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Screening of genes related to ovarian development in the swimming crab, *Portunus trituberculatus*, by suppression subtractive hybridization

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ABSTRACT. The swimming crab, *Portunus trituberculatus*, is an important marine animal and is widely cultured in China. In the present study, suppression subtractive hybridization was applied to identify the differentially expressed genes in the ovaries of mature and immature *P. trituberculatus*. One hundred and seventy six expressed sequence tag (ESTs) were identified, of which 100 were down-regulated, and 76 up-regulated. BLAST analysis identified 51 unigenes, of which 27 were down-regulated, and 24 up-regulated. Quantitative real-time reverse transcriptase polymerase chain reaction results indicated that the SSH technique is valuable in screening genes related to ovarian development. Genes identified in this study encoded proteins corresponding to a wide range of functions and included immune response protein, transcription initiation factor, metabolic proteins, chromosome, histone h3, ovarian development-related protein, and vitellogenin. In addition, 64 metabolic pathways were annotated in differentially expressed ESTs by using the

Kyoto Encyclopedia of Genes and Genomes pathway. Four annotated pathways (oxidative phosphorylation, carbon metabolism, fatty acid degradation, and protein digestion and absorption) appeared to be involved in ovarian development. In ontology analysis, 5.83% of the cellular process genes in reverse subtraction cDNA library are involved in reproduction, and 5.88% involved in developmental process. In up-regulated genes, myosin II-expressed polehole-like protein; histone h3; ovigerous-hair stripping substance; peritrophin 48; and ovarian development-related protein appeared to be involved in ovarian development. Identification of differentially expressed genes in the mature and immature ovary of the swimming crab provides new insights for further studies on the mechanism underlying ovarian development in this species.

Key words: *Portunus trituberculatus*; Ovarian development; Suppression subtractive hybridization

INTRODUCTION

Portunus trituberculatus is a commercially significant crab species and is widespread throughout Korea, Japan, China, and Southeast Asia. In China, *P. trituberculatus* is distributed throughout the marginal seas of China, including the Bohai, Huanghai, East China, and South China seas. Due to its high nutritional value, *P. trituberculatus* is a species that is intensively explored commercially in China. With the expanding scale of *P. trituberculatus* culture, more and more mature female crabs are cultured to meet the requirement for larval crabs. Ovarian maturation is therefore a critical step in reproduction of the species within culture systems. Programs of differential gene expression mediate eukaryotic biological processes, such as cellular growth and organogenesis. Molecular mechanisms of *P. trituberculatus* reproduction remain largely unknown, and mechanisms of ovarian development are worthy of being explored.

Genes related to ovarian development in the Chinese shrimp, *Fenneropenaeus chinensis*, have been identified by suppression subtractive hybridization (SSH) (Xie et al., 2010). Several genes have been characterized in *P. trituberculatus*, but most of these are immune-related, and few are associated with sex or gonad development (Liu et al., 2011). From a physiological and behavioral standpoint, reproduction in crustaceans is a complex process (Elner and Beninger, 1992). In the swimming crab, it had been observed that ovarian development is dependent on mating (Wu et al., 2007).

Identification of genes that are differentially expressed in the ovary, at the level of transcription, may help to elucidate mechanisms of ovarian maturation, and facilitate the development of novel methods for reproduction control in *P. trituberculatus*. Subtractive cDNA hybridization has been shown to be a powerful technique for the identification and isolation of cDNA of differentially expressed genes, and several forms of subtractive hybridization have been reported (Duguid and Dinauer, 1990). The polymerase chain reaction (PCR)-based SSH technique was developed for rapid and sensitive comparisons of mRNA expression patterns between ‘tester’ and ‘driver’ populations, and has been applied to many molecular genetic and positional cloning studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes (Diatchenko et al., 1996). In the present study, PCR-based SSH was applied to

screen genes associated with ovarian maturity in the swimming crab, providing a useful molecular resource for further investigation of mechanisms of ovarian development in this species.

MATERIAL AND METHODS

Animals, culturing, and treatment

Two hundred *P. trituberculatus,* with a sex ratio of 1:1, were cultured at Xinyi Corporation of Ningbo, China. Each crab was cultured in a separate plastic basket, except for mating. To control ovarian maturation, one group, containing 50 female crabs, were allowed to mate (the tester group); a second group was not mated (the driver group). Crabs in these two groups were cultured for three months under the same conditions, before the tester group was mated. Ovarian maturity was then assessed by collection of ovarian tissues for RNA isolation and construction of differential expression cDNA libraries between the tester and driver groups.

