

GROWTH FACTORS, HEAT-SHOCK PROTEINS AND REGENERATION IN ECHINODERMS

M. PATRUNO¹, M. C. THORNDYKE^{1,*}, M. D. CANDIA CARNEVALI², F. BONASORO² AND P. W. BEESLEY¹

¹*School of Biological Science, Royal Holloway, University of London, Egham TW20 OEX, UK and*

²*Department of Biology, University of Milan, Milano Via G. Celoria 26, 20133 Italy*

*Author for correspondence (e-mail: m.thorndyke@rhul.ac.uk)

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Summary

The study of regeneration in armed echinoderm species, including crinoids, ophiuroids and asteroids, is attracting increasing attention. Recent interest has focused on the presence and potential role of growth factors, including members of the nerve growth factor (NGF) and transforming growth factor-beta (TGF- β) families, in the regenerative process and their possible relationship to the normal developmental (ontogenetic) regulatory cascade. In addition, the expression patterns of the heat-shock family of stress proteins (Hsps) during regeneration are also

important. Their role forms part of a normal stress response to the trauma of autotomy in combination with a putative function in tissue remodelling and associated protein turnover during regeneration. The temporal dynamics of the stress response may also be strongly indicative of environmentally adaptive pressures operating on these systems.

Key words: regeneration, echinoderm, stress protein, growth factor, bone morphogenic protein (BMP).

Introduction

One of the most familiar features of echinoderm functional morphology and physiology is their remarkable facility for regeneration (Emson and Wilkie, 1980). Regeneration in echinoderms is found throughout the phylum and forms an integral part of their adaptive repertoire. For example, in asteroids it fulfils not only a repair facility but also, in some species, allows for asexual reproduction (Emson and Wilkie, 1980; Thorndyke et al., 1999). In both ophiuroids and holothuroids it underlies an important 'sacrificial defence' purpose. When holothuroids are threatened by predators they will eject much of their gastrointestinal tract and replace it by proliferative activity arising largely from the mesenteric epithelium (Garcia-Ararras et al., 1998; Garcia-Ararras et al., 1999). The phenomenon of regeneration has perhaps reached its greatest adaptive development in some ophiuroid families. The amphiuroid group of brittlestars includes benthic species such as *Amphiura filiformis*, which form a significant and important part of the biomass (Sköld and Rosenberg, 1996). Their feeding mechanism makes them especially vulnerable to predation by flatfish and crustaceans, and they are a crucial part of the benthic food chain being a primary source of food for bottom-dwelling fish. Estimates predict that in certain areas of the Skagerrak their lost arms contribute as much as 300 metric tonnes of biomass per year (Sköld and Rosenberg, 1996). Following arm loss, the missing part is replaced rapidly by regeneration, and it is common to find natural populations in which almost every individual present shows evidence of one, two or more current arm regeneration events (Thorndyke et al.,

1999). While it is clear that regeneration is an entirely natural phenomenon, several questions deserve attention. For example, to what extent are the events of arm autotomy and the subsequent regeneration process stressful, as with other environmental challenges such as temperature variation that impact upon an animal's physiology (Feder, 1999a; Patruno et al., 2000a)?

The intracellular protein turnover experienced by echinoderms during the arm regeneration process has been recently studied (Patruno et al., 2000a). Molecules such as ubiquitin and heat-shock proteins (for example Hsp70) were used to determine whether any changes in their expression are correlated with a particular phase of regeneration. We know from other studies that Hsps, which are encoded by highly conserved families of genes, play key roles not only in the correct folding and degradation of proteins but also during development (Becker and Craig, 1994). A specific example is *Drosophila melanogaster*, where small increases in Hsp70 levels during development enhance thermotolerance (Feder, 1999b); however, if overexpression of the Hsp70 gene is induced, larval mortality increases and development slows down (Krebs and Feder, 1997). Therefore, it might be expected that Hsps are involved in regeneration 'stress' (Patruno et al., 2000a; Feder, 1999b). Furthermore, regeneration can involve rapid and considerable growth phases where, in some individuals of *A. filiformis*, rates of arm replacement can approach 0.04 mm per day (J. Mallefet and M. C. Thorndyke, unpublished observations). Here, growth factors and other

regulators of cell proliferation and differentiation, such as members of the transforming growth factor-beta family of proteins (TGF- β), probably play an important part in the process. TGF- β family members are a diverse group of secreted proteins widely involved in many aspects of growth regulation (Kingsley, 1994; Hogan, 1996). They may act as humoral, paracrine or autocrine agents in all life cycle stages from embryogenesis to adult wound healing.

