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Molecular identification of insulin-related peptide receptor and its potential role in regulating development in *Pinctada fucata*

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ABSTRACT

The insulin-like family is not only an important regulatory factor for animal growth, development, and metabolism, but also a mediator for initiating growth activity of growth hormone. It plays an important role in transferring transmembrane information and regulating cell function by binding to tyrosine kinase receptors (insulin receptors). To better understand the role of insulin-related peptide receptor (*Pfirr*) on the developmental regulation in *Pinctada fucata*, 5.326 kb encoding cDNAs for *Pfirr* have been cloned and functionally characterized. *Pfirr* displays significant homologies to *Crassostrea gigas*, and exhibits all the typical features of insulin receptors and tyrosine kinase domain structure, both of which are typical for the protein family sharing high similarity to other orthologs. Real-time PCR analyses show that *Pfirr* widely expresses in tissues and developmental stages of *P. fucata*. Expression of *Pfirr* mRNAs at different developmental stages (polar body stage, the trocophore stage and D-shaped larva stage) following treatment with agonist IGF-I(1, 2, 4 and 8 μM/L) and antagonist PQ401 (5, 15, 25, 50 and 100 μM/L) indicated that *Pfirr* may be involved in regulating the development of embryos in *P. fucata*. These results clearly demonstrate *Pfirr* is involved in regulating developmental process in *P. fucata*.

1. Introduction

The insulin-like family, one of the most widely distributed peptide families even among invertebrate species (Renteria et al., 2008), is an important regulatory factor for animal growth (Schlueter et al., 2007), development (Riehle and Brown, 2002) and metabolism (Lardans et al., 2001).

The insulin-like family includes insulin and the insulin-like growth factors (IGFs), which play distinct physiological roles in animals mediated by specifically binding to the Insulin Receptor (IR) or the type-I of Insulin-like Growth Factor Receptor (IGF-IR) (Nakae et al., 2001). The IR, the IGF-IR, and the Insulin Receptor-related Receptor (IRR) (Ebina et al., 1985; Shier and Watt, 1989; Ullrich et al., 1986) form the subclass II of the Receptor Tyrosine Kinase (RTK) superfamily (Hubbard and Till, 2000), sharing a common character of covalently-linked homodimers ($\alpha_2\beta_2$) and several structural domains (Heldin and Ostman, 1996). Vertebrates possess more than one homologous receptor of the IR family, whereas invertebrates possess only a single one—the IRR (Leevers, 2001). This single IRR regulates both growth and metabolism in invertebrates (Kimura et al., 1997; Tatar et al., 2001; Wheeler et al., 2006), whereas two different and specialized receptors combined matching ligands undertake different physiological roles in vertebrates (Kimura et al., 1997; Roovers et al., 1995).

In molluscs, the occurrence of an insulin system was first investigated in gastropod species: in the pond snail, *Lymnaea stagnalis*, seven MIPs (mollusc insulin-like peptide) expressed in the nervous ganglia have been identified and characterized (Li et al., 1992; Smit et al., 1988, 1998). In another model gastropod, the sea hare *Aplysia californica*, insulin is produced restrictedly in the central region of the cerebral ganglia, appeared to be unique, and characterized with an extended A chain compared with other invertebrate and vertebrate insulin (Floyd et al., 1999). In *Anodonta cygnea*, six ligands were identified, purified and characterized using a radioreceptor test system (Shipilov et al., 2005). Four genes coding insulin-related peptides were also identified in the complete sequencing of the *Lottia gigantea* genome (Veenstra, 2010). In bivalve species, insulin-related peptide cDNA was characterized in the Pacific oyster *Crassostrea gigas*, and was found to be expressed as three transcripts with differing lengths of 3′-untranslated region (3′-UTR) in visceral ganglia (Hamano et al., 2005), but the exact number of the genes remains unknown.

The IRR have been identified only in a few molluscs (Gricourt et al., 2006; Lardans et al., 2001; Roovers et al., 1995), with the common character of a typical tyrosine kinase (TK) domain, having high evolutionary conservation with the vertebrates, such as human (Lu et al., 2008), *Gallus gallus* (Basu et al., 2012), *Xenopus laevis* (Klein et al.,

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2002) and *Danio rerio* (Jurczyk et al., 2011). The possible role of IRR in the activating and proliferating processes observed in an embryonic cell line of the snail *Biomphalaria glabrata* has been implicated (Lardans et al., 2001). The affinity has been researched among the insulin-related peptide receptor of the mussel *Anodonta cygnea*, recombinant piscine IGF-I, and porcine insulin. The results indicate that the receptor has similar binding properties with the vertebrates (Leibush and Chistyakova, 2003). The insulin-like receptor (CIR) of Pacific oyster (*C. gigas*) shares 66.5% homology with the tyrosine kinase domain of turbot (*Psetta maxima*), and is expressed in the mantle edge and gonads, which indicates its participation in the formation of its shell and reproduction (Gricourt et al., 2003).

Numerous insulin signaling pathways from invertebrate to vertebrate species have been found to be relatively well conserved, including ligand and its receptors which were mentioned above, to final effectors (Broughton and Partridge, 2009; Claeys et al., 2002; Wu and Brown, 2006). Studies in the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* have revealed that components of the insulin signaling pathway have been highly conserved during evolution (Garofalo, 2002). On the basis of sequence conservation, three potential elements of the oyster insulin pathway were found in *C. gigas*: CgRas, CgPten and CgP70S6K (Jouaux et al., 2012). And in other molluscan species like *Mytilus trossolus* (Ciocan et al., 2006) and *Aplysia californica* (Swanson et al., 1986), Ras has been already identified. This evidence suggests that the conservation of insulin signaling system may extend throughout bivalve species.

Studies in invertebrate species have shown the potential involvement of insulin-related peptide and its receptor in many activities such as cell proliferation and developmental processes. Molluscan insulin-related neuropeptide promoted neurite outgrowth in dissociated neuronal cell cultures of *Lymnaea stagnalis* (Kits et al., 1990). Human insulin could stimulate proliferation and differentiation of *Drosophila* embryonic neural cells (Pimentel et al., 1996). In the mollusc *Haliotis tuberculata*, porcine insulin promoted the growth of primary cultures of hemocytes (Lebel et al., 1996). The possible implication of insulin-like receptor (BgIR) in the activating and proliferating processes was observed in *B. glabrata* embryonic cells during their coculture with *Schistosoma mansoni* larvae (Lardans et al., 2001). The expression of maternal CIR during the embryonic and early larval development also suggests that insulin-like peptide is involved in organogenesis (Gricourt et al., 2006).

P. fucata, a marine bivalve mollusc, cultivated in China and worldwide, has a very high economic value in pearl production. Its development process has gone through several ecologically important transitions from planktonic to the creeping stage and to the last adult, including the synergy of the soft part and shell growth. At present, there are no reports on the existence and the role of insulin-related peptide receptor in *P. fucata* (*Pfirr*). To evaluate the functional role of *Pfirr* in development regulation, firstly, the cDNA sequences encoding *Pfirr* were cloned and characterized; secondly, the tissue distribution and the expression profiles of *Pfirr* were examined at developmental stages; thirdly, mRNAs expression of *Pfirr* at different developmental stages were measured following treatment with antagonist PQ401 (the inhibitor of IGF-IR, a diaryl urea compound) and agonist IGF-I.

2. Materials and methods

2.1. Animals and chemicals

The *P. fucata* (body weight 7.21 ± 0.57 g, shell height 38.67 ± 1.38 mm) were obtained from Marine Biology Research Station at Daya Bay of Chinese Academy of Sciences (Shenzhen city, Guangdong, P.R. China) in November, 2011. The oysters were cultivated in floating net cages in the sea under natural conditions. Tissues were dissected, and frozen immediately in liquid nitrogen, then stored at −80 °C until RNA extraction. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Chinese Academy of Sciences.

Two-year-old breeders came from one cultured population at Daya Bay in China. Gametes were obtained by dissecting the gonads and were passed through a 100 μm screen to remove the large tissue debris. The eggs were fertilized with sperm in filtered seawater containing 0.006% (V/V) of ammonia water at temperatures 24 ~ 25 °C. About two hours after fertilization, the fertilized eggs developed to the 2–4 cell stage.

The inhibitor of IGF-IR, a diaryl urea compound (PQ401) was purchased from Enzo Life Sciences (Farmingdale, USA). Recombinant human IGF-I was purchased from Shanghai Prime Gene Bio-Tech. Co. Ltd. (Shanghai City, China). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO).

2.2. Cloning and sequence analysis of Pfirr

Total RNA from *P. fucata* tissues were prepared using Trizol reagent (Invitrogen). One microgram of isolated RNA was used to synthesize first-strand cDNA using the ReverTra Ace-α First-strand cDNA Synthesis Kit (TOYOBO, Japan).

A fragment cDNA of *Pfirr* was found in the transcriptome of *P. fucata*. To amplify this cDNA fragments, specific PCR primers were designed by using Primer Premier 5.00 (Palo Alto, CA) as presented in Table 1. Full-length cDNA sequences were obtained by the 5′- and 3′-rapid amplification of cDNA ends (RACE) using BD SMART RACE cDNA Amplification Kit (Clontech, USA) (Table 1).

