaned with guanidine isothiocyanate rather than sodium hypochlorite. The SM50 protein occluded in the mineral was examined by blotting proteins from intact spicules onto membranes and then staining the membranes with antibodies. SM50 was present throughout the mineral, though more concentrated in the triradiate portion and tip of the body rod. The functional meaning of this localization is unknown. Sucov et al.[43] and Makabe et al.[44] have identified and characterized upstream cis-regulatory regions of the *SM50* gene that confer the PMC-specific expression pattern of *SM50* during larval development.

Orthologues of *S. purpuratus SM50* have been isolated and characterized in *Lytechinus pictus*, designated *LSM34*,[45] *L. variegatus*,[46] and *Hemicentrotus pulcherrimus*, designated *HSM41*.[42,47] Peled-Kamar et al.[48] have shown by knocking down *LSM34* expression using antisense oligonucleotides that *LSM34* expression is necessary for spicule formation in *Lytechinus pictus* embryos. More recent experiments using morpholino antisense oligonucleotides against LSM34 and SM50 confirmed this result[49] and showed that this protein is essential throughout overt spicule formation, including deposition of the initial calcite crystal as well as elongation of the spicule elements.

The gene encoding SM30[33] was first identified as a clone from a *S. purpuratus* cDNA library. RNA blot analysis revealed that SM30 expression is a bit different from SM50. SM30 transcript accumulation starts to increase just prior to the onset of spicule formation. Sequence and biochemical characterizations reveal that SM30 is a secreted acidic glycoprotein that has a single c-type lectin domain.[31,33] Initial

genomic cloning revealed that there are at least two *SM30* genes arranged tandemly.[36] These two *SM30* genes were designated *SM30α* and *SM30β*. However, the recent release of the *S. purpuratus* genome reveals that there are six *SM30* genes. These genes are designated *SpSM30A−F*. The previously identified *SM30α* is now called *SpSM30B* and *SM30β* is now called *SpSM30C*.

SpSM30A, SpSM30B, SpSM30C, and *SpSM30D* are arranged tandemly on a single scaffold, while *SpSM30E* and *F* are linked on another scaffold. It is not clear whether *SpSM30A−D* and *SpSM30E* and *F* are linked since the *S. purpuratus* genome is not assembled into chromosomes, but rather much smaller scaffolds. Repeat regions within the genome have so far made it impossible to align the sequences into larger assemblies at this time.

Livingston et al.[8] showed using RT-PCR analysis that *SpSM30A*, *B*, *C*, and *E* are expressed in prism stage embryos, while *SpSM30C*, *D*, *E*, and *F* are expressed in adult spine. These results suggest that these genes encode integral matrix proteins and that they are differentially expressed in different mineralized structures. Kitajima and Urakami[42] also used a monoclonal antibody against SM30 and showed, as did Urry et al.[39] that SM30 is primarily an occluded protein, very little being present on the surface of the spicule. Kitajima also showed, using the spicule blot procedure mentioned earlier, that SM30 was enriched in the body rod and postoral rod, both of which are elongated along the *c* axis, and they suggested that perhaps SM30 is somehow associated with growth of the crystal faces that result in alignment along the *c* axis. However, there is some SM30 in the ventral transverse rod (*a* axis), though at lower concentrations,[41] so the possible role(s) of the various forms of SM30 is not clear.

The spicule matrix gene *PM27* was first identified as a cDNA whose cognate mRNA increased expression levels sharply at the time of PMC ingression into the blastocoel.[50] Harkey et al.[34] showed that this cDNA encodes a protein similar in organization to SM50 since it is also a secreted, nonglycosylated protein with a single c-type lectin domain and a proline-rich repeat region. However, in PM27 the order of these domains is inverted (see Figure 4). PM27 appears to be a single copy gene with just one exon and no introns. *In situ* hybridization and immunological analyses show that *PM27* expression is PMC-specific with the protein most prevalent near the growing ends of the spicule.[34] Protein blots of isolated spicule matrix proteins reacted with PM27-specific antibodies confirm that PM27 is a genuine integral matrix protein.[41]