The purpose of the current study was to explore the putative role(s) of growth factors and Hsps in echinoderm regeneration and assess the extent to which regeneration is a stress response that might be related to a natural environmental niche. That is, could there be a difference in the regeneration stress response between those animals living in a normally more stressful (changing) environment and those from more stable environments?

Materials and methods

Samples of crinoids and ophiuroids were collected from both Bangor (North Wales) and Kristineberg Marine Research Station (Sweden). Samples of *Asterias rubens* collected at Bangor from the intertidal zone during a low tide (intertidal group) were processed *in situ* or transported and maintained in a circulating artificial seawater system as described (Moss et al., 1998). Other samples of *A. rubens* collected by scuba divers in Wales and Sweden were processed immediately or kept in a circulating seawater system (benthic group).

Thermal stress

To determine the effect of thermal stress on expression of heat-shock proteins (Hsps), some normal (non-regenerating) animals were kept at a higher temperature compared to natural conditions (water temperature in the Gulf of Taranto was 15 °C at 20 m depth and at Kristineberg, 12 °C at the same depth). In order to induce a temperature-related stress response these animals were subjected to a gradual increase in temperature. They were transferred to pre-heated tanks of seawater (from 1 to 5 °C higher than their natural habitat) and left for at least 30 min followed by subsequent recovery for 1 h at normal temperature. Samples of crinoids and ophiuroids were prepared from whole arms (normal and regenerating) and, at later stages, from regenerating blastema only. Samples of asteroids were prepared from whole normal arms and whole regenerating arms. The radial nerve cord (rnc) was also dissected and analysed for ubiquitin and hsp70 immunoreactivity. 'Whole arm preparation' refers to the complete arm with the rnc removed.

Gel electrophoresis and western blotting

Samples were homogenised in 10 mmol ml⁻¹ sodium phosphate buffer (pH 7.0) containing the following protease inhibitors: leupeptin, pepstatin, chymostatin and antipain (all at 1 mg ml⁻¹ final concentration) and phenylmethylsulphonyl fluoride (0.2 mmol l⁻¹ final

concentration). The calcareous deposits present in the homogenates were sedimented by low-speed centrifugation at 1000 g_{av} for 1 min. Prior to electrophoresis, protein determinations were made using a Bio-Rad protein assay (Bio-Rad Labs). Equal amounts of protein extract (from 20 to 60 µg per lane, depending on the antibody used) were then separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Laemmli, 1970). Gels were stained with Coomassie Blue for protein visualization. Western blotting was essentially as described (Towbin et al., 1979).

Immunodevelopment of western blots with RHUB1 anti-ubiquitin monoclonal antibody (mAb, neat hybridoma supernatant) was carried out as described (Flann et al., 1997; Mimnaugh et al., 1999). The anti-Hsp72 antibody (Stressgen SPA-810) was diluted 1:500 (v/v) in blocking solution and blots incubated overnight in the primary antibody. The anti-Hsp70 antibody (Sigma, clone BRM-22) was provided by Dr V. Matranga (C.N.R. Palermo, Italy) and used as suggested by the manufacturer. Following incubation in primary antibody, blots were washed in phosphate-buffered saline (PBS) and incubated in secondary antibody, anti-mouse horseradish peroxidase-conjugate (Dako) diluted 1:2000 (v/v) in blocking solution for 1 hour. Blots were washed in PBS (1×15 min followed by 4×5 min) and immunoreactivity visualized using the enhanced chemiluminescence method (Amersham or Pierce). The nitrocellulose membrane was silver stained following immunodevelopment with antibodies (Kovarik et al., 1987). Densitometric analysis of blots (at least three replicate samples for each stage) measured the area under the densitometrically scanned peak utilising a UVP-gel documentation and analysis system. For protein extracts all values quoted are expressed relative to normal arms (P0; 100 %).

