Evo-Devo Researches on Marine Invertebrates: Insights into the Evolution of Novel Developmental Patterns

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Contents

Abstract

The molecular background of the evolution of novel developmental patterns was examined in echinoderm and lophotrochozoa. For the evolution of echinoderm pluteus larvae, the acquisition of the larval skeleton and pluteus arm were thought to be the key steps. In this study, to elucidate the evolutionary mechanism of echinoderm larvae, I investigated the expression pattern of arm or skeletogenesis-related genes in multiple echinoderm taxa. First, I observed that *vegf/vegfr* are expressed in the site of the larval skeletogenesis of brittle star, and adult skeletogenesis of starfish, but not expressed in larval stage of starfish. These patterns suggested that the activation of VEGF signaling in larval stage was the key molecular changes for evolution of larval skeleton. Next, I found the expression pattern of arm-related genes in brittle star were quite similar to that of sea urchin. Although starfish didn't show such similarity, the expression pattern of those genes in sea cucumber exhibited similarity to those of pluteus larvae. These observations proposed the possibility of the single origin of the pluteus

larva. In addition, the similarities of expression profile between pluteus arm and spine implied the system for pluteus arm formation was co-opted from the spine formation mechanism. Next, I investigated the molecular background for the establishment of "spiralian development" in lophotrochozoa. The basal character of spiralian development is the conserved pattern of generating quartet with particular fate. I first did the genomic survey of the homeobox genes in pearl oyster, *Pinctada fucata*. In the course of annotation, I found homeobox genes that were specific to lophotrochozoa. These genes exhibited transient expression in early cleavage stage in annelid and mollusk. Most genes show localization in animal or vegetal blastomeres. Functional analysis in mollusk suggested that these genes indeed perform the role for establishment for quartet properties along A-V axis. These data suggested that the gain and duplication of lophotrochozoa specific homeobox genes was the one of the key events for evolution of spiralian development in lophotrochozoa lineage.

General Introduction

To elucidate how the diverse morphology was evolved, understanding the molecular mechanism for evolution of developmental pattern is essential (Müller 2007). Evo-Devo researches have demonstrated the molecular background of the emergence of novel developmental pattern (Carroll et al. 2010). However, it was pointed out that knowledge based on a limited number of model organism could lead to "biased pictures". Thus, information from variety of animals is valuable (Jenner and Wills 2007). Though marine invertebrates show extensive diversity in their morphology, our knowledge on their development is not sufficient (Love 2009). In this study, I focused on the two groups that include marine invertebrates, echinoderms and lophotrochozoans.

The echinodermata is one of the phyla of deutrostomes (Dunn et al. 2008). They consists of five extant groups: sea urchin, starfish, brittle star, sea cucumber and sea lily (Fig. 1-1; Hyman 1955). The larvae of echinoderms can be classified into two groups: auricularia and pluteus. Because of the

genomic information, extensive developmental knowledge of sea urchin, and developed techniques for molecular study, echinoderm thought to be a nice model for elucidating the molecular background of the evolution of the development. Here, I studied the evolutionary history and its molecular background of echinoderm larvae in Chapter1-1 and Chapter1-2.

The lophotrochozoa, which includes multiple phyla such as annelid, mollusk and platyhelminthes, is the one of the three major group of the bilaterian (Dunn et al. 2008). Although knowledge of this group is critical for considering the evolution of bilaterian, the study of lophotrochozoa has not progressed than other two groups, ecdysozoa and deutrostome (Telford and Littlewood 2009). In chapter 2, I first surveyed the homeobox genes in the genome of pearl oyster *P. fucuta*. In the course of annotation, I found there were homeobox genes that were specific to lophotrochozoa. In the latter half of chapter 2, I examined the its role for spiralian development, and discussed its evolutionary significance.

Chapter 1.

Elucidation of the mechanism for the evolution of the echinoderm larvae

Chapter 1-1

Heterochronic Activation of VEGF signaling and Evolution of the Larval Skeleton in Echinoderm Larvae.

1-1.1 Abstract

The evolution of the echinoderm larval skeleton was examined from the aspect of interactions between skeletogenic mesenchyme cells and surrounding epithelium. I focused on VEGF signaling, which was reported to be essential for skeletogenesis in sea urchin larvae. Here, I examined the expression patterns of *vegf* and *vegfr* in starfish and brittle stars. During starfish embryogenesis, no expression of either *vegfr* or *vegf* was detected, which contrast with previous reports on the expression of starfish homologs of sea urchin skeletogenic genes, including Ets, FoxN2/3 and Tbr. In later stages, when adult skeletogenesis commenced, *vegfr* and *vegf* expression were upregulated in skeletogenic cells and in the adjacent epidermis, respectively. These expression patterns suggest that heterochronic

activation of VEGF signaling is one of the key molecular evolutionary steps in the evolution of the larval skeleton in echinoderm. The absence of *vegf* or *vegfr* expression during early embryogenesis in starfish suggests that the evolution of the larval skeleton requires distinct evolutionary changes, both in mesoderm cells (activation of *vegfr* expression) and in epidermal cells (activation of *vegf* expression). In brittle stars, which have well-organized skeletons like the sea urchin, *vegfr* and *vegf* were expressed in the skeletogenic mesenchyme and the overlying epidermis, respectively, in the same manner as in sea urchins. Therefore, VEGF signaling is likely involved in larval skeletogenesis in brittle stars.

1-1.2 Introduction

Echinoderm usually has planktonic larval stage, and two types of planktonic larval forms are observed in five classes of echinoderms (Fig. 1-1). Auricularia-type larvae are found in sea lily, starfish, and sea cucumber lineages, while pluteus-type larvae occur in brittle star and sea urchin lineages. Because basal echinoderm groups, such as sea lilies, have auricularia-type larvae (Nakano et al. 2003), it was thought to represent the ancestral larval form of echinoderms. This is consistent with the appearance of acorn worm larvae, a sister group of echinoderms, which have auricularia-like larva (Hyman 1959).

The most obvious difference between pluteus and auricularia larvae is the existence of larval arms, which are supported by well-organized larval skeleton. The larval skeleton in pluteus larvae shows a stereotypic branching pattern that sustains the pluteus form. The acquisition of a larval skeleton was due to the appearance of larval skeletogenic mesenchyme cells. Larval skeletogenic cells are generally accepted to emerge through

heterochronic activation of a genetic program for an adult skeleton (Gao and Davidson 2008; Ettensohn 2009). All species of echinoderms possess adult skeletons, and a genetic program for an adult skeleton was already established in the common ancestor of echinoderms. Thus, for the evolution of a larval skeleton, animals did not need to establish a genetic regulatory network for larval skeletogenesis de novo; some steps for heterochronic activation were required. Several research groups have searched for key molecules that are responsible for the heterochronic activation of the adult skeletogenic network. Unexpectedly, most of the transcription factors that have been identified through large-scale gene regulatory network analyses, such as those involved in sea urchin larval skeletogenesis, also show expression in starfish larval mesoderm cells although starfish larvae do not develop a skeleton (Shoguchi et al. 2000; Hinman and Davidson 2007; Hinman et al. 2009; Koga et al. 2010; McCauley et al. 2010). Thus, the molecular evolutionary background for the evolution of a larval skeleton remains to be resolved.

