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SM30 protein function during sea urchin larval spicule formation

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ABSTRACT

A central issue in better understanding the process of biomineralization is to elucidate the function of occluded matrix proteins present in mineralized tissues. A potent approach to addressing this issue utilizes specific inhibitors of expression of known genes. Application of antisense oligonucleotides that specifically suppress translation of a given mRNA are capable of causing aberrant biomineralization, thereby revealing, at least in part, a likely function of the protein and gene under investigation. We have applied this approach to study the possible function(s) of the SM30 family of proteins, which are found in spicules, teeth, spines, and tests of *Strongylocentrotus purpuratus* as well as other euechinoid sea urchins. It is possible using the anti-SM30 morpholino-oligonucleotides (MO's) to reduce the level of these proteins to very low levels, yet the development of skeletal spicules in the embryo shows little or no aberration. This surprising result requires re-thinking about the role of these, and possibly other occluded matrix proteins.

1. Introduction

The larval endoskeleton of sea urchins is an often studied model system for skeletal morphogenesis and biomineralization. The development of the skeletal spicules, which are secreted by primary mesenchyme cells (PMCs) in the blastocoel of the embryo, has been investigated for a long time by classical techniques of embryology and more recently by tools of modern biophysics and cell and molecular biology (Wilt and Ettensohn, 2007; Killian and Wilt, 2008).

An important set of questions is focused on the role(s) of specialized matrix proteins that are found surrounding, and/or occluded within the mineral of the forming spicules. Indeed, similar questions pervade many currently studied instances of biomineralization, including teeth, bones, shells, carapaces, and spicules found in other phyla. We consider here the role of specialized proteins, dubbed SM30 (for spicule matrix 30 kDa). The *SM30* gene was first isolated as a cDNA from *Strongylocentrotus purpuratus* embryos and used as a marker for primary mesenchyme differentiation (George et al., 1991). The protein encoded by this cDNA was shown to be an occluded spicule matrix protein (Killian and Wilt, 1996). Genomic and molecular analyses revealed that this cDNA encodes a gene that is one of six members of the *SpSM30* gene family, which are designated *SpSM30A* through *SpSM30F* (Livingston et al., 2006; Killian et al., 2010). The SM30 proteins are present in larval spicules and PMCs, as well as teeth, test, and spines, but not in other non-mineralized tissues, except for very low levels of SpSM30F in coelomocytes (Livingston et al., 2006). Three *SpSM30* family members (*SpSM30D*, *E* and *F*) contain inserts in the first exon after amino acid 40. *SpSM30D* has a 73 amino acid insert; *SpSM30 F* has an 83 amino acid insert; *SpSM30E* has a 248 amino acid insert. The alignment of the family members has been presented in work of Killian et al. (2010). The portions of these proteins that are not encoded by exon inserts are very similar to one another (>90%) (Killian et al., 2010). Each of the SpSM30 proteins contains a putative signal sequence, a consensus N-glycosylation site, and a single C-type lectin domain. Despite a high level of amino acid sequence similarity, the calculated pI of the different SpSM30 family members ranges from 5.9 to 10.0 because of the amino acid composition of the inserts.

The proteomes of several mineralized tissues of sea urchins have been recently revealed by mass spectrometry analyzes (Mann et al., 2010), and scores of proteins (more than 200 for spicules) have been found occluded within the calcite mineral; over 50 of these proteins are unique to sea urchin mineralized tissues (Mann et al., 2010; Livingston et al., 2006). There are only a few examinations of the function of proteins occluded or associated with skeletal spicules of sea urchins. These have been briefly reviewed by Adomako-Ankomah and Ettensohn (2011), and by Killian and Wilt (2008). Morpholino anti-sense oligo nucleotides have been used in four instances in studies on sea urchin spicules. The SM50 protein found on the surface and occluded within the spicules has been

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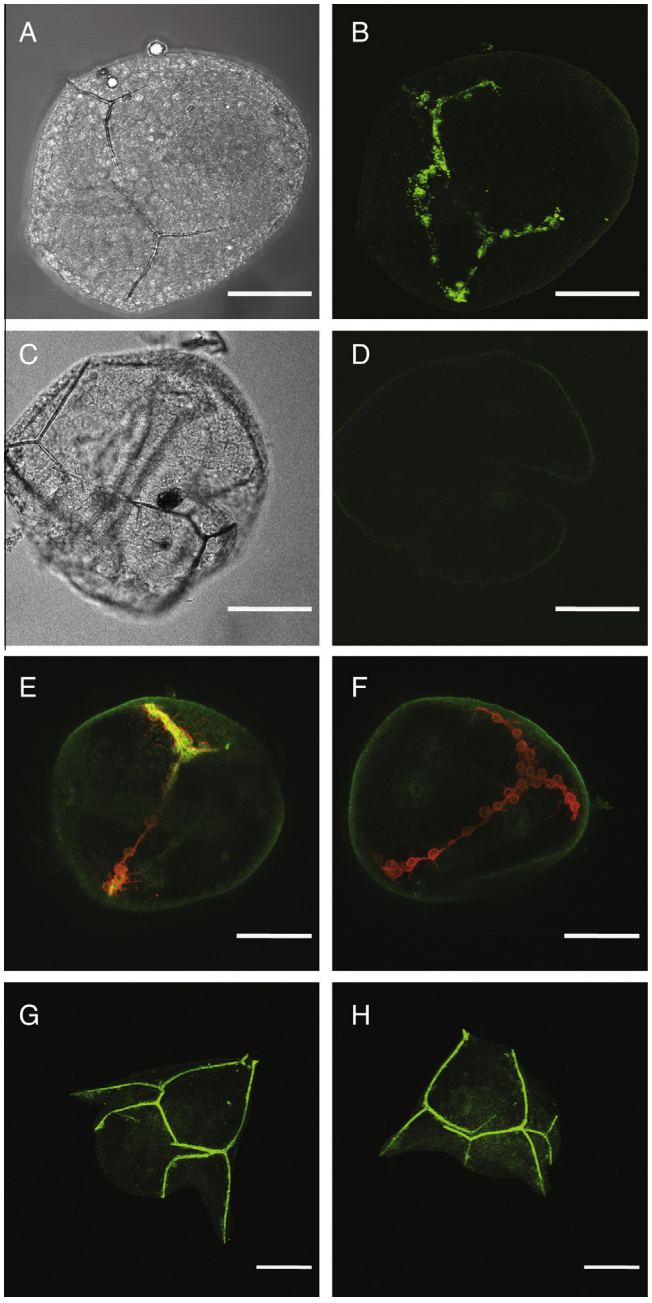

