Cah. Biol. Mar. (2013) 54 : 467-478

Cellular and molecular bases of biomineralization in sea urchin embryos

Valeria MATRANGA, Annalisa PINSINO, Rosa BONAVENTURA, Caterina COSTA, Konstantinos KARAKOSTIS, Chiara MARTINO, Roberta RUSSO and Francesca ZITO
AIstituto di Biomedicina e Immunologia Molecolare "Alberto Monroy", Consiglio Nazionale delle Ricerche, 90146 Palermo, Italy. E-mail: valeria.matranga@ibim.cnr.it

Abstract: Sea urchin embryos construct their skeleton following a precise gene-regulated time- and space-dependent programme, in concert with factors promoting cell adhesion and differentiation. The biomineral is deposited in a privileged extracellular space produced by the fused filopodia processes of the primary mesenchyme cells, the only cells producing a set of necessary matrix proteins. More than ten years ago we showed for the first time that signals from ectoderm cells promoted the expression of one of the major skeleton matrix genes by the primary mesenchyme cells. Since then, many of the crucial steps of this complex activation cascade, from ectoderm cells to embryonic spicules, have been elucidated. The experimental production of skeleton malformations, induced by the exposure to toxic metals or ionizing radiations, served as model to dissect the molecular mechanisms leading to biomineralization. With the aim of understanding the sea urchin skeleton physiology, we analysed the expression of well-known and newly-identified biomineral-related genes, including those coding for growth and transcription factors as well as for skeleton matrix proteins. This review summarizes our recent findings on sea urchin embryo skeletogenesis, with a particular attention to the role played by cellular and molecular signaling, approached by the use of experimentally induced skeleton malformations.

Résumé : *Bases cellulaires et moléculaires de la biominéralisation des embryons d'oursin.* Les embryons d'oursins construisent leur squelette selon un programme précis génétiquement régulé dans l'espace et le temps, en relation avec des facteurs permettant l'adhésion et la différenciation cellulaire. Le biominéral est déposé dans un espace extracellulaire particulier produit par les expansions filopodes fusionnées des cellules du mésenchyme primaire, les seules cellules produisant la série de protéines matricielles nécessaires. Il y a plus de 10 ans, nous avons montré pour la première fois que les signaux des cellules de l'ectoderme sont à l'origine de l'expression d'un des gènes majeurs de la matrice squelettique par les cellules du mésenchyme primaire. Depuis, beaucoup des étapes cruciales de la cascade complexe d'activation, depuis les cellules ectodermiques jusqu'aux spicules embryonnaires, ont été élucidées. La production expérimentale de malformations squelettiques, induites par l'exposition à des métaux toxiques ou des radiations ionisantes, a servi de modèle pour disséquer les mécanismes moléculaires conduisant à la biominéralisation. Dans le but de comprendre la physiologie du squelette d'oursin, nous avons analysé l'expression, à la fois de gènes bien connus et de gènes nouvellement identifiés, liés à la biominéralisation, notamment ceux codant aussi bien pour des facteurs de croissance et de transcription que pour des protéines de la matrice squelettique. Cette revue synthétise les récentes découvertes sur la genèse squelettique de l'embryon d'oursin, et porte une attention particulière au rôle joué par la signalisation cellulaire et moléculaire, obtenues par l'induction expérimentale de malformations squelettiques.

Keywords: Skeleton ● Embryo ● Biomineral ● Genes ● Signaling

Why and how to study biomineralization in echinoderms?

Among deuterostomes, only vertebrates and echinoderms form extensive biomineralized structures. Biominerals are complex polymers, which incorporate mineral and organic components, exhibiting advantageous properties compared to its inorganically formed counterpart. They are generally molded into specifically friendly spaces, in which the structure, size, shape, orientation, and assembly of the constituents are precisely controlled at several hierarchically organized molecular levels. Distinct genes governing biomineralization appeared independently in echinoderms and vertebrates during evolution and most of the skeleton matrix proteins are echinoderm-specific and vertebrate-specific (Sea Urchin Genome Sequencing Consortium, 2006). Moreover, the biointegrated mineral that constitutes the endoskeleton of echinoderms is composed mainly of calcium carbonate, while bones and teeth of vertebrates are composed of calcium phosphate. Nevertheless, cellular and molecular processes associated with earlier and later events in biomineralization appear similar in both systems. Many studies have been carried out on the biomineralization process with the aim to gain insights into its mechanisms and eventually to apply these findings for the synthesis of biominerals mimicking the natural counterparts (Cölfen & Mann, 2003; Dorozhkin, 2009). Biominerals synthesized *in vivo* are formed in an exclusive microenvironment under controlled conditions of temperature, pressure, and pH. These conditions limit the number and the type of biominerals, controlling their kinetics of nucleation, growth, and transformation by influencing gene expression (Meldrum & Cölfen, 2008). Thus, in sea urchins, a rhombohedral crystal of magnesian calcite becomes bicontinuous and sponge-like in morphology under environmental and biological control, producing biominerals with unique morphologies, such as spicules, spines or skeletal plates (Fig. 1). On the other hand, micro and macro environmental changes caused by chemical and physical pollution strongly influence the normal development and pattern of the growing crystals. During the last years, taking advantage from this notion, we analyzed the expression of biomineralization-related genes and proteins in several examples of sea urchin embryos with experimentally-induced skeleton malformations, including those produced by the exposure to toxic metals, such as cadmium or manganese, and ionizing radiation, such as UV-B and X-rays (Russo et al., 2003, 2010 & 2013**;** Roccheri et al., 2004**;** Bonaventura et al., 2005, 2006 & 2011; Pinsino et al., 2010, 2011 & 2012; Matranga et al., 2010). Besides the obvious toxicological implication, by inducing skeleton malformations we could dissect the molecular steps taking place during skeleton development and possibly understand the physiological events regulating embryonic biomineralization. In this review we will describe our most important findings related to cellular signaling and biomineral formation in the sea urchin embryo, illustrating the toxicological approach to study skeletogenesis, with the aim of proposing the use of sea urchin embryos as model to unravel the signaling pathways involved in biomineralization.

Sea urchin embryo: the most classical model used to study development

Among the echinoderms, sea urchin embryos provide an attractive and tractable model for exploring the mechanisms used for successful development, as it produces large numbers of transparent embryos exhibiting rapid cell divisions, fast morphogenesis, biochemical similarity to vertebrates and simplicity in shape and organization (Hörstadius, 1939). Additional key features accounting for sea urchin embryo success are the potent cellular mechanisms that provide them with protection, robustness, and resistance against the external environment, as well as the regulatory pathways that alter their development in response to the adverse environmental conditions encountered (Hamdoun & Epel, 2007). In the sea urchin embryo, development is controlled by gene regulatory networks (GRNs) that specify the cell fate of territories at the appropriate time and space. Currently, the sea urchin embryo endo-mesoderm GRN is the most nearly completed, validated and useful, among the GRNs available from other organisms (Peter et al., 2012). However, it is nowadays well-known that development and cell differentiation are the result of the combined action of cytoplasmic determinants and cell-cell inductions, both under the control of gene expression (Peter et al., 2012). Indeed, local interactions within the morphogenetic field allow the cells to access global information, thus leading to appropriate patterning of the tissue or embryo as a whole (Jaeger et al., 2008). Founder cells of the three germ layers, namely ectoderm, mesoderm and endoderm, are the basic units where regulatory information is localized during cleavage. Endo-mesoderm cell fate decisions are discrete and deterministic: β-catenin is required for the development of all endo-mesoderm territories, including the archenteron, the primary mesenchyme cells (PMCs) and the secondary mesenchyme cells (SMCs) (Logan et al., 1999). Cell fates are fully specified by the blastula/early gastrula stage of development, when cells have begun to express particular sets of territory-specific genes (Davidson et al., 1998). The blastula stage is characterized by the presence of a large fluid-filled blastocoel, surrounded by a single layer of cells. During gastrulation, extensive cellular rearrangements