Epidermal cells were shown to be responsible for the morphogenesis of the larval skeleton (Ettensohn and McClay 1986; Armstrong et al. 1993; Guss and Ettensohn 1997). Several genes have been identified that are involved in the interaction between the epidermis and skeletogenic mesenchyme cell, and in subsequent morphogenesis of the larval skeleton (Di Bernardo et al. 1999; Cavalieri et al. 2003; Duloquin et al. 2007; Röttinger et al. 2008; Cavalieri et al. 2011; Adomako-Ankomah and Ettensohn 2013). Moreover, recent evidence suggests that cues from the epidermis play critical roles in skeletogenesis itself. VEGF signaling has been shown to be activated by ligands from the epidermis for growth of the larval skeleton and for skeletogenesis itself (Duloquin et al. 2007;. Adomako-Ankomah and Ettensohn, 2013). Thus, to understand the mechanism that underlies pluteus evolution, the evolution of ectodermal cues is of critical importance.

In this research, I focused on VEGF signaling. I examined the expression patterns of *vegf* and *vegfr* in various echinoderm taxa and found

that *vegfr* is expressed in adult skeletogenic cells with adjacent *vegf* expression in starfish. However, expression of neither *vegf* nor *vegfr* was detected during early stages when the larval mesoderm differentiates. Brittle star embryos showed expression patterns of *vegf* and *vegfr* in larval skeletogenic cells in a manner that was similar to sea urchins. Thus, VEGF signaling is likely involved in larval skeletogenesis in brittle stars. These observations suggest that heterochronic activation of VEGF signaling is one of the key molecular evolutionary steps for the evolution of a larval skeleton. More importantly, the absence of *vegf* or *vegfr* expression in starfish suggests that the evolution of the larval skeleton required distinct evolutionary changes in both mesoderm cells (activation of *vegfr* expression) and epidermal cells (activation of *vegf* expression).

1-1.3 Materials and Methods

Fertilization and Embryo Rearing

I used adult starfish (*Patiria pectinifera*) specimens collected from Tateyama (Chiba Prefecture), Asamushi (Aomori Prefecture), and Hiraiso (Ibaraki Prefecture). Mature egg of starfish embryo were obtained by treating 1 x 10^{-6} 1-methyladenine containing ASW (Artificial Sea Water). After fertilization, embryos were reared at 22°C in ASW. Larvae were reared by feeding with *Chaetoceros calcitrans* (Marine tech)

Adult of brittle stars (*Amphipholis kochii*) were collected from Abuta (Hokkaido Prefecture) and Himi (Toyama Prefecture). The mature eggs were

obtained by cold shock (4°C) and subsequent heat shock (23°C). The embryos were reared in ASW at 23°C.

Gene Isolation

cDNAs were obtained by reverse transcription from embryonic RNA using ReverTra Ace (Toyobo). The primers that were used for amplification

are shown in Table. 1-1. The amplified DNA fragments were cloned into pGEM-T Easy vectors (Promega) and sequenced.

Phylogenetic analysis

Alignments of the sequences were performed by MAFFT (Katoh and Toh 2008). Best-fitting amino acid substitution model and Maximum Likelihood tree were inferred using RAxML 7.2.7 (Stamatakis et al. 2008). The bootstrap values were calculated from 300 replicates.

In situ hybridization

The digoxigenin (DIG) labeled antisense RNA was prepared using RNA polymerase (T7, Takara or SP6, Promega) with DIG RNA labeling mix (Roche). Fixation of embryo was done by treating 4% PFA (Paraformaldehyde) in MOPS Buffer. After several washing with PBST(Phosphate Buffered Saline with Tween 20), the solution was replaced by hybridization buffer (5 x SSC, 50% formamide, 5 x Denhalt's, 100μg/ml yeast RNA) and incubated one hour at 55°C. After prehybridization, solution was replaced the hybridization buffer with antisense RNA at 55°C for a week.

The probes were washed by several series of SSC buffers (4 x SSC, 2 x SSC or 1 x SSC with 50% formamide). After several wash of PBST, the embryo was incubated with Alkaline Phosphatase conjugated anti DIG antibody in RT (Room Tempature) for one hour. AP activity was visualized by NBT/BCIP solution (Roche). For brachiolaria larvae of starfish, samples were treated with BABB (1:2 benzyl alcohol/benzyl benzoate) after staining.

Quantitative Real-Time PCR

RNA from each stage was extracted using RNeasy with Rnase-Free DNase Kit (Qiagen KK). Reverse transcription was performed using PrimeScript (Takara). Quantitative real-time PCR (qPCR) was carried out with a Power SYBR Green Cells-to-Ct Kit (Applied Biosystems) and an ABI PRISM 7900 HT (Applied Biosystems). I examined the expression levels of *Ppvegf*, *Ppvegfr*, *PpCA1* and *Ppkroxβ* using the primers indicated in Table 1-1. Expression levels were normalized to *Ppmbf-1* using the primer indicated in Table 1-1.

1-1.4 Results

Gene Isolation and Molecular Phylogenetic Analysis

I isolated the DNA flagments of PpVEGF, PpVEGFR, AkVEGF and AkVEGFR from their cDNA. I performed phylogenetic analysis of VEGF and VEGFR genes using PDGF/VEGF domain or RTK domain, and confirmed its orthology to genes of sea urchin that have skeletogenic function (Fig. 1-2).

VEGF signaling in Starfish (*P. pectinifera*)

To examine the role of VEGF signaling in starfish, which do not possess a larval skeleton, I observed the expression patterns of *vegf* and *vegfr* in P. *pectinifera*. Before the gastrula stage, starfish larvae showed differentiation in their mesoderm cells, with some homologs of sea urchin skeletogenic genes being expressed, including *Ets Tbr, and FoxN2/3* (Shoguchi et al. 2000; Hinman and Davidson 2007; Hinman et al. 2009; Koga et al. 2010; McCauley et al. 2010). However, *Ppvegf* or *Ppvegfr* expression was not detected before the bipinnaria larval stage by in situ hybridization (Fig. 1-3 A–H).

Histologically, the first sign of adult skeletogenesis was observed during the early brachiolaria stage. Spicules were observed within aggregations of mesenchymal cells between the coelom and the epidermis on both the left and right sides (Hamanaka et al. 2011). At this stage, five spicules were observed on the left side, which corresponded to the oral side of the juvenile, while only a few spicules were observed on the right aboral side. Subsequently, at late brachiolaria stage, in addition to five spicules developed on the right side, single spicule appears in the central position of juvenile anlage (Hamanaka et al. 2011). Carbonic anhydrase (CA), which is involved in calcification in various organic tissues (Silverton 1991; Hentunen et al., 2000), is a good marker of skeletogenesis in sea urchins (Love et al. 2007). In starfish, we detected *PpCA1* expression in mesenchyme cells between the coelom and the epidermis in the early brachiolaria stage (Fig. 1-4E and G). The expression on the left side was in pentaradial manner (Fig. 1-4E), while a few clusters of cells were positive for *PpCA1* on the right side

(Fig. 1-4G, white arrowheads). This expression pattern well matched with the distribution of spicules (Fig. 1-4G and H).