Immunocytochemistry

The samples were fixed in 4 % paraformaldehyde, then processed for embedding in resin and subsequent sectioning according to established techniques (Candia Carnevali et al., 1998). After mounting semithin sections on gelatinized slides, they were treated with a resin-removing mixture (1 h in sodium ethanolate). Subsequently sections were rinsed with absolute ethanol, washed in distilled water and then immersed for 7 min in sodium periodate (fresh 1 % solution). Next, sections were treated for 30 min with 0.3 % (v/v) H₂O₂ in phosphate-buffered saline (PBS) in order to exclude the activity of endogenous peroxidases. Following several washes in PBS+Tween20 (0.05 %, v/v) and incubation in 20 % (v/v) normal goat serum (NGS) diluted in PBS+Tween20 (0.05 %, v/v) for 20 min, the specimens were incubated overnight with primary antibodies (RHUB1, neat hybridoma supernatant, or anti-Hsp72 antibody diluted 1:50 in PBS+Tween 20 (0.05 %, v/v)). After overnight incubation in primary antibody, the slides were rinsed in PBS then incubated in the appropriate secondary biotinylated antibody (Vector Labs) diluted 1:200 in PBS+Tween20 (0.05 %, v/v), followed by incubation for 1 h with avidin cross-linked biotinylated horse radish peroxidase (ABC, Vector

Labs). Visualization was by incubation in 0.05% (w/v) 3,3'-diaminobenzidine (DAB, Sigma). For fluorescence visualisation, the ABC reagent was replaced with fluorescein- or Texas Red-conjugated avidin D (Vector Labs). DAB preparations were dehydrated and mounted in DPX. Fluorescence preparations were washed and mounted in Vectashield (Vector Labs).

Observation was by a Zeiss Axioplan with fluorescence attachments and by a Leica TCS-4D confocal laser-scanning microscope (CLSM). Controls were conducted by replacing primary antiserum with non-immune sera from the animal in which the primary antiserum was raised or omitting the primary antibody and incubating in PBS and 1% (v/v) normal NGS. Nuclear labelling was confirmed by using DAPI (Molecular Probes) staining. The specificity of the anti-ubiquitin antibody is well established (Flann et al., 1997).

Some immunocytochemistry was also carried out on paraffin sections, but with the following modifications. Slides were rinsed with xylene and dehydrated in an ascending ethanol series. The step with sodium periodate was omitted and in the blocking step, NGS was used at 5% (v/v).

Results and Discussion

Is regeneration a stressful event for all echinoderms?

A complete and functional regrowth of the arm following amputation depends on many factors. Probably, one of the most important is the site of amputation along the proximal-distal axis of the arm. A traumatic amputation that does not follow the natural autotomy plane may involve more complex reparative/regenerative mechanisms and would therefore be slower than a non-traumatic autotomy (Candia Carnevali and Bonasoro, 1995). Whichever is the chosen regenerative 'pathway', substantial tissue rearrangement and

extensive cellular proliferation/differentiation is observed in all the echinoderm species so far investigated. During the arm regeneration process, these animals may experience initial stress followed by a massive intracellular protein turnover. Table 1 summarises Hsps expression detected in the echinoderm species investigated in this laboratory.

Using an immunochemical approach, two major points emerge from this study. One is the indication that regenerating tissues alone behave differently in terms of Hsp expression compared with the rest of the arm. Indeed, in rapidly regenerating tissues such as the crinoid blastema or the nerve cord in asteroids, the levels of ubiquitin conjugates increase noticeably and in general the pattern is similar to that seen in phylogenetically related systems such as the developing larvae of sea urchins (Pickart et al., 1991). In particular, there is an increase in different isoforms of ubiquitinated histones at 10 and 32 h post-fertilization in the pluteus larva stage of *Strongylocentrotus purpuratus* (Jasinskiene et al., 1995). Other examples of ubiquitinated histone upregulation are observed during spermatogenesis in the chicken (Agell and Mezquita, 1988) and in transformed human cells, in which immunoreactivity is localised to clusters within the nucleus (Vassilev et al., 1995).