Total RNA and mRNA isolation

Crabs were anesthetized by chilling, then euthanized and ovarian tissues were harvested from females for total RNA extraction, which was performed immediately using Trizol reagent, according to the manufacture protocol (Invitrogen, USA). Integrity of the extracted RNA was examined by electrophoresis on denaturing, formaldehyde 1% agarose/EtBr (w/v) gel.

Construction of SSH libraries and dot blotting

A differential expression cDNA library was constructed with SSH using a PCR-select™ cDNA subtraction kit, according to the manufacturer protocol (Clontech, Tokyo, Japan). For forward subtraction, cDNA from mature ovary was used as the tester, and cDNA of immature ovary as the driver. Conversely, cDNA from immature ovary was used as the tester, and cDNA from mature ovary as the driver for reverse subtraction, in order to maximize the sensitivity of the screening kit. Double stranded tester and driver cDNA were digested separately in *Rsa*I at 37°C for 1.5 h, and then purified prior to tester ligation to adaptors 1 and 2R (provided in the kit). Each sample was incubated at 98°C for 1.5 min before hybridization at 68°C for 8 h. Then, the two samples from the first hybridization were combined, and fresh denatured driver DNA added to further enrich for differentially expressed sequences. Then, cDNAs were directly inserted into the pMD18-T cloning vector (TaKaRa, Japan), transformed into *E. coli* DH5α-competent cells, and cultured in LB medium (containing 50 µg/mL ampicillin). Dot blotting programs were conducted according to the manufacturer instructions for the PCR-select cDNA subtraction kit.

Sequencing and analysis of expressed sequence tags (ESTs)

Clones were sequenced on an ABI3730XL automated sequencer (Applied Biosystems, Norwalk, USA). DNA from *E. coli*, vector, and adapter sequences were removed. Then, sample sequences were screened against the NCBI database (http://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/). Sequence similarities were searched by local BLASTx and BLASTn analyses against the non-redundant (nr) protein. Gene classification and function were determined by gene ontology (GO)

mapping, provided by the GO website (http://geneontology.org/). Metabolic pathways were annotated using KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/tools/kaas/).

Real-time RT-PCR analysis

Ovarian tissue from crabs in mature and immature groups (both N = 10) were collected and preserved in liquid nitrogen before RNA extraction. Total RNA was extracted as outlined above. Primers for quantitative real-time RT-PCR were designed on the basis of unique gene sequence (Table 1). Transcription levels of eight unique genes were analyzed, using *β-actin* as an internal gene control.

Table 1. Primers for determining transcription levels of selected genes.

Primer	Forward (5'-3')	Reverse (5'-3')	Length of product (bp)
β-actin	TCACACACTGTCCCCATCTACG	ACCACGCTCGGTCAGGATTTTC	114
Up-regulated genes			
Low-Density Lipoprotein Receptor (LDLR)	CCACCATAACACCGCTCACCA	TCTGGCTGGCAGCTCAGGAAT	115
Ovary development-related protein (ODR)	GCTGTTGTTTGGGCTTTGGTA	GTGGTAGTTAGTGGTGGCGCA	296
Polehole-like protein (PLP)	ACGGGCTTGACTTGTCTTTGCT	GTCCCTCTGCTACGCCAGGTA	240
Vitellogenin (VT)	TGCTGCCAAACTGTCCTTCAT	TTAATTGCCCAGGTATCGTCAT	300
Down-regulated genes			
Comm domain-containing protein 2 (CDC)	CAGTGAGGTGGAGGTCAAGGA	TCTCGGAACCAGCATCTAGGGA	164
Hemocyanin subunit 6(HC)	CTGGGTTAAACTGTCTCCTGGA	AAGCTGCTGTGGTACTCATCGA	162
Liver fatty acid binding protein (LFAB)	GGCGACAAGGACTTCACCAA	TGGTAAAGGTGCCAGTCAAGGT	124
Peroxiredoxin 6 (PRX6)	ACAAATGGGGCATCATCTTCT	GGCAGTCTTCTCATCTGGGTCA	275

RESULTS

SSH library construction and overall features of differentially expressed clones

Clones (N = 200) from the SSH library, constructed from ovary from mature and immature female crabs, were randomly picked and sequenced. In total, 176 differentially expressed cDNA clones were identified. Further analysis revealed that in mature ovary, 57.95% of SSH clones were down-regulated, and 42.05% were up-regulated.