SpSM32, SpSM29, and *SpC-lectin* are all genes encoding putative integral matrix that were identified by Illies et al.[35] These genes were identified as EST clones from a large PMC-specific EST library.[51] All three of the proteins encoded by these genes contain a consensus signal sequence and a single c-type lectin domain (see Figure 4). These proteins also have alkaline p*I*'s. RNA blot and whole mount *in situ* hybridization analyses indicate that these genes are expressed specifically by PMCs starting at the time of PMC ingression into the blastocoel. The amino acid sequence of SpSM32 is very similar to SM50. Illies et al.[35] point out that the amino acid sequence encoded by the first exon is 100% identical to that of SM50. They speculated that SpSM32 is a splice variant of SM50. The *S. purpuratus* genome sequence confirms their explanation. *SpSM50* and *SpSM32* share the same first exon and have different second exons.[8]

A genomic clone isolated by Lee et al.[37] identified another gene that is linked to *SpSM50* and *SpSM32* and likely encodes another spicule matrix gene. This gene, designated *SpSM37*, is located 12 kilobases upstream from *SpSM50/32*. The protein encoded by this gene contains a consensus signal sequence, a single c-type lectin domain, three consensus N-glycosylation sites, and a proline-rich repeat region (see Figure 4). Lee et al.[37] show that *SpSM37* and *SpSM50/32* share the same cis regulatory sequences that confer PMC-specific expression.

The observed clustering of putative integral matrix genes led Livingston et al. [8] to examine genes linked on the same scaffold as *SpSM29*.They found three genes, designated *SPU_005989, SPU_005991*, and *SPU_005992*, that encode proteins with consensus signal sequences and a single c-type lectin domain. The protein encoded by *SPU_005992* also has a proline-rich repeat region (see Figure 4). All three of these genes map to within 22 kilobases of *SpSM29* (*SPU_005990*). *SPU_005989* and *SPU_005991* are present in the large midgastrula PMC EST library generated by Zhu et al.[51] Livingston et al.[8] point out that *SPU_005992* is not present in the PMC EST library but is quite prominent in whole embryo mesenchyme blastula EST databases. This suggests that *SPU_005992* is not an embryonic spicule matrix protein but is expressed by other tissues. In addition, RT-PCR analysis (Killian and Wilt, unpublished) shows that *SPU_005992* is expressed by both adult spine and coelomocytes. Hence, *SPU_ 05992* is not uniquely present in biomineralized tissues, and its function will require further study.

Additional discussion of these spicule matrix genes, including tablular listings of the properties of the encoded proteins have been published.[5,8]

3.3. Other Biomineralization Related Genes

Livingston et al.[8] further characterized and catalogued a number of other biomineralization related genes in addition to those encoding putative integral matrix proteins. These genes included the *MSP130* gene family, cyclophilins, collagens, and carbonic anhydrases, as well as other novel genes expressed specifically by PMCs.

SpMSP130 was originally identified as a PMC-specific cell-surface glycoprotein.[52] Experimental evidence suggested that this gene is involved in Ca^{2+} uptake.[53] However, its precise role remains unclear. Illies et al.[35] identified two related proteins whose cognate gene is expressed exclusively in PMCs. These genes are designated *SpMsp130-related-1* and *SpMSP130-related-2*.

Livingston et al.[8] searched the *S. purpuratus* genome for genes similar to these three *MSP130* genes, and they found at least four other *MSP130* genes designated *SpMSP130-related-3*, *-4*, *-5*, and *-6*. Just as the integral matrix genes are often clustered, *MSP130* genes are also clustered. *SpMSP130*, *SpMSP130-related-1*, and *SpMSP130-related-3* are on the same scaffold and *SpMSP130-related-4* and *-6* are near each other on another scaffold. *SpMSP130-related-2* and *-5* are on two separate smaller scaffolds. Of the four newly identified *MSP130-related* genes, two of them, *SpMSP130-related-3* and *SpMSP130-related-5*, are shown to be PMC-specific by *in situ* hybridization analysis.[8] EST data and chip expression data suggest that *SpMSP130-related-4* and *SpMSP130-related-6* are not expressed in embryos. It is not known at this time whether these genes are expressed in adults.

