Expression of skeletogenic genes during arm regeneration in the brittle star Amphiura filiformis

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The brittle star Amphiura filiformis, which regenerates its arms post autotomy, is emerging as a useful model for studying the molecular underpinnings of regeneration, aided by the recent availability of some molecular resources. During regeneration a blastema initially is formed distally to the amputation site, and then a rapid rebuild is obtained by adding metamer units, which will eventually differentiate and become fully functional. In this work we first characterize the developmental process of the regenerating arms using two differentiation markers for muscle and skeletal structures – Afi-trop-1 and Afi-zcoll. Both genes are not expressed in the blastema and newly added undifferentiated metamer units. Their expression at different regenerating stages shows an early segregation of muscle and skeletal cells during the regenerating process, long before the metamer units become functional. We then studied the expression of a set of genes orthologous of the sea urchin transcription factors involved in the development of skeletal and non-skeletal mesoderm: Afi-ets1/2, Afi-dlx1, Afi-tbr, Afi-foxB and Afi-gataC. We found that Afi-ets1/2, Afi-dlx1, Afi-foxB and Afi-gataC are all expressed at the blastemal stage. As regeneration progresses those genes are expressed in a similar small undifferentiated domain beneath the distal growth cap, while in more advanced metamer units they become restricted to different skeletal domains. Afi-foxB becomes expressed in non-skeletal structures. This suggests that they might play a combinatorial role only in the early cell specification process and that subsequently they function independently in the differentiation of different structures. Afi-tbr is not present in the adult arm tissue at any stage of regeneration. In situ hybridization results have been confirmed with a new strategy for quantitative PCR (QPCR), using a subdivision of the three stages of regeneration into proximal (differentiated) and distal (undifferentiated) arm segments.

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One class of echinoderms in which the study of molecular mechanisms can be carried out are brittle stars (Class: Ophiuroidea) that can completely regenerate their arms post autotomy in just few weeks. However, not all species of brittle stars are equally easy to manipulate, maintain and use for experimental procedures. It has been shown that rate of regeneration depends on different environmental conditions, feeding habits and species of brittle star (Bower and Keegan, 1983; Nilsson and Sköld, 1996). Amphiura filiformis is a burrowing brittle star that regenerates its arms within only five weeks and can be easily maintained in a laboratory environment. Research into the regeneration mechanism of this particular species has so far focused largely on histogenesis and morphogenesis of the early phases of regeneration (Birelli et al., 2010) and its rate of growth and differentiation (Dupont and Thorndyke, 2006). Recently, the compilation of A. filiformis (Afi) cDNA clone microarray allowed scientists to study molecular mechanisms during the regeneration of the adult arm (Burns et al., 2012, 2011). These studies used microarray and QPCR to measure the differential expression of genes during the three pre-established stages of regeneration – blastema, 50% differentiation and 95% differentiation stage (Dupont and Thorndyke, 2006). Amongst all adult regenerating echinoderms spatial expression data has so far been obtained for three genes implicated in embryogenesis and cancer progression in sea cucumbers (Mashanov et al., 2012), the bmp2/4 gene in crinoids (Patruno et al., 2003), and two signalling molecules from the BMP family in brittle stars (Bannister et al., 1996).

To determine the suitability of this organism to study the molecular basis of regeneration, it is important to be able to draw comparisons to well-known and thoroughly studied systems. One such well-characterized molecular pathway is the sea urchin gene regulatory network (GRN) for the specification of the skeletogenic lineage in the developing embryo (Olliveri et al., 2008). Throughout the echinoderm phylum only echinoids and ophiuroids contain a larval skeleton (Strathmann, 1993). It has also been shown that many of the embryonic genes are also expressed during skeleton development in the juvenile, potentially as a result of an evolutionary adaptation where the embryonic skeleton was co-opted from the adult (Gao and Davidson, 2008). A few genes not present during the formation of the Strongylocentrotus purpuratus (Sp) adult skeleton (including transcription factors Sp-foxB, Sp-foxO and Sp-tbr) could be strictly related to the autonomously specified micromere lineage specific to sea urchin embryos (Gao and Davidson, 2008). During arm regeneration in brittle star new skeletal elements are formed, thus, the sea urchin skeletogenic GRN can provide a good source of transcription factors, signalling molecules and downstream genes with a potential role in skeletogenesis during the brittle star regeneration process.

