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Correlations Among mRNA Expression Levels of Engrailed, BMP2 and Smad3 in Mantle Cells of Pearl Oyster *Pinctada fucata**

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Abstract Molluscan engrailed proteins and bone morphogenetic proteins have attracted lots of interests in their potential important roles during embryonic shell compartment boundary formation. Engrailed is also proposed to be one of the important regulators of matrix proteins regional expressions in oyster mantle tissue, therefore determination of the regulation mechanisms on the particular expression patterns of engrailed in mollusks would be of great scientific value. However the study has been bogged down because the whole genomes of the oysters have not been sequenced completely and no oyster cell lines are available at present so that many genes of the regulators and mediators need to be cloned and classic signal pathways assays are hard to be applied. In previous study, the bone morphogenetic protein 2 (BMP2) and the oyster mothers against decapentaplegic 3 homolog (Smad3) have been identified from the pearl oyster *Pinctada fucata*. It is interesting to investigate the relationships among the oyster engrailed, BMP2 and Smad3 expressions in the oyster. Thereby a partial fragment of an engrailed homolog has been amplified from the genome template of the pearl oyster *Pinctada fucata*. Alignments with the deduced amino acids sequence show that the DNA-binding homeobox (EH4) domain of the oyster engrailed shares extremely high similarities with other engrailed proteins. Shell notching experiment and semi quantitative polymerase chain reaction results suggest that the change pattern of the oyster engrailed mRNA level is similar to that of the oyster mothers against decapentaplegic 3 homolog (Smad3) in shell repair process. Then the Smad3, the engrailed and the BMP2 mRNA expression levels in primary cultured oyster mantle tissue cells that are treated with dexamethasone (DXM) and hydrogen peroxide (H_2O_2) respectively have been examined. Real time quantitative polymerase chain reaction results show that the engrailed, the Smad3 and the BMP2 mRNA levels in the oyster mantle cells exhibit significant correlations with each other. All the results provide important clues and basis for further study on developmental and signal transduction mechanisms on oyster biomineralisation.

Key words engrailed, *Pinctada fucata*, Smad3, BMP2, shell notching, real-time PCR
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With complicated and fine structure of its nacreous layer, pearl is a typical kind of biomineralisation products. It is marvelous that matrix proteins (only less than five percent of the weight of the nacre) control the shapes and the sizes of calcium carbonate crystals in pearl and shell formation. It has been found some matrix proteins such as Nacrein, MSI7, Pearlin that exert different influences on *in vitro* calcium carbonate crystals formation have distinct expression regions in the oyster mantle tissue. It was suggested that oyster engrailed homologs may regulate the compartmentary distributions of the matrix proteins[1].

Recently, engrailed homologues have been identified from all five classes of mollusks[2]. They are highly conservative to the vertebrate engrailed

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proteins. Their expression patterns also indicate that engrailed may be important for the shell formation and their roles are widely conserved among mollusks. It is interesting that the dpp-BMP2/4 ortholog in the mollusk *Patella vulgata* is expressed in cells of the ectoderm surrounding, but not overlapping the engrailed-expressing shell-forming cells[3]. That suggests that engrailed and BMP2 are most likely involved in forming a compartment boundary between these cells, which is very similar to the situation in, for example, developing wing imaginal disc in *Drosophila*[3]. The ZfBMP4 treatment experiments reported by Kin *et al*[4] also revealed the importance of dpp (BMP ortholog in *Drosophila*) in establishing the characteristic shape of the bivalve shell anlagen.

BMP2 has been identified from the pearl oyster *Pinctada fucata*. The C-terminal 149-amino acid portion of *Pinctada fucata* BMP2 showed 66% similarity to human BMP2. This portion contained seven cysteines that other members of TGFβ family share. It is very likely that BMP2 of *Pinctada fucata* has the same functions as BMP2 in vertebrate, that is, it may participate not only in morphogenesis during development but also in the formation of hard tissue. The BMP2 gene was expressed strongly in the inner part of the mantle tissue, corresponding to the nacreous aragonite shell layer. This finding suggests that BMP2 has a key role in nacreous layer formation[5].

There is another important mediator of TGFβ signaling pathways——Smad3 that has been identified from the pearl oyster *Pinctada fucata*. Its amino acid sequence is nearly identical to the most vertebrate Smad3 proteins while the Smad3 homologues in *Drosophila* and *C. elegans* are very different. It suggests that the oyster Smad3 may play similar functions as the vertebrate Smad3 proteins[6]. It has been reported that the chondrocytes of the Smad3-deficient mice have enhanced BMP signaling[7]. It is rational that the oyster Smad3 homolog might also have influence on BMP2 expression in the oyster. Therefore it is meaningful to figure out the relationships among the oyster engrailed, BMP2 and Smad3 expressions, especially in the oyster mantle tissue, which is one of the most important tissues responsible for biomineralisation in the oyster.