Cyclophilins are a subfamily of the peptidyl prolyl cis−trans isomerases. Recently, Amore and Davidson[54] characterized a cyclophilin, *Sp-cyp1* (*Sp-cyclophilin1*) as an abundant PMC-specific transcript. They showed that *Sp-cyp1* is regulated by *ets1* and *dri*, which are two transcription factors known to be centrally involved in the PMC gene regulatory network. It seems likely that *Sp-cyp1* is involved in some post-translational modifications of proteins. However the precise function of this gene is unknown. Livingston et al.[8] discovered that eight different cyclophilins, including *Sp-cyp1*, are expressed in PMCs. *Sp-cyp1* transcript appears to be the most abundant. All the other cyclophilins are expressed at much lower levels. *Sp-cyp1* encodes a protein with a consensus signal sequence; however, most of the other cyclophilins do not.

Carbonic anhydrase is an enzyme that is thought to play a central role in the formation of the sea urchin endoskeleton. Chow and Benson[55] and other groups have reported that inhibition of carbonic anhydrase activity correspondingly blocks spicule formation or elongation. Livingston et al.[8] report that there are 19 genes in the sea urchin genome that encode a carbonic anhydrase. They found that three of these gene sequences are present in the PMC EST library generated by Zhu et al.[51] One of these genes, *SPU_012518*, was particularly prominent. *SPU_012518* encodes an α-type carbonic anhydrase with a consensus signal sequence and a C-terminal glycolipid anchor. This amino acid sequence indicates that *SPU_012518* encodes an extracellular enzyme, which, of course, is where the embryo synthesizes the spicules. Livingston et al.[8] report that this carbonic anhydrase is expressed strongly in prism stage *S. purpuratus* embryos as well as in adult spines. Love et al.[56] have cloned and characterized the orthologue of this gene in *Heliocidaris tuberculata* and *H. erythrograma*. They find that this gene is expressed in PMCs at the growing ends of spicules. Their results suggest strongly that this carbonic anhydrase plays a central role in the biomineralization of the embryonic spicules, and it may well play a role in adult tissue biomineralization. Jackson et al.[57] and Taylor et al.[58] describe a similar secreted α-type carbonic anhydrase that can be traced back to ancient metazoans and may play a role in the evolution of mineralized structures.

Steve Benson's laboratory and collaborators[59−62] have demonstrated that PMCs secrete collagens that contribute to the extracellular environment required by the PMCs to form spicules. Quite a few collagens synthesized by PMCs have been identified and characterized. These collagens include COLP1a, COLP2a, COLP3a, COLP3a, COLP4a and SpCol1.[61,63,64]

Livingston et al.[8] have identified eight genes in *S. purpuratus* that encode collagens that are expressed in PMCs. By examining the PMC database provided by Zhu et al.,[51] Livingston et al.[8] note that nonfibrillar collagens are the most abundant collagens expressed by PMCs. The nonfibrillar collagens, COL3a and SpCol1, are products of the same gene. COLP3a/SpCol1 is the most prevalent collagen mRNA in PMCs. COLP4a, also a nonfibrillar collagen, is the next most abundant. The remaining six collagen genes are expressed at much lower levels in PMCs.

SpP16 is a small transmembrane protein expressed specifically by PMCs. Its function is unknown, and similar proteins have not been found in organisms other than sea urchins. It was originally isolated by Illies et al. [35] from the PMC EST library generated by Zhu et al.[51] Cheers and Ettensohn[65] characterized its expression in *S. purpuratus* and *L. variegates* embryos. This protein is acidic with a consensus signal sequence at its N-terminus and a putative transmembrane domain near its C-terminus. During spiculogenesis, *SpP16* expression is restricted to PMCs at the growing end of the spicules. A green fluorescent protein tagged version of P16 was found localized in the membrane of PMCs along filopodia. Cheers and Ettensohn also knocked down *SpP16* and *LvP16* with morpholino antisense oligonucleotides, and they found that spicule formation was greatly disrupted, blocking spicule rod elongation.