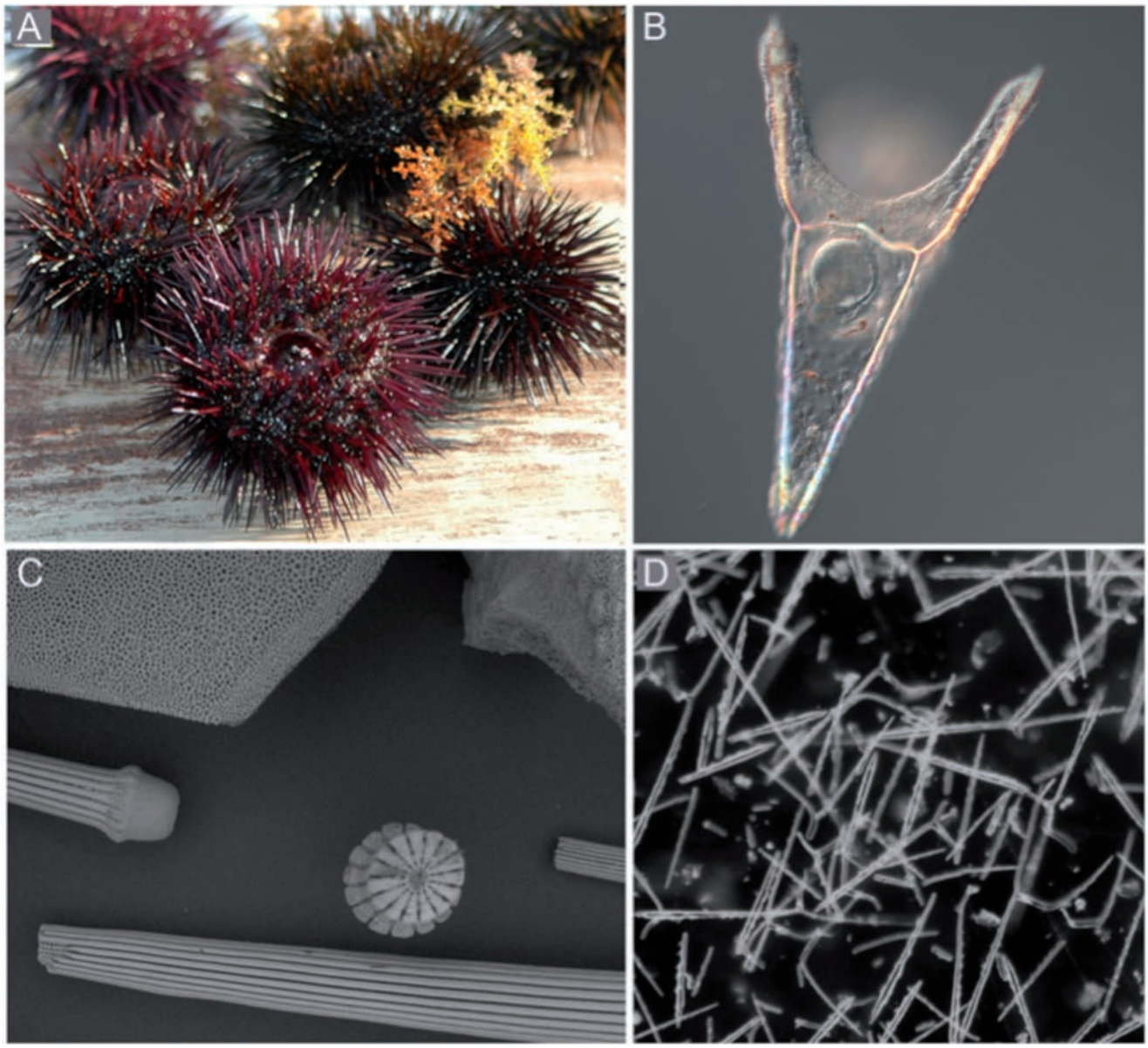

Figure 1. The sea urchin *Paracentrotus lividus*. **A.** Adult sea urchin. **B.** Interference contrast image of an embryo at the pluteus stage. **C.** Scanning electron micrograph of different parts of test and spine. **D.** Spicules purified from embryos at 60 h of culture.

occur which convert the hollow-spherical blastula into a multi-layered gastrula. Changes in shape and differentiation of embryo structures, like skeleton and intestine, lead to the formation of a pluteus, the first larval stage.

The key cellular events in sea urchin embryo skeleton formation

Skeleton development is an essential step for constructing the framework of the body of the sea urchin embryo. The PMCs control this event, using many components of the already known skeletogenic GRN, such as maternal proteins, early zygotic transcription factors, and late gene products, all of them directly regulating their morphogenetic behaviours (Livingstone et al., 2006; Ettensohn, 2009). Multiple transcription factors are activated 1-2 h before the PMCs ingression into the blastocoel, at the blastula stage, including Tel, Erg, Hex, Tgif, FoxN2/3, Dri, FoxB, FoxO, Snail, Twist (reviewed by Lyons et al., 2012). PMC ingression is an epithelial-mesenchymal transition process and involves multiple, coordinated cell biological