In this study we describe the expression pattern of a cohort of gene orthologs of sea urchin skeletogenic genes at different stages of A. filiformis arm regeneration. We show that some of the key transcription factors involved in the specification of the sea urchin embryonic skeletogenic cell lineage are likely to be also co-expressed in early phases of regeneration in the brittle star adult. These genes then identify different skeletal structures during final differentiation of the arm. Finally, we show that Afi-t-brain (Afi-tbr) and Afi-foxB are not implicated in adult brittle star skeletogenesis similarly to sea urchin juvenile spine formation. Our results show that A. filiformis can be employed to study the molecular mechanism underlying the vastly interesting and complex biological process that is regeneration.

1. Results and discussion

1.1. Adult brittle star arms: morphology of fully developed and regenerating tissues

The fully developed arm of the brittle star is formed by metameric units, each containing muscles and endoskeleton components, elements of the nervous and water vascular systems all covered by epidermis (Brusca and Brusca, 1990). There are five calcareous structures of the endoskeleton and two muscular structures in each arm segment (Fig. 1a-c and S1a-d). These tissues are divided into externally projecting spines and a pair of orally located podia, the superficial skeletal arm shields (oral, aboral and lateral), the internally localized vertebrae and a set of two intervertebral muscles. The localization of muscle tissue has been confirmed by phalloidin staining in both regenerating and non-regenerating arms (Fig. 1d), which shows strong staining of the intervertebral muscles and podia. The order in which these structures are established during the regenerative process is difficult to assess, however it seems clear from microscopic observations that the externally protruding spines and podia formed from initial buds just below the distal growth zone. Formation of the podia is specifically linked to the elongation of the radial water canal establishing a connection to the remaining water vascular system, which is the main body cavity of the animal (Brusca and Brusca, 1990). The initial phases of the regenerative process, from wound healing to blastema formation, in the brittle star A. filiformis have been previously described in some detail and shown to contain features of both epimorphic and morphallactic processes as identified by microscopic observations of histological sections. In fact, both undifferentiated pluripotent cells and the dedifferentiated myocytes contribute to the formation of the highly proliferative blastema structure, which will generate a new arm (Birelli et al., 2010). So far, little attention has been brought to either the morphogenetic and developmental processes after blastema formation or the gross anatomy of the regenerating arm. Furthermore, the well-understood anatomy of skeletogenic structures in the non-regenerating arm is not sufficient to correctly interpret any potential spatial expression patterns in the regenerating structure. In order to understand the results of a molecular study of gene expression in the process of regeneration, we first observed the initial early stages of regeneration to define the discreet organization of the blastema as well as the development of muscle and skeletal structures. Fig. S2 shows the three commonly used regenerative phases in the brittle star (Dupont and Thorndyke, 2006), which presents its growth and acquiring functional identity along the proximal–distal axis in time. In standard laboratory conditions approximately one week post amputation the blastemal bud is visible distally to the autotomy site. When the 50% differentiation stage is reached, a number of metameric units are added at the proximal end and differentiate in a gradient-like manner, while the distal tip remains highly proliferative and undifferentiated. Therefore, at this stage the miniature arm is constituted by several metameric units each of them at a slightly different developmental stage along the proximal–distal axis. The 95% stage is defined by the appearance of pronounced muscle and skeletal structures along the full length of the new arm with the exception of a few distal-most segments. The final regenerated structure contains all of the constituents of the adult non-regenerating arm. Consistent with previous findings (Birelli et al., 2010), throughout the whole process the distal-most growth zone remains proliferative and undifferentiated until the completion of regeneration. These observations lead us to subdivide the 50% and 95% stage arms into proximal and distal regions containing several segments (as shown by the blue dashed lines in Fig. S2) allowing us to better distinguish between the specification and differentiation events.

1.2. Afi-tropomyosin-1 and Afi-alpha-collagen mark muscle and skeletal structures in the regenerating arm of A. filiformis

Determining the expression pattern of genes during development and regeneration processes has been instrumental for understanding the dynamics of the events and their genetic programme.
So far, there has been limited spatial expression data available not only for this species of brittle star but for adult regeneration in echinoderms overall, while fluorescent in situ hybridization has not, to our knowledge, ever been employed for any adult echinoderms. The whole-mount in situ hybridization (WMISH) techniques used in this study have been compiled and optimized specifically for this species (see Methods).

