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RESEARCH ARTICLE

pmar1/phb homeobox genes and the evolution of the double-negative gate for endomesoderm specification in echinoderms

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ABSTRACT

In several model animals, the earliest phases of embryogenesis are regulated by lineage-specific genes, such as *Drosophila bicoid*. Sea urchin (echinoid) embryogenesis is initiated by zygotic expression of *pmar1*, a paired-class homeobox gene that has been considered to be present only in the lineage of modern urchins (euechinoids). In euechinoids, Pmar1 promotes endomesoderm specification by repressing the hairy and enhancer of split C (*hesC*) gene. Here, we have identified the basal echinoid (cidaroid) *pmar1* gene, which also promotes endomesoderm specification but not by repressing *hesC*. A further search for related genes demonstrated that other echinoderms have *pmar1*-related genes named *phb*. Functional analyses of starfish Phb proteins indicated that, similar to cidaroid Pmar1, they promote activation of endomesoderm regulatory gene orthologs via an unknown repressor that is not HesC. Based on these results, we propose that Pmar1 may have recapitulated the regulatory function of Phb during the early diversification of echinoids and that the additional repressor HesC was placed under the control of Pmar1 in the euechinoid lineage. This case provides an exceptional model for understanding how early developmental processes diverge.

KEY WORDS: Micro1, Cidaroid, Echinoid, Sea urchin, Starfish, Developmental system drift

INTRODUCTION

The rewiring of gene regulatory networks (GRNs) is essential for morphological evolution. However, not all modifications of a GRN alter the associated morphology. In some cases, different upstream developmental pathways result in a conserved developmental output, a phenomenon known as 'developmental system drift' (True and Haag, 2001; Kalinka and Tomancak, 2012), which is supported by the studies focusing on the 'hourglass model' (e.g. Irie

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and Kuratani, 2014; Hu et al., 2017). However, it is still a mystery why early developmental processes are more variable than later processes. Because morphological evolution is tightly linked with the rewiring of GRNs, a better understanding of the flexibility of GRNs would contribute to a deeper understanding of morphological evolution.

One of the factors that may contribute to early developmental diversification is lineage-specific genes, i.e. genes found in the particular lineage. The regulation of early developmental process by lineage-specific genes has been frequently observed; examples of these genes include *bicoid* in *Drosophila* (Frohnhöfer and Nüsslein-Volhard, 1986), *SPILE* in spiralians (Paps et al., 2015; Morino et al., 2017), *Siamois* in amphibians (Lemaire et al., 1995), *dharma* in zebrafish (Yamanaka et al., 1998) and *CRX*-related genes in mammals (Töhönen et al., 2015; Maeso et al., 2016). However, it is not fully understood how these lineage-specific genes acquired their functions in early embryogenesis, except in the case of the dipteran *bicoid* gene (e.g. Stauber et al., 1999; Kotkamp et al., 2010; Liu et al., 2018). Therefore, we focused on the sea urchin (echinoid) *pmar1/micro1* genes, which are key upstream factors involved in endomesoderm specification during embryogenesis, but have been identified in only one of the two echinoid lineages.

The class Echinoidea is classified into two subclasses: Cidaroida and Euechinoida (see Fig. 8A). These are estimated to have diverged 268.8 million years ago (Thompson et al., 2015). Most developmental research in this group has been performed using species of Euechinoida, such as *Strongylocentrotus purpuratus*, *Lytechinus variegatus*, *Paracentrotus lividus* and *Hemicentrotus pulcherrimus* (McClay, 2011). There are slight differences in early developmental processes between cidaroids and euechinoids; e.g. whereas mesodermal skeletogenic cells ingress before gastrulation in euechinoids, cidaroid skeletogenic mesenchyme cells delaminate after gastrulation (for further details, see Yamazaki et al., 2014). Despite these apparent differences, skeletogenic cells differentiate from cells in the vegetal region in cidaroids, resulting in pluteus larvae with a similar morphology. *pmar1/micro1* (hereafter, *pmar1*) genes have been isolated from a variety of euechinoids, including the sand dollar and heart urchin (Di Bernardo et al., 1995; Kitamura et al., 2002; Oliveri et al., 2002; Ettensohn et al., 2007; Yamazaki et al., 2010; Yamazaki and Minokawa, 2015). However, no *pmar1* gene ortholog has been identified in cidaroids (Yamazaki et al., 2012; Erkenbrack and Davidson, 2015; Dylus et al., 2016) or in other echinoderm species, such as sea cucumber (holothuroid) (McCauley et al., 2012; Thompson et al., 2017), brittle star (ophiuroid) (Dylus et al., 2016) or starfish (asteroid) (McCauley et al., 2010). Dylus et al. (2016) reported the *pplx* gene as a *pmar1*-related gene in brittle star, which we will discuss later.

Euechinoids possess multiple copies of *pmar1* genes, which are tandemly arrayed in the genome. Each of these genes encodes a transcription factor with a paired-type homeodomain that functions

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as a transcriptional repressor with engrailed homology region 1-like (eh1-like) motifs. Although unusual expression of a *P. lividus* ortholog (*hbox12*) in the ectodermal region has been reported (Di Bernardo et al., 1995), all of the other *pmar1* genes are transiently expressed in the micromere-skeletogenic cell lineage at the vegetal pole from the 16-cell stage to the mid-blastula stage as far as examined (Kitamura et al., 2002; Oliveri et al., 2002; Yamazaki et al., 2010; Yamazaki and Minokawa, 2015); i.e. *pmar1* is one of the earliest zygotically expressed genes. In embryos injected with *pmar1* mRNA, almost all cells develop into skeletogenic cells. A comprehensive GRN has been established for the skeletogenic cell lineage of euechinoids; *pmar1* is the most upstream zygotic factor in the network and is directly activated by maternal β-catenin. Pmar1 promotes the specification of skeletogenic cells by repressing a hairy gene, *hesC*, which represses downstream regulatory genes involved in skeletogenesis, such as *alx1*, *tbr*, *ets1* and *delta* (Revilla-i-Domingo et al., 2007; see reviews by Oliveri et al., 2008; Minokawa, 2017; Shashikant et al., 2018; see Fig. 8B, euechinoid). However, recent studies on two cidaroids, *Prionocidaris baculosa* and *Eucidaris tribuloides*, indicated that HesC does not repress skeletogenic regulatory gene orthologs during the early phase of endomesoderm specification; in the *hesC* knockdown cidaroid embryos, expression of *alx1*, *tbr* and *ets1* was not affected at the mid-blastula stage (Yamazaki et al., 2014), although HesC seems to exhibit a regulatory function related to *alx1* expression at the relatively later stage (Erkenbrack and Davidson, 2015), suggesting the absence of the typical double-negative gate of Pmar1 and HesC in cidaroids. Considering the recently proposed hypothesis that the larval skeletogenic cells arose in the common ancestor of eleutherozoan echinoderms (all echinoderms except crinoids) (Erkenbrack and Thompson, 2019), the Pmar1-HesC double-negative gate was likely established after the acquisition of a larval skeleton; i.e. this gate likely evolved independently of one of the novel morphologies acquired during echinoderm evolution.

The purpose of this study is to reveal the evolutionary history of the establishment of the double-negative gate of Pmar1 and HesC in echinoderms, which importantly occurred without changing the expression pattern of key endomesodermal developmental genes, such as *alx1*, *ets1* and *delta*. In the course of the comparative analysis of the GRNs among echinoderm species, we unexpectedly identified cidaroid *pmar1* gene orthologs through temporal RNA-sequencing (RNA-seq) analysis. This prompted us to further examine the *pmar1*-related genes of other echinoderms. Based on the results of expression and functional analyses of these genes, we discuss the evolution of the endomesoderm gene network in echinoderms.

RESULTS
Screening of candidate upstream gene orthologs essential for endomesoderm regulation in the cidaroid
As endomesoderm regulatory genes, *alx1* and *ets1* are expressed in skeletogenic cells in cidaroids (Yamazaki et al., 2014; Erkenbrack and Davidson, 2015). However, the upstream regulatory mechanism has not yet been revealed. To screen candidate genes responsible for regulating the onset of skeletogenic regulatory gene expression in the cidaroid, we performed RNA-seq analysis using embryos of the cidaroid *P. baculosa* at the two-cell (2 hours postfertilization; h), 16-cell (4 h), ~64-cell (6 h), ~240-cell (10 h) and ~500-cell (14 h) stages. In these embryos, the zygotic expression of *Pb-alx1* was first observed at 10 h. Based on changes in the obtained FPKM (fragments per kilobase of transcript per million mapped reads) values, 43 candidate transcription factor genes that were activated

before or simultaneously with *alx1* were selected (Table S1). The detailed criteria for selecting the candidate genes are described in the Materials and Methods section. As shown in Fig. S1, we examined the spatial expression patterns of the candidate genes and found some genes showing mesoderm-specific expression, such as *kruppel-like1* (*krl-like1*) and *kruppel-like2* (*krl-like2*), the euechinoid ortholog of which is not required for skeletogenic cell specification (Yamazaki et al., 2008). In addition, we identified a sequence that showed remarkable similarity to *S. purpuratus pmar1c*. This was unexpected because *pmar1* was thought to have emerged in the common ancestor of euechinoids (Erkenbrack and Davidson, 2015; Thompson et al., 2017). Its transient and relatively low expression may have hidden its existence in previous transcriptome data. We identified a similar sequence in another cidaroid, *Eucidaris tribuloides*, via a BLAST search using the *P. baculosa* sequence as a query against the *E. tribuloides* genome 1.0 sequence at EchinoBase (www.echinobase.org) (Kudtarkar and Cameron, 2017). The sequence was not found in the *E. tribuloides* transcriptome data obtained from EchinoBase.