In this study, we have first cloned a partial fragment of an engrailed homolog from the pearl oyster *Pinctada fucata*. It encodes the DNA-binding homeobox (EH4) domain and the EH5 domain of the oyster engrailed homolog. The EH4 domain of the oyster engrailed (Pf-EH4 for short) shares an extremely high similarity with other engrailed proteins. Shell notching experiment suggests that the change pattern of the oyster engrailed mRNA expression during the shell repair process is nearly the same to that of the oyster Smad3 but the change pattern of the oyster BMP2 is different from those of the oyster engrailed and the oyster Smad3. Hence, the relationships among the oyster engrailed, BMP2 and Smad3 should be investigated before the molecular mechanism of the compartment boundary formation in the shell formation can be fully clarified.

However, it is nontrivial to demonstrate any direct regulation in signal transduction mechanisms of the oyster *Pinctada fucata* at present. First, a signal pathway always involves many genes, such as ligands, receptors, mediators and so on. But many of the genes have not been identified from the oyster. Secondly, the oyster cell lines have not been established yet. Besides, the efficiency of transfection in primary cultured oyster cells is extremely low except for neural cells. Nevertheless, it has been reported that dexamethasone (DXM) and hydrogen peroxide (H_2O_2) can be added into the culture medium respectively to adjust the Smad3 expression level in the mammal cells[8-9]. Therefore, we have evaluated by real time quantitative PCR the expressions of the Smad3, the engrailed and the BMP2 of the *in vitro* primary cultured mantle tissue cells that are treated with DXM and H_2O_2 respectively. In the end, the potential signal transductions in the oyster that the three genes may be involved are discussed and further investigations are proposed.

1 Materials and methods

1.1 Molecular cloning of the engrailed gene from the oyster

Live adult oyster *Pinctada fucata* was obtained from the Guofa Pearl Farm in Beihai, China. The adductor muscle was separated from the oyster with sterilized knife and then ground into very fine powder in liquid nitrogen. The genome DNA was extracted with the Tissue/Cell genome isolation kit (Biodev, Beijing, China) according to the manufacturer's instructions. A pair of degenerate oligonucleotide primers Eng-2 (5′ AGCGAATTCAARMGNCCNM-GNACNDSNTT 3′) and Eng-4 (5′ TGGAAGCT-TRTANARNCCYTSNGCCAT 3′) were synthesized according to Nederbragt′s study[9]. Using the oyster

genome DNA as a template, PCR cycles were conducted in the following procedure: denaturation at 95℃ for 10 min, followed by 35 cycles at 95℃ for 1 min, 42℃ for 1 min, and 72℃ for 1 min. A final extension step was conducted at 72℃ for 7 min. The PCR product with expected size of 250 bp was cloned into pGM-T Easy Vector (Tiangen, Beijing, China) and sequenced.

The entire nucleotide sequence was analyzed using the BLAST program available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Primer Premier 5.0(PRIMER Biosoft International, Palo Alto, USA) was used to identify its encoding protein. Molecular structural simulation was performed with 3D-JIGSAW 2.0 (http://bmm.cancerresearchuk.org/～3djigsaw/). Multiple alignments were created using the Vector NTI 8.0 (InforMax Inc., Frederick, MA.)

1.2 Semi quantitative RT-PCR

Semi quantitative RT-PCR was carried out to detect the mRNA expression levels of the engrailed gene at three different stages of the embryonic development of the oyster. Total RNA from the oyster embryos at gastrula stage and D-shape larva stage and the oyster adult were isolated. Equal quantities (2 μg) of total RNA from different developmental stages were reverse-transcribed into the first strand of cDNA with Quant Reverse Transcriptase (Tiangen) following the manufacturer's instructions. Then, the synthesized cDNA was used as the template for the following PCR. The primers are listed in Table 1. The PCR procedures are as follows: 95℃ for 5 min, 30 cycles at 95℃ for 40 s, T_m for 30 s and 72℃ for 30 s, followed by 7 min incubation at 72℃. Negative controls were carried out in the absence of cDNA template to examine the cross contamination of the samples. The PCR products were determined by electrophoresis in 1.5% denaturing agarose gel and visualized by ethidium bromide (SIGMA, St. Louis, MO, USA) staining under UV light. The experiments have been repeated, and the results were consistent.