In order to understand how muscle and skeletal structures arise during the regeneration process in brittle star we first studied the expression of two conserved differentiation markers: tropomyosin-1 (Afi-trop1) and alpha-collagen (Afi-αcoll). The two genes were selected from the Amphiura publicly available sequences (NCBI) on highest sequence similarity to genes with homologous roles in other animals. Both tropomyosin-1 and alpha-collagen are widely conserved genes primarily expressed in muscle and skeletal structures respectively (Lee and Im, 2012; Steinmetz et al., 2012; Tu et al., 2012). They were thus used in this study to initially confirm the applicability of the experimental procedures (Fig. 1e–h). Vertebrate TROPOMYSIN-1 (TPM1) is an actin-binding protein found in striated muscle (Perry, 2001). We identified the homologue of vertebrate TPM1 in the brittle star by BLAST analysis considering the highest similarity score. In proximal segments of both 50% and 95% regenerating arms, Afi-trop1 shows expression in the muscle tissue only as also identified by phalloidin staining (compare Fig. 1e, f and g with Fig. 1d), namely the podia and intervertebral muscles. Notably, the intervertebral muscles are clearly visible in the fully differentiated segments of the 95% regenerating arm (Fig. 1g). In addition, the expression of Afi-trop1 in more distal segments is visible in primordial buds of podia. These metameric units are likely to be already specified, yet showing no fully differentiated structures (see first 6–7 segments expressing Afi-trop1 in Fig. 1e).

The other marker is the sea urchin Sp-fcollII/III/IIIF gene, homologous to the vertebrate col2A1 (type II collagen alpha 1) gene that has been shown to be restricted to the skeletogenic cells at late developmental stages in the embryo (Livingston et al., 2006). We identified the homologue of the sea urchin Sp-fcollII/III/IIIF in A. filiformis (Afi-αcoll). Afi-αcoll expression is localized to spines and lateral arm shields of fully differentiated arm segment as shown by both single chromogenic (Fig. 1h and S3f) and double fluorescent WMISH (Fig. 1e, f). The chromogenic in situ (Fig. 1h, Fig. S3f) shows a pattern of single cells, which is consistent with the mesh-like structure of the echinoderm skeletal elements (i.e. stereomes and spines) in which the cells are embedded (Gorzelak et al., 2011). Double fluorescent in situ of Afi-αcoll and Afi-trop1 reveals no co-localization of these two genes throughout the whole length of the regenerating arm (Fig. 1e, f) at any analysed stage, suggesting an early segregation of skeletogenic and muscle precursor cells during the regeneration process. In the distal half of the
50% regenerating arm, DAPI staining additionally reveals the presence of cells that do not express either \(Afi\)-scoll or \(Afi\)-trop. This is consistent with the presence of other cell types and an early specification and segregation of all various cell types within the metameric unit. In accordance with the differentiation role of these genes, neither \(Afi\)-scoll nor \(Afi\)-trop is expressed in the distal-most unspecified growth zone and the first few distal segments of the 50% regenerating arm (Fig. 1e, brackets). In the 95% regenerating arms only the tip does not express either gene, while \(Afi\)-trop is already visible in the first distinguishable primordial of podia of the distal segment (Fig. 1f). These data support the fact that these markers are appropriate for identifying muscle and skeletal structures during the regeneration process.

1.3. Dynamic expression of transcription factors in discreet domains

We then proceeded to study a set of transcription factor orthologs of genes involved in the specification and differentiation of skeletogenic cells in both larval and adult sea urchins (Oliveri et al., 2008; Gao and Davidson, 2008). This analysis will determine whether the role of any of these regulatory genes in skeletogenesis have been conserved not only in evolution but also between embryonic development and adult regeneration.

The genes used in these experiments are: \(Afi\)-alk1 (Ettensohn et al., 2003), \(Afi\)-foxB (Tu et al., 2006), \(Afi\)-tbr, \(Afi\)-ets1/2 (Koga et al., 2010; Oliveri et al., 2008) and \(Afi\)-gataC (Davidson et al., 2002; Solek et al., 2013). In the sea urchin GRN \(Sp\)-alk1, \(Sp\)-ets1/2 and \(Sp\)-tbr are early zygotic transcription factors that first define the skeletogenic micromere regulatory state downstream of the double-negative gate formed by \(Sp\)-hesC and \(Sp\)-pmar1 (Oliveri et al., 2002). \(Sp\)-foxB on the other hand, begins to be expressed at later stages when the skeletogenic mesenchyme ingress and is directly upstream of the differentiation gene cassette (Minokawa et al., 2004). It has been shown that there is no expression of \(Sp\)-tbr or \(Sp\)-foxB in sea urchin juvenile skeletogenic centres (Gao and Davidson, 2008). \(Sp\)-gataC is primarily a non-skeletogenic mesodermal gene that shows some skeletogenic specificity in the aboral subpopulation of skeletogenic cells at late stages in sea urchin development (Materna and Davidson, 2012; Solek et al., 2013).