Identification of cidaroid *pmar1* genes and *pmar1*-related *phb* genes from other echinoderms
Given the existence of the *pmar1* gene in cidaroids, before moving on to the functional analyses of cidaroid *pmar1*, we examined the molecular evolutionary history of *pmar1*. In the euechinoid *S. purpuratus*, the *pmar1*-related *phb1* gene was identified as a paired-class homeobox gene by Howard-Ashby et al. (2006), but a detailed analysis has not been performed. Dylus et al. (2016) reported that *phb1*-related sequences exist in cidaroids, other echinoderms and acorn worms, and demonstrated that *phb1*-like genes and *pmar1* are closely related but are recognized as distinct classes of paired homeobox genes according to phylogenetic analysis. Based on a BLAST search of the assembled transcriptome and genomic sequences (details are described in the Materials and Methods section), we identified 1, 6, 2 and 1 *pmar1*/*phb1*-like sequences in sea cucumbers, brittle stars, starfishes and feather stars (crinoids), respectively.

To evaluate the relationships between these obtained sequences and euechinoid Pmar1, we performed phylogenetic analysis using deduced homeodomain (HD) sequences with several classes of other paired-type genes according to the method of Dylus et al. (2016) (Fig. 1A). Our results showed a monophyletic clade, including the cidaroid *pmar1*-like sequences and euechinoid *pmar1* sequences with a high support value (93%). Accordingly, we designated the genes obtained from *P. baculosa* and *E. tribuloides* as *Pb-pmar1* and *Et-pmar1*, respectively. In addition, the monophyly of the clade including these sequences, *phb1*, and *pmar1* was supported with significant values, suggesting that these genes are paralogs. However, our analyses did not resolve the relationships between these genes, which is often the case when performing phylogenetic analyses with only 60 amino acids of a homeodomain. In particular, the long branches of *pmar1* genes made the tree less resolvable. Some sequences from brittle stars form a clade with sea urchin *pmar1* genes, although this clade is not supported by sufficient values, possibly owing to artificial long branch attraction. Indeed, *pmar1*/*phb1*-related genes show extensive gene duplication in the brittle star lineage, and some of the brittle star genes present an accelerated substitution rate, as reflected by their long branches (such as *Ak-phbC* and *Afi-pplx*). We designated the identified genes from nonurchin echinoderms as follows: for sea cucumbers, *phb*; for the brittle star *Amphipholis kochii*, *phbA* to *phbF*; for starfishes, *phbA* and *phbB*; and for the feather star *Oxycomanthus japonicus*, *phb*.
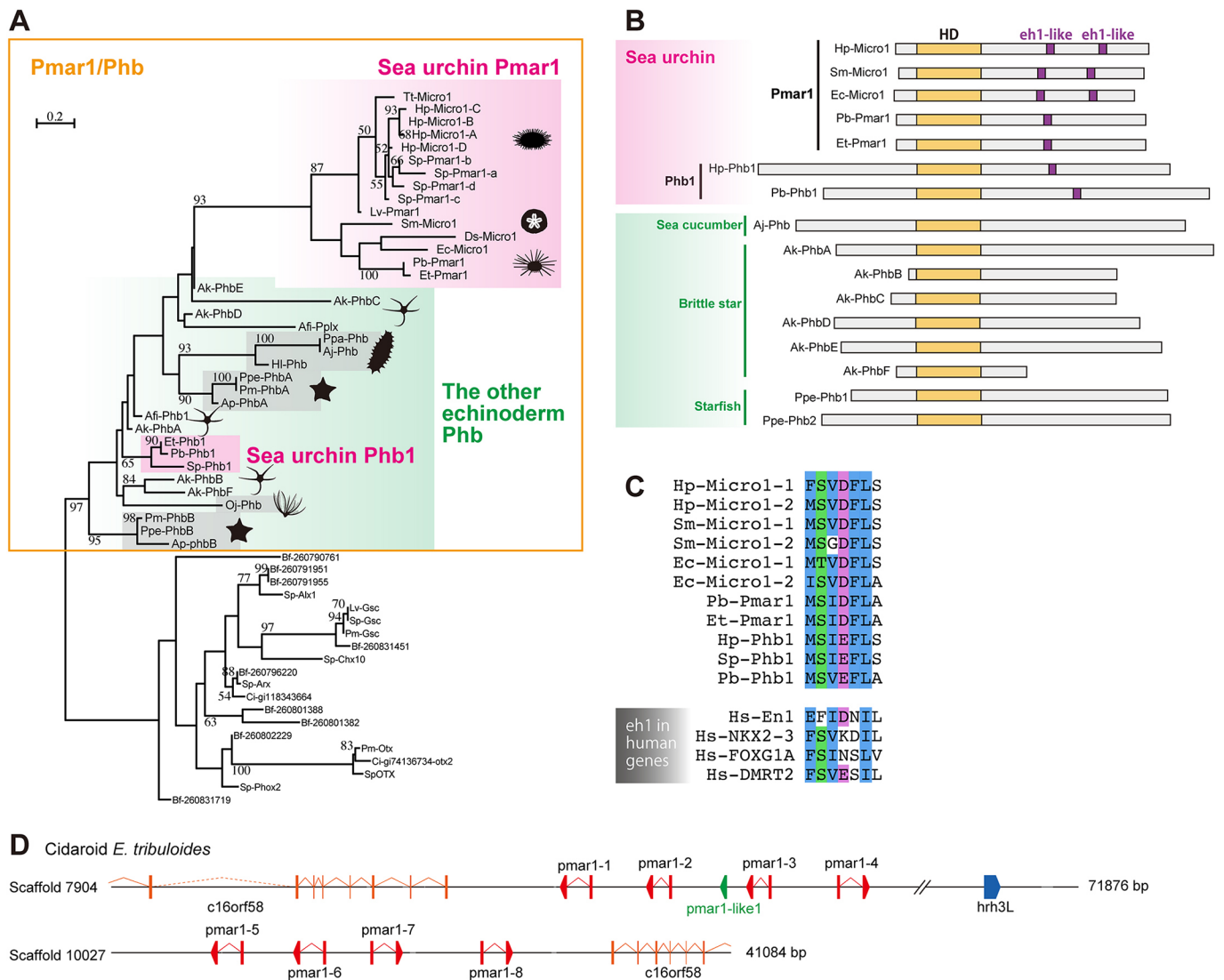
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Fig. 1. Phylogenetic relationships and protein structures of *pmar1* and *phb* in echinoderms and genomic organization of the cidaroid *pmar1*.
(A) Molecular phylogenetic tree of *pmar1* and *phb* genes. The tree was constructed based on the amino acid sequences of the homeodomains (HDs) using the maximum likelihood method. The numbers at the nodes are the bootstrap values (only values≥50% are shown). (B) Comparison of protein structure of Pmar1 and Phb. The HD is shown in orange, and engrailed homology region 1-like (eh1-like) motifs are shown in purple. (C) The amino acid sequences of eh1-like motifs. Human eh1 sequences are shown below the echinoderm sequences. (D) Two scaffolds found in the cidaroid *Eucidaris tribuloides* contain four *pmar1* sequences (red), and these sequences are orientated differently from one another. The scaffold 7904 includes one truncated *pmar1* sequence (green). *Hp*, *Hemicentrotus pulcherrimus*; *Sm*, *Scaphechinus mirabilis*; *Ec*, *Echinocardium cordatum*; *Pb*, *Prionocidaris baculosa*; *Aj*, *Apostichopus japonicus*; *Ak*, *Amphipholis kochii*; *Ppe*, *Patiria pectinifera*; *Sp*, *Strongylocentrotus purpuratus*; *Hs*, *Homo sapiens*; *Tt*, *Temnopleurus toreumaticus*; *Lv*, *Lytechinus variegatus*; *Ds*, *Diadema setosum*; *Et*, *Eucidaris tribuloides*; *Ppa*, *parastichopus parvimensis*; *Afi*, *Amphiura filiformis*; *Pm*, *Patiria miniata*; *Ap*, *Acanthaster planci*; *Oj*, *Oxycomanthus japonicus*; *Bf*, *Branchiostoma floridae*; *Ci*, *Ciona intestinalis*.

The euechinoid Pmar1 proteins commonly contain a HD in the N terminus and two engrailed homology region 1-like (eh1-like) motifs in the C terminus (Fig. 1B). The eh1 motif is a repression motif that interacts with the co-repressor groucho (Copley, 2005). Our previous study demonstrated that these eh1-like motifs are responsible for the repressive function of Pmar1 (Micro1) (Yamazaki et al., 2009). The eh1-like motifs were found only in sea urchin Pmar1 and Phb1, including those of cidaroids, but not in any of the Phb sequences of nonurchin echinoderms (Fig. 1B,C). The cidaroid Pmar1 contains only one eh1-like motif, which is highly conserved compared with euechinoid Pmar1 (Fig. 1B,C).

