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Research paper

Highly expressed EGFR in pearl sac may facilitate the pearl formation in the pearl oyster, *Pinctada fucata*

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ABSTRACT

Epidermal growth factor receptor (EGFR) plays an important role in cell growth, proliferation, differentiation and migration. Yet whether it functions in pearl formation or not is not reported. In this study, EGFR was cloned from the pearl oyster *Pinctada fucata* (named as *Pf-EGFR*) and its expression profiles were investigated. The cDNA was 2156 bp long with an ORF of 1017 bp encoding 338 amino acid residues. The deduced polypeptide contained an L domain and a cysteine-rich domain, consistent with the characteristics of ErbB family. The sequence of *Pf-EGFR* shared 22.78–56.71% identity with other EGFRs. The genomic sequence of *Pf-EGFR* consisted of six exons and five introns, being 5190 bp in total length, and expressed in all the tested tissues with a higher expression level in the pearl sac ($P < 0.05$). *In situ* hybridization showed that *Pf-EGFR* was specifically expressed on both the inner side of the outer fold and the outer side of the inner fold of the mantle, as well as on the whole pearl sac including the connective tissues. In addition, *Pf-EGFR* was markedly increased at larval metamorphosis, significantly higher than other development periods ($P < 0.05$). These results indicated that the *Pf-EGFR* may facilitate pearl formation as well as larval metamorphosis.

1. Introduction

Epidermal growth factor receptor (EGFR), a transmembrane glycoprotein, belongs to a large family of cell surface receptors with intrinsic protein tyrosine kinase (PTK) activity (Bogdan and K.C., 2001), which consisted of an external growth factor binding domain, a transmembrane domain, and an internal tyrosine kinase domain (Yarden and Ullrich, 1988). EGFR can be activated by binding of ligands, for example, epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding (HB)-EGF, amphiregulin, betacellulin and epiregulin, leading to the activation of the intrinsic kinase domain which triggers a series of signal transduction, and thus inducing cell proliferation, migration, differentiation and apoptosis (Olayioye et al., 2000; Schlessinger, 2000; Yarden and Sliwkowski, 2001; Citri and Yarden, 2006). It regulates normal physiological processes such as embryonic development, wound healing, tooth growth, eyelid opening in newborn, development of hair follicles, and mammary gland morphogenesis (Goishi et al., 2003). In addition, EGFR also contributes to pathology. For example, amplification and inappropriate activation of EGF receptor family members are associated with tumor growth (Blume-Jensen and Hunter, 2001; Yarden and Sliwkowski, 2001), psoriasis (Jost et al., 2000) and cardiomyopathy (Asakura et al., 2002; Crone et al., 2002). Yet its functions in mollusk are less reported.

EGFR has been cloned from many species, such as *Homo sapiens* (Ullrich et al., 1984), *Gallus gallus* (Lax et al., 1988), *Rattus norvegicus* (Petch et al., 1990), *Danio rerio* (Goishi et al., 2003) and so on. However, information on molecular and functional characteristics of EGFR in mollusks is rare. In mollusks, EGFR was identified in *Lymnaea stagnalis* (van Kesteren et al., 2008), *Apostichopus japonicus* (Li et al., 2012), *Haliotis diversicolor* (Bai and Ke, 2012), *Crassostrea angulata* (Qin et al., 2010) and *Crassostrea gigas* (Sun et al., 2014), respectively. EGFR plays an important role in innate immunity and differentiation (Bai and Ke, 2012) and is mainly expressed in metamorphosis stage (Han et al., 2012) and might function in the cell proliferation and migration during wound healing (Sun et al., 2014). The genomic structure was investigated in *H. sapiens* (Collins et al., 2004), *D. rerio* (Howe et al., 2013), and *Drosophila melanogaster* (Hoskins et al., 2007), but rare in aquatic

Abbreviations: EGFR, epidermal growth factor receptor; Pf-EGFR, the EGFR gene from *Pinctada fucata*; ORF, open reading frame; PTK, protein tyrosine kinase; EGF, epidermal growth factor; TGF, transforming growth factor; qRT-PCR, quantitative realtime polymerase chain reaction; RACE, rapid-amplification of cDNA ends; PKC, protein kinase C; GRAVY, grand average of hydropathicity; ISH, *in situ* hybridization; DIG, digoxigenin; PFA, paraformaldehyde.