The expression of these genes has first been analysed at the early regenerative phase (Fig. 2, Fig. S3a–c). After wound healing, in brittle star regeneration is marked by the formation of the blastema. In its earliest stage, the blastema consists of two layers—the epithelial layer and the inner layer composed of a heterogeneous population of proliferative cells (Fig. 2a; Biressi et al., 2010). According to previously published data (Biressi et al., 2010) and personal observations, as regeneration continues, the radial water canal becomes visible within the blastema and the zone of proliferation shifts to the distal end. When metameric units begin to appear distally to the amputation site, the undifferentiated growth zone is restricted to the distal tip of the blastema and remains there until the end of regeneration. To control for variability each gene expression pattern has been analysed in multiple arm samples per each stage and in at least two independent batches. Only the results that have been repeated are shown here. Interestingly, three of the four skeletogenic genes are expressed in the blastema, with the exception of \(Afi\)-tbr (Fig. 2). \(Afi\)-foxB is expressed in the outer epithelium of the blastema (Fig. 2, Fig. S3b). \(Afi\)-alk1 and \(Afi\)-gataC are both localized to the inner layers of the blastema and are absent from the outer epithelium (Fig. 2, Fig. S3a, c). \(Afi\)-ets1/2 shows expression in the inner layer of the blastema (Fig. 2). Sampling variability should be accounted for, as collected blastemas often vary in length and amount of distinguishable metameric units (Fig. 2).

Further whole-mount analysis of gene expression during later stages of regeneration (Fig. 3, Fig. S3d) reveals that \(Afi\)-tbr is in fact not expressed at any stage in any tissue of the regenerating arm, consistently with QPCR results (see below). On the contrary, \(Afi\)-alk1, \(Afi\)-ets1/2, \(Afi\)-foxB and \(Afi\)-gataC are all highly abundant in the distal arm segments directly in proximity to the undifferentiated growth zone in the intermediate thick layer of both 50% and 95% regenerating arms (Fig. 3 – Distal panels), with \(Afi\)-ets1/2 extending more broadly and distally compared to the other genes. This expression pattern implies that these genes are likely to be co-expressed in at least a subset of cells. This result is consistent with the hypothesis that they might have an early combined role in the initial specification of mesodermally derived cell populations and more specifically in the subpopulation that will give rise to the skeleton. On the contrary, in segmental units of the more advanced regeneration stage (e.g. proximal segments) \(Afi\)-alk1, \(Afi\)-ets1/2, \(Afi\)-foxB and \(Afi\)-gataC acquire differential expression patterns. \(Afi\)-alk1 in 50% proximal segments is restricted to four discrete patches that correspond to the emerging podia and spines; while in the 95% arms, \(Afi\)-alk1 is restricted to the base of the spines (effectively the lateral shield) and the whole length of the podia with higher staining at the base and tip (Fig. 3 – Proximal panels). In differentiating metameric units \(Afi\)-gataC has a strong expression in the lateral shields and becomes restricted to small groups of cells at the base of spines only at the 95% stage. \(Afi\)-ets1/2 contributes early in vertebra development of the 50% regenerating arms, where it has a strongly repetitive, patterned expression (Fig. 3, S3d). Later on, it becomes downregulated and retains a faint expression at the site of vertebrae of the 95% arms. Expression of \(Afi\)-foxB only around the bases of podia both in the 50% and 95% arms suggests that it does not play a role in regenerating any of the skeletal structures in the brittle star. The absence of expression of \(Afi\)-tbr in regenerative arms and the exclusion of \(Afi\)-foxB from skeletal structures is consistent with previous studies in other echinoderms where it has been shown that \(tbr\) and \(foxB\) are not implicated in building the juvenile skeleton (Gao and Davidson, 2008).

We show here that in the proximal segments of adult brittle star regenerating arm, those transcription factors acquire specific expression domains at the sites of the various calcerous structures. This could implicate that after initial specification of mesodermal progenitors, when all the genes are expressed together; they become involved in the differentiation of distinct skeletal tissues.