The paired-type HDs are classified into three subclasses according to the 50th amino acid (glutamine, Q; lysine, K; and serine, S), which is involved in binding sequence preference

(Wilson et al., 1993). HDs with a Q50 are shared by euechinoid Pmar1 and Phb1, whereas *pplx* from another brittle star encodes a HD with an irregular 50th amino acid, histidine (Dylus et al., 2016). Among the Pmar1/Phb sequences identified in this study, one Phb from the brittle star *A. kochii* (Ak-PhbC) includes a K50, whereas the others share a Q50.

In addition, similar to euechinoid *pmar1* (*micro1*) genes (Nishimura et al., 2004; Ettensohn et al., 2007; Cavalieri et al., 2017), the cidaroid *pmar1* gene is extensively duplicated in the genome (Fig. 1D). In the genomic sequence of *E. tribuloides* obtained from EchinoBase, we found two long scaffolds containing sequences similar to the *pmar1* gene (scaffolds 7904 and 10027). Both scaffolds include four copies of *pmar1*-related sequences, whose orientations are different, and one short *pmar1*-like sequence

without a start codon is present at the corresponding position in scaffold 7904. In summary, we identified multicopy *pmar1* genes in cidaroids and *pmar1/phb1*-related *phb* genes from nonurchin echinoderms.

Expression patterns of the cidaroid *pmar1* and *phb* genes from other echinoderms

The euechinoid *pmar1* (*micro1*) genes are commonly expressed transiently in the micromeres in the 16-cell stage and the descendant skeletogenic cells at the vegetal pole (e.g. Yamazaki et al., 2010; Yamazaki and Minokawa, 2015). To examine whether the expression patterns of *P. baculosa pmar1* are similar to those of euechinoid *pmar1*, we performed expression analysis through quantitative PCR (qPCR) and whole-mount *in situ* hybridization. Consistent with the RNA-seq analysis (Table S1), qPCR demonstrated the transient activation of *Pb-pmar1* during early stages; the expression level of *Pb-pmar1* reached a peak during the ~64-cell (6 h) and ~120-cell stages (8 h) (Fig. 2A). Whole-mount *in situ* hybridization showed the vegetal expression of *Pb-pmar1*. Almost no embryos (1/11) at the 16-cell stage (4 h) showed the whole-mount *in situ* hybridization signal (Fig. 2B), although a low level of the transcript was detected by qPCR. At the 32-cell stage (5 h), a subset of embryos (11/24) showed a signal in the smaller blastomeres (arrowheads in Fig. 2C) located in the vicinity of the vegetal pole (Yamazaki et al., 2012). The *pmar1*-expressing cells may differentiate into larval skeletogenic cells

because previous lineage-tracing experiments in another cidaroid, *E. tribuloides*, demonstrated that smaller vegetal blastomeres develop into skeletogenic cells (Wray and McClay, 1988). The signal continued to be detected at 8 h but not at 14 h (Fig. 2D,E). Thereafter, the transcripts remained undetectable up to 36 h by qPCR (Fig. 2A). This expression pattern is very similar to that of euechinoid *pmar1* genes.

We also examined the expression patterns of *phb* genes in the embryos of the starfish *Patiria pectinifera* (Fig. 2F-O), the sea cucumber *Apostichopus japonicus* (Fig. 2P, Fig. S2) and the brittle star *A. kochii* (Fig. 2Q-S, Fig. S2). The expression of the starfish *P. pectinifera phbA* and *phbB* genes (*Ppe-phbA* and *Ppe-phbB*) was first detected at the 200- to 400-cell stage (6 h) (Fig. 2G,L), and both genes showed expression at the vegetal pole of the hatched blastula (Fig. 2H,I,M,N). At the mid-gastrula stage (24 h), *phbA* expression was detected in the region encircling the blastopore (Fig. 2J), which seems to be endoderm lineage, but *phbB* expression was no longer detected (Fig. 2O). The sea cucumber and brittle star *phb* genes showed similar expression patterns: their expression was first detected at the cleavage stage or early blastula stage, and was subsequently maintained in either the mesoderm or endoderm lineage of cells (Fig. 2P-S, Fig. S2). Thus, our analyses showed that all *phb* genes examined are expressed in the endomesoderm region at the vegetal pole. Some of the *phb* genes (*Ppe-phbA*, *Aj-phb* and *Ak-phbA/C*) were detected in the presumptive endoderm region
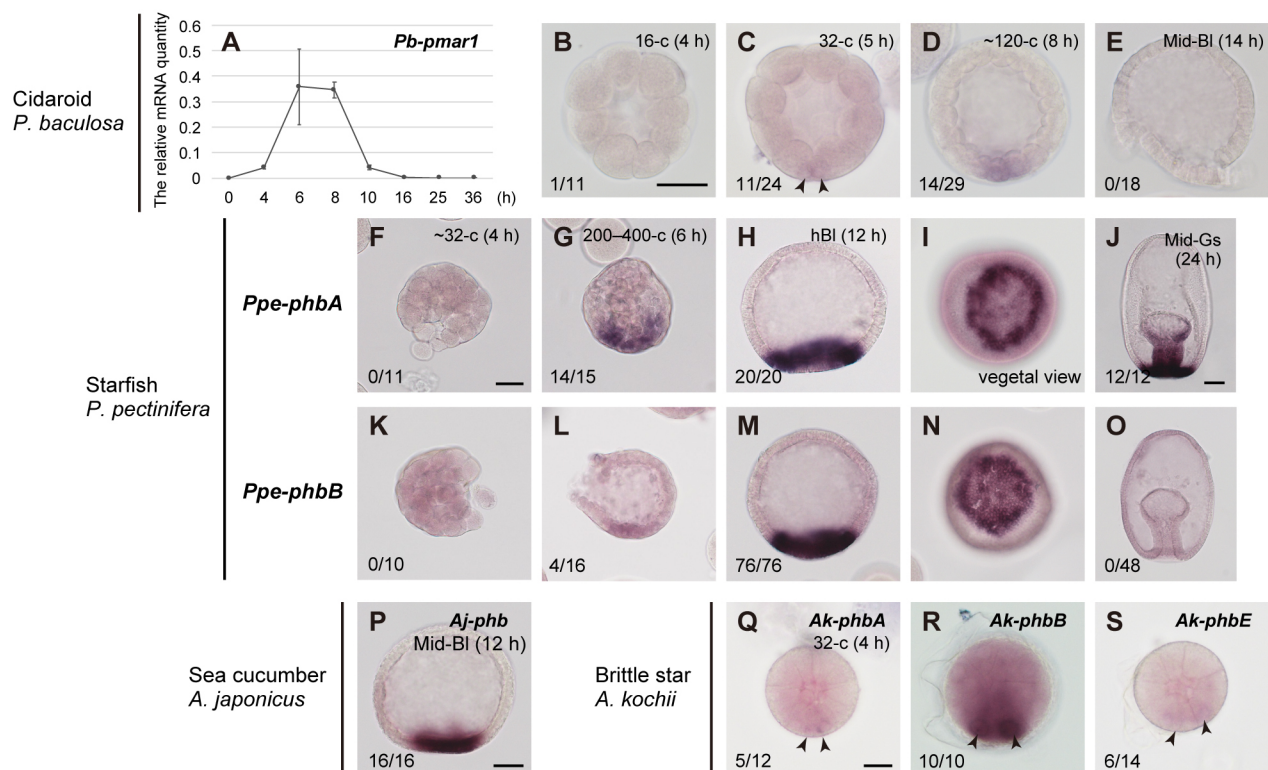

Fig. 2. Embryonic expression of *pmar1* and *phb* in echinoderms. (A-E) Expression of the cidaroid *P. baculosa pmar1* (*Pb-pmar1*). (A) The transcript levels of *Pb-pmar1* were measured by quantitative real-time PCR (qPCR). Data are mean±s.d. (B-E) Expression patterns of *Pb-pmar1* were examined using whole-mount *in situ* hybridization. The signal was detected in the smaller blastomeres at the vegetal pole (arrowheads) of the 32-cell stage (C) and in the presumptive descendant cells at the ~120-cell stage (D). (F-S) Spatial expression of the starfish *P. pectinifera phbA* (*Ppe-phbA*; F-J) and *phbB* (*Ppe-phbB*; K-O), the sea cucumber *A. japonicus phb* (*Aj-phb*; P), and the brittle star *A. kochii phb* genes (*Ak-phbA*, *Ak-phbB* and *Ak-phbE*; Q-S) was examined by whole-mount *in situ* hybridization. Expression of *Ppe-phbA*, *Ppe-phbB* and *Aj-phb* was detected at the vegetal pole of the hatched blastula stage (H,I,M,N,P). Three *phb* genes of the brittle star (*Ak-phbA*, *Ak-phbB* and *Ak-phbE*) were expressed in several blastomeres of the 32-cell stage (arrowheads in Q-S). The numbers shown in the lower left corner of each image are the number of embryos showing whole-mount *in situ* hybridization signals/total number of examined embryos from one batch. Scale bars: 50 μm.

during relatively later stage, which is clearly distinct from the expression patterns of euechinoid *pmar1* genes.