Table 1 Sequences of designed primers

Gene name	GenBank No.	Primer	Sequence	Temperature/℃	Amplification/bp
Pf-engrailed	#	ES1	5′ CGGCATTCTCTAACGATCAGC 3′	42	198
		ES2	5′ CCATGAGATGTAACGCTAACAAG 3′		
Pf-GAPDH	AB205404	GAPDHF	5′ GATGGTGCCGAGTATGTGGTA 3′	53	229
		GAPDHR	5′ CGTTGATTATCTTGGCGAGTG 3′		
Pf-Smad3	EU137731	SB1	5′ TTCCCCATTTACTCCACCCATC 3′	62	350
		SB2	5′ TCGTATTGAAGCACCAGTGTTGC 3′		
Pf-BMP2	AB176952	BMP2F	5′ GCGGTCGAAGAACTAAAA 3′	51	460
		BMP2R	5′ ATCCGCATCCTTCAACAA 3′		

#: GQ241736 for the fragment coding the EH4 and EH5 motifs of the oyster *Pinctada fucata*.

1.3 Shell notching experiments and semi quantitative PCR

Shell notching experiments were performed according to the method of Mount *et al.*[10] with some modifications. A V-shaped notch was cut on the shell margin close to the adductor muscle of the oysters without hurting the mantle tissue, and then divided into six groups randomly, each of which contained five individuals. The six groups were returned to seawater tanks for 0, 6, 12, 24, 36 and 48 h, respectively, and then sacrificed. About 1 cm^2 area of the mantle tissue around the cut was separated. The mantles of the same group were placed together and ground in the liquid nitrogen. Meanwhile, the mantles of five individuals without shell notching were separated in the same way and used as a control. The total RNA was extracted, the cDNA first strand was synthesized and semi quantitative PCR was performed as described above. The shell notching and succedent RT-PCR experiments were repeated to make sure that the results were consistent.

1.4 The oyster mantle tissue cells culture and real time quantitative PCR

The culture of the cells migrated from the mantle tissue of the oyster *Pinctada fucata* was performed following the procedure as described in previous study[11].

Five days after the culture initiation, dexamethasone or hydrogen peroxide were added into the culture medium to the final concentration of 10^{-7} mol/L or 10^{-4} mol/L, respectively. The NaCl solution (60 μl of 150 mmol/L) was added into another 6 ml cultured primary mantle cells as blank control. The cells were collected at 24 h after the treatment with drugs. The total RNA of the cells migrated from the oyster mantle tissue challenged with different solutions was extracted. The cDNA was synthesized as described above. Real-time quantitative PCR reactions were carried out in 25 μl volume of a solution containing 12.5 μl SYBR Premix Ex Taq (TaKaRa, Dalian, China), 10 μmol/L each primer (sense and antisense), 0.5 μl ROX reference Dye Ⅱ, 2 μl 10-fold diluted cDNA template and 9 μl sterile distilled water. The primer pairs were the same as described above. The assays were performed on Mx3000P™ RT-PCR System (Stratagene, USA). The procedures are as following: 95℃ 5 min, 40 cycles at 95℃ for 5 s, 60℃ for 20 s, and 60℃ for 1 s, and measurements were done at the end of the 60℃ annealing step. The Ct value of the oyster GAPDH (the internal control) in each cDNA template was tested to ensure that each template had the same input total RNA. Standard curves were generated using cDNA (10^{-4} mg to 1 mg). For each reaction, the crossing point (CP) was optimized using Mx3000P software (version 2.0; Stratagene, La Jolla, USA). The PCR efficiency (E) was calculated by the standard curve method: $E=10^{(-1/\text{slope})}-1$; this value was optimized for each primer pair by performing standard curves from serial dilutions of each positive cDNA control to ensure that E ranged from 0.8 to 1.2. All of the real time PCR reactions were performed in quadruplicate. The gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method[12], and normalized by dividing the calculated values for the mRNA samples by that of GAPDH mRNA at the same time point. The data from the experiments were analyzed using the analysis of variance (ANOVA) in Origin 7.0 (OriginLab Corporation, USA).

2 Results

2.1 Cloning of the EH4 conserved motif of the engrailed homolog in the pearl oyster

Figure 1 presents the 244 bp fragment encoding a part of the cDNA sequence of the engrailed homolog in the pearl oyster, *Pinctada fucata*. It was cloned from the oyster genome and sequenced. Its mRNA sequence has been submitted to GenBank with the accession number of GQ241736. By aligning its deduced amino acid sequence (81 residues) with other engrailed proteins, the EH4 homeobox motif (59 residues) and the EH5 motif (19 residues) of the engrailed homolog of the oyster were predicted. The sequence of the first eight amino acids of the EH4 motif of the oyster engrailed homolog was deduced from the forward primer that was used to clone the conserved region of the engrailed homolog in oyster *Pinctada fucata* (Pf-en). There just ten amino acids of the EH5 motif of the oyster engrailed homolog are determined.