1.4. Quantitative analysis of expression

Quantification of mRNA transcripts has been previously carried out on complete blastemas, 50% and 95% regenerating arms of \(A\). \(filiforms\) that have been pooled together (Burns et al., 2011). This study aimed to find genes associated with specific stages of regeneration, although those results could not be used to identify the possible roles of mRNAs in the specification or differentiation of distinct structures. We thus subdivided the arms at the 50% and 95% regeneration stage into more refined domains with the intention to separate the proximal differentiated segments from the distal undifferentiated or less differentiated segments (see Fig. S2). This partition was used on samples collected for QPCR and resulted in a more sensitive experimental procedure to identify dynamic changes in expression patterns throughout the arm regeneration process and to validate the observed expression pattern. It needs to be noted that the proximal and middle samples of the regenerates have a larger heterogeneity than the blastemas due to the lateral water canal, and are absent from the outer epithelium (Fig. 2). The data are expressed as relative abundance of transcripts for at least two independent sample batches. \(Afi\)-trop1 temporal expression complements the observable spatial pattern showing upregulation (from 1% in the
Our QPCR data shows that additionally highly expressed in the distal segments of 50% and 95% arms of the samples is highly diluted, even of the cells of the regenerative arms. Thus, the number of transcripts within the total RNA of the samples is highly diluted, even of the cells of the regenerative arms. This is partially consistent with the WMISH data and is likely caused by the expression being restricted to a population of cells at the base of the spines, which represent a very small percentage of cells. The arm samples were collected for RNA extraction or in situ hybridization according to the scheme in Fig. S2. Thirty individual arm samples (2 arms/animal) were pooled together and stored in 1 ml RLT buffer (Qiagen) at 4°C, then washed three times with phosphate-buffered saline (PBS) overnight at 4°C, then washed three times with phosphate-buffered Tween-20 (PBT), and finally transferred to 100% methanol for long-term storage at −20°C.

2. Experimental procedures

2.1. A. filiformis animals and samples

Animals and regenerating arm samples were obtained from the Sven Lovén Centre for Marine Biology in Kristineberg, Sweden. Maintenance and collection was performed as previously described (Bannister et al., 2005). The animals were left to regenerate post-acclimatization for approximately 1 week (for blastemas), 3 weeks (50% differentiation index) and 5 weeks (95% differentiation index) to obtain standardized stages (Dupont and Thorndyke, 2006), after which the arm samples were collected for RNA extraction or in situ hybridization according to the scheme in Fig. S2. Thirty individual arm samples (2 arms/animal) were pooled together and stored in 1 ml RLT buffer (Qiagen) at −80°C for later RNA extraction. Samples for in situ hybridization were fixed in 4% paraformaldehyde (PFA) in 1× phosphate-buffered saline (PBS) overnight at 4°C, then washed three times with phosphate-buffered Tween-20 (PBT), and finally transferred to 100% methanol for long-term storage at −20°C.

2.2. RNA extraction, cDNA synthesis and antisense RNA probe synthesizing

Total RNA was extracted from blastemas, 50% proximal and distal, 95% proximal, middle and distal segments (n = 30) and 10 whole non-regenerating arms using the RNeasy® micro kit (Qiagen) in accordance with manufacturer’s protocol. First strand cDNA was synthesised using the equivalent of 10 arms total RNA with the iScript™ cDNA synthesis kit (BIORAD), in 20 μl reaction according to manufacturer’s guidelines. Specific PCR fragments were amplified using the High fidelity PCR system (Roche) or by rapid amplification of cDNA ends (RACE) using KAPA Hifi DNA polymerase (Kapa Biosystems). The RACE library was constructed using the First Choice RLM-RACE kit (Invitrogen) from 5, 27 and 72 h cDNA stages. PCR products were then ligated respectively into pGEM®-T Easy (Promega) or pCR®-Blunt II-TOPO® vectors (Invitrogen). Identity and orientation of cloned fragments has been verified by sequencing. All primers were designed for A. filiformis.
sequences using Primer3 software (http://frodo.wi.mit.edu/), all sequences can be found in NCBI Genbank under accession numbers listed in Supplementary material Fig. s4.

Stable cDNA was transcribed in the presence of DIG or fluorescein-conjugated nucleotides to produce RNA antisense probes for fluorescent and chromogenic in situ hybridization (probe sizes ranged from 300 bp to 1.5k bp).