Function of Pmar1 in the cidaroid: conservation and diversification of protein function between cidaroids and euechinoids

The result of the above expression analysis suggests that the function of cidaroid Pmar1 is similar to that of euechinoid Pmar1. To examine whether Pmar1 also controls skeletogenic cell specification in cidaroid embryos, we performed overexpression analysis using *P. baculosa* embryos (Fig. 3). The phenotype of *pmar1* mRNA-injected cidaroid embryos was not identical to that observed in euechinoids (i.e. fate conversion to the skeletogenic cell phenotype in almost all cells), although excess mesoderm cell differentiation was observed. When the control embryos developed into elongated swimming blastulae, Pmar1-overexpressing embryos showed a rather spherical morphology (16 h; Fig. 3A,E). During gastrulation, a broader area of the vegetal side invaginated in
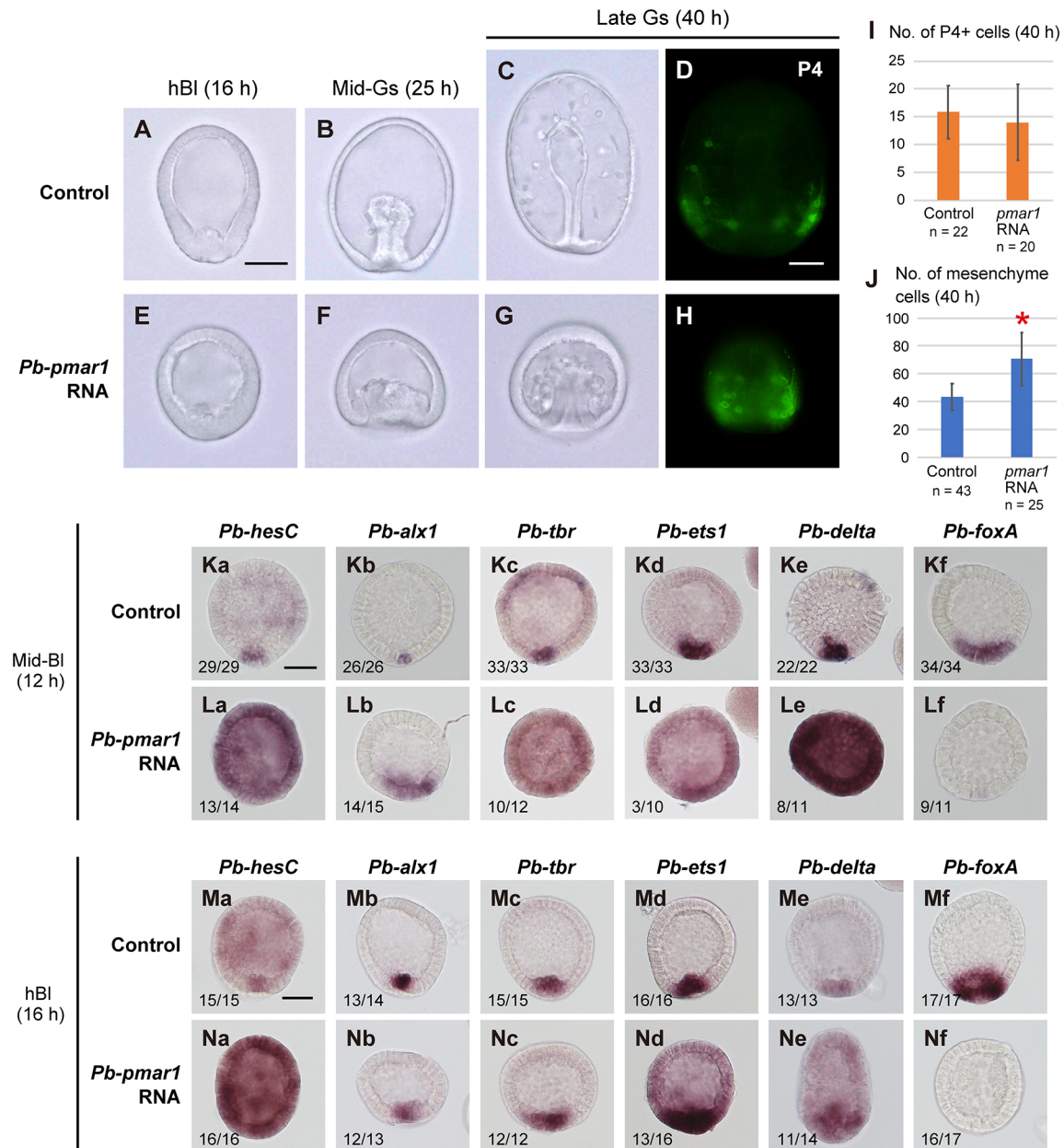

Fig. 3. Pmar1 regulates endomesoderm regulatory gene orthologs but not via HesC in the cidaroid embryos. (A-J) Effects of Pb-Pmar1 overexpression were examined in the cidaroid *P. baculosa* embryos. (A-D) Control embryos injected with 0.2 M KCl. (E-H) Embryos injected with *Pb-pmar1* mRNA. (A-C,E-G) Living embryos. (D,H) Fluorescence images of embryos examined by immunohistochemistry using skeletogenic cell-specific P4 antibody. (I,J) The average numbers of presumptive skeletogenic cells expressing P4 (I) and total mesenchyme cells (J) were examined at 40 h. The results from two batches are shown. Data are mean±s.d. The red asterisk in J indicates the significant difference between control and Pb-Pmar1-overexpressing embryos ($P<0.05$, Mann–Whitney U-test). (K-N) Expression of endomesoderm regulatory gene orthologs was examined by whole-mount *in situ* hybridization using embryos at the two blastula, mid-blastula (12 h) (K,L) and hatched blastula (hBl) (16 h) stages (M,N). (K,M) Control embryos. (L,N) Embryos injected with *Pb-pmar1* mRNA. The numbers shown in the lower left corner of each image are the number of embryos showing typical staining pattern/total number of examined embryos from two batches. Scale bars: 50 µm.

overexpressing embryos (Fig. 3B,F). To examine the effects of skeletogenic and nonskeletogenic mesenchyme cell formation, we counted the mesenchyme cell number at the late gastrula stage (40 h) (Fig. 3C,D,G-J). The number of presumptive skeletogenic cells expressing the skeletogenic cell marker P4 did not increase significantly in embryos overexpressing Pmar1 (Fig. 3I). On the other hand, the number of mesenchyme cells showed a significant increase in Pmar1-overexpressing embryos (Fig. 3J).

The observed phenotypic difference suggests that the regulatory function of Pmar1 differs between cidaroids and euechinoids. Thus, we examined the effect of cidaroid Pmar1 on skeletogenic gene orthologs by assessing the expression of *hesC*, *alx1*, *tbr*, *ets1* and *delta*, as well as the putative endoderm regulatory gene *foxA* (Erkenbrack et al., 2018) by whole-mount *in situ* hybridization in the blastula stage (Fig. 3K-N). *Pb-pmar1* mRNA-injected embryos showed global activation of *hesC* (Fig. 3Ka,La,Ma,Na). This effect on *hesC* expression was opposite to that observed in euechinoids, in which overexpression of Pmar1 suppresses the expression of *hesC* (Revilla-i-Domingo et al., 2007). On the other hand, the effects on other genes were similar to those found in euechinoids. The expression of *tbr*, *ets1* and *delta* was expanded throughout the entire embryo (Fig. 3Kc-e,Lc-e,Mc-e,Nc-e). In contrast, *foxA* expression disappeared in Pmar1-overexpressing embryos (Fig. 3Kf,Lf,Mf, Nf). The expression of *alx1* was also moderately expanded in Pmar1-overexpressing embryos at the earlier stage (Fig. 3Kb,Lb), but ectopic expression was not observed at the later stage (Fig. 3Mb, Nb), which is consistent with the lack of an increase in skeletogenic cell numbers in Pmar1-overexpressing embryos at the gastrula stage. It should be noted that the expression pattern of *alx1* was different from those of the other genes in the cidaroid embryos. During the blastula stage, the expression of *tbr*, *ets1* and *delta* was detected uniformly in the vegetal region of normal *P. baculosa* embryos (Fig. S3B-D,F-H), whereas patchy expression of *alx1* was frequently observed (Fig. S3A,E). This suggests that an additional mechanism, possibly related to a molecular or mechanical bias in the earlier stage, exists for *alx1* regulation in this species. In summary, cidaroid Pmar1 promotes the activation of endomesodermal/skeletogenic regulatory gene orthologs (*alx1*, *tbr*, *ets1* and *delta*) but not by repressing *hesC*.