Sequencing and analysis of ESTs

Sequences were screened against the UniVec database. In total, 176 high quality EST sequences were obtained. These genes were all functionally annotated by blasting against the NCBI database, and 51 differentially expressed genes were annotated. Of these, 24 were up-regulated (Table 2) and 27 down-regulated (Table 3).

Gene ontology

Gene ontology terms for the 176 unique sequences were obtained. Of these, 76 were up-regulated, and 100 down-regulated. Sequence descriptions and GO numbers are summarized in Tables 4 and 5. Under the biological process GO terms in the reverse library, 5.83% were cellular processes involved in reproduction. In the forward library, 5.88% were involved in developmental processes; no sequences involved in reproduction were observed. In the molecular function category, 20% of genes in the reverse library, and 15% in the forward library, were classified to

have structural molecule activities. As to the cellular component category, 9.65% in the reverse, and 11.33% in the forward libraries were related directly to intracellular function.

Table 2. Putative up-regulated genes in mature ovary.

Sample No.	Homologous to genes in database	Organism	E-value
D08-A6	Zinc proteinase Mpc1 ABD65301	*Litopenaeus vannamei*	8.83e-103
A07-A5	Adenine nucleotide translocase AEZ68611	*Litopenaeus vannamei*	5.51e-57
A03-C9	Alpha 2-macroglobulin AEC50080	*Pacifastacus leniusculus*	3.37e-27
F09-C12	Chromosome 4 open reading frame 34 NP_001016053	*Xenopus* (*Silurana*) *tropicalis*	2.49e-22
B03-B9	Chymotrypsin-like serine proteinase ACC68669	*Fenneropenaeus chinensis*	3.76e-104
F10-D1	Cytochrome b BAD16771	*Acromyrmex echinatior*	7.23e-28
G03-D8	Cytochrome c oxidase polypeptide vb AFV69126	*Euphausia superba*	3.01e-25
C04-C3	Eukaryotic translation initiation factor 3 subunit d	*Scylla paramamosain*	5.09e-76
F05-C8	Fatty acid-binding protein AGO02161	*Scylla paramamosain*	5.79e-22
F12-B8	Histone h3 CAH61024	*Philocelis karlingi*	1.35e-44
F06-C4	Peritrophin-48 XP_001999045	*Acromyrmex echinatior*	8.36e-08
E01-A9	Low Density Lipoprotein Receptor XP_003690198	*Apis florea*	1.25e-14
G02-D6	Lectin c ADG85667	*Marsupenaeus japonicus*	1.41e-08
E09-B7	NADH dehydrogenase XP_001654149	*Aedes aegypti*	4.95e-26
C05-D7	Nfx1-type zinc finger-containing protein 1 EFX77800	*Daphnia pulex*	1.86e-33
D05-A3	Ovary development-related protein AAO73308	*Eriocheir sinensis*	2.02e-52
D09-A7	Pdgf vegf-related factor 1 ADF87936	*Eriocheir sinensis*	7.44e-29
B12-C12	Polehole-like protein ACV60547	*Penaeus monodon*	4.43e-09
F04-C7	Ovigerous-hair stripping substance AAP57670	*Chiromantes haematocheir*	4.24e-55
F10-C12	Receptor for activated protein kinase c1 ABU49887	*Penaeus monodon*	7.27e-76
D09-C7	Short-chain dehydrogenease reductase EJY57385	*Aedes aegypti*	2.95e-99
F01-B11	Splicing arginine serine-rich 7 ACO12997	*Lepeophtheirus salmonis*	8.06e-37
H07-C6	von Willebrand factor AEC22817	*Macrobrachium nipponense*	7.87e-31
A04-G9	Vitellogenin ABX89617	*Callinectes sapidus*	9.25e-164

Table 3. Putative down-regulated genes in mature ovary.