Livingston et al.[8] found genes clustered near the *SpP16* gene that have interesting properties. *SPU_018403* is located 129 kb upstream of SpP16 (*SPU_018408*), and it encodes an acidic protein with a consensus signal sequence at its N-terminus and a transmembrane domain at its C-terminus. *In situ* hybridization analysis shows that *SPU_018403* expression is restricted to PMCs. Another gene, *SPU_018407*, that is 37 kilobases upstream from SpP16, also has a consensus signal sequence at its N-terminus and a transmembrane domain at its C-terminus. However, the amino acid sequence encodes a more alkaline protein. *In situ* hybridization analysis also shows this gene's expression is limited to PMCs. *SPU_018405* is yet another gene clustered near *SpP16* that appears to encode a small secreted acidic protein that is present in low copy numbers in PMC EST databases, and it has not been further characterized.

SpP19 is a small protein expressed specifically by PMCs that was originally cloned by Illies et al.[35] This protein is acidic and does not contain a consensus signal sequence. While it does contain a possible nuclear localization sequence (RKKK), a green fluorescent protein tagged version of SpP19 localizes in the cytoplasm.[62] The function of this protein is unknown, and it has not been found in other organisms. Illies et al.[35] note that the cognate gene sequence for *SpP19* is very prevalent in the midgastrula PMC EST library generated by Zhu et al.[51] *SpP19* expression in PMCs is detectable several hours before the ingression of the PMCs into the blastocoel. Livingston et al.[8] found that this protein is encoded by a single copy gene (*SPU_004136*).

3.4. Biosynthesis and Secretion

Over 99% of the mass of hypochlorite-cleaned spicules isolated from pluteus larvae is calcite. Using ^{45}Ca tracer, Nakano et al.[66] showed that seawater, which is 10 mM in $CaCl_2$, is the ultimate source of the calcium, and several authors have studied the effects of inhibition of calcium transport on spicule formation.[67,68] Lennarz and his colleagues[67] have directly shown that ^{45}Ca is transported into PMCs and this transport is sensitive to calcium channel blockers. Most calcium transport takes place with high-affinity, low-capacity pumps and channels, and there has been no published work on identification of possible low-affinity, high-capacity systems for transport. Calreticulin and endoplasmin, both proteins of the endoplasmic reticulum, have been implicated in movement of massive amounts of calcium through ameloblasts[69] involved in formation of vertebrate teeth, but their role in spicule formation is unknown. Likewise, an L type calcium pump protein has been identified in scleractinian coral,[70] and similar genes do exist sea urchin; their role is not yet known.

Intracellular precipitated accumulations of calcium salts have been shown in fixed PMCs engaged in spicule building by Decker et al.[12] and Beniash et al.[71] In both cases,

considerable processing and fixation preceded visualization, leaving open the question of artifact. Importantly, Beniash et al.[71] showed that the visualized granules were probably amorphous calcium carbonate and that heating converted the granules to calcite. Recent studies[72] from the Wilt laboratory used a vital fluorescent dye, calcein, to tag calcium precipitates in PMCs *in vitro* that were secreting spicules. They observed punctate labeling of cells after a brief pulse of dye. After washing of the cells and continued culture, they observed that the cellular fluorescence had disappeared while the newly secreted spicule became labeled. This is consistent with a precursor of intracellular ACC as the proximate source for the secreted calcium that is incorporated into the spicule.

In contrast to intracellular calcium being localized in punctate deposits visible in the light microscope (i.e., approximately 200 nm or more), immunostaining of spicule matrix proteins SpSM30B and SpSM50 show broad swatches of perinuclear intracellular localization without any evidence of punctate appearance.[41] Immuno-electron microscope studies[73] have shown that both of these proteins are present at high levels in the Golgi apparatus and also in small (<50 nm) intracellular post-Golgi transport vesicles. Hence, calcium and these two matrix proteins are localized in cellular compartments of different sizes and are probably transported and delivered vectorially via different trafficking vehicles. The secreted calcium and these two matrix proteins are also directed to different positions in the spicule; this has been confirmed by following green fluorescent protein tagged SpSM30B and SpSM50.[67,68,72] The tagged matrix proteins are secreted and retained near the PMC that synthesizes them, while secreted calcium, followed by calcein labeling, moves quickly through the syncytium to the extending tip of the spicule. Ingersoll and Wilt[74] also showed that inhibition of metalloproteases interfered with secretion of both calcium and matrix protein; however the mechanism of action of proteases in this regard is unknown.[75,76]

We should add that the secretion of calcium and matrix proteins is targeted. These components end up in the spicule; hence, trafficking must be vectorial. PMCs secrete many other proteins and proteoglycans,[61] as well as the previously mentioned nonfibrillar collagens, that populate the blastocoel but are not found in spicules.