Because the GRN downstream of Pmar1 is likely to differ between cidaroids and euechinoids, we asked whether any biochemical features of Pmar1 have changed during sea urchin

evolution. To address this issue, we examined whether cidaroid Pmar1 can perform similar functions in euechinoid embryos. We injected the cidaroid *P. baculosa pmar1* mRNA into the euechinoid *H. pulcherrimus* and observed the resultant phenotype (Fig. 4). The phenotype of embryos overexpressing Pb-Pmar1 was identical to that of embryos overexpressing the euechinoid Pmar1. The Pmar1-overexpressing embryos showed global expression of *alx1* and *ets1* at the hatched blastula stage (Fig. 4F-H), whereas these genes were expressed specifically in the skeletogenic cell region of control embryos (Fig. 4A-C). Until the gastrula stage, almost all cells of the Pmar1-overexpressing embryos developed into mesodermal mesenchyme cells that expressed the skeletogenic cell marker P4 (Fig. 4D,E,I,J). These observations suggest that cidaroid Pmar1 exhibits euechinoid Pmar1-like activity in euechinoid embryos, probably through the repression of *hesC*, and may function as a repressor.

Starfish Phb regulates endomesoderm regulatory genes as a repressor

To estimate the function of the ancestral genes of *pmar1* and *phb*, we further examined the functions of two *phb* genes (*phbA* and *phbB*) in the starfish *P. pectinifera* (Fig. 5). We performed knockdown and overexpression analyses using morpholino antisense-oligos (MOs) and synthesized mRNAs, respectively. In embryos injected with *phbA* and/or *phbB* MOs, gastrulation and mesenchyme formation were inhibited (Fig. 5E-H) compared with these processes in control embryos (Fig. 5A-D,M). At the gastrula stage during normal development, the archenteron is subdivided into two regions: the endoderm region, which shows alkaline phosphatase (AP) activity; and the AP-negative mesodermal region (Kuraishi and Osanai, 1994). All mesenchyme cells express the antigen of the MC5 monoclonal antibody (Hamanaka et al., 2011). In the knockdown embryos, AP activity was significantly reduced (Fig. 5C,G), and the total number of mesenchyme cells recognized by the MC5 antibody decreased (Fig. S4). Double-knockdown caused more-severe effects (see the detailed observations of archenteron and mesenchyme cell formation in Fig. S4), implying that the two *phb* genes function redundantly. In contrast, the embryos overexpressing PhbA and PhbB formed an enlarged AP-positive region and subsequently developed into an exogastrula (Fig. 5I-L). These observations suggest that, similar to echinoid Pmar1, the starfish Phb proteins are required for the formation of vegetal tissues.
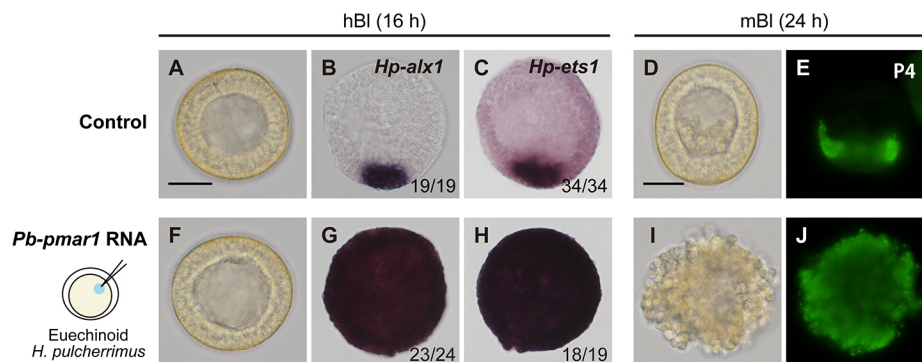

| | hBl (16 h) | | | mBl (24 h) | |

Fig. 4. The regulatory activity of cidaroid Pmar1 is similar to that of euechinoid Pmar1. Cidaroid *pmar1* (*Pb-pmar1*) mRNA was injected into fertilized eggs of an euechinoid *Hemicentrotus pulcherrimus* (*Hp*). (A-E) Control embryos injected with 0.2 M KCl. (F-J) Embryos injected with *Pb-pmar1* mRNA. (A-C,F-H) Embryos of hatched blastula (hBl) stage. (D,E,I,J) Embryos of mesenchyme blastula (mBl) stage. (A,D,F,I) Living embryos. (B,C,G,H) Embryos examined by whole-mount *in situ* hybridization using RNA probes for *Hp-alx1* (B,G) and *Hp-ets1* (C,H). (E,J) Fluorescence images of embryos examined by immunohistochemistry using the skeletogenic cell-specific P4 antibody. The numbers shown in the lower right corner of B, C, G and H are the number of embryos showing typical staining pattern/total number of examined embryos from two batches. Scale bars: 50 μm.
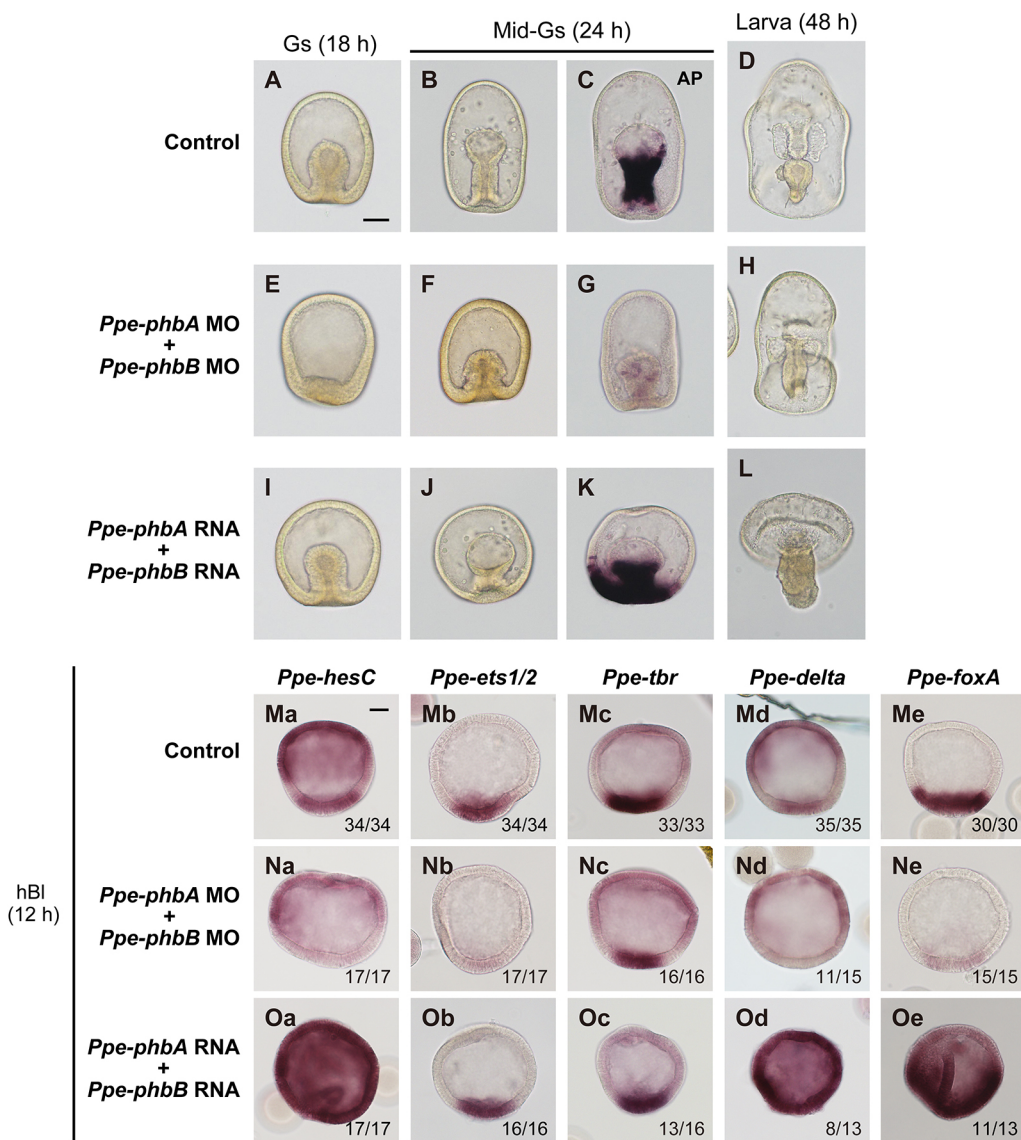
Fig. 5. Phb regulates endomesoderm regulatory gene orthologs in the starfish embryos. The knockdown and overexpression analyses of starfish *phbA* and *phbB* were performed using sequence-specific morpholino antisense-oligos (MOs) and synthesized mRNA in the starfish *P. pectinifera* embryos. (A-D) Control embryos injected with 0.2 M KCl (control for overexpression experiments) or the MO for the limpet homeobox gene (control for knockdown experiments). (E-H) Embryos injected with *Ppe-phbA* MO and *Ppe-phbB* MO. (I-L) Embryos injected with *Ppe-phbA* and *Ppe-phbB* mRNA. (C,G,K) Embryos stained for alkaline phosphatase (AP) activity. (M-O) Gene expression was investigated at the hatched blastula (hBl) stage (12 h) by whole-mount *in situ* hybridization. (M) Control embryos injected with 0.2 M KCl or control MO for the limpet homeobox gene. (N) Knockdown embryos. (O) Overexpressing embryos. The numbers shown in the lower right corner of M-O indicate the number of embryos showing typical expression patterns/total number of examined embryos from two batches. Scale bars: 50 μm.