A

GAGATTGACAAGCGACCCCGAACGGCATTCTCTAACGATCAGCTTCAAAGATTAAAGCGG

E I D K R P R T A F S N D Q L Q R L K R

GAATTTGATTCATGTCAATATCTCACAGAACAACGCAGACGAGATTTGGCTCGAGAACTG

E F D S C Q Y L T E Q R R R D L A R E L

CAGTTATCGGAAGGACAAATTAAAATTTGGTTTCAAAACAAAAGGGCTAAAGTGAAAAAA

Q L S E G Q I K I W F Q N K R A K V K K

TCCGCGGGATCCAAAAACTTGTTAGCGTTACATCTCATGGCGCAGGGATTGTACAAGCTT

S A G S K N L L A L H L M A Q G L Y K L

CCA

P

Fig. 1 The cDNA sequence and deduced amino acid sequence of the EH4 motif of the engrailed from the pearl oyster, *Pinctada fucata*

The primers used in cloning of the conserved region of the oyster engrailed homolog are boxed. The EH4 motif (59 aa) is shadowed and the EH5 motif (19 aa) is underlined.

2.2 Sequence alignments of the oyster EH4 motif with the engrailed proteins in other species

The results of the sequence alignments of the oyster EH4 motif with the engrailed proteins in other species show that the identity of the EH4 motif of the oyster *Pinctada fucata* (Pf-EH4) is over 50% identical and nearly 90% positive to the EH4 motifs of the engrailed proteins in other species (Figure 2). It suggests that the EH4 motif of the oyster engrailed protein may also able to bind specific sequence for engrailed in certain promoters and then regulate the expressions of the downstream genes. The EH4 motif of the oyster *Pinctada fucata* (bivalve, mollusca) is most similar (79%) to the EH4 motifs of the engrailed proteins of *Haliotis asinine* and *Lymnaea stagnalis* (gastropod, mollusca) (Figure 2). The alignments

results are in accordance with the evolutionary relationship. Since the EH4 domain may be just 5% of the whole engrailed protein in the oyster, it is not meaningful to do a polyphylogenetic tree analysis on the EH4 amino sequence of the oyster engrailed alone.

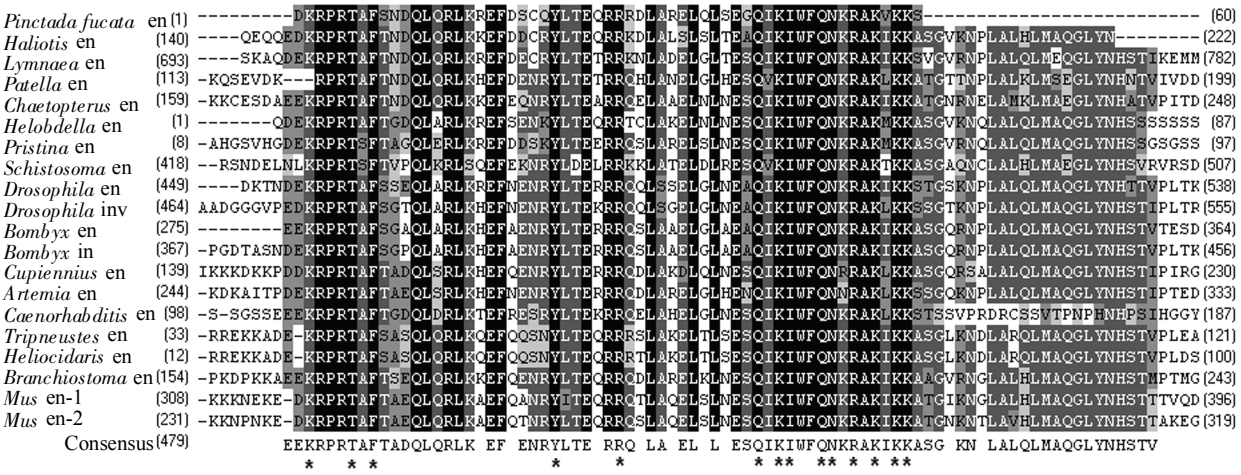

Fig. 2　Comparison of the amino acid sequences of the EH4 motif of *Pinctada fucata* engrailed(GQ241736) and other engrailed proteins