For all PCR reactions in the present study, amplifications were performed as follows: denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 15 s, 52–58 °C for 15 s and 72 °C for 1–1.5 min. The reaction was ended by a further extension of 10 min at 72 °C. The amplification products were purified using the E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA) and subcloned into the pTZ57R/T vector (Fermentas, USA). Three different individual positive clones were sequenced on an ABI 3700 sequencer (Applied Biosystems).

Predictions of export-directing signal sequences, transmembrane regions and domains involved in signal transduction processes were done as described (Schultz et al., 1998) using the simple modular architecture research tool (SMART) available under http://smart.embl-heidelberg.de. Predictions of serine/threonine phosphorylation sites were performed as described (Blom et al., 1999) using the NetPhos 2.0 prediction server available under http://www.cbs.dtu.dk/services/NetPhos. Multiple sequence alignments of amino acids were performed with ClustalX (1.81). Protein phylogenetic analysis was conducted with MEGA4 using the neighbor-joining method.

2.3. Tissue distribution of Pfirr in P. fucata

The tissue expression pattern of *Pfirr* mRNA in the various tissues was analyzed by real-time PCR. Total RNA was isolated from 6 tissues, including mantle, digestive gland, adductor muscle, heart, ovary during the previtellogenic arrest stage and testis at multiplicative stage (n = 3).

Quantitative real-time PCR was performed on a Roche LightCycler 480 real time PCR system using SYBR® *Premix Ex Taq*™ (TAKARA, Japan) according to the manufacturer's protocol. Real-time PCR conditions were as follows: denaturation at 94 °C for 1 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 15 s and 72 °C for 60 s. *Pfirr* transcript levels were normalized against *gapdh* transcripts levels. *Gadph* was expressed equally in all the tested tissues. Real-time PCR primers for *Pfirr* and *gapdh* were present in Table 1. The efficiency of primers for *Pfirr* was 1.963 and that for *gapdh* 1.901.

2.4. Expression of Pfirr mRNA at the developmental stages of P. fucata

For analyzing the developmental expression patterns of *Pfirr*, six developmental stages: fertilized eggs, embryos at polar body stage,

Table 1
Gene-specific primers[a] used for RT-PCR analysis.

Gene	Primer	Primer direction	5′ to 3′ sequence
Pfirr	Partial	F1	ACAAGGAAGGACCGTATT
		R1	CGTAATGATTTCGTCGTG
	5′ RACE	UPM mixture	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
			CTAATACGACTCACTATAGGGC
		R2	CGGAGTGAGCGTTATGTGGA
		NUP	AAGCAGTGGTATCAACGCAGAGT
		R2nest	CGACGATTCTCCACGATGAC
	3′ RACE	F2	AAAATGGACAAGCCAGAAGG
		UPM mixture	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
			CTAATACGACTCACTATAGGGC
		F2nest	ACAGTGAAGTTTGTGGTGCCG
		NUP	AAGCAGTGGTATCAACGCAGAGT
	Real-time	irrF	AGACGGAGACGGGAAAGAAG
	PCR	irrR	CCCCCAACAGACGTACAACA
gapdh	Real-time	gapdhF	TGGCATTGAGGAAGGTTTG
	PCR	gapdhR	GTGGAGGATGGTATGATGTTAGA

[a] All primer pairs were designed to originate in different exons to exclude false positive bands in case of potential genomic DNA contamination.

the trocophores, D-shaped larvae, umbo larvae and metamorphosis of larvae were collected and stored at −80 °C. The total RNA extraction and quantitative real-time PCR analyses were the same as described above. *Gadph* was expressed stably in all tested developmental stages. Three repetitions of the reaction were performed.

2.5. Effects of PQ401 and IGF-I on the development of P. fucata

To evaluate the effects of PQ401 and IGF-I on development of *P. fucata*, the embryos at polar body stage, the trocophore and D-shaped larva stage were treated with different concentrations of PQ401 and IGF-I for an hour. Every treatment had three replicates.

IGF-I was dissolved in PBS (0.2 M) at a concentration of 0.02 mM/L, and then the embryos at polar body stage (about 5000 embryos/ml) were separately treated with a final concentration of 1 μM/L, 2 μM/L, 4 μM/L and 8 μM/L of IGF-I for an hour. Control embryos were treated with seawater only. After being treated for one hour, seawater was suctioned by siphon for fresh filtered seawater. Two hours later, the total number of embryos developed to the 2–4 cell stage was counted under a microscope. The mRNA expression of *Pfirr* was analyzed by real-time PCR as described above.

PQ401 was dissolved in DMSO at a concentration of 10 mM/L, and then the embryos at polar body stage (about 1000 embryos/ml) were separately treated with a final concentration of 5 μM/L, 15 μM/L, 25 μM/L, 50 μM/L and 100 μM/L of PQ401 for one hour. The embryos at polar body stage were the reference sample. Two hours later, the total number of embryos developed to the 2–4 cell stage was counted under a microscope. The fresh trocophore larva and fresh D-shaped larva (about 1000 embryos/ml) were treated with a final concentration of 0.5 μM/L of PQ401 for one hour, then collected by centrifugation. Control embryos were treated with or without DMSO (10 mM/L) only.

2.6. Statistical analysis

The total number of developed embryos and the undeveloped ones were counted under a microscope by using a counter. Embryos were counted in five different visual fields every time.

The trocophores and D-shaped larvae, floating in the seawater after treatments and being active observed under a microscope, were identified as survivors. The embryos were observed and counted under a microscope, majority of which developed to the next developmental stage was identified as the development of the embroys.

Quantitative data were expressed as mean ± S.E.M. Statistical differences were estimated by one-way ANOVA followed by Duncan's multiple range tests. A probability level less than 0.05 was used to indicate significance. All statistics were performed using SPSS 13.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Cloning and sequence analysis of Pfirr

A full-length cDNA encoding *Pfirr* was isolated from *P. fucata* (GenBank accession number: JX121113), the cDNA sequence of *Pfirr* was 5326 bp, containing an open reading frame of 4617 bp and 5′ UTR of 709 bp, encoding a precursor protein of 1538 amino acids (aa), with an N-terminal putative signal peptide sequence of 20 aa. The second hydrophobic region, comprising residues 1003–1025, was the putative transmembrane domain (Fig. 1).

The highly conserved domains for insulin receptors are also present in PfIRR, including L1–Cys–L2 tyrosine kinase-domains separated by three fibronectin III regions, and a dibasic KK-motif (Fig. 1). In the intracellular region, a putative tyrosine kinase domain was present between residues 1065 and 1343 (Fig. 1). Several sequence motifs in the intracellular domain were crucial for enzymatic function and downstream signaling, such as a NP × Y motif (NPDY), a canonical GxGxxG-motif (GQGSFGMV), the invariant V, the catalytic loop (HRDLAARN) and the signature pattern (D-I-Y-x(3)-Y-Y-R) (Fig. 1).

An amino acid sequence alignment of tyrosine kinase catalytic domain of insulin receptor homologs from different species was shown in Fig. 2. In the tyrosine kinase domain, PfIRR displayed homologies of 45 to 81% identical to aligned insulin receptor homologs. As the highly conserved region between species, the central core of the catalytic domain (from P1185 to G1293) effectively exhibited the greatest frequency of identical residues. In this region, several essential motifs exhibited high homology, such as the invariant K1101, and the consensus sequences DLAARN and PVRWMAPE (Fig. 2).

Phylogenetic analysis showed that IR family was clustered into two separate clades: the vertebrata and the invertebrata. Invertebrates posed only a single homologous receptor (IRR) of the IR family, whereas three individual sets (IR, IGF-IR and IRR) of orthologous sequences were present in the vertebrata branch. The IRR of *P. fucata* was most closely related to that of *C. gigas*, which also belonged to bivalves; and closer to that of gastropods *B. glabrata* and *L. stagnalis* (Fig. 3).

3.2. Tissue distribution of Pfirr in P. fucata

The *Pfirr* mRNA was expressed in all tissues examined, with a high level in testis at proliferating stage and adductor muscle, moderate level in digestive gland and mantle, low level in heart and in previtellogenic ovary (Fig. 4).