Cidaroid Pmar1 leads to the activation of endomesoderm regulatory genes (e.g. *alx1* and *ets1*) and *hesC*, as mentioned above. To evaluate the regulatory function of the starfish Phb proteins, we also analyzed the expression of endomesoderm regulatory gene orthologs in PhbA/B-perturbed *P. pectinifera* embryos. We examined the expression of *hesC*, *ets1/2* (*ets1* ortholog), *tbr*, *delta* and *foxA* in experimental embryos at the hatched blastula stage (12 h) (Fig. 5M-O). In the Phb-knockdown embryos, the expression of *hesC*, *ets1/2*, *delta* and *foxA* was significantly reduced at the vegetal pole (Fig. 5Na,b,d,e), whereas *tbr* expression was not affected (Fig. 5Nc). Conversely, in Phb-overexpressing embryos, *hesC* and *delta* expression was expanded throughout the embryos (Fig. 5Oa,d), which is similar to that in Pmar1-overexpressing cidaroid embryos (see Fig. 3). However, *ets1/2* and *tbr* expression did not appear to be affected (Fig. 5Ob,c), and expansion of *foxA* expression was observed in the Phb-overexpressing starfish embryos (Fig. 5Oe), suggesting that the regulatory functions of starfish PhbA/B for *foxA*, *ets1/2* and *tbr* are distinct from that of cidaroid Pmar1. Nonetheless, these observations suggest that, similar to cidaroid Pmar1, starfish PhbA/B leads to the activation of *hesC*, *ets1/2* and *delta*, although

an additional factor(s) is needed for *ets1/2* expression. Based on these results, we suggest that starfish PhbA/B exhibit a regulatory function similar to that of cidaroid Pmar1.

To determine whether the biochemical activity of starfish Phb proteins is comparable with that of echinoid Pmar1, we examined the activity of starfish Phb when expressed in euechinoid embryos. We injected the *P. pectinifera phbA* and *phbB* mRNAs into the eggs of the euechinoid *H. pulcherrimus* (Fig. S5). When the control embryos developed into mesenchyme blastulae, the embryos overexpressing starfish PhbA showed a moderate increase in skeletogenic cells (Fig. S5A,B). In contrast, no obvious effects were observed in the embryos expressing starfish PhbB (Fig. S5C). These observations suggest that at least one starfish Phb protein can exhibit some degree of Pmar1-like activity in euechinoid embryos.

Because starfish Phbs have no typical eh1-like motifs (see Fig. 1B), we asked whether starfish Phb proteins function as repressors. We overexpressed the mRNAs encoding the two types of proteins: PhbA/B fused to the *Drosophila* Engrailed repression domain (EnR) or to the VP16 activation domain (VP16AD) (Fig. 6). The Phb proteins fused with the EnR domain caused phenotypes similar to those caused by the wild-type proteins (Fig. 6C,D).
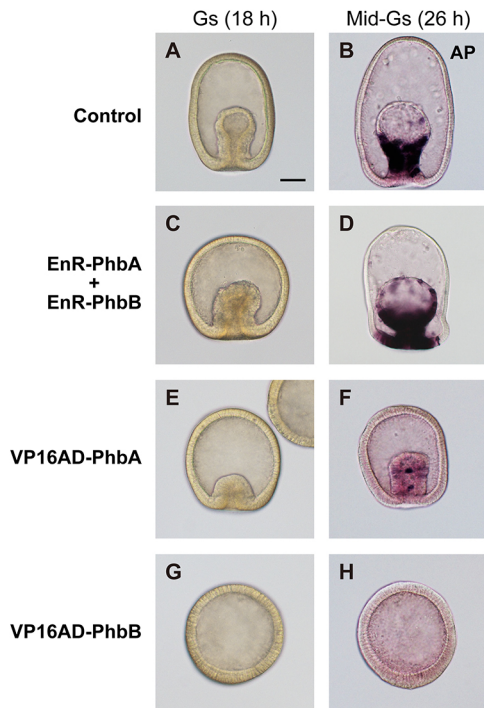
Fig. 6. Starfish Phb proteins function as repressors. (A,B) Control *P. pectinifera* embryos injected with 0.2 M KCl. (C-H) Embryos overexpressing starfish PhbA/B (Ppe-PhbA and Ppe-PhbB) fused to the *Drosophila* Engrailed repression region (EnR) (C,D), Ppe-PhbA fused to VP16 activation domain (VP16AD) (E,F) and Ppe-PhbB fused to VP16AD (G,H). (A,C,E,G) Images of living embryos at the gastrula stage (18 h). (B,D,F,H) Embryos stained for AP activity at the mid-gastrula stage (26 h). All experiments were conducted in more than three batches. Scale bar: 50 μm.

In contrast, overexpression of PhbA- or PhbB-VP16AD retarded the development of endomesodermal tissues (Fig. 6E-H). These results suggest that starfish PhbA and PhbB function as repressors similar to euechinoid Pmar1.

Vegetal expression of *hesC* is regulated by Delta-Notch signaling in cidaroid and starfish embryos

Our data indicate that, in contrast to euechinoid Pmar1, Pmar1/Phb leads to the activation of *hesC* in both cidaroid and starfish embryos, although this promotion probably occurs indirectly because both Pmar1 and Phb may function as repressors. Erkenbrack and Davidson (2015) demonstrated that a Delta signal is required for *hesC* expression in the vegetal embryo of another cidaroid, *E. tribuloides*. Here, we confirmed the same result in the cidaroid *P. baculosa* and the starfish *P. pectinifera*. To estimate the function of Delta signaling, we treated the embryos with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), which inhibits Notch signaling, according to Erkenbrack et al. (2018) (Fig. 7). In the DAPT-treated *P. baculosa* embryos, the vegetal expression of *hesC* disappeared, while *tbr*, *ets1* and *foxA* expression was not affected (Fig. 7F,H-J). Because the experimental embryos showed a slightly increased signal intensity of *alx1* (compare Fig. 7G with Fig. 7B), we counted the number of cells expressing *alx1* (Fig. 7K). The average number increased moderately but significantly; the control and experimental embryos showed *alx1* expression in 12.2±1.3 cells (*n*=13) and 14.5±2.1 cells (*n*=13) on average, respectively. We also found that the number of skeletogenic cells expressing P4 increased moderately in Delta signaling-deficient embryos (Fig. S6A-E),

suggesting that *P. baculosa* HesC represses skeletogenic fate in the cells around the vegetal pole, as noted by Erkenbrack and Davidson (2015).

A significant decrease in *hesC* mRNA at the vegetal pole was also found in the DAPT-treated starfish embryos (Fig. 7L,O), whereas the expression of *ets1/2* and *foxA* was not altered (Fig. 7M,N,P,Q). We also confirmed the results of Hinman and Davidson (2007), showing that depletion of Delta function resulted in the activation of *ets1/2* in the starfish gastrula (compare Fig. S6I and Fig. S6N). In the DAPT-treated embryos, cell conversion into globular mesenchyme cells was observed in the whole upper region of the archenteron in the mid-gastrula stage (Fig. S6G,L). This implies that Delta-Notch signaling affects the genes responsible for the epithelial-mesenchymal transition of the mesoderm region in starfish embryos but may be regulated independently of HesC because HesC-knockdown in starfish embryos had no effect on mesoderm differentiation, as further discussed. In summary, the above data suggest that cidaroid and starfish *hesC* genes are regulated by Delta-Notch signaling. This system appears to be the ancestral mode of *hesC* regulation in eleutherozoans.

DISCUSSION

The sea urchin Pmar1-HesC double-negative gate for endomesoderm specification has been considered to have been newly acquired during echinoid evolution. However, it is still unknown how the system was established because *pmar1*-related genes and their upstream regulators have not been examined in noneuechinoid echinoderms. Through experiments using various echinoderms, we provide a hypothetical evolutionary scenario for the *pmar1* gene (Fig. 8A) and models of the upstream GRN for endomesoderm in starfish and cidaroids comparable with that of the euechinoid *S. purpuratus* (Fig. 8B). The Pmar1-HesC regulatory system provides a remarkable opportunity to understand the diversification of the early developmental GRN.

Molecular evolution of the *pmar1* homeobox gene

Our screening of upstream regulators of endomesoderm development in noneuechinoid echinoderms revealed that cidaroids have *pmar1* genes and that other echinoderms possess *pmar1/phb1*-related *phb* genes (Fig. 1). Our phylogenetic analyses supported the hypothesis that duplication of an ancient *phb1* gene (referred to as *phb* genes in this study) led to the emergence of the *pmar1* gene, which was reported by Dylus et al. (2016) (Fig. 8A). We note that the eh1-like motif is shared by Phb1 and Pmar1 in echinoids. Thus, we prefer the evolutionary history that *pmar1* and *phb1* were derived from an ancestral gene in which the eh1-like motif evolved in the common ancestor of echinoids, although the relationships between *pmar1*, *phb1* and other *phb* genes have not been revealed by phylogenetic analysis. After the emergence of the *pmar1* gene, tandem duplications of the *pmar1* gene occurred in the genome, and the substitution rate of Pmar1 was accelerated, a phenomenon known as asymmetric evolution (Holland et al., 2017). This asymmetric evolution made our phylogenetic analyses less resolvable. Thus, we cannot exclude the alternative phylogenetic history in which the common ancestors of echinoderms possessed both *phb* and *pmar1*, and gene loss occurred in multiple lineages, although this scenario is less parsimonious.