Sample No.	Homologous to genes in database	Organism	E-value
G04-H5	Ribosomal protein AAV71145	*Callinectes sapidus*	4.08e-153
B02-G3	40s ribosomal protein s12 CCE46016	*Nephrops norvegicus*	4.33e-82
A02-G7	Alcohol dehydrogenase class-3-like XP_002738071	*Saccoglossus kowalevskii*	3.21e-58
E02-E5	Aldehyde dehydrogenase XP_002425846	*Pediculus humanus corporis*	7.23e-86
G08-F1	Anionic trypsin-2 precursor XP_002126930	*Ciona intestinalis*	1.50e-12
G08-F5	Arginine kinase AEZ68729	*Pagrus major*	7.99e-142
D12-E3	Carboxypeptidase a2 EFX80631	*Daphnia pulex*	7.49e-71
H05-F6	Activated protein kinase C receptor AAO73307	*Eriocheir sinensis*	1.32e-41
B12-G6	Chitinase ABY85409	*Scylla serrata s*	7.17e-25
G04-E6	Chromadorea alt protein XP_002587162	*Branchiostoma floridae*	4.60e-08
E02-E4	Chymotrypsin-like protein AEE25770	*Scylla paramamosain*	4.65e-135
G06-E9	E3 sumo-protein ligase nse2 AFP23392	*Scylla paramamosain*	5.06e-58
A12-E3	Ferritin AEK81609	*Portunus trituberculatus*	9.38e-115
C07-H4	Glutathione s-transferase mu 3 XP_003444865	*Oreochromis niloticus*	7.07e-104
A11-F4	Heme binding protein 2 BAA99544	*Amphibalanus amphitrite*	1.22e-08
F04-F12	Hemocyanin subunit 6 AAA96966	*Metacarcinus magister*	3.34e-100
F02-F9	Intracellular fatty acid binding protein ABE77153	*Pacifastacus leniusculus*	1.60e-04
F12-H1	Liver fatty acid binding protein ADP05225	*Eriocheir sinensis*	3.13e-52
H09-F12	Macrophage mannose receptor 1-like AAX63905	*Fenneropenaeus chinensis*	1.15e-05
H02-F1	Myosin regulatory light chain 2 smooth muscle ACY66440	*Scylla paramamosain*	4.02e-106
H10-C2	Comm domain-containing protein 2 EGI62544	*Scylla paramamosain*	1.97e-30
A03-E7	Peroxiredoxin 6 ACJ53746	*Scylla paramamosain*	1.85e-22
E09-F10	Prefoldin subunit 5 BAM18170	*Papilio xuthus*	3.25e-56
F05-G1	Proteasome beta 4 subunit EFX84494	*Daphnia pulex*	9.18e-35
B05-G7	Vitelline membrane outer layer protein 1 homolog NP_493786	*Caenorhabditis elegans*	1.42e-15
H08-F11	Zinc finger protein-like 2.09E-17	*Saccoglossus kowalevskii*	2.09e-17
C03-G9	Zinc finger matrin-type protein 5 XP_002596619	*Branchiostoma floridae*	3.73e-17

Table 4. GO results from reverse subtraction cDNA library.

Annotation	GO No.	EST No.	Percentage (%)
Cellular component	GO:0005575	49	17.60
Intracellular part	GO:0044424	39	14.42
Cell part	GO:0044464	40	14.01
Intracellular	GO:0005622	40	9.65
Cell	GO:0005623	40	8.40
Intracellular organelle part	GO:0044446	22	8.37
Macromolecular complex	GO:0032991	27	7.61
Extracellular region	GO:0005576	13	7.53
Organelle	GO:0043226	30	6.28
Extracellular region part	GO:0044421	9	5.88
Plasma membrane	GO:0005886	8	5.65
Organelle part	GO:0044422	22	5.40
Membrane	GO:0016020	13	5.34
Non-membrane-bounded organelle	GO:0043228	24	5.28
Extracellular space	GO:0005615	5	5.00
Molecular function	GO:0003674	71	26.58
Structural molecule activity	GO:0005198	20	20.00
Binding	GO:0005488	35	15.78
Structural constituent of ribosome	GO:0003735	20	20.00
Lipid transporter activity	GO:0005319	12	12.00
Protein binding	GO:0005515	13	9.08
Substrate-specific transporter activity	GO:0022892	12	7.20
Transporter activity	GO:0005215	13	5.32
Hydrolase activity	GO:0016787	19	5.13
RNA binding	GO:0003723	9	5.08
Ribosome binding	GO:0043022	5	5.00
Laminin binding	GO:0043236	5	5.00
Catalytic activity	GO:0003824	28	5.00
Biological process	GO:0008150	67	16.69
Gene expression	GO:0010467	26	19.31
Protein metabolic process	GO:0019538	37	17.28
Cellular process	GO:0009987	37	17.00
Cellular macromolecule metabolic process	GO:0044260	27	15.48
Cellular biosynthetic process	GO:0044249	25	14.40
Macromolecule metabolic process	GO:0043170	39	14.23
Metabolic process	GO:0008152	48	13.06
Primary metabolic process	GO:0044238	39	12.24
Macromolecule biosynthetic process	GO:0009059	25	11.16
Transport	GO:0006810	29	10.09
Biosynthetic process	GO:0009058	25	8.64
Cellular metabolic process	GO:0044237	30	8.39
Lipid localization	GO:0010876	12	7.20
Establishment of localization	GO:0051234	29	6.06
Cellular process involved in reproduction	GO:0048610	11	5.83
Oxidation-reduction process	GO:0055114	7	5.56
Macromolecule localization	GO:0033036	22	5.01