2.3. Whole mount in situ hybridization (WMISH)

The WMISH protocol was optimized from previously described protocols used in different species (Harkey et al., 1988; Tautz and Pfeifle, 1989; Zhu et al., 2001). Three to five regenerating arms were re-hydrated at room temperature (RT) with descending concentrations of ethanol/H$_2$O (70%, 50% and 30%) and then washed 5 times in PBT. The samples were pre-hybridized for 1 h at 45 °C in hybridization buffer (HB) (50% Deionized formamide, 10% PEG, 0.6 M NaCl, 0.02 M Tris pH 7.5, 0.5 mg/ml yeast tRNA, 0.01% Tween-20, 0.05 M EDTA, 1× Denhardst, DEPC-treated H$_2$O) then transferred to hybridization buffer containing 0.02 ng/µl of labelled probe and hybridized overnight at 45 °C. Excess of probe was washed 1× with 75% HB in PBT, 50% HB in PBT and 25% HB in PBT at 45 °C, 2× more in PBT and then three times in 0.1× SSC pH 7.0 (0.015 M NaCl, 0.0015 M sodium citrate, DEPC-treated H$_2$O) at room temperature. The hybridized probe was detected with an appropriate antibody diluted 1:1000 for 1 h at RT. For chromogenic detection the anti-DIG-AP or anti-Fluorescein-AP FAB fragments (Roche) were used as previously described (Minokawa et al., 2004). The progression of staining was frequently checked under the stereomicroscope. For fluorescent staining the Tyramide Signal Amplification (TSA) system (Perkin Elmer; POD-TSA detection system) was used as described in (Croce and McClay, 2006). In both cases, following staining the regenerating arms were washed with 1× PBT containing 0.05 M EDTA and 4× in PBT and stored at 4 °C.

Phalloidin staining was conducted using fixed arms but before storage in methanol. Samples were washed 2× in PBT and 3× in PBS then incubated in 1:500 dilution of phalloidin-Atto 488

![Fig. 3. Spatial expression of transcription factors Afi-alx1, Afi-ets1/2, Afi-foxB, Afi-tbr and Afi-gataC in the regenerating arm. Afi-tbr is not expressed at all in the regenerating arm. All four remaining genes are strongly upregulated directly beneath the undifferentiated distal cap. Proximal expression of Afi-alx1 is localized to the bases of the spines and podia and Afi-gataC only to the base of the spines, both at the 50% and 95% differentiation stages. Afi-ets1/2 shows a repetitive pattern of expression restricted to the site of developing vertebrae in the proximal regions of the regenerating arm. Expression of Afi-foxB is localized to the non-skeletogenic structures (around the base of the podia). Scale bar: 100 µm. P – proximal, D – distal. Arrowheads – site of developing spines, arrows – site of developing podia, asterisks – intervertebral muscles.](image-url)
Sigma solution at 4°C overnight. The next day the samples were washed 4× in PBS. All images were obtained using the AxioImager A1 fluorescent microscope (Zeiss).

2.4. QPCR

Quantitative PCR was carried out on cDNA synthesized from regenerating arm samples in accordance to the method employed for sea urchin embryos (Rast et al., 2002). At least four experimental replicas were used for each combination of cDNA and primers and data were considered only if the replicas have a standard deviation less than 1.00. 18S rRNA was used as the internal standard and negative controls were included in each run. Power SYBR® Green QPCR master mix (Applied Biosystems) was employed along with primers (2.5 pmol/μl) designed as described before (Rast et al., 2002). The QPCR was run on a 384-well plate using the 7900HT Fast Real-Time PCR system (Applied Biosystems).

3. Conclusion

In this study we characterize the dynamics of the developmental processes during A. filiformis arm regeneration and the spatio-temporal expression pattern of several genes. The data described here shows that brittle stars can be employed for studying molecular regulation of the regenerative process.