The DNA binding sites in the consensus sequence are marked with asterisk. The species with accession numbers in parentheses are the mollusc *Haliotis asinine* (ABC00198), *Lymnaea stagnalis* (BAF96782), *Patella vulgata* (AF440096); the polychaete annelid *Chaetopterus* sp. (AAK67707); the clittelate annelids *Helobdella triserialis* (P23397) and *Pristina leidyi* (AAK64219); the platyhelmint *Schistosoma mansoni* (AAA29929); arthropods: the insects *Drosophila melanogaster* (engrailed, P02836 and invected, P05527) and *Bombyx* (engrailed, AAA62704 and invected, P27610), the chelicerate *Cupiennius salei* (CAA07503) and the crustacean *Artemia fransiscana* (CAA50279); the nematode *Caenorhabditis elegans* (T15488); the echinoderms *Tripneustes gratilla* (P09532) and *Heliocidaris erythrogramma* (AAD55133); the cephalochordate *Branchiostoma floridae* (AAB40144); the (vertebrate) chordate *Mus musculus* (en-1, NP_034263 and en-2, D48423).

Another notable characteristic is that all the residues that are predicted to be the DNA binding sites in the EH4 motif of the oyster engrailed homolog according to the conserved domain (GenBank CDD No. 28970) are identical to the DNA binding sites of the other engrailed proteins (Figure 2). It suggests that the DNA binding sequence for the oyster engrailed protein may be highly similar to the DNA sequences that the other ever-known engrailed proteins select to bind.

2.3　Pf-en mRNA expression levels in three developmental stages

Figure 3 displays the results of the semi quantitative RT-PCR of Pf-en mRNA expression levels in three different developmental stages of the oyster *Pinctada fucata*. The engrailed is expressed at such a low level in the gastrula stage that the PCR product of it was undetectable in the gel. However, the oyster engrailed expression increases dramatically to a high level in the D-shape larva stage of the oyster embryo development process. It is from the D-shape larva stage that the shell production begins [13]. Therefore, the Smad3 and engrailed gene may be used as the marker genes for shell formation. The PCR

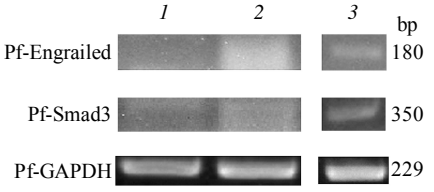

Fig. 3　Pf-en mRNA expression in three different developmental stages of the oyster *Pinctada fucata*

The total RNAs were extracted from three different developmental stages of the oyster *Pinctada fucata* to synthesize cDNAs. Then semi quantitative PCR were conducted to analyse the mRNA expression levels of the oyster engrailed and Smad3. The housekeeping gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is included as a positive control to make sure the amounts of the cDNAs used in all the samples are equal. *1*: Gastrula stage; *2*: D-shape larva stage; *3*: Adult.

product for the adult samples can be more clearly seen in the gel. It indicates the mRNA transcripts of engrailed in adult are more stable. The expression pattern of the oyster engrailed is consistent with that of the oyster Smad3 in different development stages. It suggests that there may be a correlation between the engrailed and the Smad3 expressions in the oyster.

2.4 Pf-Smad3, Pf-en and Pf-BMP2 mRNA expressions after shell notching

As shown in Figure 4, the mRNA level of Pf-Smad3 increases gradually and reaches a peak at 12 h after the shell notching. Then, the Pf-Smad3 mRNA level decreases gradually and reaches the lowest level at 36 h after the shell notching. However, the mRNA expression of Pf-Smad3 increases to the highest level again at 48 h after the shell notching. These results suggest that the oyster Smad3 participates in shell repair. The change pattern of the mRNA levels of Pf-en is similar to that of Pf-Smad3 except that the Pf-en mRNA level reaches the highest level at 24 h after shell notching. The mRNA level of Pf-BMP2 changes in a different pattern. It increases to its highest expression level at 6 h after the shell notching and stays at that level until 36 h after the shell notching. These results indicate that engrailed and BMP2 also participate in shell repair and the response of BMP2 is faster than those of the other two genes in the oyster.

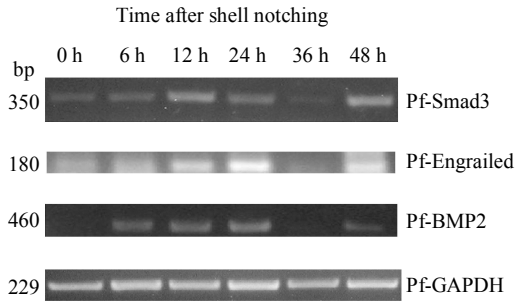

Fig. 4 The oyster engrailed, Smad3 and BMP2 mRNA expressions after shell notching

The shell margin close to the adductor muscle of the oysters was cut in a V-shaped nick without hurting the mantle tissue. The oysters were then divided into six five-individual groups randomly and returned to seawater tanks for 0, 6, 12, 24, 36 and 48 h, respectively. About 1 cm^2 area of the mantle tissue around the cut of the same group were excised, placed together. The total RNA was extracted, the cDNA first strand was synthesized and semi quantitative PCR was performed. The shell notching and succedent RT-PCR experiments were repeated to make sure that the results were consistent.