4. Biomineralization in Adults

The five extant classes of echinoderms all possess a system of internal skeletal support comprised of calcareous plates, or ossicles, formed by the mesodermally derived dermis. The chordates are the only other animal phylum with skeletal support enclosed by a covering epithelium. The sea urchins (Class Echinoidea) have been most studied, and they possess the most extensive endoskeleton. In addition to the test and spines, which may appear to be on the surface but are covered by a thin epidermis, the adults possess elaborate teeth and small appendages called pedicellariae. Even the tube feet have calcareous ossicles at their distal tips and small spicules embedded in the walls of the tube foot.

Though the process of metamorphosis has been described in the classical zoological literature, little is known about the origins of the adult endoskeleton.[77] The developing juvenile, known as the echinus rudiment, forms primarily from coelomic tissue adjacent to the gut of the larva. Mamiko Yajima has carried out experiments designed to determine which cells in the larva give rise to stem cells in the adult that differentiate into adult biomineralized tissues. Initial studies[78] demonstrated that both the additional skeletal elements of late larval development and the developing spines, tube feet, and test plates of the juvenile are formed by cells that stain with monoclonal antibodies directed against cell-surface-specific antigens found in the PMCs of the embryo and larva. On the other hand, the skeletogenic cells of the developing juvenile, while morphologically similar to PMCs, only gradually display the antigen just prior to overt calcification; this argues that fully differentiated PMC descendants are not involved in biomineralization of juvenile skeletal elements. There was no evidence that already differentiated, brightly staining PMCs were responsible for forming adult structures.

This work was followed up[79] by transplantation of cells between two species (*P. depressus* and *H. pulcherrimus*) that possess distinguishable skeletal phenotypes. Both skeletogenic PMCs and another mesenchymal population called secondary mesenchyme cells (SMCs) were transplanted. Transplanted PMCs only conferred a donor phenotype on skeletons of early larvae, but transplantation of SMCs conferred a skeleton of donor phenotype in late larvae and metamorphosing animals. This is consistent with previous work[80,81] showing that if PMCs are removed from an early embryo, the SMCs can transdifferentiate and form a complete, normal larval skeleton. Yajima marked cells by transplanting PMCs from embryos containing a transgene for green fluorescent protein, a reliable and widely used cell lineage tracer. When tagged PMCs were transplanted, they could be seen engaged in skeletogenesis in developing larvae, but after metamorphosis, they were undetectable. In contrast, tagged SMCs participated in late larval skeletogenesis and could also be demonstrated participating in skeletogenesis during metamorphosis. These results were confirmed by PCR analysis and make a strong case for participation, perhaps exclusive, of SMC descendants in development of biomineralized skeletal elements of the adult. This hypothesis was supported by experiments using embryos of the sand dollar, *Peronella japonica*.[82] When the micromeres, which form the PMCs but not SMCs, are removed, the larva did not form arms with spicules, but after metamorphosis normal juveniles with adult skeletal elements were formed.

Earlier studies on the *SpSM50* and *SpSM30B* genes showed that they are expressed in mineralized tissues of the adult but not in nonmineralized tissues.[83−85] Ameye et al.[86] showed by using transmission immuno-electron microscopy that *SpSM30B* and *SpSM50* are expressed in scleroblasts (sometimes called calcoblasts) of test plates and developing pedicellariae and in the odontoblasts of teeth. The proteins were localized in Golgi stacks, Golgi-derived vesicles, and the matrix of several adult mineralized tissues. Subsequent work[87] using scanning electron microscopy combined with immunolabeling clearly showed expression of *SpSM30B* in spine trabeculae.