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invertebrates. So it is very important to isolate and characterize more EGFR genes from other aquatic invertebrates to understand the evolution of EGFR genomic structure.

Pearls are gemstones produced by shellfish, such as pearl oysters (*Pinctada fucata*), and have long been prized worldwide because of their rarity and beauty. Pearl culture is an important industry in China and many other countries in the world (Masaoka et al., 2013). Pearl culture contains four steps: 1) pre-operative conditioning, 2) nucleus implantation, 3) post-operative care, and 4) culturing and harvest (Wada, 1999; Lucas and Southgate, 2008). Nucleus implantation (at the beginning of the culturing process) is the most important step; the pearl oyster is forced open, and a nucleus is implanted into the gonad with a mantle graft. Around the nucleus, a pearl sac (PS) is formed by proliferation of the outer mantle epithelial cells of the mantle graft (Inoue et al., 2010). Mantle tissue in bivalves has a wide array of functions including nutrient storage, direction of feeding currents and sensorial capacity (Zandee et al., 1980). Among others, a major function of the mantle is biomineralization in which specialized secretory cells produce the shell (Simkiss and Wilbur, 1989). It is the biomineralization that makes it possible to culture pearls artificially. The *P. fucata* shell consists of two mineralized layers: a nacreous layer made from aragonite, and a prismatic layer made from calcite. The nacreous and prismatic layers are formed in the mantle center (MC) and mantle edge (ME), respectively, by epithelial cell secretion (Sudo et al., 1997; Wada, 1999). So mantle and pearl sac play an important role in pearl formation. Since the mantle and pearl sac consisted mainly of epithelial cells, then what functions the EGFR plays during the pearl culture are worth studying.

In this study, the cDNA sequence and the genomic structure of EGFR in pearl oyster were investigated and its expression profile in different tissues was analyzed as well. The precise positions of the gene expressions in the mantle—the main tissue responsible for shell formation, and pearl sac—the main organ responsible for pearl formation, were detected *via in situ* hybridization to see whether EGFR relates to pearl formation or not. Finally, to better understand its potential roles in larval development, its expression levels in different developmental stages of the pearl oyster were investigated.

2. Materials and methods

2.1. Sample preparation

Healthy pearl oysters aged 1–2 years old with a shell length range of 60–70 mm, shells of about 50–60 mm in height and 40–50 g in wet mass were obtained from the pearl oyster culture base of the South China Sea Fisheries Research Institute (Xincun Port, Hainan Province, China). Nuclear implantation was carried out by experienced technicians in June 2013 with similar method used in conventional pearl culture. A total of 20 implanted oysters were sampled in September 2014. The twenty individuals were kept in an 80 l aerated sand-filtered seawater at 25 °C and fed twice daily with *Chlorella vulgaris* for one week before dissection. For expression analysis, various tissues including mantle, adductor muscle, gill, hepatopancreas, pearl sac, intestine and gonad were separately sampled from three individuals out of the twenty and preserved in 2.0 ml tubes with sample protector (TaKaRa, Dalian, China) until RNA extraction. Among others, the pearl sacs were excised from host oysters by removing the outer layers with a surgical blade until a thin (<0.5 mm) layer tissue surrounding the pearls remained. All samples were immediately protected in sample protector and taken to the laboratory in Guangzhou and stored in −80 °C. All larval stages of oysters were reared in 4 m × 6 m × 1.6 m concrete tanks, with temperature maintained between 25 °C and 30 °C, and salinity at 25.0 to 28.9. The veliger larvae were fed a mixture of *Platymonas subcordiformis* and *Dicrateria zhanjiangenis*. The following stages were collected: trochophore (8 h after fertilization); D-veliger larvae (24 h after fertilization); umbo veliger larvae (10 days after fertilization); pediveliger larvae (17 days after fertilization); and larvae during metamorphosis (24 h after settlement). Different stage larvals were observed under binocular microscope to determine the exact stage and harvested using a 40 μm nylon mesh membrane, larval samples were washed with 1 × PBS (phosphate-buffered saline) and immediately protected in 2.0 ml tube with sample protector (TaKaRa, Dalian, China) until RNA extraction. Adductor muscle was collected and kept in 100% ethanol for DNA extraction. Mantle and pearl sac were removed from the adult *P. fucata* in similar process above and immediately fixed in 4% paraformaldehyde in 0.2 M PBS overnight for *in situ* hybridization.