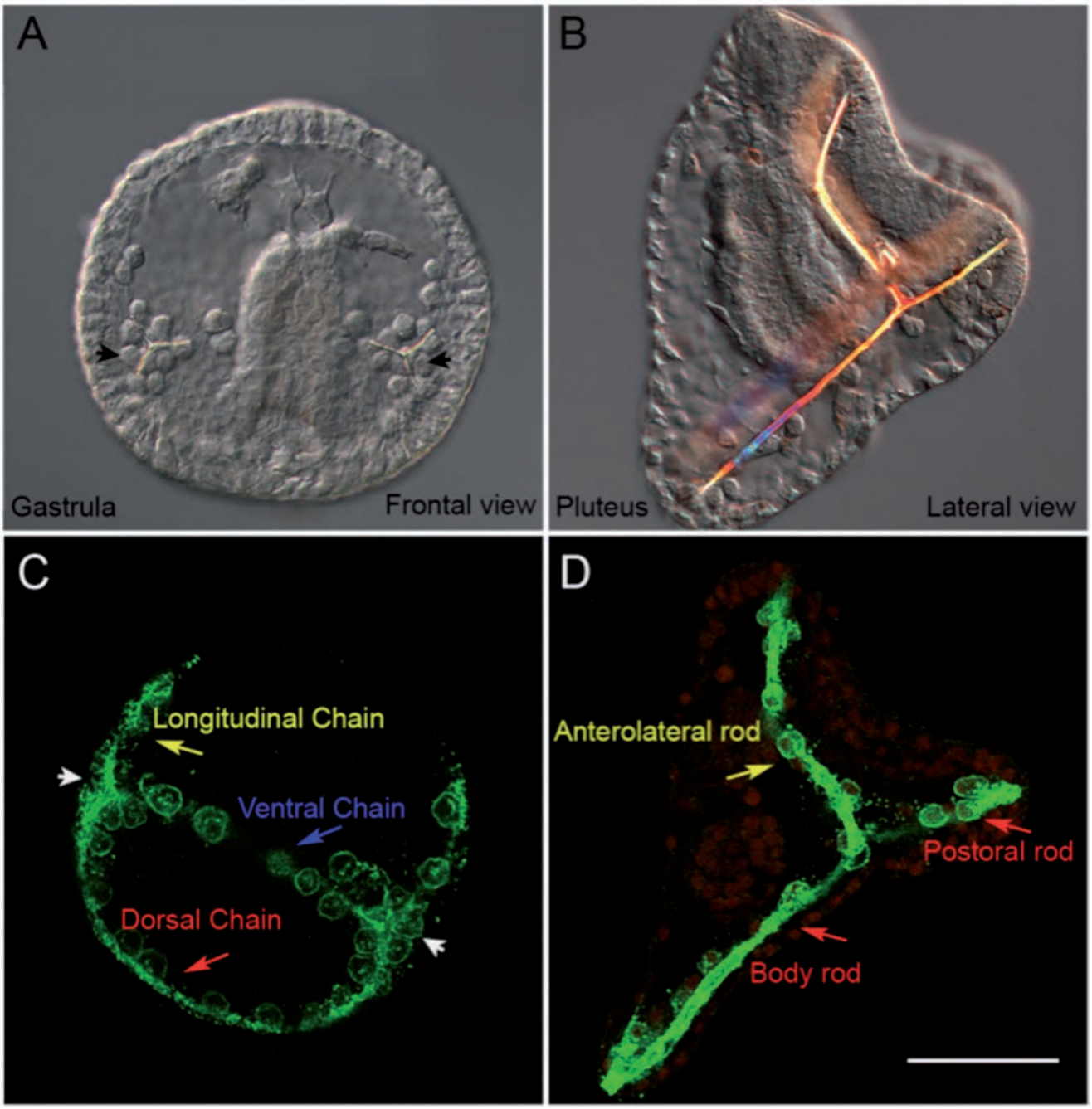

Figure 2. Development of *Paracentrotus lividus* skeleton. Interference contrast and immunostaining of PMCs at late gastrula, frontal view (**A & C**), and pluteus, lateral view (**B & D**). Immunostaining with 1D5 mAb recognizes the antigen present on the PMCs cell surface, the protein known as MSP130 (mesenchyme surface protein 130). **A.** Gastrula showing PMCs aggregates (ventrolateral clusters) enclosing the spicule triradiate rudiments (see arrow heads). **B.** Early pluteus showing the typical three-dimensional endoskeleton pattern (lateral view) originated directly from the elongation and branching of a spicule triradiate rudiment. **C.** Gastrula immunostaining shows the typical PMC ring pattern, with two ventrolateral PMC aggregate-forming cells, enclosing the spicule triradiate rudiments (see white arrow heads), and the longitudinal (yellow arrow), dorsal (red arrow) and ventral (blue arrow) chains. **D.** Late pluteus immunostaining detects PMCs forming the anterolateral rod (yellow arrow), body and postoral rod (red arrow), originated directly from the longitudinal chain and dorsal chain. Bar = 50 μm.

events that causes morphogenetic changes to the PMC precursors as: i) the loss of connections to their neighbours; ii) the loss of affinity to the apical lamina; iii) the gain of affinity to the basal lamina and blastocoelar matrix; iv) the change in cell adhesion properties; v) the change in cell shape; vi) the acquisition of mobility (Lyons et al., 2012). PMCs emerge on the vegetal floor of the blastocoel as a result of ingression, and migrate along the wall of the blastocoel towards the animal pole. Progressively, they gather to form a characteristic ring-like pattern, around the blastocoel wall just inside the boundary between ectoderm and endoderm; extend thin filopodia in every direction and cells fuse to form a syncytial network with two ventrolateral clusters of PMCs (Fig. 2). Skeletogenesis begins with the accumulation and secretion of the biomineral within a privileged extracellular space enshrouded by the fused PMCs filopodial processes of each ventrolateral cluster (Dubois & Chen, 1989; Wilt, 2002 & 2005). The two spicule rudiments elongate and branch in a three-dimensional endoskeleton composed of magnesian calcite and spicule matrix proteins (Killian & Wilt, 1996 & 2008). Many of the proteins involved in the sea urchin skeletogenesis are members of small families of co-ordinately expressed genes which are clustered in the genome, including the spicule matrix proteins SM30, SM50, P19 and the cell surface proteins MSP130, P16 (Livingston et al., 2006; Costa et al., 2012). At gastrulation, the PMCs transmit an inhibitory signal to the SMCs, also known as non-skeletogenic mesoderm (NSM), preventing their differentiation into skeletogenic mesenchyme, thus promoting the production of a variety of differentiated mesodermal cells: pigment cells, circumesophageal muscle cells, blastocoelar cells, and coelomic pouch cells, suggesting that SMCs function as multipotent stem cells (Kiyomoto et al., 2007; Zito & Matranga, 2009). However, some NSM cells have the potential to express a skeletogenic phenotype under appropriate experimental conditions (Kiyomoto et al., 2007; Zito & Matranga, 2009).

Ever since Hans Driesch's famous experiments on sea urchin embryos, it has been evident that embryonic pattern formation depends on the positional information a cell receives (Driesch, 1892). PMCs require several types of cues, including axial, temporal and scalar signals, provided by the overlying ectoderm and the apical extracellular matrix (ECM) in order to organize the proper animal-vegetal and oral-aboral position, formation, and orientation of the skeleton (Zito et al., 2003; Kiyomoto et al., 2004; Duloquin et al., 2007; Röttinger et al., 2008; Matranga et al., 2011).