Firstly, in situ hybridization analysis reveals that upstream transcription factors orthologous to sea urchin embryonic skeletogenesis genes (Afi-alx1 and Afi-ets1/2) are also expressed during the formation of skeletal structures (e.g. vertebrae, spines and shields) in the brittle star regenerating arm. The only exception is Afi-tbr, which is not expressed in A. filiformis arm regeneration. This is consistent with the idea that the co-option of tbr in the skeleton forming cells only is specific for sea urchin embryos and its ancestral regulatory role is not in specification or differentiation of the skeletogenic cells. In fact, Sp-tbr is generally expressed in endomesodermal cell types in other echinoderms including sand dollars (Minemura et al., 2009) and it is not expressed in juvenile skeletogenesis (Gao and Davidson, 2008), which represents the plesiomorphic character of the Echinodermata. Regulatory network analysis in S. purpuratus (Oliveri et al., 2008; Rafiq et al., 2012) shows that Sp-tbr gene only inputs in a few “skeletogenic” genes (e.g. p16rel1 and 2) in agreement with a relatively recent role during echinoid skeletogenesis. The remaining transcription factor genes (Afi-ets1/2, Afi-alx1, Afi-gataC and Afi-foxB) are highly upregulated in the blastema and the distal, differentiating segments of the 50% and
95% arms. Although Afi-foxI is present in adult regenerating tissue, it is not localized in skeletogenic tissue/cells during differentiation. This result complements previous studies, which show the lack of expression of this gene during the building of the adult skeleton (Gao and Davidson, 2008). The expression of Afi-ak1, Afi-ets1/2 and Afi-gataC beneath the growth cap suggests that these genes could have a role in early specification of mesodermal cell types and more specifically in skeletogenic lineage progenitors. On the contrary, in proximal segments characterized by a more advanced developmental stage and the appearance of muscle, skeletal and other more differentiated structures, the three genes acquire specific expression patterns and mark distinct calcereous tissue suggesting a non-combinatorial role of these genes. Afi-ak1 is specifically expressed in the base of spines and podia, while Afi-ets1/2 is highly transcribed in cells that coincide with the shape and position of the vertebrae. Afi-gataC is primarily localized to the lateral shields and just the base of spines in the late regenerative phase. On the contrary, the combinatorial regulatory role of Afi-ak1 and Afi-ets1/2 provides inputs in most of the sea urchin embryonic “skeletogenic” differentiation genes (e.g. SMO5, p19) as shown by GRN analysis (Oliveri et al. 2008; Rafiq et al. 2012); it is also present during sea urchin juvenile skeletogenesis; and it is evolutionary conserved in other echinoderm classes (Gao and Davidson, 2008).

Secondly, the optimized staging method to distinguish between proximal (differentiated) and distal (undifferentiated) segments used in QPCR experiments has provided informative data on the dynamics of expression along the whole length of the regenerating arm and complements spatial patterns. Further development of techniques for functional gene analysis will certainly enrich the molecular toolbox available for A. filiformis, although the results of this study already provide a good starting point for future research into deuterostome regeneration and increase our understanding of the evolution and mechanisms of this process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gep.2013.09.002.

References

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud’homme, B., Rosa, R., 2000. The new animal phylogeny: reliability and implications. Proceedings of the National Academy of Sciences of the United States of America 97, 1–4.

Bannister, R., McConnell, I., Graham, A., Thorndyke, M., Bressey, P., 2005. Afuni, a novel transforming growth factor-beta gene is involved in arm regeneration by the brittle star Amphiura filiformis. Developmental Genes and Evolution 215, 1–9.

Bannister, R., McConnell, I., Graham, A., Thorndyke, M., Bressey, P., 2008. Coelomic expression of a novel bone morphogenetic protein in regenerating arms of the brittle star Amphiura filiformis. Development Genes and Evolution 218, 1–6.

Biressi, A., Zou, T., Dupont, S., Dahlberg, C., Benedetto, C., Bonasoro, F., Thorndyke, M., Carnevali, M., 2010. Wound healing and arm regeneration in Ophiodermia longicaudata and Amphiura filiformis (Ophiuroidea, Echinodermata): comparison of histology and histogenesis. Zoomorphology 129, 1–10.

Bowmer, T., Keegan, B., 1983. Field survey of the occurrence and significance of regeneration in Amphiura filiformis (Echinodermata: Ophiuroidea) from Galway Bay, west coast of Ireland. Marine Biology 74, 1–7.

Brusa, R.C., Brusa, G.J., 1990. Invertebrates. Sinauer Associates, Sunderland, MA.

Burns, G., Ortega-Martinez, O., Dupont, S., Thorndyke, M., Peck, L., Clark, M., 2012. Intrinsic gene expression during regeneration in arm explants of Amphiura filiformis. Journal of Experimental Marine Biology and Ecology 413, 1–7.

Carnevali, M., Bonasoro, F., 2001. Microscopic overview of crinoid regeneration. Microscopy Research and Technique 55, 1–24.

Carnevali, M.D., 2006. Regeneration in echinoderms: repair, regrowth, cloning. IJS 55, 403–426.

Crocce, J., McClay, D., 2006. The canonical Wnt pathway in embryonic polarity. Seminars in Cell & Developmental Biology 17, 168–174.