Although *pmar1* shows an accelerated substitution rate, its basic biochemical nature and developmental function have not changed. Pmar1 remains a transcriptional repressor, and it may have obtained an additional eh1-like motif in the echinoid lineage. In addition to the asymmetric evolution of *pmar1*, another important evolutionary
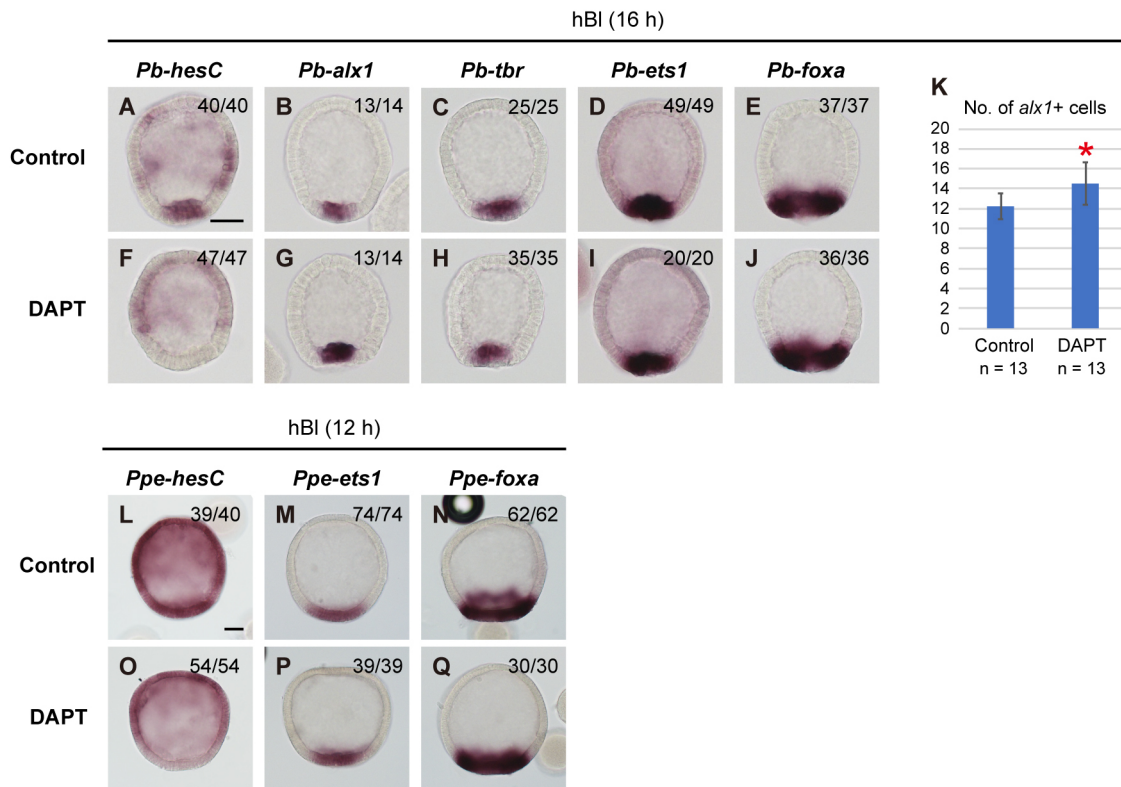
Fig. 7. Delta-Notch signaling regulates vegetal *hesC* expression in cidaroid and starfish embryos. Delta-Notch-inhibited cidaroid (*P. baculosa*) and starfish (*P. pectinifera*) embryos were examined by whole-mount *in situ* hybridization. (A-E) Control cidaroid embryos treated with dimethyl sulfoxide (DMSO). (F-J) Cidaroid embryos treated with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT). (K) The average numbers (mean±s.d.) of *alx1*-expressing cells in the experimental embryos of *P. baculosa*. Red asterisk indicates the significant difference between control and DAPT-treated embryos (*P*<0.05, Mann–Whitney *U*-test). (L-N) Control starfish embryos treated with DMSO. (O-Q) Starfish embryos treated with DAPT. Embryos were examined at the hBl stage (16 h and 12 h for the cidaroid and starfish, respectively). The numbers shown in the top right corner of each image indicate the numbers of embryos showing typical expression patterns/total number of examined embryos from two batches. Scale bars: 50 μm.

change is the positioning of *hesC* as a downstream target gene, as mentioned below. However, our analysis revealed that there was a time lag between the extensive duplication of *phb*/*pmar1* and the acquisition of *hesC* as a target gene. The asymmetric gene duplication occurred before the cidaroid-euechinoid divergence, but HesC regulation was placed under the control of Pmar1 in the common ancestor of euechinoids.

Evolution of the Pmar1-HesC double-negative gate

Our models of the endomesoderm GRN in cidaroids and starfish provide valuable information for understanding the evolutionary scenario of the upstream GRN (Fig. 8B). Our results support the idea that in the common ancestor of starfish and sea urchins (eleutherozoan), the *phb* gene exhibited a regulatory function in endomesoderm specification by regulating *ets1* (*ets1/2*) and *delta* (Fig. 8A). Because Phb likely functions as a transcriptional repressor, starfish *ets1* and *delta* may be regulated by an unknown repressor that is repressed by Phb proteins (X in Fig. 8B). Because HesC also acts as a repressor of *alx1* expression in cidaroids, this interaction of HesC with *alx1* dates back to the common ancestor of echinoids, even though *hesC* is activated indirectly, and not repressed, by Pmar1. Thus, the presumptive GRN in cidaroids seems to represent an intermediate state in the evolution of the Pmar1-HesC double-negative gate; i.e. HesC is just beginning to regulate *alx1* to specify mesodermal skeletogenic cells in this GRN. After euechinoids diverged from the cidaroid lineages, *hesC* regulation was placed under the control of Pmar1. Our cross-species analysis suggests that

the positioning of *hesC* downstream of Pmar1 occurred through the modification of the *cis*-regulatory sequence of *hesC* and not through the alteration of the coding sequence of Pmar1.

These results bring us to the idea that HesC was one of the genes whose developmental role was most drastically changed during echinoderm evolution. McCauley et al. (2010) demonstrated that, in embryos of the starfish *P. miniata*, HesC knockdown caused no obvious effects on the expression of *ets1/2* or *tbr* or endomesoderm formation. Similarly, we observed that the embryos of the starfish *P. pectinifera* injected with two distinct *hesC*-specific MOs showed no defects in the vegetal tissues until the late gastrula stage (Fig. S7), suggesting that HesC is not essential for early endomesoderm specification. On the other hand, in cidaroid embryos, HesC appears to repress *alx1* expression to some extent and skeletogenic cell fate (Erkenbrack and Davidson, 2015; this study). It is only in the euechinoid lineage that multiple other endomesodermal regulatory genes, such as *ets1* and *delta*, are regulated by HesC (Fig. 8B). The next issue to address to understand the changes in the GRN will be how the multiple endomesodermal regulatory genes were placed under the control of HesC, which may have occurred through the modification of *cis*-regulatory motifs of target genes or those in the coding sequences of HesC. It should be noted that this event is expected to have occurred in a coordinate manner with the addition of *hesC* regulation under Pmar1 control.

Regarding the evolutionary change in the Pmar1-target gene repertoire, we consider the possibility that the target may not have
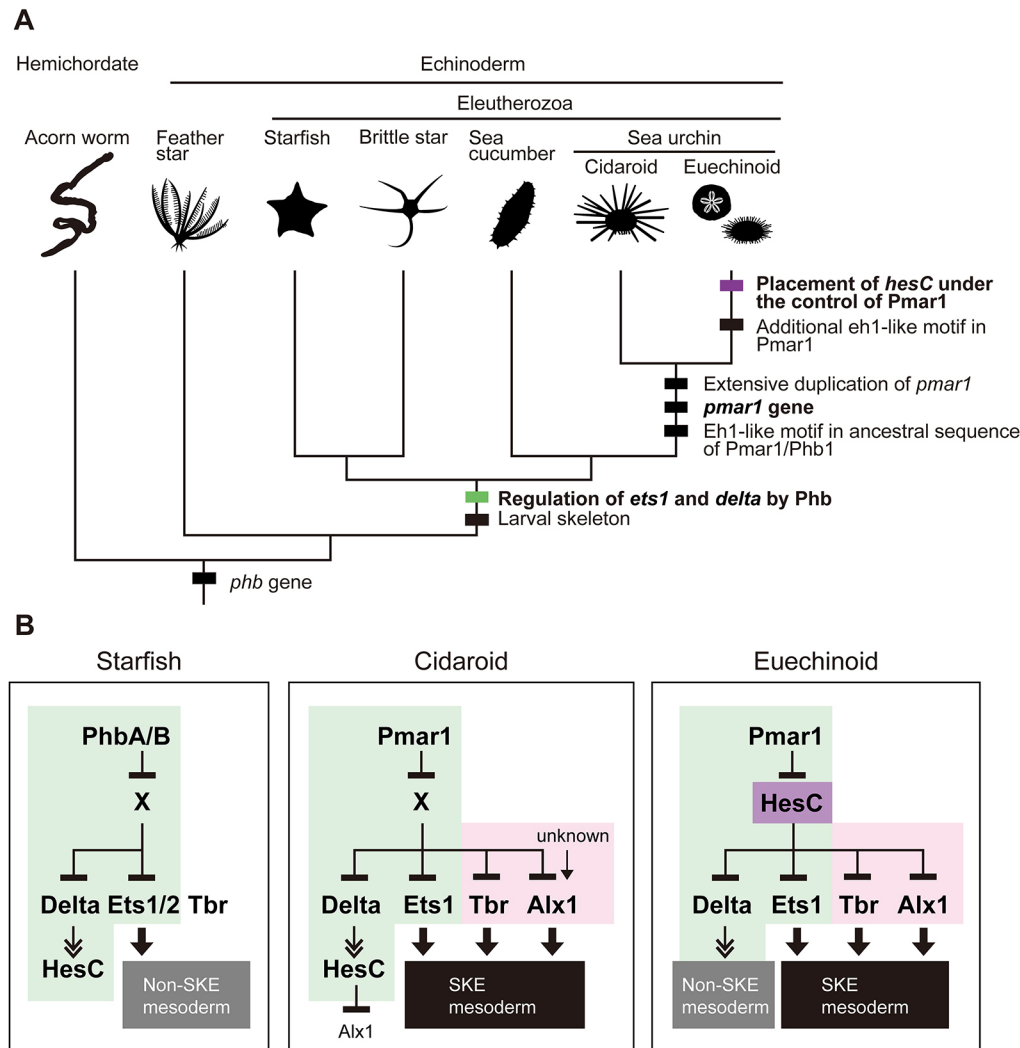
DEVELOPMENT