Role of the ECM and growth factors in sea urchin skeleton formation

In general, the ECM is the non-cellular component present within cells and tissues; in development and morphogenesis it functions as adhesive substrate, provides structural support, is a reservoir of growth factors, presents growth factors to their receptors, senses and transduces mechanical signals, is source of spatial cues (Rozario & Desimone, 2010). During sea urchin embryo skeletogenesis, the ECM provides several signaling molecules regulating cell recruitment and migration to the site of skeleton formation, and promoting differentiation and gene expression. It is possible to distinguish an apical (outside the embryo) and a basal (inside the blastocoel) ECM (Zito et al., 2005; Zito, 2012): among the components present in the blastocoel, only ECM molecules that support and/or direct PMC movements have been well described, such as: i) Pamlin, isolated from the basal lamina of *Hemicentrotus pulcherrimus* (Katow, 1995); ii) ECM3, detected in the basal lamina adjacent to the ectoderm in all regions except for the animal pole of *Lytechinus variegates* (Wessel & Berg, 1995); iii) collagen, studied in *Strongylocentrotus purpuratus* (Wessel at al., 1991); iv) *Pl*-200K, identified in *Paracentrotus lividus* (Tesoro et al., 1998). The apical ECM is a very complex structure, consisting of a number of layers with many different elements (for a review see Alliegro et al., 1992). There are a number of evidences of indirect roles played by apical ECM molecules during the formation of the skeleton but, actually, no ECM molecule with an active direct role has been identified in sea urchin embryo. In our laboratory, we isolated and characterized *Pl*-nectin, which is located on the apical surface of ectoderm and endoderm cells from the blastula and gastrula stage onwards and has been shown to mediate the adhesion of blastula cells to the substrate (Matranga et al., 1992). Its functional role during embryogenesis has been highlighted by means of a monoclonal antibody (McAb), which interfered with *Pl*-nectin *in vivo* (Zito et al., 1998). Indeed, the addition of McAb to *Pl*-nectin to embryo cultures inhibited ECM-ectoderm cell interactions and caused a dramatic impairment of skeletogenesis, offering a good model for the study of factor(s) involved in skeleton elongation and patterning. The protein is a discoidin family member, whose complete sequence and domain architecture has been recently characterized (Costa et al., 2010). *Pl*-nectin consists of 6 tandemly-repeated discoin domains, which provide with the ability of: 1) binding to ECM molecules bearing galactose and N-acetyl-glucosamine carbohydrate moieties (including collagen, or cell membrane surface glycoproteins), 2) binding to cell surface proteins, such as tyrosine kinase receptors and G protein-coupled receptors, 3) forming multimeric structures

by self binding of the discoidin domains (Costa et al., 2010). By *in vitro* immunoprecipitation and affinity chromatography experiments, it has been shown that *Pl*-nectin binds to the *βC* integrin subunit, suggesting that the interaction of *Pl*-nectin with ectoderm cells is mediated by a *βC*-containing integrin receptor (Zito et al., 2010). *Pl*-nectin is an "indirect actor" in the ecto-mesoderm signaling. In fact, ectoderm cells require contacts with *Pl*-nectin in order to send to PMCs those inductive signals that are needed for correct skeletogenesis (Zito et al., 1998). The attractive idea that skeleton formation is regulated by the ectoderm was firstly proposed more than 60 years ago (Von Ubish, 1937), although the molecular cues implicated in such interactions are being identified only in recent years. Several signals released by the ectoderm have been identified among growth factors, including EGF, univin, VEGF and FGF (Grimwade et al., 1991; Zito et al., 2003; Duloquin et al., 2007; Röttinger et al., 2008). Univin was the first gene encoding a member of the TGF-β superfamily to be identified in the sea urchin embryo (Stenzel et al., 1994). Ten years ago, we demonstrated that univin produced by ectoderm cells is the inductive signal responsible for skeleton growth. In fact, we found that skeleton-defective embryos exposed to *Pl*-nectin McAb showed a strong reduction in the levels of expression of *Pl*-univin, which paralleled a downregulation of SM30, encoding for one of the major skeleton matrix proteins (Zito et al., 2003). Based on these results we proposed for the first time, a regulative model where we postulated that the secretion of univin, or other growth factor(s), into the blastocoel by some ectodermal cells drives PMCs to synthesize SM30 and other spicule matrix proteins required for spicule growth (Fig. 3). Later, Duloquin et al. (2007) confirmed the guidance role of VEGF/VEGFR signaling for the positioning and differentiation of migrating PMCs during gastrulation of the sea urchin embryo. In the same way, Röttinger et al. (2008) have described the role played in skeletogenesis by another growth factor, the FGF-A, and its receptor FGFR-1 and FGFR-2, demonstrating that this signaling pathway regulated PMCs cell migration, skeleton differentiation, as well as gastrulation. In conclusion, results from different laboratories suggest that there is an interplay between ectoderm and mesenchyme cells and that different growth factors associated with the ECM seem to be independent and not functionally redundant, each of them being required for controlling skeleton morphogenesis.

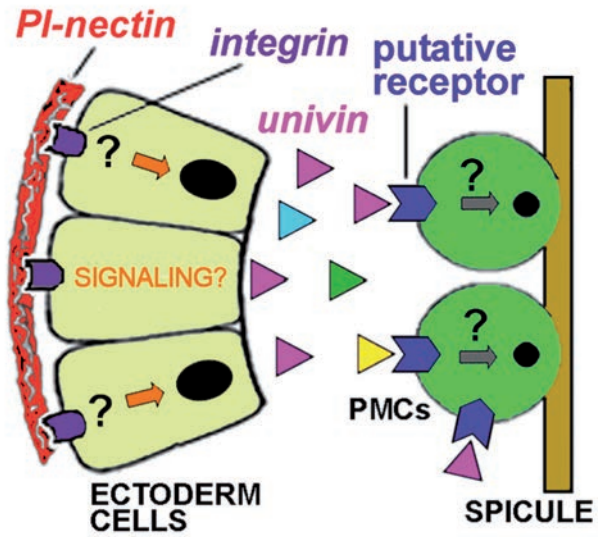

Figure 3. Schematic drawing illustrating ecto-mesoderm signaling leading to spicule formation. Endoderm cells interact with *Pl*-nectin in the outer extracellular matrix, and secrete into the blastocoel *univin*, a member of the transforming growth factor-beta superfamily, which in turn signals PMCs to synthesise the spicules via a hypothetical receptor. The interaction of ectoderm cells with *Pl*-nectin is possibly mediated by an integrin receptor, and it activates a yet unknown signaling pathway.

Skeletogenic gene expression and signaling pathways in sea urchin embryos with experimentally induced skeleton malformations

As previously mentioned, an integrated network of genes, proteins and pathways are normally functioning during sea urchin embryo development, as integral part of their developmental program. On the other hand, such complex networks of factors allows sea urchin embryos to defend themselves against various types of stressors, suggesting a dual function, regulating both defence and development. In addition, genes involved in signal transduction often respond to environmental stress, activating alternative signaling pathways as a defence strategy for survival (Hamdoun & Epel, 2007). During the last years, to understand basic principles of skeleton formation we analysed the expression of well-known and newly-identified biomineral-related genes and signaling pathways in several examples of embryos with experimentally-induced skeleton malformations (Fig. 4). Such embryos were produced by the exposure to toxic metals, such as manganese or cadmium, as well as to ionizing radiation, such as UV-B and X-rays (Russo et al., 2003; Roccheri et al., 2004; Bonaventura et al., 2005, 2006 & 2011; Matranga et al., 2010; Russo et al., 2010; Pinsino et al., 2010 & 2011). Manganese-exposed embryo were characterized by the lack of skeleton (triradiate spicule rudiments), although all the other morphological features remained amazingly unperturbed, even at the prism/pluteus stages. PMCs maintained the capacity to migrate and pattern inside the blastocoel, but showed a strong depletion of calcium in the Golgi regions, suggesting that manganese competes with