2.5 The Pf-Smad3, Pf-en and Pf-BMP2 mRNA expressions in primary cultured oyster mantle cells

In order to regulate the Smad3 mRNA expression level of the oyster mantle tissue cells, dexamethasone and hydrogen peroxide were added into the culture medium of the *in vitro* primary cultured oyster mantle tissue cells respectively. The migrated oyster mantle tissue cells were collected and real time quantitative RT-PCR was performed subsequently. The results (Figure 5) show that the Pf-engrailed mRNA, Pf-Smad3 and Pf-BMP2 expressions slightly decrease when the final concentration of dexamethasone in the culture medium is 10^{-7} mol/L, compared to their mRNA expressions in the control group that is treated with NaCl solution. On the contrary, the Pf-Smad3, Pf-Engrailed and Pf-BMP2 mRNA transcripts remarkably increase in the cells that are treated with H_2O_2 whose final concentration in the culture medium is 10^{-4} mol/L. These results demonstrate that dexamethasone and hydrogen peroxide can not only

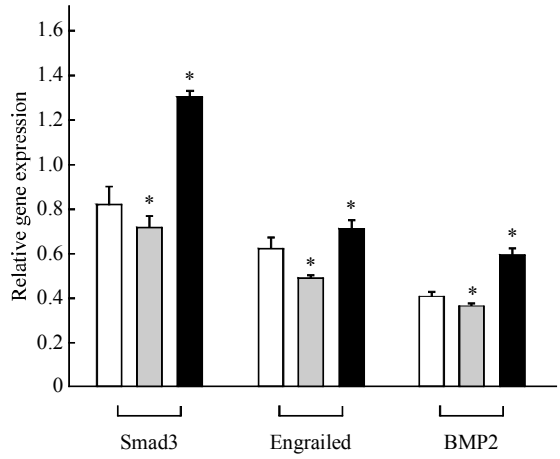

Fig. 5 Relative expression levels of the oyster Smad3, engrailed and BMP2 mRNAs

Five days after the *in vitro* culture of the mantle tissue of the oyster *Pinctada fucata*, dexamethasone (DXM) were added into the culture medium to the final concentration of 10^{-7} mol/L. Another group of samples were added with hydrogen peroxide (H_2O_2) to 10^{-4} mol/L. The NaCl solution was used as blank control. The cells were collected at 24 h after the treatment with drugs. The total RNA was extracted and the cDNA was synthesized as described above. Real-time quantitative PCR reactions were carried out by using SYBR Premix Ex Taq according to the manufacturer's instructions. The gene expression levels were calculated and normalized by dividing the calculated values for the mRNA samples by that of GAPDH mRNA at the same time point. Data are means and standard errors of four independent experiments. Asterisks indicate that significant difference exists between the experimental samples and the control samples (*$P < 0.01$). □: NaCl; ▨: DXM; ■: H_2O_2.

adjust the oyster Smad3 mRNA level but also can influence the engrailed and BMP2 mRNA levels in the oyster. The effects of the drugs are similar to their effects in mammals. It suggests there may be similar signal transduction pathways in the oyster.

2.6 Sequence alignments of Pf-EH4 with HOX proteins

Figure 6 presents the result of the sequence alignments of Pf-EH4 with HOX9c (GenBank No.NP_008828), HOXA13(NP_032290), and HOXD13 (NP_032301). The Pf-EH4 is 98.3% positive but only 28.8% identical to the homologous region of the other three HOX proteins. While, the fragment "IWFQNK-RAKVKK" in the N-terminal of Pf-EH4 is 75.0% identical. It suggests that the Pf-EH4 might have similar functions as the HOX proteins.

Fig. 6　Comparison of amino acid sequences of the EH4 motif of *Pinctada fucata* engrailed (GQ241736) and other three types of HOX proteins

The HOX proteins represented with accession numbers are HOX9c (GenBank No. NP_008828), HOXA13 (NP_032290), and HOXD13 (NP_032301).

2.7 Structural simulation of Pf-EH4

Figure 7 shows the simulated molecular structures for the EH4 motif of the engrailed homolog in the oyster (Pf-EH4) (shown in black) and the human En-1 protein (GenBank No. NP_001417, shown in gray). The 3-D simulated structure of the highly conserved EH4 motif of the oyster is greatly similar to that of the human, consisting of three α-helixes that are characteristics of all the homeodomains. It indicates that the EH4 motif of the engrailed homolog in the oyster may play the similar function as the other engrailed proteins that can bind TATA box to regulate gene expression.