The other major point is that the prolonged expression period of Hsp72 (Table 2) during regeneration in intertidal asteroids may reflect the more stressful amputation experienced by starfish compared to brittlestars or featherstars. In crinoids and ophiuroids, Hsp72 is present only briefly after amputation. This short expression period suggests that these animals are not dramatically affected by arm amputation, since this is a more natural phenomenon resulting from high rates of fish predation (Sköld and Rosenberg, 1996). The constitutive form of the Hsp70 family (Hsp73) remained constant throughout the regenerative process, confirming its basic role in cellular metabolism (Table 2).

Table 1. Summary of the changes in levels of ubiquitin conjugates from different regenerating tissues and in heat-treated animals

Time post amputation	Crinoids						Asteroids						Ophiuroids						
	0	24 h	1 w	4 w	6 w	HS	0	48 h	1 w	4 w	6 w	HS	0	24 h	1 w	4 w	6 w	HS	
Regenerating whole arms																			
HMW		+	+	NC	NC	++		NC	NC	NC	NC	++		NC	NC	NC	NC	++	
LMW		--	-	NC	NC	--		--	NC	NC	NC	--		NC	NC	NC	NC	--	
Regenerating blastema																			
HMW		NA	++	++	++	NA	/							NA	NA	+	+	NC	NA
LMW		NA	++	++	++	NA	/							NA	NA	+	+	NC	NA
Regenerating rnc																			
HMW	/							++	++	++	++	NA	/						
LMW	/							++	++	++	++	NA	/						

Ubiquitin conjugates were detected by western blotting.

+, moderate increase compared with control; ++, large increase compared with control; -, moderate decrease compared with control; --, large decrease compared with control; HS, heat-shocked animals; NC, no change compared with control; NA, not assessed; cross-hatching indicates observations not appropriate for this particular species; w, weeks; rnc, radial nerve cord; HMW, high molecular mass ubiquitin conjugates (30–200 kDa); LMW, low molecular mass conjugates (0–20 kDa).

Table 2. Summary of Hsp levels during the arm regenerative process and in heat-shocked *Asterias rubens* from benthic and intertidal zones

Time post amputation	<i>Asterias rubens</i> (Intertidal)								<i>Asterias rubens</i> (Benthic)							
	0	24 h	48 h	1 w	3 w	4 w	6 w	HS	0	24 h	48 h	1 w	3 w	4 w	6 w	HS
Hsp 73	(+)	NC	NC	NC	NA	NA	NA	NA	(+)	NC	NC	NC	NA	NA	NA	NA
Hsp 72	(+)	++	++	++	++	++	NC	++	-	++	++	+	-	-	-	++

(+), presence of Hsp72-Hsp73; +, moderate increase compared with control; ++, large increase compared with control; NC, no change compared with control; -, not detected; NA, not assessed.
HS, heat-shocked animals.

Is the expression of Hsps in echinoderms dependent on an environmental gradient of stress?

In nature, organisms show a variation in the stress response that is dependent on an environmental gradient of stress. Indeed, one important matter is whether organisms from environments with little stress have a different stress response from that of animals living in a more changeable environment. Although some studies (reviewed by Feder and Hofmann, 1999) confirmed that increasing levels of expression of Hsps are positively correlated with stressful environments, the picture is not clear. For example, Hsp expression varies seasonally in some fish (Feder and Hofmann, 1999) and intertidal invertebrates (Hofmann and Somero, 1995). Furthermore, studies on ubiquitin conjugation and Hsp72 expression in *Asterias rubens* collected from a stable (benthic) or variable (intertidal) environment showed (1) no differences in the level and pattern of ubiquitin conjugates between the two populations, (2) a marked elevation of Hsp72 expression in the intertidal compared with the benthic animals and (3), following amputation, that Hsp72 levels were elevated for longer in the intertidal than in the benthic animals. In contrast, crinoid and ophiuroid samples, collected from different (North Sea and Mediterranean Sea) but stable environments, showed no difference in Hsp expression during normal growth and regeneration. These data confirm that the stress response may indeed be environment-dependent in echinoderms as proposed by Feder and Hoffman (Feder and Hoffman, 1999; Hoffman, 1999), but also show how such a conserved mechanism is intimately correlated with multiple patterns of Hsp expression in all phyla.

TGF- β homologues are present in crinoid echinoderms

Growth factors are a comprehensive group of polypeptides, known as multifunctional hormones, which are involved in fundamental cell activities such as proliferation, differentiation and maintenance (Cross and Dexter, 1991). A peculiar characteristic of growth factors is their ability to display multiple properties depending on what has been called the 'cellular context', e.g. according to the type of target cells involved and their synergistic/antagonistic interactions with other factors (Massagué and Wotton, 2000).