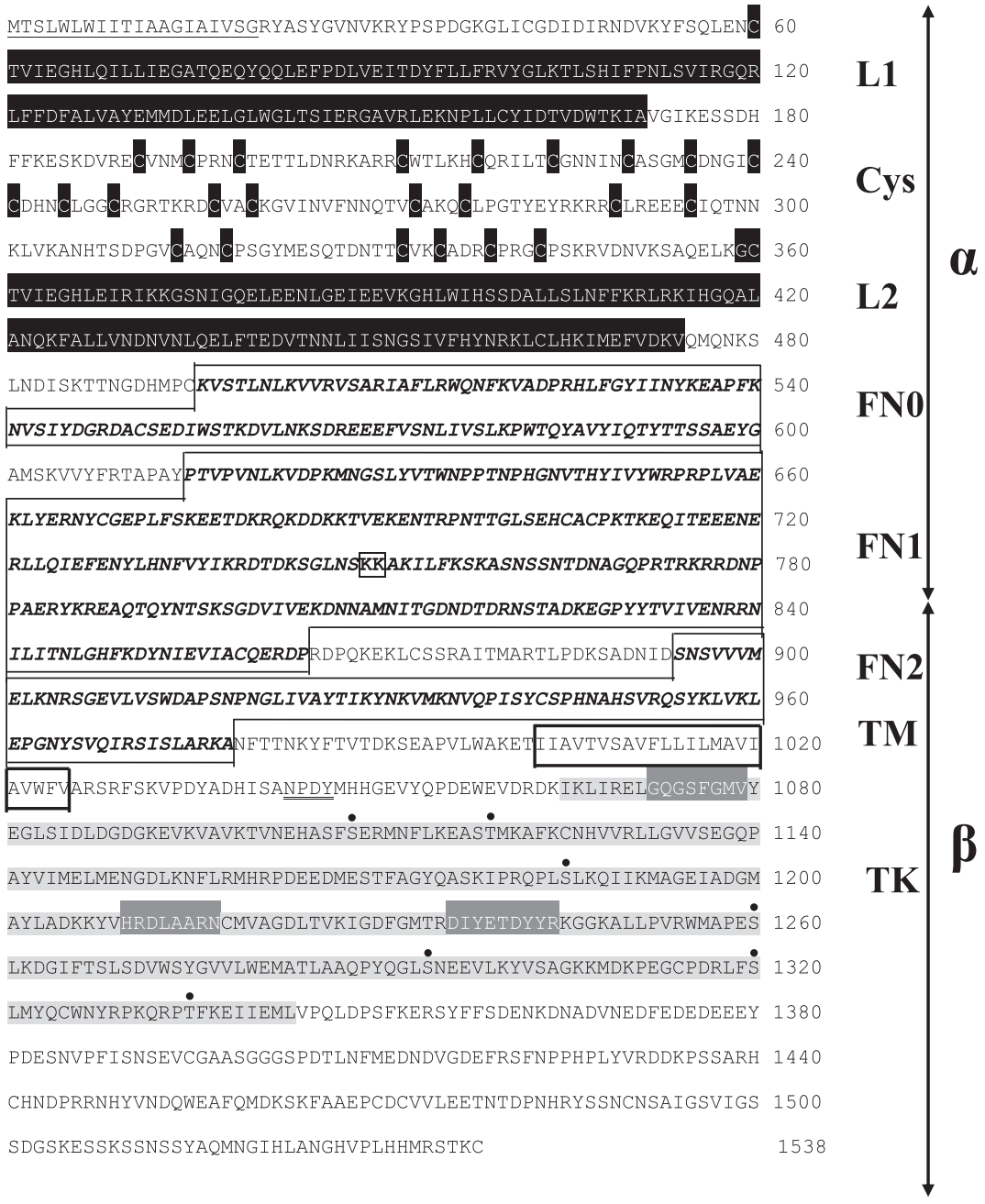

Fig. 1. Amino acid sequence and structural features of PfiRR. The putative export-directing signal sequence is underlined. Indicated are identified receptor L domains and cysteine residues of the Cys-rich region (black boxes with white lettering) fibronectin III domains (boxed with bold letters), and the tyrosine kinase domain (boxed with light gray background). The transmembrane region is boxed. Catalytically important residues of the tyrosine kinase domain are marked by white letters on a dark gray background. Juxtamembrane NP × Y motive is double underlined. Further indicated are the dibasic, potential cleavage site for proteolytic processing (boxed KK-motif) as well as residues with a putative function in disulfide bonding between the α and the β domain. Putative serine/threonine phosphorylation site of this region are marked by dots.

3.3. Expression of Pfirr at the developmental stages of P. fucata

The *Pfirr* mRNAs were expressed at all developmental stages examined. The highest transcript levels of *Pfirr* appeared at fertilized egg, and remained high levels till the trocophore stage, then dropped significantly at the D-shaped larva stage, and kept at a low level after hatching to the metamorphosis of larva stage (Fig. 5).

3.4. Expression of Pfirr mRNA at the developmental stages following treatment with IGF-I and PQ401

Pfirr mRNA levels increased significantly to more than 3-fold higher than controls at polar body stage after 1 h post-treatment with 1 μM/L of IGF-I, whereas no significant difference was found among other treatments with 2 μM/L, 4 μM/L and 8 μM/L of IGF-I (Fig. 6B). Two hours later, only 13.13 ± 2.03% of the embryos treated with the dose of 8 μM/L has developed to the 2–4 cell stage. The flocculant appearing in the seawater was the dying embryos. The seawater with control embryos was clear and the embryos were at the bottom of the vessel. The rates of 2-4 cell embryos treated with 1 μM/L, 2 μM/L and 4 μM/L showed no significant difference with the control (Fig. 6A).

The DMSO control significantly decreased the *Pfirr* mRNA expression. Treatment with 5 μM/L, 25 μM/L, 50 μM/L and 100 μM/L of PQ401 except for 15 μM/L distinct from DMSO significantly decreased *Pfirr* mRNA levels at polar body stage. No significant difference was found among the effect of the 5 dose of PQ401, and the dose of

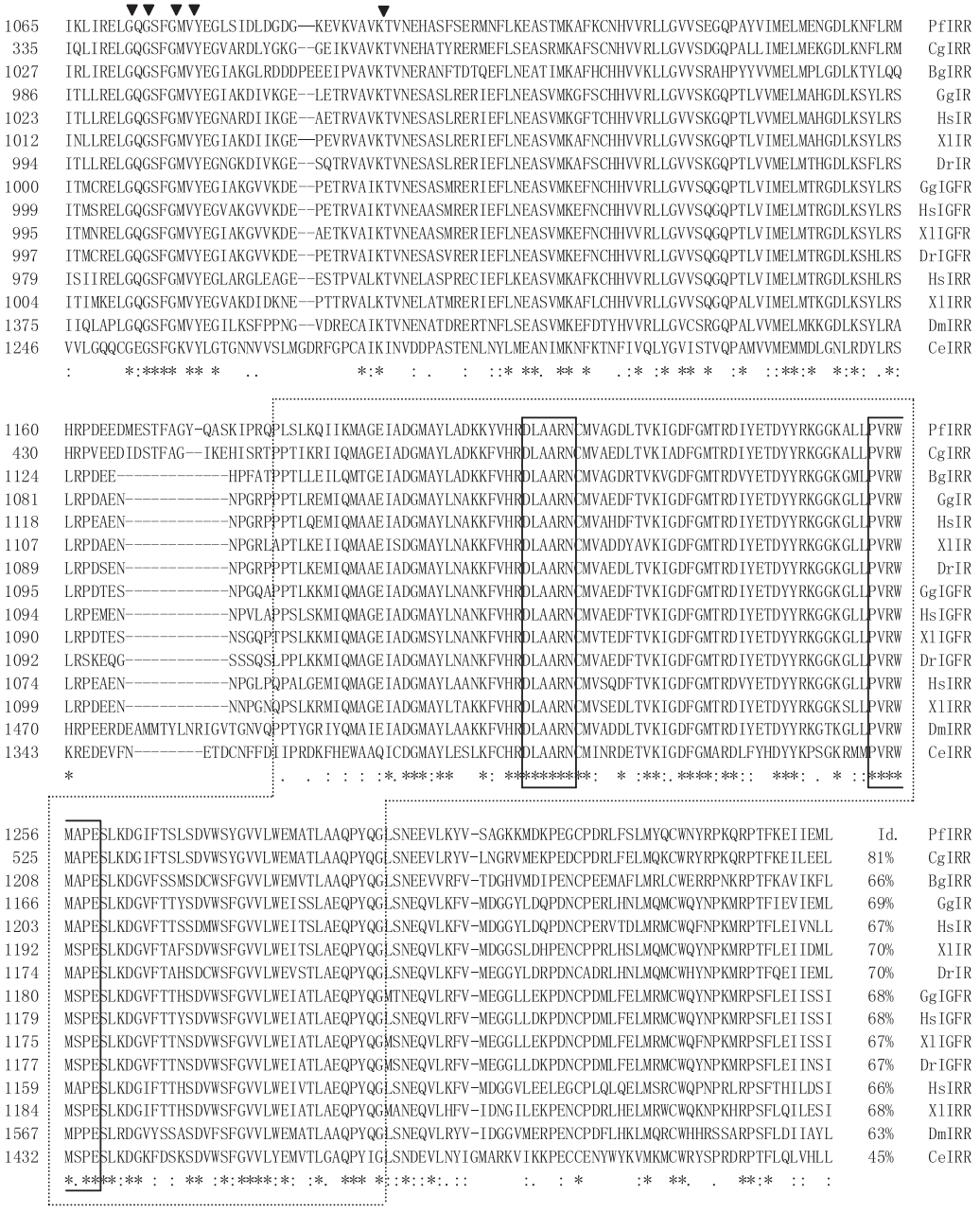

Fig. 2. Amino acid sequence alignment of tyrosine kinase domains from insulin receptors of different species. The PfIRR amino acid sequence were aligned by the Clustal method with tyrosine kinase domains of Cg (*Crassostrea gigas* IRR: AJ535669); Bg (*Biomphalaria glabrata* IRR: AF101195); Gg (*Gallus gallus* IR: XP_418250.2 IGF-IR: NP_990363.1); Hs (*Homo sapiens* IR: AAA59174.1 IGF-IR: AAB22215 IRR: NP_055030); Xl (*Xenopus laevis* IR: NP_001081702 IGF-IR: NP_001081734.1 IRR: NP_001083465); Dr (*Danio rerio* IR: NP_001136144 IGF-IR: NP_694500); Dm (*Drosophila melanogaster* IRR: U18351); Ce (*Caenorhabditis elegans* IRR: AF012437). Amino acids which have been shown to be highly conserved were marked by stars, those which were not conserved were marked by colon and dot. Black triangles indicate the conserved residues of the ATP binding site. The central core of the catalytic domain is boxed as were the two consensus sequences for tyrosine substrate specificity. Percentages refer to identity values between PfiRR and the remaining sequences in the tyrosine kinase region.