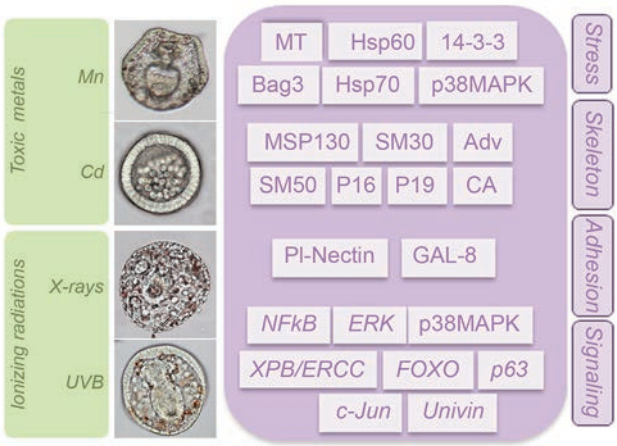

Figure 4. Summary of skeletogenic genes and signaling pathways studied in *P. lividus* sea urchin embryos with experimentally induced skeleton malformations. On the left: malformed embryos exposed to manganese, cadmium, UV-B and X-rays. On the right: genes and proteins investigated in our studies were divided into four categories (stress, skeleton, adhesion, signaling). Met, metallothionein; Hsp60, Heat shock protein 60; 14-3-3, 14-3-3 protein; Bag 3, Bcl-2 associated athanogene 3; Hsp70, Heat shock protein 70; p38MAPK, 38 mitogen-activated protein kinase; Msp130, Matrix spicule protein; SM30, Spicule matrix 30; Adv, Advillin; SM50, Spicule matrix 50; P16, P16 protein; P19, P19 protein; CA, Carbonic anhydrase; *Pl*-Nectin, *Parcentrotus lividus* nectin protein; Gal-8, Galectin 8; NFkB, Nuclear factor kappa-light-chain-enhancer of activated B cells; ERK, Extracellular signal-regulated kinase; XPB/ERCC, xeroderma pigmentosum B/excision repair cross-complementing; FOXO, Forkhead box protein.

calcium uptake and internalization (Pinsino et al., 2011). Moreover, we found steady-stable Extracellular signal-Regulated Kinase (ERK) MapK phosphorylated levels, together with the miss-regulation of the skeletogenic genes *Pl*-msp130 and *Pl*-sm30. Our results suggest that skeleton elongation and patterning is controlled by calcium signaling and internalization (stores) through the transient modulation of the ERK signaling that regulates skeletogenic gene expression (Pinsino et al., 2011). We extended further our studies, focusing on the temporal activation of the p38 mitogen-activated protein kinase (p38MAPK) and its correlation with the proteolytic activities of metalloproteinases (MMPs). We found that Mn affects both the physiological dynamic activation of the p38MAPK and the dynamic proteolytic activities of a few specific MMPs (90-85 kDa and 68-58 kDa), suggesting that their enzymatic activities could be dependent on the p38MAPK signaling during skeleton development (Pinsino et al., 2013).

The effects of Cd on sea urchin embryos and larvae have been studied extensively in our laboratory examining developmental malformations, specific genes expression, stress proteins induction, apoptosis (for a review see Roccheri & Matranga, 2010). The defects observed in embryos continuously exposed to high sublethal Cd concentrations mostly consisted in gut and skeletal abnormalities (elongation and patterning), as well as differentiation impairment till a complete block of development (Russo et al., 2003; Roccheri et al., 2004). After cadmium removal embryos partially recovered a normal morphology showing a general delay in development (Roccheri et al., 2004). However, at least 30% of the scored embryos were found with aberrant skeleton morphologies (Roccheri et al., 2004). Lower doses of Cd for longer periods of exposure produced larvae, which could eventually continue development (8-arm pluteus), although major defects consisted in the reduced size or lack of arms (Filosto et al., 2008).

Similarly, severe phenotypes were found in embryo cultures exposed to high doses of X-rays and UV-B: in general, we found a dose-dependent delay in the developmental schedule, as well as major abnormalities in specific embryonic territories and tissues, namely skeleton and intestine. In fact, exposed embryos had no spicules, PMCs were delocalized inside the blastocoel, gastrulation was highly impaired, and no elongation of the archenteron was produced. These results, together with a reduced expression of SM30 indicated that UV-B and X-rays strongly affect sea urchin embryo biomineralization (Bonaventura et al., 2005 & 2006; Matranga et al., 2010).

Cadmium (Russo et al., 2003), UV-B (Bonaventura et al., 2005), and X-rays (Matranga et al., 2010) not only affected skeleton elongation and patterning but also caused the miss-regulation of other developmental structures, such as mesoderm, endoderm, and ectoderm. Moreover, defects in biomineralization have been correlated to the expression of stress proteins, such as hsp60 and hsp70 (Roccheri et al., 2004; Bonaventura et al., 2005 & 2006; Pinsino et al., 2010), kinases responsive to stress stimuli, such as p38 MAPK (Bonaventura et al., 2005), Bag3 and p63 (Bonaventura et al., 2011), apoptosis (Filosto et al., 2008), mRNA over-expression, such as Metallothionein (MT) and *Pl*-14-3-3ε (Russo et al., 2003 & 2010). A very recent study performed in our laboratory analyzed the expression of stress response genes, namely XPB/ERCC3, NF-kB, FOXO; c-Jun, demonstrating a time- and dose- dependent modulation in response to UVB radiation (Russo et al., 2013).

The finding of an increase of the p38MAPK activated form in UVB irradiated embryos (Bonaventura et al., 2005) is in agreement with reports describing the protective anti-apoptotic role of p38MAPK activation in UVB irradiated keratinocytes. On the other hand, it has been previously reported that the p38MAPK activation is required for sea urchin embryo skeletogenesis and oral specification

(Bradham & McClay, 2006), in agreement with our findings in Mn exposed embryos (Pinsino et al., 2013). Thus, the p38MAPK activation after UVB irradiation might have a substantial role in the regulation of sea urchin skeletogenesis. Most proteins and genes analyzed so far in sea urchin embryos with experimentally induced skeleton malformations often have at least a dual function: protection against environmental hazards and execution of the developmental program. For example, this is the case of *Pl*-MT, whose mRNA has been found over-expressed in embryos treated with cadmium (Russo et al., 2003) and temporally regulated in distinct tissues during normal development (Russo et al., 2013). Current trends claim that the idea of "one gene - one protein - one function" has become obsolete, as increasing numbers of proteins have been found to have two or more different functions. As a result, the notion of moonlighting proteins has developed since the early nineties, adding another dimension to cellular complexity (for a recent review, see Copley, 2012). The question remains how cells switch between distinct functions of the moonlighting proteins, complicating the attempts in understanding regulatory networks, as well as physiological and pathological processes.

Sea urchin skeletal nano-bio-materials for bio-medical applications

Currently, one of the most demanding challenges for the production of new biomaterials is the exploitation of bioceramic nanopowders obtained from naturally derived raw materials. The recent trend in bioceramic research is to develop nanopowders with precise control of particle size, morphology, crystallinity degree and chemical composition. For example, a few studies were aimed at producing hydroxyapatite nanopowders from marine invertebrates, such as mollusk and sea urchin, using chemical approaches (Lemos et al., 2006; Ağaoğullari et al., 2012). As previously mentioned in this review, proteins control structural formation of the skeleton and become integral parts of the bio-composite. Among others, these include silicateins and silicase in sponges, amelogenin and collagen in mammalian bone, calcite- or aragonite-forming proteins in mollusc shells, magnetite-forming proteins in magnetotactic bacteria, calcite-forming proteins in sea urchins (Livingstone et al., 2006; Arakaki et al., 2008; Furuhashi et al., 2009; Deshpande et al., 2010; Wang et al., 2012). Yet, it has been extremely difficult to determine the role of individual players of the biological crystal growth machinery. With regard to the sea urchin embryo, a good strategy could be to reproduce *in vivo* the synthesis of single crystals by culturing isolated PMCs and single recombinant proteins, or their mixtures. An interesting recent report demonstrated that recombinant sea urchin vascular endothelial growth factor (VEGF) directs single-crystal growth and branching *in vitro* (Knaap et al., 2012), confirming the validity of such an approach.