Fig. 8. The evolutionary history of the endomesoderm specification system and comparison of gene regulatory network (GRN) models. (A) The relationships of echinoderms and hypothetical evolutionary events for endomesoderm specification mechanism. Because acorn worm has a *phb1*-like sequence (Dylus et al., 2016), the origin of *phb* genes can be traced back to the common ancestor of the hemichordate and echinoderm. The larval skeletogenic cells likely arose in the common ancestor of eleutherozoans (Erkenbrack and Thompson, 2019). Additional details can be found in the text. (B) The GRN models of starfish, cidaroid and euechinoid. *Delta*, *ets1* (*ets1/2*) or *alx1* regulates skeletogenic mesenchyme cell (SKE mesoderm) and/or nonskeletogenic mesenchyme cell (non-SKE mesoderm) differentiations. The green area is conserved among eleutherozoans, whereas pink and purple areas indicate changes in *alx1/tbr* and *hesC*, respectively, that occurred during echinoderm diversification.

been simply transferred from an unknown factor (X) to HesC. The present study shows that Phb/Pmar1 repress an unknown repressor other than HesC in starfish and cidaroid embryos (Fig. 8B). On the other hand, our previous study using two distantly related euechinoid species suggested the existence of an additional repressor downstream of euechinoid Pmar1. In euechinoid embryos injected with *pmar1* (*micro1*) mRNA, the fate conversion of almost all cells into putative skeletogenic cells is commonly observed (Oliveri et al., 2002; Nishimura et al., 2004; Yamazaki et al., 2009), which suggests that Pmar1 represses the global repressor(s). In contrast, *hesC* MO-embryos show only a moderate expansion of skeletogenic cell region, although the transient global activation of some micromere/skeletogenic regulatory genes (*delta* and *ets1*) is observed at the earlier stage (Yamazaki and Minokawa, 2016). Furthermore, the Pmar1-overexpressing embryos show almost no expression of a nonskeletogenic regulatory gene *gcm* at the blastula stage, whereas *hesC* MO-embryos of two euechinoid species show expanded expression of *gcm* (Yamazaki and Minokawa, 2016), i.e. the regulatory states in these embryos are clearly distinguishable. This difference implies that an additional repressor of skeletogenic regulatory genes is present to repress skeletogenic cell fate in the animal region of euechinoids, which is supported by structure-function correlation analysis of the Pmar1 (Micro1) protein (Yamazaki et al., 2009). Accordingly, we predict that there is an unknown repressor (X) shared by eleutherozoans downstream of Phb/

Pmar1; i.e. an additional target of Pmar1, *hesC*, had been added in the euechinoid lineage. Our data suggest that the gene encoding the unknown repressor in the cidaroid is not a member of the hairy gene family because we found no hairy genes showing expression patterns similar to euechinoid *hesC* in the cidaroid *P. baculosa* (i.e. nonvegetal ectodermal expression during the blastula stage). Two hairy genes (*hesA* and *hesD*) showed zygotic expression during the early stages of *P. baculosa* (Fig. S1); strong expression of *hesA* was detected in whole embryos, whereas *hesD* showed only faint expression at the blastula stage. To understand how the upstream GRN has been rewired, it is crucial to identify the unknown repressor X in these animals. Further study of the sea urchin *phb1* genes may also be informative to determine how the ancestral function of *phb* was modified after the emergence of *pmar1*.

Our results illuminate the evolutionary history of the echinoderm GRN, in which the upstream GRN recruited a new component (i.e. *hesC*) without changing the developmental outcome. The stepwise rewiring of transcription networks via the addition of target genes is one of the general evolutionary pathways observed in the transcription network of yeast. Li and Johnson (2010) proposed that transition through intermediate states would not decrease fitness, which is the key to understanding the evolutionary process of GRN rewiring. In the case of GRN modification in euechinoids, the following two changes must have occurred: (1) recruitment of new repressor

targets under the control of HesC; and (2) recruitment of *hesC* as a repressor target of Pmar1, irrespective of the order. The future questions that we need to address using the experimental system of echinoderms are as follows: what is the intermediate state that enabled GRN rewiring without causing catastrophe, and what sort of molecular evolution occurred during this stepwise process?

MATERIALS AND METHODS
Animals and embryos
The collection of adult *P. baculosa* and *H. pulcherrimus* and the handling of gametes and embryos were performed according to a previously described method (Yamazaki et al., 2010; Hibino et al., 2019). The collection and handing of gametes of *P. pectinifera* and *A. kochii* were performed according to Koga et al. (2010). The adults of *A. japonicus* were collected around the Misaki Marine Biological Station, University of Tokyo. The gametes were obtained according to the method of Kikuchi et al. (2015).

Screening of upstream gene candidates for larval skeleton formation in the cidaroid *P. baculosa* and collection of *pmar1/phb*-related genes from other echinoderms
To survey the candidate upstream genes in the endomesoderm GRN of the cidaroid *P. baculosa*, we performed screening based on temporal expression patterns. We first performed RNA-seq using samples from embryos at five developmental stages (2 h, 4 h, 6 h, 10 h, and 14 h). The criteria for the selection of candidate genes were as follows: (1) zygotic activation (more than a fivefold increase in the FPKM value compared with the value of maternal expression) earlier than or simultaneously with expression of *alx1*, (2) encoding a DNA-binding domain according to the HMMER search (Johnson et al., 2010), and (3) an FPKM value greater than 5 at the onset of *Pb-alx1* activation (10 h). To identify these obtained sequences, we checked the top BLAST hit sequence in the sea urchin *S. purpuratus* gene database (named Sp genes) of EchinoBase (www.echinobase.org/Echinobase/). The amplified sequence of *P. baculosa pmar1* has been deposited in DDBJ (accession number LC483152), and the sequences of the other genes are shown in the supplementary Materials and Methods. We further identified *pmar1*, *phb1* and *phb* genes from the other echinoids and echinoderms. See supplementary Materials and Methods for more details.

Molecular phylogenetic analysis
The genes used for the outgroup were selected according to the previous analysis (Dylus et al., 2016). See supplementary Materials and Methods for more details.

Whole-mount *in situ* hybridization
Whole-mount *in situ* hybridization was performed as described previously (Koga et al., 2010; Yamazaki et al., 2010; Morino et al., 2012). See Supplementary Materials and Methods and Table S2 for more details.

Perturbation experiments
The overexpression and translational perturbation analyses were performed using synthesized mRNAs and MOs, respectively. See Supplementary Materials and Methods for more details.

QPCR
The qPCR analysis was performed as described previously (Yamazaki et al., 2012) using CFX Connect Real-Time PCR Detection System (Bio-Rad). The *EF1alpha* gene was used for the internal reference standard according to Koga et al. (2016). The primer sequences used are follows: *Pb-pmar1*-qF, 5′-CGATATCGACGTGCGAGAAA-3′; *Pb-pmar1*-qR, 5′-TGAAACCA-GACCTGTATTCTC-3′; *Pb-EF1a*-qF, 5′-GCGTGAGCGAGGTATCAC-AAT-3′; and *Pb-EF1a*-qR, 5′-ACAATCAGCACCGCACAATC-3′.

Evaluation of mesenchyme cell and endoderm differentiation
The differentiation of skeletogenic cells, mesenchyme cells and endoderm was evaluated using fixed embryos of *P. baculosa*, *H. pulcherrimus* and *P. pectinifera*. See Supplementary Materials and Methods for more details.

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