5 μM/L of PQ401 had the most apparent effect on *Pfirr* mRNA levels. (Fig. 7B). The rate of embryos at polar body stage developing to 2–4 cell stage apparently reduced to 10.09 ± 4.3% after treatment with 100 μM/L of PQ401 (Fig. 7A).

After treatment of PQ401 on trocophores and D-shaped larvae for one hour, the larva density decreased, and some larvae sank to the bottom of the vessel, which was easily observed by visualization (no statistical data). These sunken larvae cannot swim like the normal ones when observed under a microscope. The control larvae were floating in the seawater and swimming when observed under a microscope. At trocophore stage (Fig. 8A), the DMSO control did not affect the expression of *Pfirr* when compared with the control, and mRNA expression of *Pfirr* was significantly reduced. At the D-shaped larva stage (Fig. 8B), no significant effect could be observed.

4. Discussion

In the present study, we have cloned the sequence of *Pfirr* cDNAs in *P. fucata*, a bivalve mollusc. The vertebrates express three different paralogs of insulin receptor family: the IR, the IGF-IR, and the IRR, but invertebrates usually contain only one ortholog (De Meyts, 2004). *P. fucata* contains one insulin receptor encoding gene, the IRR, which has also been demonstrated for *C. gigas*, *B. glabrata, L. stagnalis* and

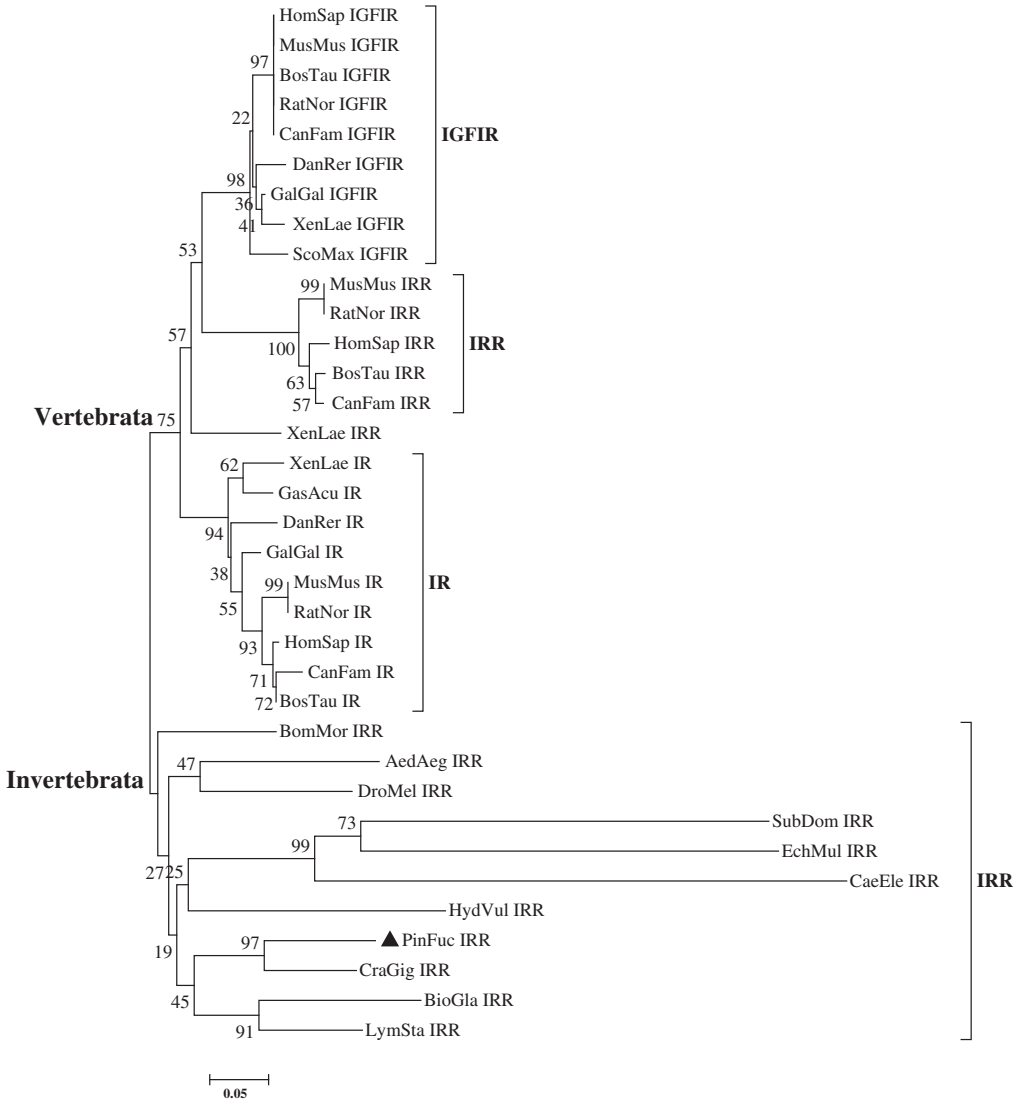

Fig. 3. Phylogenetic analysis of the insulin receptor family. The phylogenetic tree was constructed by MEGA 4.0.2 using the neighbor-joining method with 1000 bootstrap replicates. The number shown at each branch indicates the bootstrap value (%). GenBank accession numbers or ENSEMBL databases for insulin receptors (IR), insulin-like growth factor receptor (IGF-IR), IR-related receptor (IRR) in vertebrate and insulin-related peptide receptor (IRR) in invertebrate: BosTau (*Bos taurus* IR: XP_590552 IGF-IR: NP_001231541 IRR: NP_001178164); CanFam (*Canis familiaris* IR: XP_542108 IGF-IR: XP_545828.2 IRR: XP_547526); GasAcu (*Gasterosteus aculeatus* IR: ENSGACP00000013853); MusMus (*Mus musculus* IR: NP_034698.2 IGF-IR: NP_034643.2 IRR: NP_035962); RatNor (*Rattus norvegicus* IR: EDL74923 IGF-IR: NP_434694.1 IRR: NP_071548); ScoMax (*Scophthalmus maximus* IGF-IR: CAA12278); LymSta (*Lymnaea stagnalis* IRR: Q25410); HydVul (*Hydra vulgaris* IRR: M64612); PinFuc (*Pinctada fucata* IRR: JX121113); AedAeg (*Aedes aegypti* IRR: U72939); DomSil (*Domestic silkworm* IRR: AF025542); EchMul (*Echinococcus multilocularis* IRR: AJ458426); SubDom (*Suberites domuncula* IRR:Y17880).

other invertebrates (Gricourt et al., 2006; Lardans et al., 2001; Roovers et al., 1995).

In the extracellular portion, the 'L1–Cys–L2' structure was two receptor L-domains separated by a cysteine rich region, which was a typical feature of the insulin receptor family where it formed ligand binding domain. Three fibronectin type III domains (FN0, FN1, and FN2), another highly conserved feature, were identified between the L1–Cys–L2 region and the transmembrane segment. A dibasic KK-motif at position 749–750 instead of a tetrabasic motif at a corresponding position could possibly be serving as a modified proteolytic processing site (Fig. 1). In the intracellular region, a putative tyrosine kinase domain was present between residues 1065 and 1343, which was the first requisite for the identification of *Pfirr* as an insulin-related peptide receptor.