The expression of *Ppvegfr* in the early brachiolaria stage was detected in mesenchymes located between the epidermis and the coelom around the stomach and intestine (Fig. 1-4A-D) Expression was detected on both the left and right sides (Fig. 1-4B, black arrows and white arrowheads). Five clusters of *Ppvegfr*-positive cells were clearly observed on the left side (Fig. 5D, black arrows). Expression on the right side was observed as a few clusters of mesenchyme cells (Fig. 1-4C, white arrowheads). This expression pattern was quite similar to that of *PpCA1*, although the *Ppvegfr* positive region was a bit broader than that of *PpCA1*. Therefore, I judged that *Ppvegfr* expression overlapped with the skeleton in the early brachiolaria stage. In the late brachiolaria, new expressions was came out in the aboral side of the juvenile anlage, and now *Ppvegfr* expression was observed in 11 clusters: five clusters on the outer and oral side (Fig. 1-4I and J, black arrows), five clusters on the inner and aboral side (Fig. 1-4I and K, white arrowheads),

and a central cluster (Fig. 1-4I and K, black arrowhead). This pattern matches the distribution of the adult skeleton (Hamanaka et al. 2011).

The earliest expression of *Ppvegf* was detected during the early brachiolaria stage in the epidermis around the stomach and intestine (Fig. 1-4F). These *Ppvegf*-positive cells were adjacent to *Ppvegfr*-positive cells. This epidermal expression was maintained in most of late brachioraria larvae (Fig. 1-4L), but some larvae showed *Ppvegf* expression only in mesenchyme cells (Fig. 1-4M). This variation was probably due to variation in the progress of development, although I could not distinguish developmental progress morphologically. In summary, expression of *Ppvegfr* was detected in skeletogenic cells and *Ppvegf* was expressed in cells that were adjacent to *Ppvegfr*-positive cells.

To examine quantitative expression levels of *Ppvegf* and *Ppvegfr,* we performed qPCR for *Ppvegf* and *Ppvegfr* (Fig. 1-5A). The relative expression levels of *Ppvegf* and *Ppvegfr* in embryos and during the bipinnaria larval stage were notably lower compared with levels after the early brachiolaria

stage. These temporal expression profiles were consistent with *in situ* hybridization data, although both *Ppvegf* and *Ppvegfr* expression levels began to increase during the bipinnaria stage based on qPCR, I did not detect these levels of expression by *in situ* hybridization. When these expression profiles are compared with that of *Ppkroxβ* that was shown to be upregulated and function after gastrulation (Hinman and Davidson 2003), expression levels of *Ppvegf* or *Ppvegfr* in early stages was comparable with that of *Ppkroxβ* before activation (Fig. 1-5B). Therefore, I interpreted that the expression levels of *Ppvegf* or *Ppvegfr* in early embryogenesis are below the level of biologically significant expression. Importantly, the timing of the upregulation of *Ppvegf* and *Ppvegfr* was highly coincident with adult skeletogenesis, and with the upregulation of skeletogenic effector gene: *PpCA1* (Fig. 1-5B).

Considering these patterns, the echinoderm ancestor likely possessed a skeleton only in the adult stage; the expression patterns of *vegf* and *vegfr* in starfish likely represent the ancestral condition, and supports the idea that

heterochronic activation of adult skeletogenic program led the evolution of larval skeleton (Gao and Davidson 2008; Ettensohn 2009). In other words, one may reasonably assume that VEGF signaling was involved in adult skeletogenesis in echinoderm ancestors, and that heterochronic activation of VEGF signaling in larval stages was one of the essential evolutionary events that led to the acquisition of a larval skeleton in sea urchins. One important fact is that neither *vegf* nor *vegfr* expression was observed during starfish embryogenesis. This suggests that heterochronic activation is required for the upregulation of both *vegf* in the epidermis and *vegfr* in mesenchyme cells.

VEGF signaling in Brittle Star (*A. kochii*)

Well-developed larval skeletons are also observed in brittle stars (Hyman 1955). Thus, I asked whether heterochronic activation of a skeletogenic developmental program via VEGF signaling was achieved in a similar manner in brittle stars and examined the expression patterns of *vegf* and *vegfr*. Skeletogenesis in brittle star larvae proceeded in a similar

manner to the process in sea urchins. Primary mesenchymal cells (PMCs) emigrate from the vegetal pole to the blastcoel before gastrulation at the blastula stage (Yamashita 1985; Gliznutsa and Dautov 2005). Subsequently, PMCs aggregate bilaterally by the posterior end of the archenteron in the gastrula stage, where the first larval spicules develop (Yamashita 1985; Gliznutsa and Dautov 2005).

In the brittle star *A. kochii*, the earliest expression of *Akvegfr* was detected during the blastula stage in one end of the blastula wall (Fig. 1-6A). Judging from later expression, I thought this expression marked the vegetal side of the embryo, although I was not completely certain because no morphological landmark exists with which to orient the embryo at this stage. After the emigration of the PMCs, *Akvegfr* expression was detected in PMCs (Fig. 1-6B). After gastrulation, *Akvegfr* signals were restricted to the PMC clusters (Fig. 1-6C), where first spicule formation occurs (Yamashita 1985; Gliznutsa and Dautov 2005). In subsequent stages, expression was observed in skeletogenic cells of the larval arms (Fig. 1-6D).

The earliest expression of *Akvegf* was also detected during the blastula stage in cells of the subequatorial region (Fig. 1-6E). During this stage, expression was not restricted along the dorsoventral axis but was observed as a circle in either the vegetal or animal view (Fig. 1-6E, inset). However, after emigration of the PMCs, epidermal expression was restricted in bilateral cell populations (Fig. 1-6F). Later on, bilateral epidermal expression was inherited in epidermal cells at the tips of the larval arms (Figs. 1-6G and H).

These expression patterns for *vegfr* in skeletogenic cells and *vegf* in adjacent epidermal cells suggest that VEGF signaling is also involved in skeletogenesis in brittle stars.

1-1.6 Discussion

Conserved Expression Patterns of *vegf* and *vegfr* in Skeletogenic Sites among Echinoderms

I examined the temporal and spatial expression patterns of *vegf* and *vegfr* in starfish and brittle stars. In sea urchins, *vegfr* is reportedly expressed in larval skeletogenic mesenchyme cells as well as in adult skeletogenic cells (Duloquin et al. 2007; Gao and Davidson 2008). *vegf* is expressed in epidermal cells that are adjacent to *vegfr*-positive cells during larval skeletogenesis (Duloquin et al. 2007; Adomako-Ankomah and Ettensohn, 2013). My analysis showed that *vegfr* was expressed in both of the skeletogenic cells examined, i.e., adult skeletogenic cells of starfish and larval skeletogenic cells of brittle stars. In addition, *vegf* expression was detected in adjacent epidermal cells. These observations suggest the idea that VEGF signaling has a conserved role in skeletogenesis in echinoderms, at least in eleutherozoans. This in turn raises an interesting question about the role of VEGF signaling in the sister group hemichordates, which possess

mineralized skeletons within epidermis of adult body (Cameron and Bishop, 2012). An analysis of VEGF signaling in acorn worms may shed new light on the evolutionary origins of the echinoderm skeleton.