Politi and her collaborators[88] studied regeneration of the spine. They characterized the newly regenerated material after gentle etching of the material in water. Electron microscopy and Fourier transform infrared spectroscopy showed that the newly forming tip of the regenerate is composed of hydrated, amorphous, precipitated calcium carbonate. During regeneration, the hydrated ACC is transformed to an anhydrous form of ACC, which in turn is transformed to the more stable crystalline calcite. This important observation is consistent with what is known about spicule formation in the embryo, and the progression of

hydrated amorphous calcium carbonate through an anhydrous state, which is then transformed to calcite, may be a widespread scenario of biomineralization. Calcite is the lowest energy state of precipitated calcium carbonate, so the conversion of ACC to calcite is favored thermodynamically. The details of these changes at the atomic level are under study. There are several interesting and puzzling questions: What is the nature of conversion of hydrated to nonhydrated ACC and what is the fate of the released water? Do organic components play an important role in this conversion? How does perfect crystallinity become initiated and propagated in the anhydrous ACC, and what are the roles of organic molecules in this conversion? The answers to these questions are unknown. What is known is that hydrated ACC can persist in natural materials for a very long time, presumably because of the activity of matrix proteins.[24] Anhydrous ACC found in spicules and regenerating spines has a structure close to calcite but still is sufficiently disordered to prevent coherent diffraction of X-rays.[22] Its transformation to calcite is also regulated and occurs over hours or days rather than the seconds taken when pure ACC is formed and transforms to calcite in the laboratory.

The tooth of the adult sea urchin is composed of high magnesium calcite. The tooth contains several different calcitic structural elements. It is continuously abraded at its distal (adoral) tip. The histology of the tooth was studied using light and electron microscopy in pioneering studies by Markel and his colleagues.[89,90] The structure, composition, and mechanical properties have been described by Wang et al.[91] The tooth is a complex assemblage of single-crystal calcite plates, needles of calcite, and very high magnesium (~40%) microcrystals of calcite. There is also an organic matrix surrounding, and possibly embedded within, the various elements of the tooth. It is astonishing that the whole tooth behaves as two single crystals when viewed in the light microscope with polarized light.

Arthur Veis and his colleagues have carried out a histological analysis[92,93] of this complex organ. The proliferative soft tissue, called the plumula, is found in the proximal portion of the tooth. Mineralization begins here, but then ceases. The cells of the plumula continuously migrate distally along the forming tooth, forming a cellular syncytium that deposits channels of extracellular membranous material. In the tooth proper, just distal to the plumula, these channels become mineralized with high magnesium (~10−15%) calcite; subsequently, adjacent plates become connected with very high magnesium (up to 40%) polycrystalline calcite, which binds the plates together.[90] The crystallographic axes of these elements are well-aligned so that the assemblage diffracts as a single crystal. Small cavities, ranging from 10−220 nm diameter, possibly filled with hydrated extracellular material, are scattered throughout the mature tooth.[94] The tooth is a continuously growing and complex structure. The cells and some mineral form in the plumula. The highly mineralized distal, incisal edge of the tooth is constantly abraded and lost by the scraping that occurs during feeding. Even the highly cellular plumula is composed of different histological and functional zones. Veis and his colleagues speculate that the mineral formed in the proliferative zone of the plumula may serve an entirely different purpose than the calcified elements of the tooth, possibly providing skeletal support for the soft plumula rather than dentition.

The Veis group has used micro-computer-tomographic techniques to map the distribution of calcium and magnesium in the tooth structures,[95] as well as the mineralized supporting organ, Aristotle's lantern.[96] They have also extracted water-soluble proteins, as well as proteins released by demineralization of the tooth, and fractionated them. There are a very large number of proteins, and of particular interest is the finding of proteins with phosphorylated serine and a high content of aspartic acid in mineralized plates of the tooth.[97] Another novel form of mapping of Ca, Mg, and protein was carried out by secondary ion mass spectroscopy (SIMS), and it was shown that aspartic acid fragments colocalized well with very high magnesium containing calcite.[98]

Recent studies from the Weizmann laboratory[99] show that the formation of calcite needles of the tooth utilizes an ACC precursor phase, and the formation of the calcite plates may involve a similar mechanism since ACC can be identified in the center of plates. The ACC of the developing needle is gradually transformed to calcite. So the strategy of utilization of ACC as precursor to biomineralized calcite can now be extended to include the spicule of the embryo, the regenerating spine, and the continuously growing tooth.