Metabolic pathway annotation

Sixty-four metabolic pathways were annotated, including 20 in up-regulated and 44 in down-regulated genes (Table 6). Four pathways (oxidative phosphorylation, carbon metabolism, fatty acid degradation, and protein digestion and absorption) were suggested to be involved in ovarian development.

Transcription of selected genes by real-time PCR

Expression of eight differential genes, including four up-regulated and four down-regulated genes, were detected by quantitative real-time RT-PCR (Figure 1). Most of the detected genes

were showed similar expression characterization to the result of SSH library, except genes PLP and LFAB, which showed insignificant differences in mature ovary and immature ovary.

Table 5. GO results from forward subtraction cDNA library.

Annotation	GO No.	EST No.	Percentage (%)
Cellular component	GO:0005575	41	17.44
Cell	GO:0005623	37	14.73
Intracellular part	GO:0044424	34	13.88
Macromolecular complex	GO:0032991	25	12.12
Intracellular	GO:0005622	35	11.33
Cell part	GO:0044464	36	11.21
Protein complex	GO:0043234	10	10.00
Non-membrane-bounded organelle	GO:0043228	23	8.64
Organelle	GO:0043226	32	7.01
Molecular function	GO:0003674	53	36.95
Binding	GO:0005488	25	20.15
Catalytic activity	GO:0003824	25	15.30
Structural molecule activity	GO:0005198	15	15.00
Hydrolase activity	GO:0016787	18	12.48
Peptidase activity	GO:0008233	9	9.00
Protein binding	GO:0005515	10	8.72
Nucleic acid binding	GO:0003676	13	8.00
RNA binding	GO:0003723	10	8.00
Nucleotide binding	GO:0000166	7	7.00
Transporter activity	GO:0005215	7	6.13
Biological process	GO:0008150	50	60.17
Metabolic process	GO:0008152	36	24.81
Protein metabolic process	GO:0019538	27	16.12
Regulation of biological process	GO:0050789	15	16.08
Primary metabolic process	GO:0044238	28	14.69
Catabolic process	GO:0009056	14	14.00
Transport	GO:0006810	15	13.20
Cellular process	GO:0009987	32	11.36
Gene expression	GO:0010467	17	10.20
Macromolecule metabolic process	GO:0043170	27	10.03
Cellular metabolic process	GO:0044237	25	9.68
Biological regulation	GO:0065007	15	9.65
Nucleobase-containing compound metabolic process	GO:0006139	8	8.36
Establishment of localization	GO:0051234	15	7.92
Cellular component organization	GO:0016043	9	7.66
Cellular macromolecule metabolic process	GO:0044260	17	6.72
Biosynthetic process	GO:0009058	20	6.67
Macromolecule biosynthetic process	GO:0009059	17	6.12
Cellular biosynthetic process	GO:0044249	17	6.12
Developmental process	GO:0032502	6	5.88

DISCUSSION

Understanding gonad development is necessary to control reproductive maturation in the economically important species (Leelatanawit et al., 2009). As an important measure for understanding molecular mechanisms of physiological traits such as gland maturation and sex differentiation, a large number of EST projects on crustaceans have been recently reported (Zou et al., 2011). Suppressive subtractive hybridization was reported to be an applicable technique to many molecular genetic and positional cloning studies, for the identification of disease, developmental, tissue specific, or other differentially expressed genes (Diatchenko et al., 1996). Screening of an ovarian cDNA library for shrimp, *Marsupenaeus japonicus*, using SSH-enriched probes has been reported (Zhang et al., 2007), and more than 20 differentially expressed genes were identified in

Table 6. KEGG annotation of differentially expressed genes.