Heterochrony of VEGF Signaling for Pluteus Evolution

The better understanding larval skeletogenesis in sea urchins prompted us to examine the molecular evolutionary background of the acquisition of a larval skeleton. However, several transcription factors that were characterized as essential for sea urchin larval skeletogenesis are also expressed in mesoderm cells in starfish embryos, including *Ets*, *Tbr and FoxN2/3* (Shoguchi et al. 2000; Hinman and Davidson 2007; Hinman et al. 2009; Koga et al. 2010; McCauley et al. 2010). VEGF signaling is the first that appears to be essential for sea urchin skeletogenesis, and expression was not detected by *in situ* hybridization during embryogenesis in starfish. I have provided evidences from qPCR that quantitative upregulation of *vegf* and *vegfr* occurred only during the adult skeletogenic stage. Therefore, I propose that heterochronic activation of VEGF signaling is one of the

essential evolutionary events that led to the acquisition of a larval skeleton in sea urchins.

One interesting aspect of the heterochronic activation of VEGF signaling is that both *vegfr* expression in mesoderm cells and *vegf* expression in the epidermis are required for activation. These heterochronic activations cannot be a consequence of a single molecular evolutionary event. Therefore, during the evolution of a larval skeleton, I may have to assume that a condition existed in which *vegf* was expressed in the epidermis, but that no cells could respond because none of the surrounding cells expressed *vegfr*. Alternatively, mesoderm cells expressed *vegfr*, but no receptors were activated due to the absence of *vegf* ligand expression around them. Either condition seems to be less advantageous than the ancestral condition in which neither gene is expressed, when considering the costs of transcription and translation. One of the central questions of evolutionary biology is how organisms overcome valleys in the adaptive landscape during evolution (Futuyma, 2013). The evolution of VEGF signaling in echinoderms provides

a unique experimental system with which to explore this issue.

A more interesting observation is that activation of VEGF signaling in larval stage has also observed in brittle stars. This indicates that distinct heterochronic activation occurred both in the epidermis for *vegf* and the mesoderm for *vegfr*. If the process for heterochrony is simple, it is not unlikely to have occurred through two independent lineages. However, I proposed that the evolution of the larval skeleton is not simple genetic change; it must have involved two distinct evolutionary steps, namely the activation of *vegfr* in mesoderm cells and the activation of *vegf* in the epidermis. Furthermore, whether the activation of VEGF signaling is sufficient for the evolution of a larval skeleton is not certain. Although VEGF signaling is essential for larval skeletogenesis in sea urchins, inhibition of VEGF signaling by means of morpholino injections against *vegf* or *vegfr* do not suppress the expressions of all of the skeletogenic markers. For example, one of the skeletogenic effector genes, msp130, was not suppressed (Duloquin et al. 2007). This may be explained by assuming that heterochronic

activation occurred for an upstream regulator of *vegfr*, which is also involved in msp130 expression. Thus, complicated evolutionary steps may have occurred.

It is not likely that multiple and similar evolutionary steps occur in two distinct lineages. Therefore, I should carefully consider another possibility for larval origin. In next section, I discuss the possibility of single origin of pluteus larvae.

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Tables

Table 1-1. Primers for Gene isolation and qPCR.

Primers for Gene Isolataion		Accsession Number
Gene Name	Primer Sequence	
PpVEGF	F:TCAAATCACAAGTCCGATGGAACGAAA R:TAAAGAAGGTTATATGAGGGGATTGGACA	AB705445
PpVEGFR	F:TCAGGAGGAAGAAGCAGAAGTA R:TCAGAAGTGAACAGTGACCACT	AB705446
AkVEGF	F:TGYAARAAYGGNGGNTGYTG R:GGRCARAANDHNGGYCC	AB705448
AkVEGFR	F:TGYGARCAYYTNCCNTAYGAY R:ATNWSYTCNGGNGCCATCCAYTT	AB705449

Primers for qPCR		
Gene Name	Primer Sequence	PCR efficiency
PpVEGF	F:GTGACTGCCTGACCAACAAC R:CCGCACATTCCTCTCCTTAT	199.2%
PpVEGFR	F:GAACACCGAGGCTAAGGAAA R:GGCCCTTCTCAATGTACTGG	194.9%
PpMBF-1	F:GGAGAGCAGATGGTCATGGT R:CAGTGTTTCCGTCATTTTGC	196.8%
PpCA1	F:CATGGACGGTGTTCAAAATG R:AATCGGGATGTCAGGAGATG	196.4%
PpKroxb	F:GTGTCTGCGTCTGCGTCTAT R:TCACAGCCCATCTTGGTACA	190.9%

Figures

Figure 1-1. Phylogeny and the morphology of the echinoderm larvae.

The phylogenetic tree was based on several molecular phylogenetic and palaeontological studies (Paul and Smith 1984; Wada and Satoh 1994; Littlewood et al. 1997; Janies 2001; Janies et al. 2011; Telford et al. 2014). Pluteus type of larva is observed in sea urchins and brittle stars although they are not closely related each other. Because the acorn worm and the basal echinoderms, sea lily produce auricularia larvae, this type is regarded as ancestral.