PfIRR contains all crucial residues and motifs for binding ligands and enzymatic activity, thus suggesting that it is a functional insulin receptor. For example, a NP × Y motif (NPDY) in the juxtamembrane region was essential for the interaction with insulin receptor substrate (IRS) factors. A canonical GxGxxG-motif (GQGSFGMV) was important for ATP/Mg^{2+}- binding. The invariant V was necessary for the correct positioning of conserved glycines and their interaction with the ATP molecule, the catalytic loop (HRDLAARN) (Hanks et al., 1988). And the signature pattern (D-I-Y-x(3)-Y-Y-R) for class II receptor tyrosine kinases (DIYETDYYR) contained the putative site for autophosphorylation (Yarden and Ullrich, 1988), the D-I-Y not D-V-Y may be an altered signature present in *P. fucata*. The two conserved hydrophobic regions, the hydrophobic signal peptide (SP) (Nielsen et al., 1997) at the N-terminus and the putative transmembrane (TM) domain (von Heijne, 1992), have also been confirmed in PfIRR. Usually, the insulin receptor has a tetrabasic motif known to be essential for proteolytical cleavage of the receptor to its active $\alpha_2\beta_2$ hetero-tetrameric form (De Meyts, 2004; Ottensmeyer et al., 2000; Sparrow et al., 1997), but it is absent in PfIRR, and instead contains a dibasic KK-motif which is similar with *Echinococcus multilocularis* IR (Konrad et al., 2003). This may be explained by the species diversity, or that PfIRR may be processed by other proteases. This possibility requires further investigation.

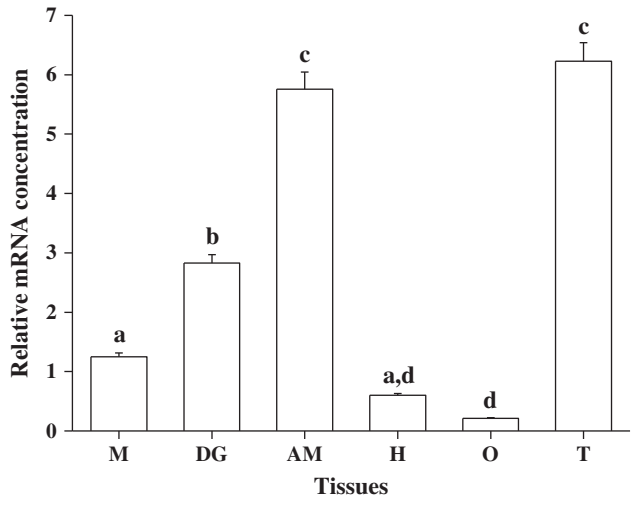

Fig. 4. Expressions of *Pfirr* mRNA in various tissues of *Pinctada fucata*. The mRNA levels were quantified by real-time RT-PCR in: 1. M, mantle, 2. DG, digestive gland, 3. AM, adductor muscle, 4. H, heart, 5. O, ovary, 6. T, testis. The results are expressed as fold-changeValues without common notation (a, b, c, d) differ significantly ($p < 0.05$).

Alignment of TK catalytic domains from different species provides evidence of PfIRR as an insulin-related peptide receptor. The invariant K1101, which was essential for enzyme activity and directly involved in the phospho-transfer reaction; and the consensus sequences DLAARN and PVRWMAPE that were strong indicators of tyrosine substrate specificity confirmed the tyrosine kinase nature of the mollusc receptor (Hanks et al., 1988). PfIRR displays homologies of 45–81% identical to aligned insulin receptor homologs, and showed the highest homology with that of *C. gigas* (Gricourt et al., 2006), which is consensus with the result of phylogenetic analysis.

In order to study the phylogenetic relationships between *Pfirr* and other distinct members of the IR family, a multiple sequence alignment including 35 homologous sequences was constructed by MEGA 4.0.2. Three independent branches (IR, IGF-IR and IRR) are present in the vertebrates, and the IRR seemed to have a closer relationship to the IGF-IR as compared with the IR. It suggests that the IGF-IR and IRR share a common ancestor. This result is consistent

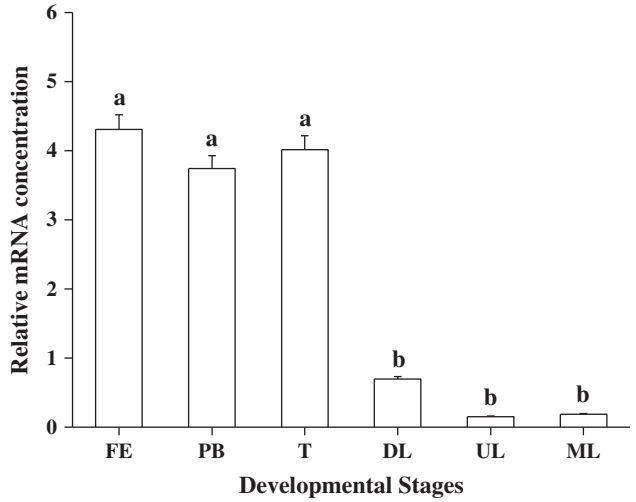

Fig. 5. Expression of *Pfirr* mRNA at the developmental stages of *Pinctada fucata*. The mRNA levels were quantified by real-time RT-PCR in: 1. FE, fertilized egg; 2. PB, polar body stage; 3. T, trocophore; 4. DL, D-shaped larva; 5. UL, umbo larva; 6. ML, metamorphosis of larva. The results are expressed as fold-changeValues without common notation (a, b) differ significantly ($p < 0.05$).

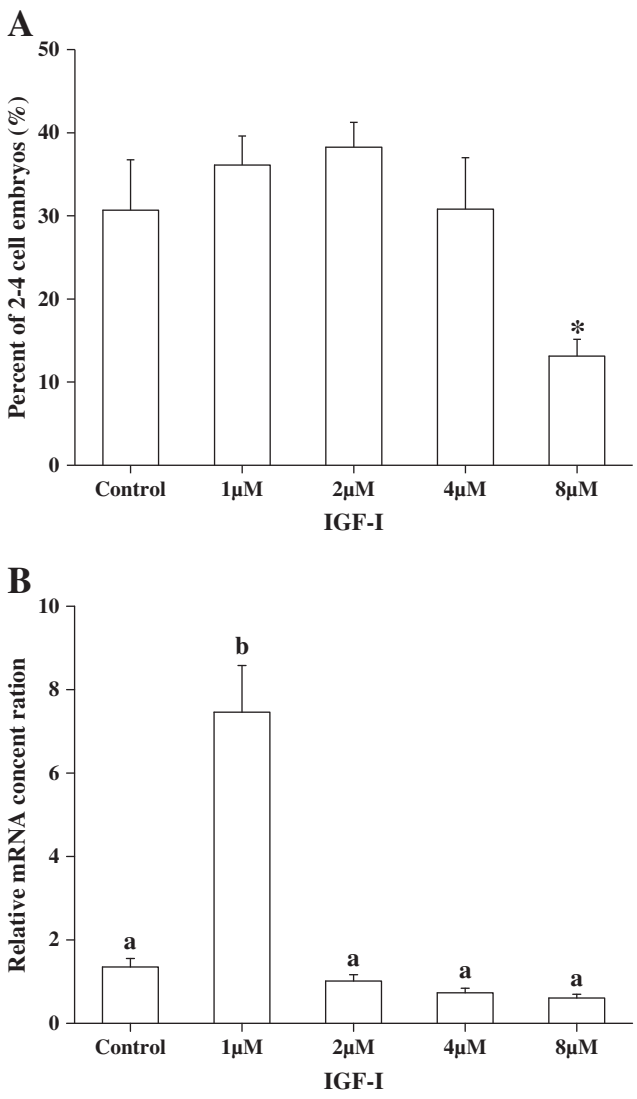

Fig. 6. Effects of IGF-I on the development of embryos at polar body stage of *P. fucata*. (A) Percent of embryos at polar body stage developed to 2–4 cell stage. Results are expressed as a percentage of the mean of three replicate concentrations obtained in one experiment. Significant statistical differences compared to the control are indicated by *, $p < 0.05$. (B) Expression of *Pfirr* mRNA following treatment with different concentrations of IGF-I. Each bar represents the mean ± S.E.M. of 3 samples. The results are expressed as fold-change in relative mRNA expression. Statistical differences were estimated by one-way ANOVA followed post tests. Values without common notation (a, b) differ significantly ($p < 0.05$). (a: developmental data, b: mortality rates and c: mRNA expression).

with previous studies showing that the IGF-IR and IRR is the result of a second gene duplication event in evolutionary history of the IR family (Maures et al., 2002; Renteria et al., 2008; Schlueter et al., 2006).