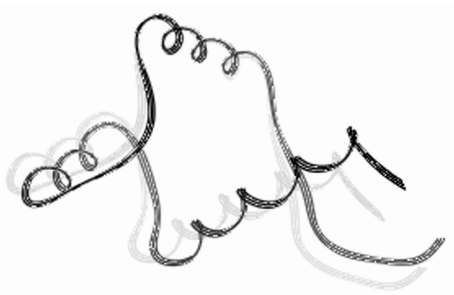

Fig. 7　The simulated molecular structures

The EH4 motif of the engrailed homolog in the oyster (in black) was predicted by software 3D-JIGSAW 2.0. The EH4 motif of the human En-1 protein (GenBank No. NP_001417) (in gray) was similar to that of the oyster.

3 Discussion

In this paper, we have investigated the effects of dexamethasone and hydrogen peroxide on the Smad3, the engrailed and the BMP2 mRNA expression levels in primary cultured oyster mantle tissue cells. First, a part of the cDNA that encodes the oyster engrailed homolog was cloned from the genome of the pearl oyster *Pinctada fucata* and sequenced. The deduced amino acid sequence of the fragment contains the engrailed homeobox (EH4) domain and part of its EH5 domain. Sequence alignment shows that the EH4 domain of the oyster engrailed protein bears extremely high similarities to the engrailed proteins in vertebrate, suggesting that the engrailed homolog may play important roles in the oysters in a conservative way.

Semi quantitative reverse-transcription PCR was applied to measure the mRNA expression levels of the engrailed and the Smad3 during three developmental stages of the oyster. The results show that both the oyster engrailed and Smad3 mRNAs increase significantly at the D-shape stage. The remarkable increases of the Smad3 and the engrailed gene expressions would regulate expressions of many other genes as they are so highly conserved transcriptional factors. Therefore, the oyster Smad3 and the engrailed may play important roles in many developmental events in the D-shape stage when the shell production

begins[14]. The D-shape stage is also the point where hemocytes first appear in the development of the oyster[13]. Hemocytes play important roles in the inflammation caused by encapsulation, thus being crucial for shell formation, which is a well established mechanism for the production of cultured pearls[14]. It has been reported by Mount *et al.*[10] that crystalblastic cells are found at the mineralization front, some of which are observed releasing crystals. Thus, the potential functions of the oyster Smad3 and engrailed in hemocytes production in the oyster are worthy to be investigated since regulating hemocytes production may be one of the important functions in shell formation.

The shell notching experiments results of this study show that the change pattern of the engrailed mRNA expression level is very similar to the Smad3, too. But BMP2 is different from the two in the oyster during the shell repair process. These results indicate that the Smad3, the engrailed and the BMP2 all participate in shell repair process and there may be correlation between the Smad3 and the engrailed. However, by now the signal transduction mechanisms on the relationships among the oyster Smad3, engrailed and BMP2 expressions have not been well clarified due to the lacking of the oyster cell lines and the incomplete sequencing of the oyster genome.

It has been known that dexamethasone (DXM) suppresses Smad3 pathway in osteoblastic cells[8] and hydrogen peroxide increases the expression of Smad3 in A549 cells[9] and significantly increased endothelial expression of BMP2[15]. DXM is also proved to inhibit canonical Wnt signaling[16] and have suppressive effect on BMP2 expression[17]. Therefore, DXM and H_2O_2 are added into the culture medium of *in vitro* primary cultured oyster mantle cells respectively. Real time quantitative PCR results show that the oyster engrailed, Smad3 and BMP2 expression levels are all changed in the drug treated groups. Dexamethasone represses the mRNA expressions of the engrailed, the Smad3 and the BMP2 of the oyster mantle cells while hydrogen peroxide increases the three genes mRNA expressions. These results demonstrate there are significant correlations among the engrailed, the Smad3 and the BMP2 mRNA expressions in the oyster mantle tissue. Smad3 might influence the oyster Wnt pathway[18], while engrailed may be one of the targets of the oyster Wnt signaling pathway as in the vertebrate[19]. The interaction of the oyster Smad3 and BMP2 would be more complicated. The similar effects of DXM and H_2O_2 on the oyster mantle cells as on the vertebrate cells indicate there may be similar signal pathways that regulate the mRNA expressions of the engrailed, the Smad3 and the BMP2 in the oyster as those in the vertebrate.