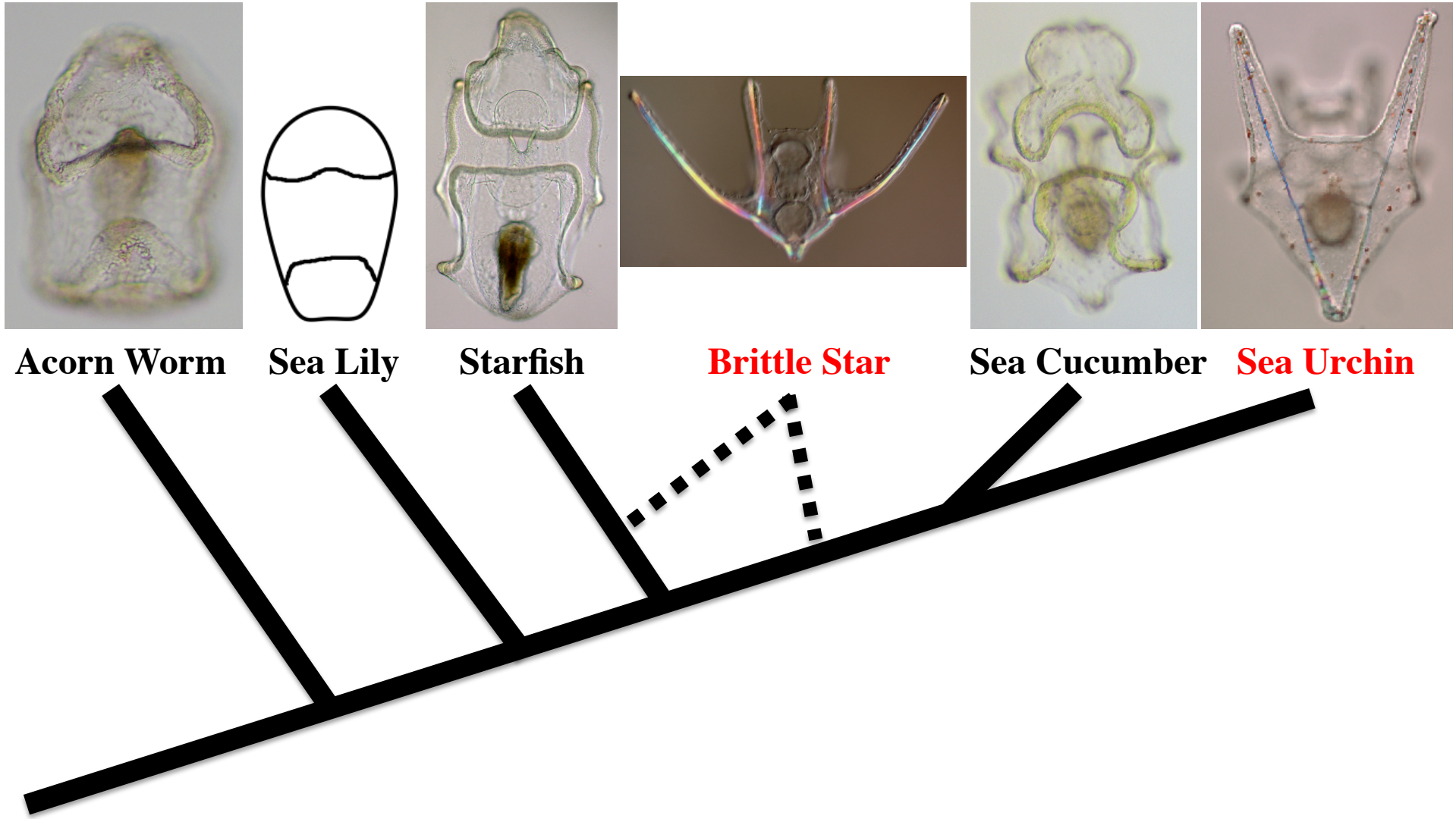

Acorn Worm
Sea Lily
Starfish
Brittle Star
Sea Cucumber
Sea Urchin

Figure 1-2. Molecular phylogenetic trees for gene ontology of VEGF (A) and VEGFR(B)

The selected amino acid substitution models were DCMut+F (A), rtREV+F (B).

The numbers on the nodes show the percentage of successful bootstrap replicate (n = 300) for each node (percentage higher than 50 are shown).

Mm: *Mus musculus*. Sp: *Strongylocentrotus purpuratus*. Pl: *Paracentrotus lividus*. Hl: *Holothuria leucospilota*. Ak: *Amphipholis kochii*. Pp: *Patiria pectinifera*. Pm: *Patiria miniata*.

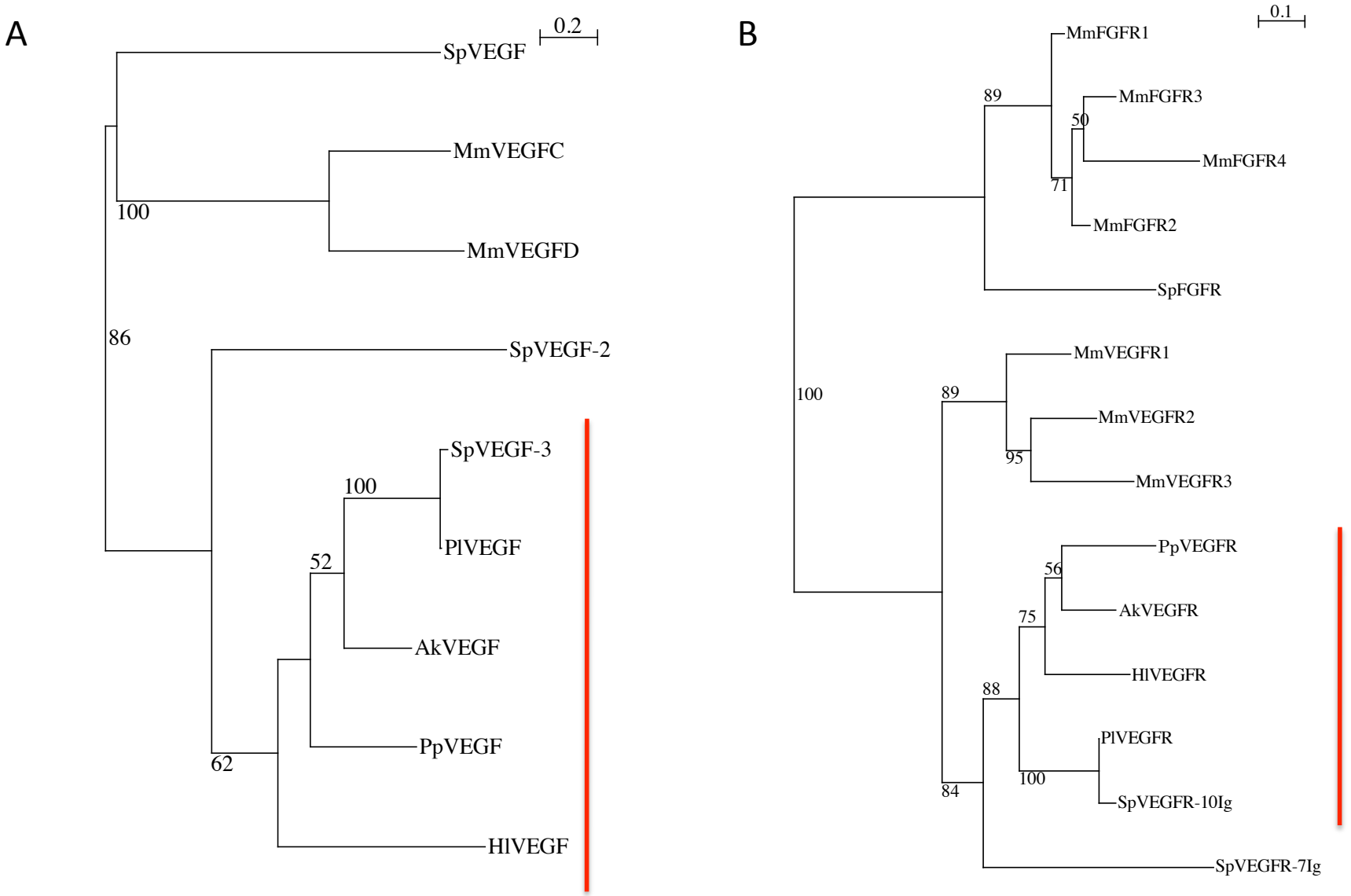

A
0.2
SpVEGF
MmVEGFC
100
MmVEGFD
86
SpVEGF-2
SpVEGF-3
100
PlVEGF
52
AkVEGF
PpVEGF
62
HlVEGF
B
0.1
MmFGFR1
89
MmFGFR3
50
MmFGFR4
71
MmFGFR2
SpFGFR
MmVEGFR1
100
89
MmVEGFR2
95
MmVEGFR3
PpVEGFR
56
AkVEGFR
75
HlVEGFR
88
PlVEGFR
100
SpVEGFR-10Ig
84
SpVEGFR-7Ig