Various antibodies directed against vertebrate dentin and bone proteins and antibodies directed against SM30 and SM50 proteins of embryonic spicules have been employed to detect cross-reactions with proteins of the tooth. Cross reactivity with vertebrate dentin matrix protein and the SM50 protein have been demonstrated. As mentioned previously, Ameye et al.[86] demonstrated cross-reacting SM30- and SM50-like epitopes in mineralizing portions of the tooth. Recently the Veis laboratory has constructed cDNA libraries of plumula and tooth tissue.[100] These libraries probably contain cDNA that encode a very large variety of proteins, not all of them necessarily related to biomineralization. They identified a homologue of a vertebrate protein called mortalin, which is a member of the hsp70 group of proteins that serve a variety of functions in humans and other vertebrates. The protein is expressed in odontoblasts and matrix of the plumula in the region where syncytium formation takes place. The use of cDNA libraries[101] of plumula and mineralized tooth tissues portends good progress for identification and characterization of tooth matrix proteins.

5. Evolution of Integral Matrix Proteins

The putative sea urchin integral matrix proteins identified so far all contain a single c-type lectin domain. There may well be other types of proteins that will be identified as also comprising the integral matrix. However, the prevalence of c-type lectins raises the question: why have c-type lectins been co-opted by sea urchins for use during biomineralization? This question has intriguing evolutionary implications. Bottjer et al.[102] point out that the calcitic stereom (as the fenestrated skeleton of the adult is called) present in sea urchins represents a synapomorphy (common trait) that is present in all echinoderms and that these integral matrix proteins may be a window into how echinoderms first formed mineralized tissues. Paleogenomics, the study of ancient genomes through the analysis of extant organisms, is an approach to this problem. However, sequences of the integral matrix proteins do not yet exist for direct examination of this question. Nevertheless, comparisons of sea urchin integral matrix proteins to those of animals in other phyla are possible and offer a few preliminary clues.[8,101]

Biomineralization in vertebrates is widely studied and a number of integral matrix proteins that are involved in the formation of vertebrate teeth and bones have been identified. The literature is too extensive to review here. However, among the most widely studied matrix proteins are the so-called secreted calcium binding phosphoproteins (SCPPs). SCPPs include dentin sialophosphoproteins (DSPPs), secreted phosphoproteins 1 (SPP1), small integrin-binding ligand N-linked glycoprotein (SIBLING), and secreted protein, acidic, rich in cysteine (SPARC). Kawasaki and Weiss[103] and Fisher et al.[104] have looked at SCPP protein sequences, as well as the genomic organization of their cognate genes. They have hypothesized that despite the diverse amino acid composition of the encoded proteins, genomic organization of SCPP genes points to a common origin. They proffer the idea that this rapidly evolving class of proteins is derived from a SPARC gene that was present in protostomes and deuterostomes (see Kawasaki and Weiss[105] for review).

Sea urchins do not utilize SCPPs for biomineralization despite the fact that they do have a *SPARC* gene. *SpSPARQ* is utilized for other functions in sea urchins.[8] Are there similarities in the role of SCPPs in vertebrates and c-type lectins in sea urchins? It is unclear whether c-type lectins and SCPPs mediate mineralization in the same way. However the two classes of proteins do have some shared physical characteristics. C-type lectins have regions that tolerate large amounts of sequence variability while still maintaining the c-type lectin fold.[106] SCPPs also tolerate wide sequence variability, yet keep their structural integrity.[103] Perhaps genes that are able to diversify quickly but retain structural integrity were more easily coopted to act as integral matrix proteins.