In the frame of the EU 7th FP Biomintec project "Biomineralization: Understanding of basic mechanisms for the design of novel strategies in nanobiotechnology", we produced a toolkit of molecular probes, recombinant proteins and antibodies that will be probably applied in the field of nanotechnology. In fact, we identified, cloned and characterized a few genes involved in the biomineralization process; these include carbonic anhydrase, P16 and P19 (small acidic proteins), galectin-8, advillin (lectins), SM30a, SM50 (C-type lectins) and tetraspanin (membrane spanning protein). We studied their expression during sea urchin embryonic development by qPCR and *in situ* hybridization and produced recombinant proteins and specific antibodies for two of them, namely carbonic anhydrase and galectin-8.

We demonstrated that P16 and P19, previously identified by proteomic analysis in adult tooth, spine and test (Mann et al., 2008a & b), are involved in the formation and elongation of the embryonic skeleton (Costa et al., 2012). In particular, we found that P19 has a high similarity, in its C-terminal region, with the dentin matrix protein-1 (DMP-1), a protein highly expressed in human osteoblasts. Since in general it is known that proteins with a total acidic charge (pI 3.5-4.5) and a low molecular weight (16-19 kDa) are important in the formation of the biomineral as well as in phosphates homeostasis during mineralization, and, considering the homology between P19 and DMP-1, the production of the P19 recombinant protein is of potential interest for a therapeutic action in bone remodelling in osteoarticular diseases.

We identified and characterized, for the first time in sea urchins, galectin-8, a new member of the tandem repeat galectin family, in the assumption that it might be involved in biomineralization such as other members of the galectin family found in mammalian osteoblasts and osteocytes. We measured the carbohydrate binding activity of the recombinant protein as well as its ability to promote cell adhesion, indicating that it might operate as a ligand involved in cell-matrix interactions. Interestingly, Galectin-8 has been identified by proteomic analysis in adult adhesive organs (tube feet), thus indicating that the protein is being used for substrate attachment in different life stages (Santos et al., 2013), and suggesting its potential medical application as new dental bio-adhesive.

Similarly, in our laboratory carbonic anhydrase has been molecularly and biologically characterized in the sea urchin embryo. Its expression in the PMCs, and occurrence in the proteome of adults (Mann et al., 2008a) strongly suggested a role in biomineralization. In fact, the sea urchin carbonic

anhydrase would promote the formation of solid $CaCO_3$ through the acceleration of CO_2 hydration rate, which is naturally a slow process, resulting in calcite crystal formation. Further analysis on its biological activity is under way.

In conclusion, recombinant galectin and carbonic anhydrase, as well as other proteins described herewith to be involved in sea urchin skeletogenesis, might inspire future biomedical applications, thus proving to be a great promise for dental or orthopaedic applications such as bone grafts, tendon/ligament repair, regenerative medicine.

Concluding Remarks

Classical studies on the physiology on biomineral formation in the sea urchin embryo state that spicules are formed inside a syncytium produced by specialized cells (PMCs), deposited within a privileged extracellular space created by the fused filopodial processes of the PMCs (Wilt, 2005). However, despite many studies for more than a hundred years, unravelling the complexity of this well orchestrated phenomenon has not been simple. Thus, more studies are needed to understand the cellular events playing a role in biomineralization, including those related to the mechanisms of transformation of amorphous calcium carbonate into calcite, recently well addressed in a few laboratories (Politi et al., 2008). In addition, the cells that produce these elements express specific genes under a complex signaling control of transcription and growth factors, whose networks (GRN) description is increasing in dimension and complexity over the years (Ettenhson, 2009 & 2013). In this review we described the approach we used in our laboratory to dissect the molecular bases of biomineralization, which is the experimental production of skeleton malformations induced by the exposure of early embryos to toxic metals or ionizing radiations. Besides, our last work was directed to identify relevant skelegenesis-related cDNAs, cloning and characterize their cDNA sequences, studying the developmental spatial and temporal gene expression, perform phylogenetic analysis, produce recombinant proteins and specific antibodies, carry out functional assays. A molecular toolset of genes (cDNA), recombinant proteins and specific antibodies is now ready for molecular, biochemical, cellular and physiological studies, with which we expect to contribute to the understanding of the sea urchin embryo biomineralization process.

Finally, we hope to develop new biotechnological approaches to the treatment of biomineral-associated pathologies in humans. This research field seems to be very promising for applications in the treatment of skeletal diseases as demonstrated for example by the osteogenic potential on human osteosarcoma cells (SaOS-2), used as an in vitro model, of recombinant proteins of marine organism origin (Wiens et al., 2010). In fact, these induced the differentiation and expression of genes which are known to control the interaction (cross-talk) of anabolic (osteoblasts) and catabolic (osteoclasts) pathways of human bone cells. Experiments are in progress in our laboratory aimed at the production of recombinant proteins of interest for their use in functional assays in homologous and heterologous systems.

Acknowledgments

The work described has been fully supported by the 7FP EU-ITN BIOMINTEC Project, contract number 215507 to VM. KK has been the recipient of a *Marie Curie* ITN program fellowship in the frame of the above mentioned project. Partial support was obtained by the CNR Flagship Project POM-FBdQ 2011-2013. Authors wish to thank the STEMBIO Department of the University of Palermo, Italy, for providing access to the confocal microscopy facility, and Prof. F. Marin for access and help in the use of the SEM at the UMR 5561 CNRS Biogéosciences, Université de Bourgogne, France. The technical assistance of Mr. Mauro Biondo is also acknowledged.

References

Ağaoğullari D., Kelb D., Gökçea H., Dumana I., Öveçoğlua M.L., Akarsubaşi A.T., Bilgiç D. & Oktard F.N. 2012. Bioceramic Production from Sea Urchins. *Acta Physica Polonica A*, **121**: 23-26.

Alliegro M.C., Black S.D. & McClay D.R. 1992. Deployment of extracellular matrix proteins in sea urchin embryogenesis. *Microscopy Research and Technique,* **22**: 2-10.

Arakaki A., Nakazawa H., Nemoto M., Mori T. & Matsunaga T. 2008. Formation of magnetite by bacteria and its application. *Journal of the Royal Society Interface*, **5**: 977-99.

Bonaventura R., Poma V., Costa C. & Matranga V. 2005. UVB radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. *Biochemical and Biophysical Research Communications*, **328**: 150-157.

Bonaventura R., Poma V., Russo R., Zito F. & Matranga V. 2006. Effects of UV-B radiation on the development and hsp 70 expression in sea urchin cleavage embryos. *Marine Biology*, **149**: 79-86. Erratum in: *Marine Biology* (2007), **150**: 1051

Bonaventura R., Zito F., Costa C., Giarrusso S., Celi F. & Matranga V. 2011. Stress response gene activation protects sea urchin embryos exposed to X-rays. *Cell Stress and Chaperones*, **16**: 681-687.

Bradham C.A. & McClay D.R. 2006. p38 MAPK is essential for secondary axis specification and patterning in sea urchin embryos. *Development*, **133**: 21-32.