The mRNA expression of *Pfirr* has been found in all tissues examined, which suggests a wide distribution in the adult body. It is in good agreement with previous studies of *C. gigas* (Jouaux et al., 2012) CIR, *B. glabrata* IRR (Lardans et al., 2001), *Xenopus laevis* IR and *Gallus gallus* IRR (Hernandez-Sanchez et al., 2008), and other animals. High expression levels in adductor muscle strongly suggest the possible role of *Pfirr* in glycogen metabolism (Berthelin et al., 2000). The moderate expression level in digestive gland was in accordance with *C. gigas*, and may be related to the paracrine function of insulin-like peptide produced during digestion (Jouaux et al., 2012). The low expression level in mantel was also detected in *C. gigas* (Jouaux et al., 2012). Other tissues like mantle and heart were also potential targets of insulin-like signaling (Berthelin et al., 2000; Gomez-Mendikute et al.,

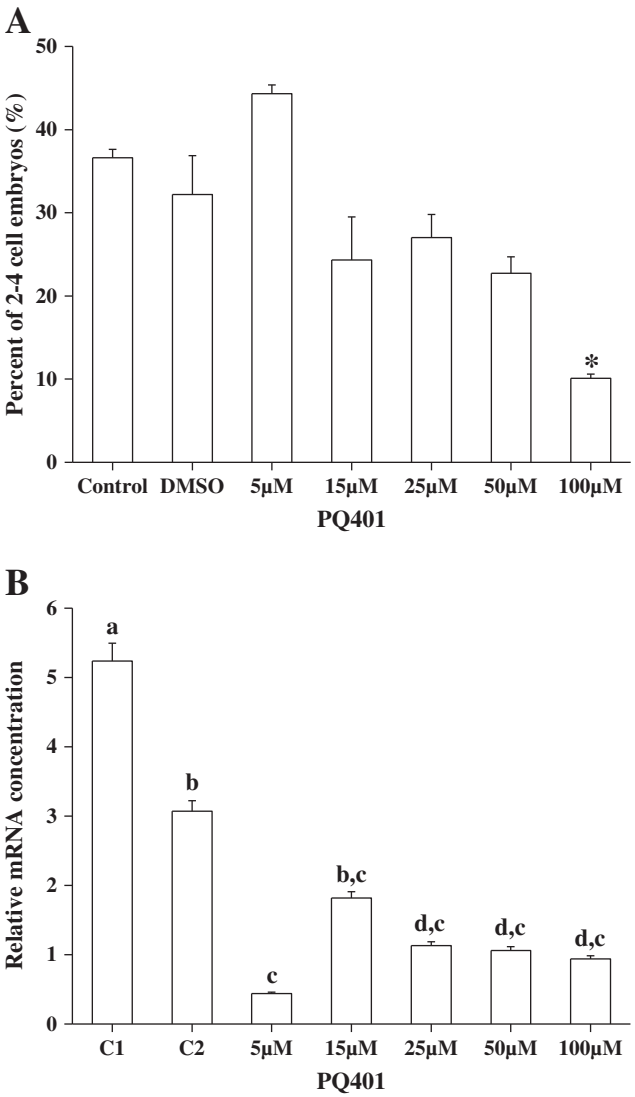

Fig. 7. Effects of PQ401 on the development of embryos at polar body stage of *P. fucata*. (A) Percent of embryos at polar body stage developed to 2–4 cell stage. Results are expressed as a percentage of the mean of three replicate values obtained in one experiment. Significant statistical differences compared to the control are indicated by *, $p < 0.05$. (B) Expression of *Pfirr* mRNA following treatment with different concentrations of PQ401. C1. Control; C2. DMSO control. Each bar represents the mean ± S.E.M. of 3 samples. The results are expressed as fold-change in relative mRNA expression relative to the control. Statistical differences were estimated by one-way ANOVA followed Tukey's post tests. Values without common notation (a, b, c, d) differ significantly ($p < 0.05$).

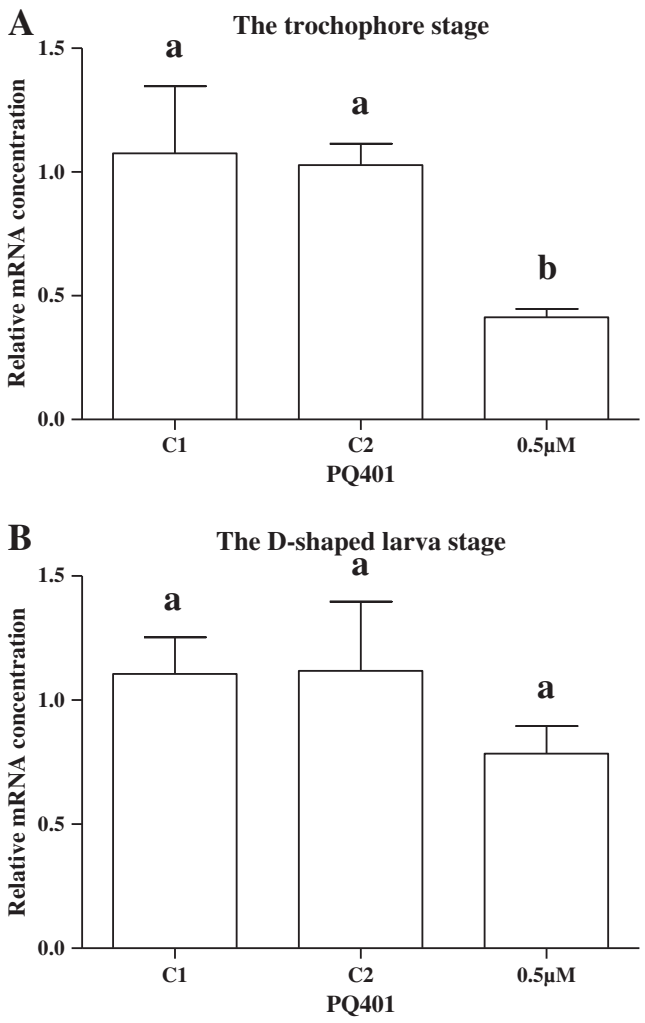

Fig. 8. Expression of *Pfirr* mRNA at the trocophore stage (A) and D-shaped larva stage (B) following treatment with different concentration of PQ401. C1. Control; C2. DMSO control. Each bar represents the mean ± S.E.M. of 3 samples. The results are expressed as fold-change in relative mRNA expression relative to the control. Statistical differences were estimated by one-way ANOVA followed Tukey's post tests. Values without common notation (a, b) differ significantly ($p < 0.05$).

2005). *Pfirr* mRNA is expressed at low levels during the previtellogenic arrest stage, which is consistent with the observation in mosquito *Aedes aegypti* (Riehle and Brown, 2002) and *C. gigas* (Jouaux et al., 2012). Similar to trout (Le Gac et al., 1996), boars (Minagawa et al., 2012), *Drosophila* (Ueishi et al., 2009) and *C. gigas* (Jouaux et al., 2012), the high level expression in testis at multiplicative stage may implicate the possible role of *Pfirr* in the regulation of testicular function.

The *Pfirr* mRNA was also expressed at high levels in embryonic stages with low levels in the larva stages, the expression pattern suggests that *Pfirr* may play a crucial role in early development. And it might be involved in regulating organogenesis and neurogenesis (Bateman and McNeill, 2006), or energy accumulation previously developed to larva. Similar expression pattern has also been found in *C. gigas* (Gricourt et al., 2006), but the mRNA expression of *CIR* gradually increases from D-type larvae to 11 days veliger stage, declines at the metamorphosis stage, which could be due to the specific role that this receptor is playing in molecular signaling during cell proliferation/differentiation. The possible involvement of insulin receptor in early development is also identified in *D. melanogaster* (Garofalo and Rosen, 1988; Petruzzelli et al., 1986) and seems to function by promoting cellular proliferation and/or neurogenesis (Fernandez et al., 1995).

The extracellular part of PfIRR has typical features of the insulin receptor family, but specific binding for insulin was found to be lower than IGF-I in invertebrates. Previous studies demonstrated that hrIGF-I could stimulate protein synthesis more efficient than insulin in vertebrates and invertebrates such as *C. gigas* (Gricourt et al., 2003), *H. tuberculata* (Lebel et al., 1996), *P. maximus* (Giard et al., 1998) and *H. duryi* (Sevala et al., 1993). In this present study, high concentrations of IGF-I do not significantly affect the mRNA expression of *Pfirr*, but do cause low rate of embryo development to 2–4 cell stage and part of embryos at polar body stage to death. High concentrations of IGF-I may lead to desensitization between IGF-I and *Pfirr*. The most efficient dose of IGF-I to increase *Pfirr* mRNA expression is 1 μM/L in the tested concentration range, with a slight (but not significant) increase on the rate of embryos developed to 2–4 cells. This lowest dose of IGF-I may be the most reasonable and physiological dose among tested different concentration to increase mRNA

expression of *Pfirr* to stimulate the development, as the other higher concentrations led to mortalities due to the overall toxicity. In mouse, IGF-I could increase blastocyst formation and total blastocyst cell number at early embryo development in culture (Lin et al., 2003). The proliferating effect of insulin-like substances was also demonstrated in the oyster *C. gigas* in relation with a homologous insulin receptor-related receptor expression (Gricourt et al., 2006). Thus, we suggest that IGF-I can effectively increase the mRNA expression of *Pfirr* and that may lead to stimulate cell proliferation in *P. fucata*.