2.2. RNA isolation and first-strand synthesis

Total RNA was extracted from each tissue of four adult oysters with the TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity was determined by separating on a 1.2% formaldehyde-denatured agarose gel. The quantity of RNA was determined by measuring at an OD of 260 nm with a NanoDrop ND-1000 UV–Visible Spectrophotometer. Then 1 μg of total RNA from each tissue was used as the template for the reverse transcription reaction with a Prime ScriptII 1st strand cDNA Synthesis kit (Takara, Dalian, China). The first-strand cDNA was synthesized and used as the template for further PCR analysis.

2.3. cDNA cloning of the Pf-EGFR from P. fucata

cDNA sequence of EGFR gene was obtained from the transcriptome sequences, through BlastX search with the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). The accuracy of *Pf-EGFR* sequence was confirmed by using two pairs of gene-specific primers EGFR-F1/EGFR-R1 and EGFR-F2/EGFR-R2 (Table 1), and the full length of the cDNA was cloned using 3′RACE method with TAKARA 3′-Full RACE Core Set with PrimerScript™ RTase (TaKaRa, Dalian, China) and two pairs of gene specific nested primers (Table 1). The RACE PCR was performed in a 20 μl reaction volume, containing 2 μl of 10 × Ex Taq buffer, 1.6 μl of dNTP Mix (2.5 mmol l^{-1}), 0.8 μl of each primer (10 mmol l^{-1}), 13.8 μl of double-distilled water, 0.2 μl of Ex Taq (TaKaRa, Dalian, China) and 0.8 μl of cDNA as template. The PCR program was as follows: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; a final elongation step of 72 °C for 10 min. The PCR products were isolated using DNA Gel Extraction Kit (OMEGA, USA) and cloned into PMD18-T vector (TaKaRa, Dalian, China). The recombinant vector was transformed into competent *Escherichia coli* DH5a cells and sequenced after recombinant identification.

Table 1
Primers used for cloning and characterization of *Pf-EGFR* gene.

Primer name	Sequences (5′-3′)	Application
EGFR-F1	CGCTAGGCAAACCCTTAAA	cDNA cloning
EGFR-R1	CAATCGTTCGGGAGGTAGT	cDNA cloning
EGFR-F2	GATGCGTTCCTTCGTGTCC	cDNA cloning
EGFR-R2	CAGACGAATCATCGGCTCA	cDNA cloning
EGFR-3′-GSP1	AAAGCAACTTGATCCCTCG	3′RACE
EGFR-3′-GSP2	TGACTCGGGTTCCATCCAT	3′RACE
3′Adaptor outer	TACCGTCGTTCCACTAGTGATTT	3′RACE
3′Adaptor inner	CGCGGATCCTCCACTAGTGATTTCACTATAGG	3′RACE
gEGFR-F1	TCGTAAGAACGGAAAGATGCG	Genome cloning
gEGFR-R1	CAATCCCAGAAGCACATAACCA	Genome cloning
gEGFR-F2	CGCATCGACCATCGTGTT	Genome cloning
gEGFR-R2	CTCCGAAAATAACAGGGTA	Genome cloning
gEGFR-F3	GGAGAGAATGCTTATGACAA	Genome cloning
gEGFR-R3	AAAGTGAGGAAGGCAAACC	Genome cloning
qEGFR-F1	CGTGGAAGGAAACTTAGAG	qRT-PCR
qEGFR-R1	ATCCCAGAAGCACATAACC	qRT-PCR
18 s-rRNA-F	TGTCTGCCCTATCAACTTTC	qRT-PCR
18 s-rRNA-R	TGTGGTAGCCGTTTCTCA	qRT-PCR
YEGFR-F1	CCCGATTACCACTATGAGATG	ISH
YEGFR-R1	CAACAATAAGGTTTACCGTCC	ISH