Fig.1. Effects of anti-LpSM30 morpholino injection on *L. pictus* embryo development. One-cell *L. pictus* zygotes were injected with morpholino solutions and examined at 48 hr [A–F] and 72 h [G and H] post-fertilization for effects on development using bright field microscopy as well as fluorescence microscopy of immunostaining. [A] control 48 h embryo; [B] control 48 h embryo stained using anti-LpSM30 antibody; [C] 48 h embryo that was injected with MO_lpsm30; [D] 48 h embryo that was injected with MO_lpsm30 and stained using anti-LpSM30 antibody; [E] control 48 h embryo doubly stained with antibody against LpSM30 (green) and 6a9, an antibody diagnostic for PMC differentiation (red); [F] 48 h embryo that was injected with MO_lpsm30 and doubly stained by reacting with antibody against LpSM30 (green) and 6a9 PMC marker (red); [G] control 72 h embryo reacted with anti-LSM34 antibody (LSM34 is the ortholog of SM50 found in *L. pictus*); [H] 72 h embryo that was injected with MO_lpsm30 and reacted with anti-LSM34 antibody. Scale bars are 50 μm.

shown by this method to be absolutely required for spicule formation (Peled-Kamar et al., 2002; Wilt et al., 2008). Three cell surface proteins found uniquely on PMCs as well as in the spicule matrix, SpP-16, SpP58A, and SpP58B (Cheers and Ettensohn, 2005; Mann et al., 2010; Adomako-Ankomah and Ettensohn, 2011), have also been found to be essential for normal spicule development. Even though these genes encode transmembrane proteins, it is possible that SpP-16, SpP58A and SpP58B could function by proteolytic release of their ectodomain into the mineral phase. A family of glycolipid anchored glycoproteins, the msp130 family, has also been implicated in spicule formation. An antibody directed against the polysaccharide moiety of msp130 was shown to inhibit spicule formation (Carson et al., 1985).

The experiments reported here employed morpholino antisense oligonucleotides (MO) to study SpSM30 function. We find that knock down of SpSM30 protein levels to undetectable levels does not stop biomineralization, spicule formation, or elongation. Some implications of these findings are discussed.

2. Materials and methods

2.1. Embryos and microinjections

S. purpuratus adults were collected from intertidal and subtidal areas near Bodega Bay, California. Adult *Lytechinus pictus* were purchased from Marinus, Inc., Venice, California. Gametes were obtained by injection of 0.5 M KCl. into the coelom. Standard techniques of fertilization and culture of embryos were used (Foltz et al., 2004). Microinjection of oligonucleotides followed the conventional methods for sea urchin eggs (McMahon et al. 1985; Cheers and Ettensohn, 2004). Solutions containing morpholino anti-sense or control oligonucleotides were used at various concentrations (<2 mM) described in the results. Oligonucleotides were obtained from Gene Tools Inc., Philomath, Oregon. Rhodamine B-dextran (10,000 MW neutral) obtained from Molecular Probes, Eugene, Oregon, was included in microinjection solutions (3 mg/ml) in order to judge the relative amounts of solution actually injected into the embryo.

2.2. Morpholino oligonucleotides

Translation blocking morpholinos directed against the start site of translation were used in all cases. Negative controls were carried out by injection of solution without morpholino, or containing a scrambled sequence provided by Gene Tools.

Table 1
Effects of anti-SM30 morpholinos on spicule development.

Prism stage embryos (48 h)			
MO[1]	No. of gastrulae[*]	Spicules (%)[**]	
		Abnormal	Normal
Control	397	4	96
A	47	–	100
B/C	346	–	100
E	39	–	100
A, B/C	49	2	100
A, B/C, E	44	8	92
Pluteus stage embryos (72 h)			
Control	281	4	96
A	65	3	97
B/C	41	2	98
E	26	–	100
A, B/C	94	4	96
A, B/C, E	162	7	93

[1] The concentration of each MO used for microinjection was 1.5 mM, except that injections of two MO's used 1.0 mM for each, and injections of three MOs used 0.7 mM each.

[*] Only embryos that initiated gastrulation within 2 h of companion controls were considered.

[**] Normal spicules: all 3 rays present in the "a" plane + elongated body rod and post-oral rod. Ventral transverse rod initiated. Spicules are birefringent.