Davidson, E., Rast, J., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.-H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otm, O., Brown, C., Livi, C., Lee, P., Revilla, R., Schultra, M., Clarke, P., Rust, A., Pan, Z., Aronne, M., Rowen, L., Cameron, R., McClay, D., Hood, L., Bolouri, H., 2002. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. Developmental Biology 246, 1–29.

Dolmatov, I., Ginnamon, T., 2001. Muscle regeneration in holothurians. Microscopy Research and Technique 55, 1–12.

Dubois, P., Ameye, L., 2001. Regeneration of spines and pedicellariae in echinoderms: a review. Microscopy Research and Technique 55, 1–11.

Dupont, S., Thorndyke, M., 2006. Growth or differentiation? Adaptive regeneration in the brittlestar Amphiura filiformis. Journal of Experimental Biology 209, 1–9.

Edmondson, C.H., 1915. Autonomy and regeneration of Hawaiian starfishes. Bishop Museum Occasional Papers 11, 3–20.

Ettensohn, C.A., Ili, M.R., Oliveri, P., De Jong, D.L., 2003. Alx1, a member of the Cdx/Pdx/Alx subfamily of Paired-class homeodomain proteins, is an essential component of the gene network controlling skeletogenic fate specification in the sea urchin embryo. Development 130, 2917–2928.

Gao, F., Davidson, E., 2008. Transfer of a large gene regulatory apparatus to a new echinoderm. Development Genes and Evolution 218, 1–6.

Garey, J., Swalla, B., 2000. Special feature: exploring the deuterostome regeneration and increase our understanding of the evolution and mechanisms of this process.

García-Arias, J., Greenberg, M., 2001. Visceral regeneration in holothurians. Microscopy Research and Technique 55, 1–14.

Gorzelak, P., Stolarski, J., Palczewski, C., Myung, A., 2011. 3Hm labeling of the sea urchin regenerating spine: Insights into echinoderm biomineralization process. Journal of Structural Biology 176, 1–8.

Harkey, M., Whiteley, H., Whiteley, A., 1988. Coordinate accumulation of five homeobox genes in pre-gastrular sea urchin embryos. Developmental Biology 132, 1–12.

Hinman, V., Arenas-Mena, C., Otm, O., Brown, C., Livi, C., Lee, P., Revilla, R., Schultra, M., Clarke, P., Rust, A., Pan, Z., Aronne, M., Rowen, L., Cameron, R., McClay, D., Hood, L., Bolouri, H., 2002. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. Developmental Biology 246, 1–29.

Lee, J.-M., Im, G.-I., 2012. PTH/PTHrP isoforms have differing effect on chondrogenic differentiation and hypertrophy of mesenchymal stem cells. Biochemical and Biophysical Research Communications 421, 1–6.

Livingston, B., Kilian, C., Wilt, F., Cameron, A., Landrum, M., Ermolaeva, O., Sapojnikov, V., Maglott, D., Buchanan, A., Ettensohn, C., 2006. A genome-wide analysis of biomineralization-related proteins in the sea urchin Strongylocentrotus purpuratus. Developmental Biology 300, 335–348.

Mashanov, V., Zueva, O., García-Arias, J., 2012. Expression of Wnt9, TCTP, and Cart1/Alx3/Alx4 subfamily of Paired-class homeodomain proteins, is an evolutionary conserved in other echinoderm classes. Journal of Structural Biology 176, 1–8.

Minemura, K., Yamaguchi, M., Minokawa, T., 2009. Evolutionary modification of T/Bmp1/Tll in sea cucumber visceral regeneration. Gene expression patterns: GEP 4, 403–426.

Miyamoto, N., Komatsu, M., Kiyomoto, M., Koga, H., Matsubara, M., Fujitani, H., Miyamoto, N., Komatsu, M., Kiyomoto, M., Akasaka, K., Wada, H., 2010. Functional evolution of Ets in echinoderms with focus on the evolution of echinoderm larval skeletons. Development Genes and Evolution 220, 107–115.

Nilsson, H., Skold, M., 1996. Arm regeneration and spawning in the brittle star Amphiura filiformis (Echinodermata: Ophiuroidea) during hypoxia. Journal of Experimental Marine Biology and Ecology 413, 1–7.

Oliveri, P., Carrick, D.M., Davidson, E.H., 2002. A regulatory gene network that directs micromere specification in the sea urchin embryo. Developmental Biology 246, 209–228.

Oliveri, P., Tu, Q., Davidson, E., 2008. Global regulatory logic for specification of an echinoderm cell lineage. Proceedings of the National Academy of Sciences of the United States of America 105, 1–8.