Figure 1-3. Expression Patterns of *vegf* and *vegfr* in Starfish (*P. pectinifera*)

(A)-(D) No expression of *Ppvegfr* was detected in blastula (13.5h, A), gastrula (20h, B) and bipinnaria larva stage (2dpf and 5dpf. C, D). (E)-(H) Expression of *Ppvegf* was not detected in blastula (E), gastrula (F) and bipinnaria larva stage (G, H)

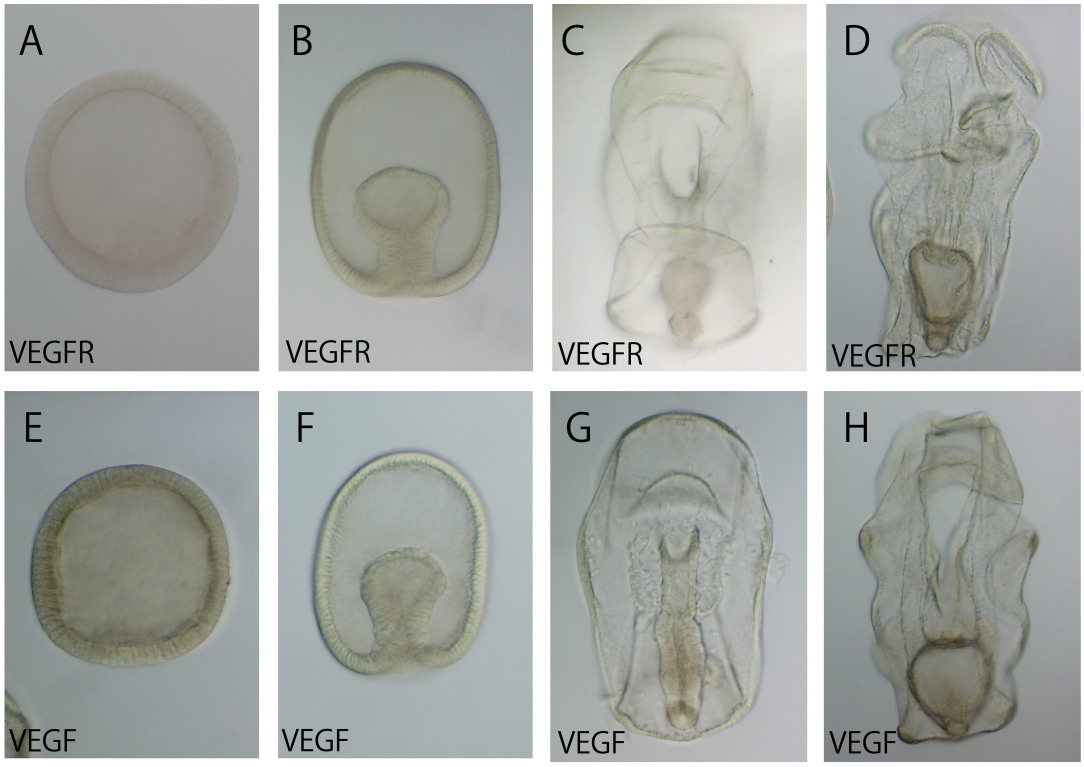

A
VEGFR
B
VEGFR
C
VEGFR
D
VEGFR
E
VEGF
F
VEGF
G
VEGF
H
VEGF

Figure 1-4. Expression Patterns of *vegf*, *vegfr* and *CA1* during adult skeletogenesis of Starfish (*P. pectinifera*).

(A)-(D) Expression of *Ppvegfr* in early brachiolaria. (A) View from the left side. Expression was detected in mesenchymes between coelom and epidermis. (B) View from the dorsal side with higher magnification. Expression was observed on both the left (black arrows) and right side (white arrowheads). (C) View from the right side. *Ppvegfr* expression on right side was observed in a few clusters of cells (white arrowheads). (D) Higher magnification of Fig. 1-4A. Expression of *Ppvegfr* on the left side was in pentaradial manner (black arrows). (E) Expression of *PpCA1* in early brachiolaria stage. View from the left side. Expression of *PpCA1* was also in pentaradial manner. (F) Expression of *Ppvegf* in early brachiolaria stage. View from the left side. Expression of *Ppvegf* was observed in epidermis around stomach and intestine. (G) Dorsal view of *PpCA1* expression in early brachiolaria stage. Expression was detected in mesenchymes between epidermis and coelom on the left side (black arrows) and right side (white arrowheads). The each position of mesenchymes on both left and right side were seemed to correspond to the position of spicules indicated (H). (H) Spiculogenesis in brachilaria stage. View from dorsal side. Spicules were observed within the mesenchyme clusters on both the left (black arrows) and right side (white arrowheads). (I)-(K) Expression of *Ppvegf* in late brachiolaria (aboral view). Expression was observed in mesenchyme cells within the adult rudiment. Black arrows indicate five clusters on the outer and oral side (I, J), white arrowheads point out five clusters on the inner and aboral side (I, K) and a black arrowhead indicates a central cluster (I, K). (J, K) show the same specimens shown in (I) with higher maginifacation, and different foci. (L, M) *Ppvegf* expression in late brachiolaria stage. (L) Expression was detected in epidermis of adult rudiment in most of late brachiolaria larvae. (M) In some larvae, *Ppvegf* expression was observed only in mesenchyme cells within adult rudiment.