Our recent investigation of 'putative' growth factors important for the regenerative process in crinoids has concentrated on TGF- β 1 because of its importance in wound healing in both embryonic and adult vertebrates (Nodder and Martin, 1997; O'Kane and Ferguson, 1997). Other studies support the idea that TGF- β is an important molecule involved in epithelial-mesenchymal interactions, which are fundamental for pattern determination in regenerating systems (Ferretti and Géraudie, 1998). One of the most familiar roles for TGF- β is in wound repair following injury. Here, it plays a part in a variety of associated processes including the regulation of extracellular matrix (ECM) structure and function, cell migration and adhesion. These factors are, therefore, prime candidates for a role in regeneration. The considerable amino acid identity exhibited by mature proteins of the TGF- β superfamily (Fig. 1) has allowed the characterization of echinoderm homologues. To summarise, it seems that TGF- β 1 is a good candidate for one of the growth factors involved in the initialization of wound

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1 CRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCFPFLADHLNSTNHAI VQTLVNSVNS-SI PKACCVPELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
2 CKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGDCFPFLADHLNSTNHAI VQTLVNSVNS-KI PKACCVPELSAISMLYLDENEKVV LKNYQDMVVEGCGCR
3 CKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGDCFPFLADHLNSTNHAI VQTLVNSVNS-KI PKACCVPELSAISMLYLDENEKVV LKNYQDMVVEGCGCR
4 CRRHSLYVDFSDVGWNDWIVAPPGYQAYYCHGDCFPFLADHLNSTNHAI VQTLVNSVNSPLAVPKACCVPTDLSPI SMLYLNENDQVVLKNYQDMVVEGCGCR
5 CQRDLYVDFSDVNWDDWIVAPHG YHAFYCNCECFPLAEYMNATNHAI VQTLVNSVNSDPSLTPKPCCVPELSPIAMLYVDECELVLKTYQQMAVEGCGCR
6 CRRHSLYVDFSDVGWDDWIVAPLGYDAYYCHGDCFPFLADHFNSTNHAVVQTLVNNMNP GKVPKACCVPTQLDSVAMLYLNDQSTVVLKNYQEMTVVGGCGR
7 CRRHPLYVDFREVGWDDWIVAPPGYEGWYCHGDCFPFLSAHMNSTNHAVVQTLVNSMNPGLVPKACCVPTQLTSISMLYLDENEKVV LKNYHEMVA VGGCGR
8 CQRRLPFVDFADVGWSDWIVAPHG YDAYY CQGD CFPFLSDHLNGT NHAI VQTLVNSVNSPAAVPKACCVPTQLSSI SMLYMDENVNVLKNYQDMVVEGCGCR
9 CQRHRLFVSRDVGWENWI IAPMGYQAYYCDGDCFPFLGERLNGT NHAI I QTLVNSIDNRAV PKVCCAPT KLSGISMLYFDNNENVL RQYEDMVEACGCR
*:* :*:.* :*.*:*** **.:**.*.*****. :*.****:***:*.:: ** ** **.* :****.: .***: *:* * .****

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Fig. 1. Alignment of the C-terminal regions of some TGF- β superfamily members. Alignment begins at the first conserved cysteine of each molecule and extends to the C terminus. GenBank accession numbers are: (1) *Homo sapiens* BMP-4, BAA06410.1; (2) *Oryctolagus cuniculus*, AAB96785.1; (3) *Dama species* BMP-2, CAA05033.1; (4) *Branchiostoma floridae*, AAC97488.1; (5) *Halocynthia roretzi*, BAA31132.1; (6) *Drosophila melanogaster* DPP, AAC47552.1; (7) *Schistocerca americana*, AAA81169.1; (8) *Junonia coenia*, AAB46367.1; (9) *Strongylocentrotus purpuratus*-UNIVIN, P48970. Asterisks indicate amino acid identity; dots indicate similarities.