Recently, a new class of small-molecules called diaryl ureas that inhibit the IGF-IR was identified (Gable et al., 2006). One diaryl urea compound, PQ401, was found to antagonize IGF-IR autophosphorylation of the IGF-IR in cultured human MCF-7 cells with an IC_{50} of 12 μM/L and the growth of cultured breast cancer cells in serum at 10 μM/L in previous studies. Since the autophosphorylation region of IRR and IGF-IR exhibited high conservation, we used PQ401 to inhibit PfiRR. Our studies indicate that PQ401 can decrease the mRNA expression of *Pfirr* and lead to the developmental arrest of embryos in *P. fucata*, reduced *Pfirr* expression may lead partial embryos at polar body stage to cleavage, developmental arrest and poor floating. We used PQ401 at concentration in the range of 5 to 100 μM/L, among the different treatments. The results indicate that 5 μM/L may be the most efficient dose to decrease *Pfirr* expression without disturbing development whereas 100 μM/L clearly affects it. The lowest dose may be the most reasonable and physiological dose among the different tested concentrations to decrease mRNA expression of *Pfirr* to delay the development. The reduction of expression by other higher doses of PQ401 may be due to the overall toxicity causing mortality and not to a direct effect of PQ401 over *Pfirr*. However, the mechanism under this result needs to be clarified in the future.

Since the lower dose of PQ401 (5 μM/L) seems to be the most efficient to inhibit *Pfirr* mRNA expression at polar body stage, 3 low doses (0.5, 1 and 1.25 μM/L) were compared to test effect of PQ401 on embryos at trocophore and D-shaped larva stages. The results are illustrated here with the dose 0.5 μM/L leading to higher survival. The results show that the trocophore is sensitive to this dose of PQ401, and 0.5 μM/L is the efficient concentration of PQ401 to significantly decrease *Pfirr* mRNA expression. However, D-shaped larvae seem less sensitive to this dose of PQ401. We have also conducted other higher concentrations of PQ401 to treat D-shaped larvae, but the results are similar to that of 0.5 μM/L, *Pfirr* mRNA expression decreased without significant differences (data not shown). So we submit that embryos at D-shaped stage may be the most sensitive stage to PQ401, the decreased expression of PQ401 without significant differences could lead most of the D-shaped larvae to death. The appearance of flocculation was obvious to observe when more than half of the trocophores or the D-shaped larvae were dying. And the trocophores or the D-shaped larvae that settled at the bottom of the vessel were also observed under a microscope to confirm death. Although the precise mortality rates were not recorded, this study can demonstrate that the inhibition of *Pfirr* affects the development of *P. fucata*. In other animals, inhibitions of insulin receptor have also been shown to affect development and growth. For example, mutation of an insulin receptor family member of *C. elegans*, DAF-2, leads to developmental arrest at the dauer larval stage and an increase in life span (Kimura et al., 1997). IGF-IR inhibition resulted in reduced zebrafish embryonic growth, arrested development and increased lethality (Schlueter et al., 2007). IGF-IR null mutant mice exhibited severe growth retardation at birth (45% of wild-type littermates) and died shortly after birth from respiratory failure (Liu et al., 1993).

In conclusion, the present study shows that *Pfirr* exhibits all the typical features of insulin receptors; the tissue distribution and the expression profiles of *Pfirr* at developmental stages suggest its wide distribution and crucial role in development at embryonic stages other than larval stage; expression of *Pfirr* mRNAs at different developmental stages following treatment with agonist IGF-I and antagonist PQ401 indicated that *Pfirr* may be involved in regulating the development of embryos in *P. fucata*. In the future, the mechanism of how *Pfirr* regulated the development and the high mRNA expression of *Pfirr* before D-shaped larva stage in the developmental process of *P. fucata* need to be investigated.

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References

Basu, S., Rajakaruna, S., Menko, A.S., 2012. Insulin-like growth factor receptor-1 and nuclear factor kappaB are crucial survival signals that regulate caspase-3-mediated lens epithelial cell differentiation initiation. Journal of Biological Chemistry 287, 8384–8397.

Bateman, J.M., McNeill, H., 2006. Insulin/IGF signalling in neurogenesis. Cellular and Molecular Life Sciences 63, 1701–1705.

Berthelin, C., Kellner, K., Mathieu, M., 2000. Histological characterization and glucose incorporation into glycogen of the Pacific oyster *Crassostrea gigas* storage cells. Marine Biotechnology (New York, N.Y.) 2, 136–145.

Blom, N., Gammeltoft, S., Brunak, S., 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. Journal of Molecular Biology 294, 1351–1362.

Broughton, S., Partridge, L., 2009. Insulin/IGF-like signalling, the central nervous system and aging. Biochemical Journal 418, 1–12.

Ciocan, C.M., Moore, J.D., Rotchell, J.M., 2006. The role of Ras gene in the development of haemic neoplasia in *Mytilus trossulus*. Marine Environmental Research 62, S147–S150 (Suppl.).

Claeys, I., Simonet, G., Poels, J., Van Loy, T., Vercammen, L., De Loof, A., Vanden, B.J., 2002. Insulin-related peptides and their conserved signal transduction pathway. Peptides 23, 807–816.

De Meyts, P., 2004. Insulin and its receptor: structure, function and evolution. Bioessays 26, 1351–1362.

Ebina, Y., Edery, M., Ellis, L., Standring, D., Beaudoin, J., Roth, R.A., Rutter, W.J., 1985. Expression of a functional human insulin receptor from a cloned cDNA in Chinese hamster ovary cells. Proceedings of the National Academy of Sciences of the United States of America 82, 8014–8018.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., Schlessinger, J., 1995. The *Drosophila* insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. EMBO Journal 14, 3373–3384.

Floyd, P.D., Li, L., Rubakhin, S.S., Sweedler, J.V., Horn, C.C., Kupfermann, I., Alexeeva, V.Y., Ellis, T.A., Dembrow, N.C., Weiss, K.R., Vilim, F.S., 1999. Insulin prohormone processing, distribution, and relation to metabolism in *Aplysia californica*. Journal of Neuroscience 19, 7732–7741.

Gable, K.L., Maddux, B.A., Penaranda, C., Zavodovskaya, M., Campbell, M.J., Lobo, M., Robinson, L., Schow, S., Kerner, J.A., Goldfine, I.D., Youngren, J.F., 2006. Diarylureas are small-molecule inhibitors of insulin-like growth factor I receptor signaling and breast cancer cell growth. Molecular Cancer Therapeutics 5, 1079–1086.

Garofalo, R.S., 2002. Genetic analysis of insulin signaling in *Drosophila*. Trends in Endocrinology and Metabolism 13, 156–162.

Garofalo, R.S., Rosen, O.M., 1988. Tissue localization of *Drosophila melanogaster* insulin receptor transcripts during development. Molecular and Cellular Biology 8, 1638–1647.

Giard, W., Lebel, J.M., Boucaud-Camou, E., Favrel, P., 1998. Effects of vertebrate growth factors on digestive gland cells from the mollusc *Pecten maximus* L.: an in vitro study. Journal of Comparative Physiology. B 168, 81–86.

Gomez-Mendikute, A., Elizondo, M., Venier, P., Cajaraville, M.P., 2005. Characterization of mussel gill cells in vivo and in vitro. Cell and Tissue Research 321, 131–140.

Gricourt, L., Bonnec, G., Boujard, D., Mathieu, M., Kellner, K., 2003. Insulin-like system and growth regulation in the Pacific oyster *Crassostrea gigas*: hrIGF-1 effect on protein synthesis of mantle edge cells and expression of an homologous insulin receptor-related receptor. General and Comparative Endocrinology 134, 44–56.

Gricourt, L., Mathieu, M., Kellner, K., 2006. An insulin-like system involved in the control of Pacific oyster *Crassostrea gigas* reproduction: hrIGF-1 effect on germinal cell proliferation and maturation associated with expression of an homologous insulin receptor-related receptor. Aquaculture 251, 85–98.

Hamano, K., Awaji, M., Usuki, H., 2005. cDNA structure of an insulin-related peptide in the Pacific oyster and seasonal changes in the gene expression. Journal of Endocrinology 187, 55–67.

Hanks, S.K., Quinn, A.M., Hunter, T., 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241, 42–52.

Heldin, C.H., Ostman, A., 1996. Ligand-induced dimerization of growth factor receptors: variations on the theme. Cytokine & Growth Factor Reviews 7, 3–10.