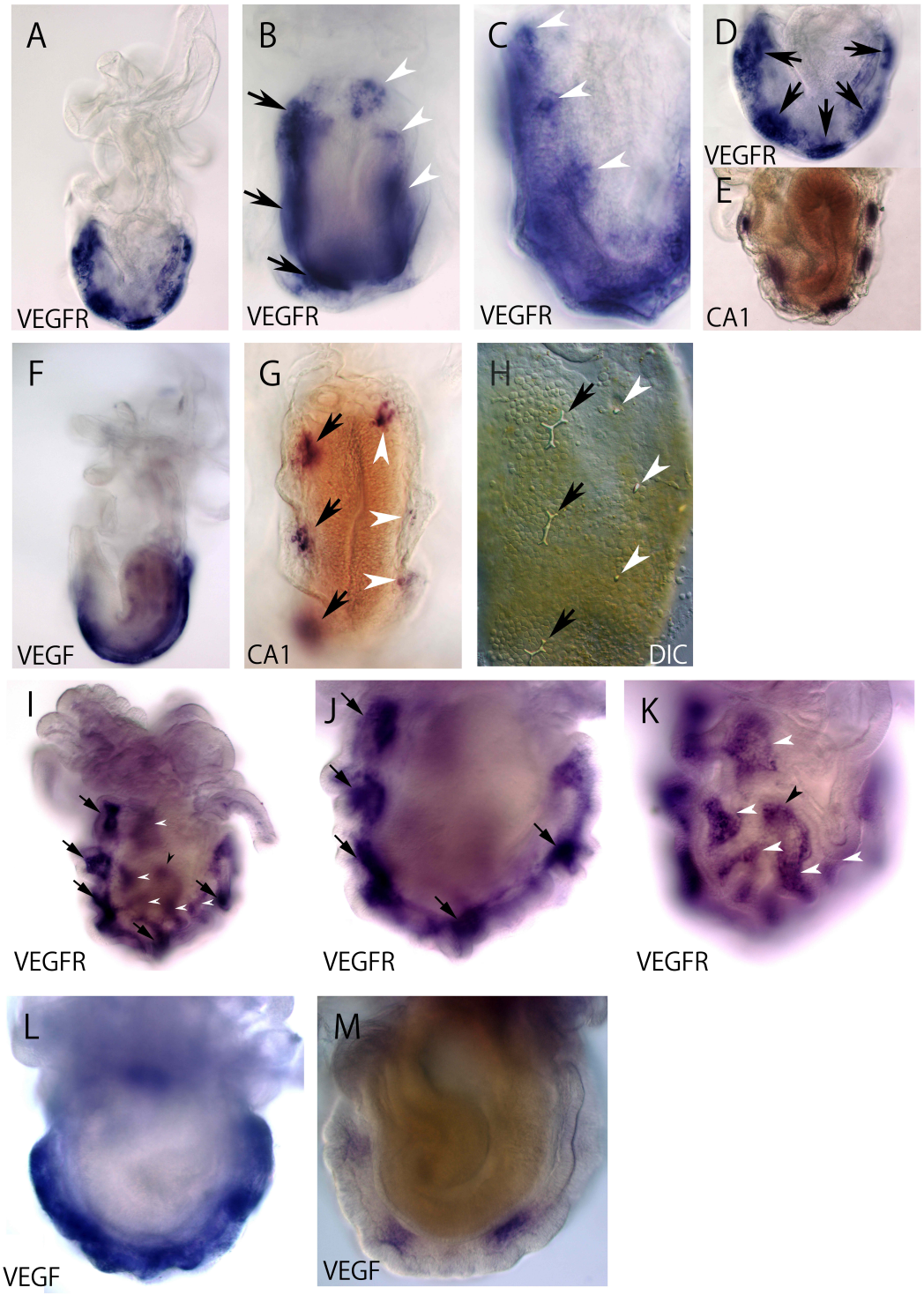

A
VEGFR
B
VEGFR
C
VEGFR
D
VEGFR
E
CA1
F
VEGF
G
CA1
H
DIC
I
VEGFR
J
VEGFR
K
VEGFR
L
VEGF
M
VEGF

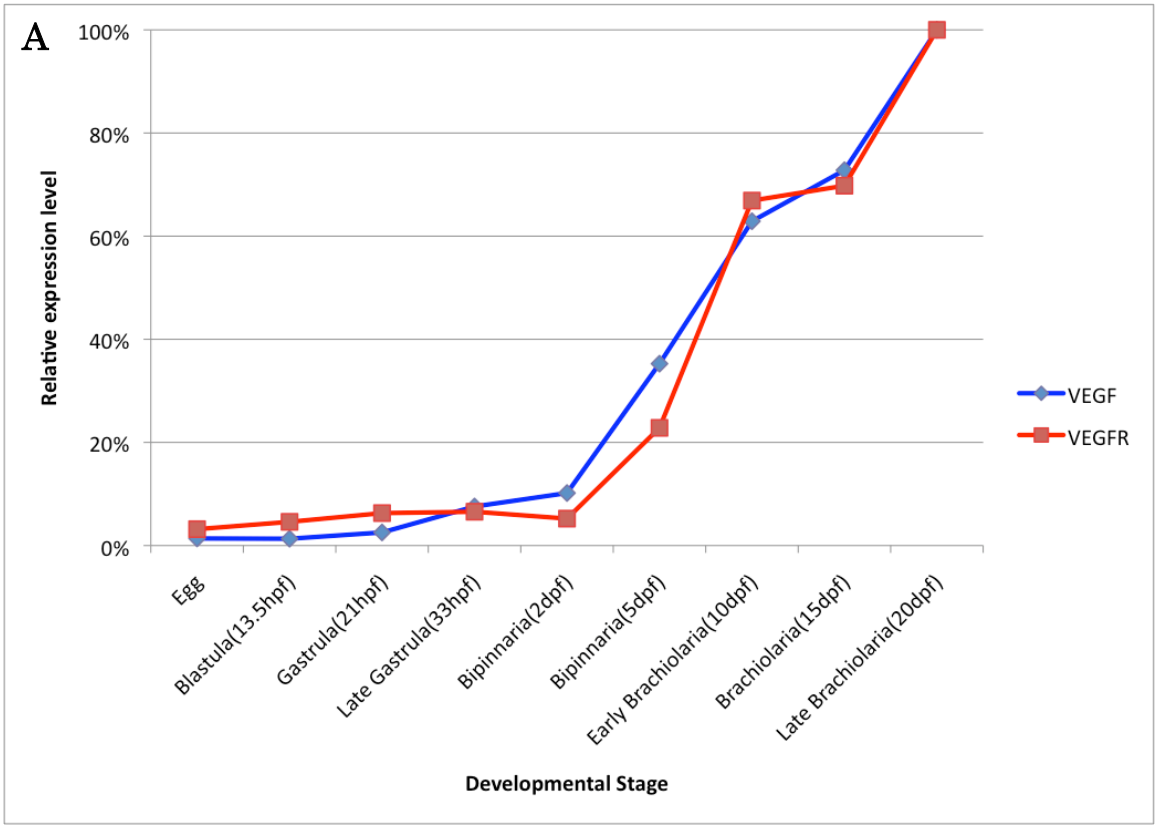
Figure 1-5. Temporal expression profile of *Ppvegf* and *Ppvegfr*, *PpCA1* and *Ppkroxβ* by qPCR.

(A) Temporal expression profile of *Ppvegf* and *Ppvegfr* by qPCR.

Values are normalized by *Ppmbf-1* expression level, and represented as percentage of signal intensity of late brachiolaria stage.

(B) Temporal expression profile of *Ppvegf* and *Ppvegfr*, *PpCA1* and *Ppkroxβ*.

Values are normalized by *Ppmbf-1* expression level.


A
100%
80%
60%
40%
20%
0%
Relative expression level
Egg
Blastula(13.5hpf)
Gastrula(21hpf)
Late Gastrula(33hpf)
Bipinnaria(2dpf)
Bipinnaria(5dpf)
Early Brachiolaria(10dpf)
Brachiolaria(15dpf)
Late Brachiolaria(20dpf)
Developmental Stage
VEGF
VEGFR