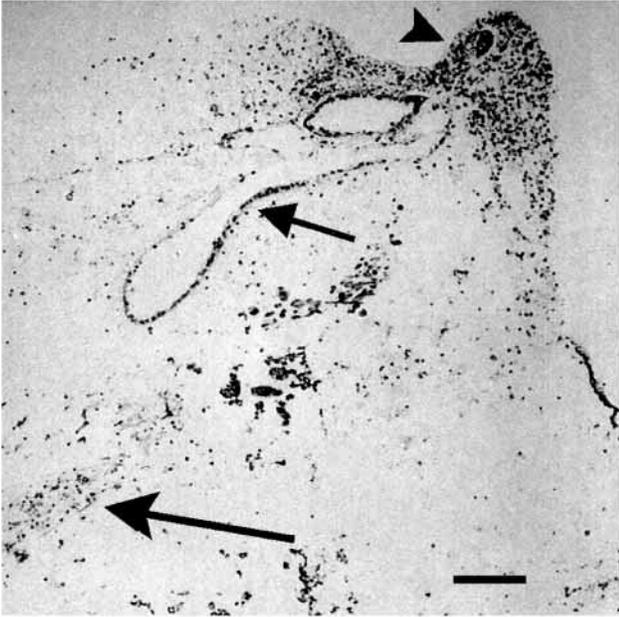


Fig. 2. Immunocytochemical localization of TGF- β 1 using the (MAS729) antibody in regenerating arms of *Antedon mediterranea*. Strong immunoreactivity is observed in the regenerating blastema (arrowhead) and weaker immunoreactivity in the brachial nerve (long arrow) and coelomic epithelium (short arrow) at 1 week post-amputation. Scale bar, 100 μ m.

healing and also for activating the proliferation of migratory elements.

The presence and distribution in normal and regenerating arms of *Antedon mediterranea* and *Antedon bifida* of both a TGF- β -like molecule and its receptors have been detected by immunochemical studies (Patrino et al., 2000b). Increasing amounts of immunolabelling and western blot detection of these molecules at early stages of regeneration suggest that their involvement is important at early regenerative stages (Fig. 2; M. Patrino, M. Thorndyke and P. Beesley, unpublished observations). Less obvious candidates, though possibly more significant, are members of the bone morphogenetic protein (BMP) subfamily, since these are a group of secreted morphogens that play a crucial and central role early in the establishment of body axes (Hogan, 1996). Moreover, they later participate in the regulation of patterning and cell identity in the developing nervous system (Dale et al., 1999; Nguyen et al., 2000). It is not surprising then that BMPs have been identified in echinoderms. Here, emphasis has been placed on the very early stages of development, where BMPs have been shown to be involved in regulating the position of the ectoderm/endoderm boundary as well as epidermal/nonepidermal differentiation (Angerer et al., 2000). To date, BMP homologues have been found only in four echinoderm species. Three BMP2/4 homologues have been identified in echinoids *Triploneustes gratilla* (*TgBMP2/4*) (Hwang et al., 1997), *Strongylocentrotus purpuratus* (*SpBMP2/4*) (Angerer et al., 2000) and *Lytechinus vulgaris* (*LvBMP2/4*) (C. Y. Logan and D. R. McClay, unpublished; Accession Number

AF119712); one BMP2/4 homologue from the asteroid *Asterias rubens* (C. Lelong, M. Mathieu and P. Favrel, unpublished; Accession Number CAB63584); and one BMP5/7 homologue from *Strongylocentrotus purpuratus* (Ponce et al., 1999) and *univin* (Stenzel et al., 1994), also from *Strongylocentrotus purpuratus*. *In situ* hybridisation and mRNA microinjection in *S. purpuratus* confirmed that BMP signalling might be considered as a developmental coordination system homologous with that of vertebrates. In particular, these experiments suggest that BMP2/4 might have a significant role in establishing morphogenetic gradients in echinoderms (Angerer et al., 2000; Angerer and Angerer, 2000). In contrast, the functional role of BMPs during growth in adult echinoderms is still unknown. In this respect, however, our work reported here implicates a role for TGF- β -like factors in adult regeneration. Clearly then, such factors are prime candidates for a role (or roles) in regenerative development. Indeed, new data suggest that a recently characterised crinoid BMP homologue (*AnBMP2/4*) (Patrino et al., 2000b) might be upregulated during regeneration and so play a part in the regulation of patterning and cell lineage specification that occurs at this time.

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