Cölfen H. & Mann S. 2003. Higher-order organization by

mesoscale self-assembly and transformation of hybrid nanostructures. *Angewandte Chemie International Edition*, **42**: 2350-2365.

Copley S.D. 2012. Moonlighting is mainstream: paradigm adjustment required. *Bioessays*, **34**: 578-588.

Costa C., Cavalcante C., Zito F., Yokota Y. & Matranga V. 2010. Phylogenetic analysis and homology modelling of *Paracentrotus lividus* nectin. *Molecular Diversity*, **14**: 653-65.

Costa C., Karakostis K., Zito F. & Matranga V. 2012. Phylogenetic analysis and expression patterns of p16 and p19 in *Paracentrotus lividus* embryos. *Development Genes and Evolution*, **222**: 245-51.

Davidson E.H., Cameron R.A. & Ransick A. 1998. Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. *Development*, **125**: 3269-3290.

Deshpande A.S., Fang P.A., Simmer J.P., Margolis H.C. & Beniash E. 2010. Amelogenin-collagen interactions regulate calcium phosphate mineralization in vitro. *The Journal of Biological Chemistry*, **285**: 19277-19287.

Dorozhkin S.V. 2009. Nanodimensional and nanocrystalline apatites and other calcium orthophosphates in biomedical engineering, biology and medicine. *Materials*, **2**: 1975-2045.

Driesch H. 1892. Entwicklungsmechanische Studien I. Der Werth der beiden ersten Furchungszellen in der Echinodermentwicklung. Experimentelle Erzeugung von Theil- und Doppelbildungen. Zeitschrift für wissenschaftliche. *Zoologie*, **53**: 160-178.

Dubois P. & Chen C. 1989. Calcification in echinoderms. In: *Echinoderm Studies* (M. Jangoux & J.M. Lawrence eds), pp.109-178. AA Balkema: Rotterdam.

Duloquin L., Lhomond G. & Gache C. 2007. Localized VEGF signaling from ectoderm to mesenchyme cells controls morphogenesis of the sea urchin embryo skeleton. *Development*, **134**: 2293-2302.

Ettensohn C.A. 2009. Lessons from a gene regulatory network: echinoderm skeletogenesis provides insights into evolution, plasticity and morphogenesis. *Development*, **136**: 11-21

Filosto S., Roccheri M.C., Bonaventura R. & Matranga V. 2008. Environmentally relevant cadmium concentrations affect development and induce apoptosis of *Paracentrotus lividus* larvae cultured in vitro. *Cell Biology and Toxicology*, **24**: 603-610.

Furuhashi T., Schwarzinger C., Miksik I., Smrz M. & Beran A. 2009. Molluscan shell evolution with review of shell calcification hypothesis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **154**: 351-371.

Grimwade J.E., Gagnon M.L., Yang Q., Angerer R.C. & Angerer L.M. 1991. Expression of two mRNAs encoding EGF-related proteins identifies subregions of sea urchin embryonic ectoderm. *Developmental Biology*, **143**: 44-57.

Hamdoun A. & Epel D. 2007. Embryo stability and vulnerability in an always changing world. *Proceedings of the National Academy of Sciences*, **104**: 1745-1750.

Hörstadius S. 1939. The mechanism of sea urchin development studied by operative methods. *Biological Reviews*, **14**: 132-179.

Jaeger J., Irons D. & Monk N. 2008. Regulative feedback in pattern formation: towards a general relativistic theory of positional information. *Development*, **135**: 3175-3183.

Katow H. 1995. Pamlin, a primary mesenchyme cell adhesion protein, in the basal lamina of the sea urchin embryo. *Experimental Cell Research*, **218**: 469-478.

Killian C.E. & Wilt F.H. 1996. Characterization of the proteins comprising the integral matrix of *Strongylocentrotus purpuratus* embryonic spicules. *The Journal of Biological Chemistry*, **271**: 9150-9159.

Killian C.E., Wilt F.H. 2008. Molecular aspects of biomineralization of the echinoderm endoskeleton. *Chemical Reviews*, **108**: 4463-4474.

Knapp R.T., Wu C.H., Mobilia K.C. & Joester D. 2012. Recombinant sea urchin vascular endothelial growth factor directs single-crystal growth and branching in vitro. *Journal of the American Chemical Society*, **134**: 17908-17911.

Kiyomoto M., Zito F., Sciarrino S. & Matranga V. 2004. Commitment and response to inductive signals of primary mesenchyme cells of the sea urchin embryo. *Development, Growth and Differentiation*, **46**: 107-114.

Kiyomoto M., Zito F., Costa C., Poma V., Sciarrino S. & Matranga V. 2007. Skeletogenesis by transfated secondary mesenchyme cells is dependent on extracellular matrix–ectoderm interactions in *Paracentrotus lividus* sea urchin embryos. *Development, Growth and Differentiation*, **49**: 731-741.

Lemos A.F., Rocha J.H.G., Quaresma S.S.F., Kannan S., Oktara F.N., Agathopoulosa S. & Ferreira, J.M.F. 2006. Hydroxyapatite nano-powders produced hydrothermally from nacreous material. *Journal of the European Ceramic Society*, **26**: 3639-3646.

Livingston B.T., Killian C.E., Wilt F., Cameron A., Landrum M.J., Ermolaeva O., Sapojnikov V., Maglott D.R., Buchanan A.M. & Ettensohn C.A. 2006. A genome-wide analysis of biomineralization related proteins in the sea urchin *Strongylocentrotus purpuratus*. *Developmental Biology*, **300**: 335-348.

Logan C.Y., Miller J.R., Ferkowicz M.J. & McClay D.R. 1999. Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development*, **126**:345-357.

Lyons D.C., Kaltenbach S.L. & McClay D.R. 2012. Morphogenesis in sea urchin embryos: linking cellular events to gene regulatory network states. *WIREs Developmental Biology*, **1**: 231-252.

Mann K., Poustka A.J. & Mann M. 2008a. The sea urchin (*Strongylocentrotus purpuratus*) test and spine proteomes. *Proteome Science*, **6**: 22.

Mann K., Poustka A.J. & Mann M. 2008b. In-depth, high-accuracy proteomics of sea urchin tooth organic matrix. *Proteome Science*, **6**: 33.

Matranga V., Di Ferro D., Zito F., Cervello M. & Nakano E. 1992. A new extracellular matrix protein of the sea urchin embryo with properties of a substrate adhesion molecule. *Development Genes and Evolution*, **201**: 173-178.

Matranga V., Zito F., Costa C., Bonaventura R., Giarrusso S. & Celi F. 2010. Embryonic development and skeletogenic gene expression affected by X-rays in the Mediterranean sea urchin *Paracentrotus lividus*. *Ecotoxicology*, **19**: 530-537.

Matranga V., Bonaventura R., Costa C., Karakostis K., Pinsino A., Russo R. & Zito F. 2011. Echinoderms as blueprints for biocalcification: regulation of skeletogenic genes and matrices. *Progress in Molecular and Subcellular Biology*, **52**: 225-248.

Meldrum F.C. & Cölfen H. 2008. Controlling mineral morphologies and structures in biological and synthetic systems. *Chemical Reviews*, **108**: 4332.