As for further research, in case that the oyster cell lines are difficult to acquire, EMSA, SPR, footprinting and other DNA-Protein interaction methods can be used to determine any engrailed binding sites in the promoters of the oyster genes, thus providing direct proofs of gene regulation in the oyster that the engrailed is involved. The recombinant protein GST-Pf-EH4 is expressed in *E. coli* and can be purified (unpublished data). The consensus sequences specific for EH4 homeodomain have been characterized as "YAATYANB"[20] in vertebrate. However, no putative engrailed binding sites have been found in the promoters of mollusks since only a few promoters have been predicted from the mollusks. A Hoxd-8 binding site with the sequence as "GTTTTATT" has been found in the promoter region of the activin-like receptor 1 in *C. gigas* whose genome has been mostly sequenced[21]. This sequence is very similar to engrailed binding sites. Figure 6 shows that the EH4 domain of the oyster is similar to HOX proteins. It suggests that the oyster engrailed protein may be able to bind the promoter of its ALR-1 homolog. There are engrailed binding sites in the promoters of many TGF-β superfamily members in vertebrate. For example, the human bone morphogenic protein (BMP)-5 gene promoter contains an engrailed recognition sequences (–549～–541)[22]. Therefore, it is most likely that there are also regulation effects of engrailed on TGF-β signal pathways.

Many experiments demonstrated that the MH1 domain of Smad4 binds N-terminal residues of the homeodomain of Hoxc9[23] and HOX proteins interact with the MH2 domain of R-Smads[24]. It is presumptive that the oyster engrailed may also bind to its Smad proteins. Engrailed in vertebrate is able to bind directly to many other transcriptional factors, such as Pax6[25], and FOX[26].

In conclusion, we cloned a highly conserved fragment of the cDNA that encodes the engrailed homolog in the pearl oyster *Pinctada fucata*. The change patterns of the engrailed during three developmental stages of the oyster and the shell repair process are very similar to that of the oyster Smad3 in

respective. Dexamethasone (DXM) and hydrogen peroxide experiments indicate that the mRNA expression of the engrailed, the Smad3 and the BMP2 are correlated and there may be similar signal transduction mechanism on these genes expressions. These genes are most likely to play important roles in the oyster biomineralisation although they are also important to other physiological process. This study is the initial step to give light on the signal transduction and developmental mechanisms on molluscan shell formation. Further identification of the DNA binding sequences of the engrailed and the Smads in the promoter regions of the matrix proteins and genes that are related to shell formation in the oyster will provide more clues to explore the functions of the engrailed and the TGF-β pathways in oyster biomineralisation. Since DXM and H_2O_2 can regulate the expression of the Smad3, engrailed and BMP2, the drugs may be used to adjust pearl sac formation and nacre secretion.

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合浦珠母贝外套膜细胞 engrailed、BMP2 和 Smad3 的 mRNA 表达水平的相关性 *

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摘要 软体动物 engrailed 蛋白和骨形成相关蛋白对胚胎贝壳区域边界形成可能具有重要作用，engrailed 还被推测为调节基质蛋白在外套膜组织区域化表达的重要调控因子．因此，弄清调控 engrailed 在软体动物中特征表达的分子机制有着重要的研究意义．但是，由于贝类基因组测序尚不完整，目前也没有建立获得贝类细胞系，以致于许多预测可能参与调控的基因需要通过克隆来鉴定，而且经典的研究细胞信号通路的方法也很难得到应用．目前，在中国南海广泛养殖的合浦珠母贝中，已获知其 BMP2 和 Smad3 的 cDNA 全长，以该贝的基因组为模板，PCR 扩增获得了一段 engrailed 编码区片段．经软件分析，该片段含有 EH4 结构域，且与其他物种 engrailed 蛋白具有很高的同源性．研究的贝中，特别是外套膜组织中，engrailed、BMP2 和 Smad3 三者表达之间的相关性，将有助于我们理解贝壳形成的分子机制．贝壳缺刻后半定量 PCR 试验结果表明，三者均参与贝壳修复，且在贝壳缺刻后的修复过程中，engrailed 和 Smad3 的 mRNA 表达变化规律非常相似，提示它们之间可能存在相互影响的联系．用地塞米松(DXM)和过氧化氢(H_2O_2)分别处理原代培养的贝外套膜组织迁出细胞，实时相对定量 PCR 检测 engrailed、BMP2 和 Smad3 的 mRNA 表达水平，统计分析结果表明，三者具有显著的相关性．上述所有结果为进一步研究贝类生物矿化的发育和信号转导机制提供了新的思路和基础．

关键词 engrailed，合浦珠母贝，Smad3，BMP2，贝壳蚀刻实验，实时 PCR

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