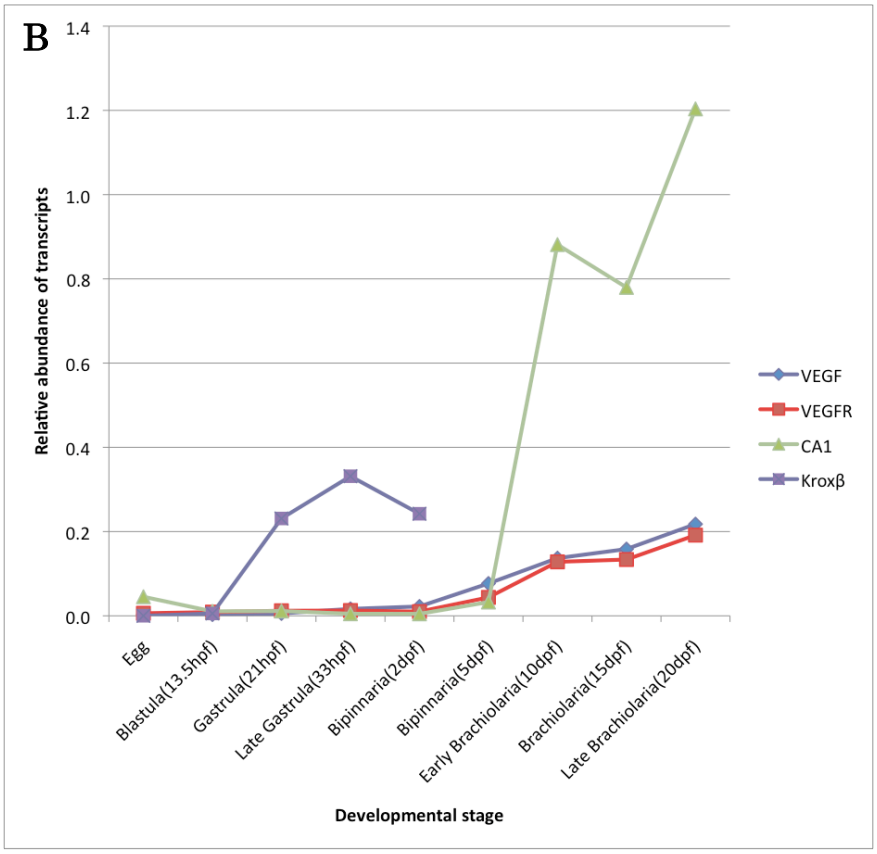
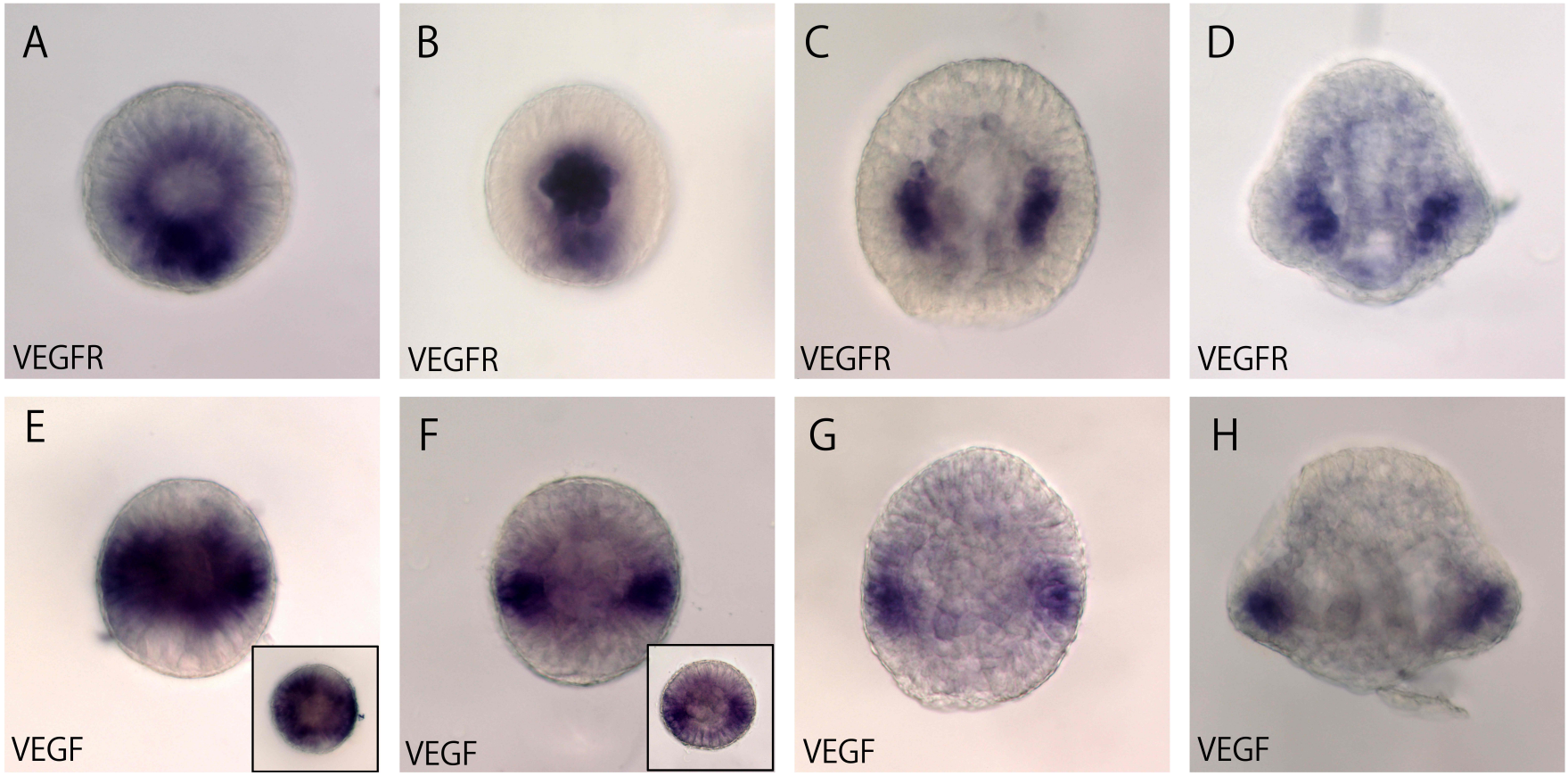

B
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative abundance of transcripts
Egg
Blastula(13.5hpf)
Gastrula(21hpf)
Late Gastrula(33hpf)
Bipinnaria(2dpf)
Bipinnaria(5dpf)
Early Brachiolaria(10dpf)
Brachiolaria(15dpf)
Late Brachiolaria(20dpf)
Developmental stage
VEGF
VEGFR
CA1
Kroxβ

Figure 1-6. Expression patterns of *vegf* and *vegfr* in brittle star (*A.kochii*)

(A)-(D) Expression of *Akvegfr*. (A) Expression was detected in part of blastula wall at hatching blastula. (B) Expression was observed in whole PMC in mesenchyme blastula. (C) In gastrula stage, expression was observed in PMC clusters. (D) Expression was maintained in PMC cluster in early pluteus stage. (E)-(H) Expression of *Akvegf.* (E) Expression was detected in cells subequatorial region of ectoderm cells in hatching blastula. (Inset) Expression viewed from either the vegetal or animal view. (F) Expression was observed in bilateral cell populations in ectoderm of mesenchyme blastula. (Inset) Expression viewed from either the vegetal or animal view. (G) Expression was seen bilaterally in ectoderm of gastrula. These *Akvegf* positive cells lie adjacent to PMC clusters. (H) In early pluteus stage, expression was maintained in epidermal cells at the tips of larval arms.


A
VEGFR
B
VEGFR
C
VEGFR
D
VEGFR
E
VEGF
F
VEGF
G
VEGF
H
VEGF