Hernandez-Sanchez, C., Mansilla, A., de Pablo, F., Zardoya, R., 2008. Evolution of the insulin receptor family and receptor isoform expression in vertebrates. Molecular Biology and Evolution 25, 1043–1053.
Hubbard, S.R., Till, J.H., 2000. Protein tyrosine kinase structure and function. Annual Review of Biochemistry 69, 373–398.
Jouaux, A., Franco, A., Heude-Berthelin, C., Sourdaine, P., Blin, J.L., Mathieu, M., Kellner, K., 2012. Identification of Ras, Pten and p70S6K homologs in the Pacific oyster *Crassostrea gigas* and diet control of insulin pathway. General and Comparative Endocrinology 176, 28–38.
Jurczyk, A., Roy, N., Bajwa, R., Gut, P., Lipson, K., Yang, C., Covassin, L., Racki, W.J., Rossini, A.A., Phillips, N., Stainier, D.Y., Greiner, D.L., Brehm, M.A., Bortell, R., Dilorio, P., 2011. Dynamic glucoregulation and mammalian-like responses to metabolic and developmental disruption in zebrafish. General and Comparative Endocrinology 170, 334–345.
Kimura, K.D., Tissenbaum, H.A., Liu, Y., Ruvkun, G., 1997. daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science 277, 942–946.
Kits, K.S., de Vries, N.J., Ebberink, R.H., 1990. Molluscan insulin-related neuropeptide promotes neurite outgrowth in dissociated neuronal cell cultures. Neuroscience Letters 109, 253–258.
Klein, S.L., Strausberg, R.L., Wagner, L., Pontius, J., Clifton, S.W., Richardson, P., 2002. Genetic and genomic tools for *Xenopus* research: The NIH *Xenopus* Initiative. Developmental Dynamics 225, 384–391.
Konrad, C., Kroner, A., Spiliotis, M., Zavala-Gongora, R., Brehm, K., 2003. Identification and molecular characterisation of a gene encoding a member of the insulin receptor family in *Echinococcus multilocularis*. International Journal of Parasitology 33, 301–312.
Lardans, V., Coppin, J.F., Vicogne, J., Aroca, E., Delcroix, M., Dissous, C., 2001. Characterization of an insulin receptor-related receptor in *Biomphalaria glabrata* embryonic cells. Biochimica et Biophysica Acta 1510, 321–329.
Le Gac, F., Loir, M., Le Bail, P., Ollitrault, M., 1996. Insulin-like growth factor (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and Sertoli cells. Molecular Reproduction and Development 44, 23–35.
Lebel, J.M., Giard, W., Favrel, P., Boucaud-Camou, E., 1996. Effects of different vertebrate growth factors on primary cultures of hemocytes from the gastropod mollusc, *Haliotis tuberculata*. Biology of the Cell 86, 67–72.
Leevers, S.J., 2001. Growth control: invertebrate insulin surprises! Current Biology 11, R209–R212.
Leibush, B.N., Chistyakova, O.V., 2003. Receptor of insulin-like growth factor 1 in tissues of the mollusc *Anodonta cygnea*. Journal of Evolutionary Biochemistry and Physiology 39, 169.
Li, K.W., Geraerts, W.P., Joosse, J., 1992. Purification and sequencing of molluscan insulin-related peptide II from the neuroendocrine light green cells in *Lymnaea stagnalis*. Endocrinology 130, 3427–3432.
Lin, T.C., Yen, J.M., Gong, K.B., Hsu, T.T., Chen, L.R., 2003. IGF-1/IGFBP-1 increases blastocyst formation and total blastocyst cell number in mouse embryo culture and facilitates the establishment of a stem-cell line. BMC Cell Biology 4, 14.
Liu, J.P., Baker, J., Perkins, A.S., Robertson, E.J., Efstratiadis, A., 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75, 59–72.
Lu, Y., Dolle, M.E., Imholz, S., van T, S.R., Verschuren, W.M., Wijmenga, C., Feskens, E.J., Boer, J.M., 2008. Multiple genetic variants along candidate pathways influence plasma high-density lipoprotein cholesterol concentrations. Journal of Lipid Research 49, 2582–2589.
Maures, T., Chan, S.J., Xu, B., Sun, H., Ding, J., Duan, C., 2002. Structural, biochemical, and expression analysis of two distinct insulin-like growth factor I receptors and their ligands in zebrafish. Endocrinology 143, 1858–1871.
Minagawa, I., Fukuda, M., Ishige, H., Kohriki, H., Shibata, M., Park, E.Y., Kawarasaki, T., Kohsaka, T., 2012. Relaxin-like factor (RLF)/insulin-like peptide 3 (INSL3) is secreted from testicular Leydig cells as a monomeric protein comprising three domains B-C-A with full biological activity in boars. Biochemical Journal 441, 265–273.
Nakae, J., Kido, Y., Accili, D., 2001. Distinct and overlapping functions of insulin and IGF-I receptors. Endocrine Reviews 22, 818–835.
Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10, 1–6.
Ottensmeyer, F.P., Beniac, D.R., Luo, R.Z., Yip, C.C., 2000. Mechanism of transmembrane signaling: insulin binding and the insulin receptor. Biochemistry-Us 39, 12103–12112.
Petruzzelli, L., Herrera, R., Arenas-Garcia, R., Fernandez, R., Birnbaum, M.J., Rosen, O.M., 1986. Isolation of a *Drosophila* genomic sequence homologous to the kinase domain of the human insulin receptor and detection of the phosphorylated *Drosophila* receptor with an anti-peptide antibody. Proceedings of the National Academy of Sciences of the United States of America 83, 4710–4714.
Pimentel, B., de la Rosa, E.J., de Pablo, F., 1996. Insulin acts as an embryonic growth factor for *Drosophila* neural cells. Biochemical and Biophysical Research Communications 226, 855–861.
Renteria, M.E., Gandhi, N.S., Vinuesa, P., Helmerhorst, E., Mancera, R.L., 2008. A comparative structural bioinformatics analysis of the insulin receptor family ectodomain based on phylogenetic information. PLoS One 3, e3667.
Riehle, M.A., Brown, M.R., 2002. Insulin receptor expression during development and a reproductive cycle in the ovary of the mosquito *Aedes aegypti*. Cell and Tissue Research 308, 409–420.
Roovers, E., Vincent, M.E., van Kesteren, E., Geraerts, W.P., Planta, R.J., Vreugdenhil, E., van Heerikhuizen, H., 1995. Characterization of a putative molluscan insulin-related peptide receptor. Gene 162, 181–188.
Schlueter, P.J., Royer, T., Farah, M.H., Laser, B., Chan, S.J., Steiner, D.F., Duan, C., 2006. Gene duplication and functional divergence of the zebrafish insulin-like growth factor 1 receptors. The FASEB Journal 20, 1230–1232.
Schlueter, P.J., Peng, G., Westerfield, M., Duan, C., 2007. Insulin-like growth factor signaling regulates zebrafish embryonic growth and development by promoting cell survival and cell cycle progression. Cell Death and Differentiation 14, 1095–1105.
Schultz, J., Milpetz, F., Bork, P., Ponting, C.P., 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proceedings of the National Academy of Sciences of the United States of America 95, 5857–5864.
Sevala, V.M., Sevala, V.L., Saleuddin, A., 1993. Hemolymph insulin-like peptides (ILP) titers and the influence of ILP and mammalian insulin on the amino acid incorporation in the mantle collar in vitro in *Helisoma* (Mollusca). Biology Bulletin 185, 140–148.
Shier, P., Watt, V.M., 1989. Primary structure of a putative receptor for a ligand of the insulin family. Journal of Biological Chemistry 264, 14605–14608.
Shipilov, V.N., Shpakov, A.O., Rusakov, Y.I., 2005. Pleiotropic action of insulin-like peptides of mollusk, *Anodonta cygnea*. Annals of the New York Academy of Sciences 1040, 464–465.
Smit, A.B., Vreugdenhil, E., Ebberink, R.H., Geraerts, W.P., Klootwijk, J., Joosse, J., 1988. Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide. Nature 331, 535–538.
Smit, A.B., van Kesteren, R.E., Li, K.W., Van Minnen, J., Spijker, S., Van Heerikhuizen, H., Geraerts, W.P., 1998. Towards understanding the role of insulin in the brain: lessons from insulin-related signaling systems in the invertebrate brain. Progress in Neurobiology 54, 35–54.
Sparrow, L.G., McKern, N.M., Gorman, J.J., Strike, P.M., Robinson, C.P., Bentley, J.D., Ward, C.W., 1997. The disulfide bonds in the C-terminal domains of the human insulin receptor ectodomain. Journal of Biological Chemistry 272, 29460–29467.
Swanson, M.E., Elste, A.M., Greenberg, S.M., Schwartz, J.H., Aldrich, T.H., Furth, M.E., 1986. Abundant expression of Ras proteins in *Aplysia* neurons. The Journal of Cell Biology 103, 485–492.
Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M., Garofalo, R.S., 2001. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science 292, 107–110.
Ueishi, S., Shimizu, H., H, I.Y., 2009. Male germline stem cell division and spermatocyte growth require insulin signaling in *Drosophila*. Cell Structure and Function 34, 61–69.
Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Et, A., 1986. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO Journal 5, 2503–2512.
Veenstra, J.A., 2010. Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other mollusks and insects. General and Comparative Endocrinology 167, 86–103.
von Heijne, G., 1992. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. Journal of Molecular Biology 225, 487–494.
Wheeler, D.E., Buck, N., Evans, J.D., 2006. Expression of insulin pathway genes during the period of caste determination in the honey bee, *Apis mellifera*. Insect Molecular Biology 15, 597–602.
Wu, Q., Brown, M.R., 2006. Signaling and function of insulin-like peptides in insects. Annual Review of Entomology 51, 1–24.
Yarden, Y., Ullrich, A., 1988. Growth factor receptor tyrosine kinases. Annual Review of Biochemistry 57, 443–478.