Peter I.S., Faure E. & Davidson E.H. 2012. Predictive computation of genomic logic processing functions in embryonic development. *Proceedings of the National Academy of Sciences*, **109**: 16434-16442.

Pinsino A., Matranga V., Trinchella F. & Roccheri M.C. 2010. Sea urchin embryos as an in vivo model for the assessment of manganese toxicity: developmental and stress response effects. *Ecotoxicology*, **19**: 555-562.

Pinsino A., Roccheri M.C., Costa C. & Matranga V. 2011. Manganese interferes with calcium, perturbs ERK signaling, and produces embryos with no skeleton. *Toxicological Sciences*, **123**: 217-30.

Pinsino A., Matranga V. & Roccheri M.C. 2012. Manganese: a new Emerging Contaminant in the Environment. In: *Environmental Contamination* (Jatin Srivastava ed), pp. 17-36. InTech Open Access Publisher: Rijeka, Croatia.

Pinsino A., Roccheri M.C. & Matranga V. 2013. Manganese overload affects p38 MAPK phosphorylation and metalloproteinase activity during sea urchin embryonic development. *Marine environmental research,* doi: 10.1016/j.marenvres.2013.08.004.

Politi Y., Metzler R.A., Abrecht M., Gilbert B., Wilt F.H., Sagi I., Addadi L., Weiner S. & Gilbert P.U. 2008. Transformation mechanism of amorphous calcium carbonate into calcite in the sea urchin larval spicule. *Proceedings of the National Academy of Sciences*, **105**: 17362-17366.

Roccheri M.C., Agnello M., Bonaventura R. & Matranga V. 2004. Cadmium induces the expression of specific stress proteins in sea urchin embryos. *Biochemical and Biophysical Research Communications*, **321**: 80-87.

Roccheri M.C. & Matranga V. 2010. Cellular, Biochemical and molecular effects of cadmium on marine invertebrates: focus on *Paracentrotus lividus* sea urchin development. In: *Cadmium in the Environment* (R.G. Parvau ed), pp. 337-366, Nova Science Publishers, Inc.: New York.

Röttinger E., Saudemont A., Duboc V., Besnardeau L., McClay D. & Lepage T. 2008. FGF signals guide migration of mesenchymal cells, control skeletal morphogenesis [corrected] and regulate gastrulation during sea urchin development. *Development*, **135**: 353-365.

Rozario T. & DeSimone D.W. 2010. The extracellular matrix in development and morphogenesis: A dynamic view. *Developmental Biology*, **341**: 126-140.

Russo R., Bonaventura R., Zito F., Schröder H.C., Müller I., Müller W.E.G. & Matranga V. 2003. Stress to cadmium monitored by metallothionein gene induction in *Paracentrotus lividus* embryos. *Cell Stress and Chaperones*, **8**: 232-241.

Russo R., Zito F., Costa C., Bonaventura R. & Matranga V. 2010. Transcriptional increase and misexpression of 14-3-3 epsilon in sea urchin embryos exposed to UV-B. *Cell Stress and Chaperones,* **15**: 993-1001.

Russo R., Zito F. & Matranga V. 2013. Tissue-specificity and phylogenetics of Pl-MT mRNA during *Paracentrotus lividus* embryogenesis. *Gene*, **519**: 305-310.

Russo R., Bonaventura R. & Matranga V. 2013. Time- and dose-dependent gene expression in sea urchin embryos exposed to UVB. *Marine Environmental Research,* doi: 10.1016/j.marenvres.2013.08.006.

Santos R., Barreto A., Franco C. & Coelho A.V. 2013. Mapping sea urchins tube feet proteome - A unique hydraulic mechanosensory adhesive organ. *Journal of Proteomics*, **79**: 100-113.

Sea Urchin Genome Sequencing Consortium 2006. The genome of the sea urchin *Strongylocentrotus purpuratus*. *Science*, **314**: 941-952.

Stenzel P., Angerer L.M., Smith B.J., Angerer R.C. & Vale W.W. 1994. The univin gene encodes a member of the transforming growth factor-beta superfamily with restricted expression in the sea urchin embryo. *Developmental Biology*, **166**: 149-158.

Tesoro V., Zito F., Yokota Y., Nakano E., Sciarrino S. & Matranga V. 1998. A protein of the basal lamina of the sea urchin embryo. *Development, Growth and Differentiation*, **40**: 527-35.

Von Ubisch L. 1937. Di Normale Skelettbildung bei *Echinocyamus pusillus* und *Psamechinus miliaris* und die Bedeutung dieser Vorgänge für die Analyse der Skelette von Keimblatt-Chimären. *Zeitschrift für wissenschaftliche Zoologie,* **149**: 402-476.

Wang X., Schloßmacher U., Wiens M., Batel R., Schröder H.C. & Müller W.E. 2012. Silicateins, silicatein interactors and cellular interplay in sponge skeletogenesis: formation of glass fiber-like spicules. *FEBS Journal*, **279**: 1721-1736.

Wessel G.M., Etkin M. & Benson S. 1991. Primary mesenchyme cells of the sea urchin embryo require an autonomously produced, nonfibrillar collagen for spiculogenesis. *Developmental Biology*, **148**: 261-272.

Wessel G. & Berg L. 1995. A spatially restricted molecule of the extracellular matrix is contributed both maternally and zygotically in the sea urchin embryo. *Development, Growth and Differentiation*, **37**: 517-527.

Wiens M., Wang X.H., Schloßmacher U., Lieberwirth I., Glasser G., Ushijima H., Schreoder H.C. & Mueller W.E.G. 2010. Osteogenic potential of biosilica on human osteoblast-like (SaOS-2) cells. *Calcified Tissue International*, **87**: 513-524.

Wilt F.H. 2002. Biomineralization of the spicules of sea urchin embryos. *Zoological Science*, **19**: 253-261.

Wilt F.H. 2005. Developmental biology meets materials science: Morphogenesis of biomineralized structures. *Developmental Biology*, **280**: 15-25.

Zito F., Tesoro V., McClay D.R., Nakano E. & Matranga V. 1998. Ectoderm cell–ECM interaction is essential for sea urchin embryo skeletogenesis. *Developmental Biology*, **196**: 184-192.

Zito F., Costa C., Sciarrino S., Poma V., Russo R., Angerer L.M. & Matranga V. 2003. Expression of univin, a TGF-beta growth factor, requires ectoderm-ECM interaction and promotes skeletal growth in the sea urchin embryo. *Developmental Biology*, **264**:217-227.

Zito F., Costa C., Sciarrino S., Cavalcante C., Poma V. &

Matranga V. 2005. Cell adhesion and communication: a lesson from echinoderm embryos for the exploitation of new therapeutic tools. *Progress in Molecular and Subcellular Biology*, **39**: 7-44.

Zito F. & Matranga V. 2009. Secondary mesenchyme cells as potential stem cells of the sea urchin embryo. In: *Stem Cells in Marine Organisms* (B.Rinkevic & V. Matranga eds), pp. 187-213. Springer: Dordrecht.

Zito F, Burke R.D. & Matranga V. 2010. Pl-nectin, a discoidin family member, is a ligand for betaC integrins in the sea urchin embryo. *Matrix Biology*, **29**: 341-345.

Zito F. 2012. Role of extracellular matrix in regulating embryonic epithelial-mesenchymal transition. *Biomolecular Concepts*, **3**: 333-344.