During the Cambrian expansion of metazoan body plan diversity some 544 million years ago (Mya), there was the relatively sudden appearance of skeletons and carapaces.[107,108] Since mineralized skeletons appear over such a relatively brief period of time, the hypothesis has been put forth that the Cambrian soft-bodied organisms must have "recruited" proteins that were already serving other functions. In other words, variants of existing proteins were used to help regulate formation of mineralized composites that could be used for structural function.[107,109]

A number of reports[107,110,111] have suggested that seawater chemistry may have dictated the choice of mineralogy of the skeletons of clades when mineralized structures first appeared during the Cambrian period. Porter[110] found a close correlation of seawater Mg^{2+}/Ca^{2+} ratios with the mineralogy of calcium carbonate of the mineralized skeleton first acquired by various clades. Seawater from the Ediacaran (635−542 Mya) and Nemakit-Dalynian (542−525 Mya) periods favored the initial formation of aragonite (another stable form of crystalline $CaCO_3$) skeletons, while seawater from the Tommotian (525−521 Mya) and the Atdabanian (521−519 Mya) through the Toyonian (519−513 Mya) period favored the initial formation of calcite skeletons. Once a clade formed a skeleton, changes in the Mg^{2+}/Ca^{2+} ratio had no effect on the mineralogy of the skeleton formed. Porter[110] suggests that the initial skeleton formation in particular taxa was a response to the environment. Once the skeleton was formed, subsequent biomineralization was an intracellular process, which is less influenced by the environment. Echinoderm skeleton formation fits in with this theory put forth by Porter and others. The echinoderms first formed calcitic skeletons 520 Mya during the Atdabanian period, which was during a time when the seawater chemistry favored the initial formation of calcitic skeletons.

The sea urchin genome contains genes that encode over a hundred small c-type lectins that have one or two c-type lectin domains.[112,113] These sorts of proteins are involved in innate immunity and function as opsonins. Opsonins bind particles and enhance the phagocytosis of the particle. Smith et al.[114] report that sea urchin coelomic fluid contains a large number of small c-type lectins. The coelomocytes are the cells of the sea urchin adult that are at the center of the sea urchin immune response.[114−116] In addition, RT-PCR analyses show that the cognate transcripts of the putative integral matrix proteins SpSM30F and SpSM29 are found in coelomocytes.[8] The protein encoded by *SpSM30F* is also similar in structure to the collectins.[112] The collectins are a family of proteins that are a component of the complement system, which results in the opsonization of foreign particles.[117] These observations suggest an unexplored tie between proteins involved in biomineralization and innate immunity in the sea urchin. Dubois et al.[118] have also suggested that cells of mesothelial origin could possibly serve both immune and skeleton-forming roles.

Taken together, these various observations raise the possibility that the early echinoderms first co-opted rapidly diversifying c-type lectins of the immune system to regulate calcite mineralization during times of changing seawater chemistry in the early Cambrian period. Comparisons of gene sequences encoding integral matrix proteins from other echinoderms, when they become available, should help clarify whether this hypothesis is viable.

6. Concluding Remarks

The developing sea urchin offers splendid opportunities to observe biomineralization in real time. The background of biological information about the development of the skeleton in these embryos is excellent, and there is a substantial literature on the formation of the spicule. The test and spines of the adult have also been studied, and similar occluded matrix molecules seem to be found in the adult and embryo. The introduction of modern molecular biology and genomics brings more power to studies of biomineralization in this system.

What do we know from study of this system? The number of matrix molecules and their biochemical complexity is greater than originally envisaged. Limited functional studies of these matrix proteins is now possible using molecular biological techniques, and at least one of them, SM50, has been shown to be essential for spicule formation. We can expect this approach to be fruitful for study of other matrix molecules. The relationship of matrix to the material properties of the spicule is of great interest; progress has been slow, but application of newer techniques of structural analysis should give us some insight in the near future.

Comparisons of echinoderm biomineralization with vertebrates now make clear that from the point of view of occluded matrix molecules, the bones and teeth of vertebrates are constructed from a different suite of organic molecules. Detailed comparisons of echinoderms with mollusk shells should be of great interest and can now be done with additional genomic information. We predict that comparative studies that utilize genomics will clarify the evolution of biomineralization of animal skeletons.

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