Down-regulated genes	Up-regulated genes
01200 Carbon metabolism (1)	00190 Oxidative phosphorylation (3)
01220 Degradation of aromatic compounds (1)	00910 Nitrogen metabolism (1)
00010 Glycolysis / Gluconeogenesis (2)	03040 Spliceosome (1)
00040 Pentose and glucuronate interconversions (1)	03013 RNA transport (2)
00053 Ascorbate and aldarate metabolism (1)	03050 Proteasome (1)
00620 Pyruvate metabolism (1)	02020 Two-component system (1)
00640 Propanoate metabolism (1)	04340 Hedgehog signaling pathway (1)
00680 Methane metabolism (1)	04020 Calcium signaling pathway (1)
00071 Fatty acid degradation (2)	04918 Thyroid hormone synthesis (1)
00561 Glycerolipid metabolism (1)	04260 Cardiac muscle contraction (2)
00280 Valine, leucine and isoleucine degradation (1)	05202 Transcriptional misregulation in cancers (1)
00310 Lysine degradation (1)	05322 Systemic lupus erythematosus (1)
00330 Arginine and proline metabolism (2)	05010 Alzheimer's disease (3)
00340 Histidine metabolism (1)	05012 Parkinson's disease (4)
00350 Tyrosine metabolism (2)	05016 Huntington's disease (4)
00360 Phenylalanine metabolism (1)	05034 Alcoholism (1)
00380 Tryptophan metabolism (1)	05166 HTLV-I infection (1)
00410 beta-Alanine metabolism (1)	05162 Measles (1)
00480 Glutathione metabolism (1)	05168 Herpes simplex infection (1)
00740 Riboflavin metabolism (1)	05169 Epstein-Barr virus infection (1)
00830 Retinol metabolism (1)	
00860 Porphyrin and chlorophyll metabolism (1)	
00903 Limonene and pinene degradation (1)	
00940 Phenylpropanoid biosynthesis (1)	
00950 Isoquinoline alkaloid biosynthesis (1)	
00965 Betalain biosynthesis (1)	
00625 Chloroalkane and chloroalkene degradation (2)	
00626 Naphthalene degradation (1)	
00980 Metabolism of xenobiotics by cytochrome P450 (2)	
00982 Drug metabolism-cytochrome P450 (2)	
03010 Ribosome (2)	
03050 Proteasome (1)	
04080 Neuroactive ligand-receptor interaction (1)	
04810 Regulation of actin cytoskeleton (1)	
04510 Focal adhesion (1)	
04530 Tight junction (1)	
04670 Leukocyte transendothelial migration (1)	
04916 Melanogenesis (1)	
04972 Pancreatic secretion (2)	
04974 Protein digestion and absorption (2)	
04978 Mineral absorption (1)	
05204 Chemical carcinogenesis (2)	
05162 Measles (1)	
05164 Influenza A (1)	

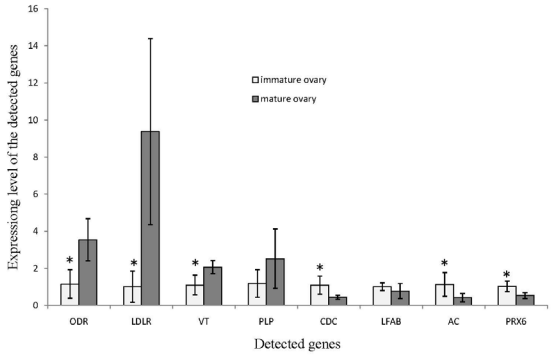

Figure 1. Expression levels of selected genes. Bars represent mean ± SEM. Significant differences (P < 0.05) between mature and immature ovaries are indicated by an asterisk.

ovary and testis. To date, no cDNA library has been constructed to select development-related genes in *P. trituberculatus*. In the present study, differentially expressed genes in mature and immature ovary from *P. trituberculatus* were analyzed using SSH. Expression characterization of 8 genes was also carried out by quantitative real-time PCR, which confirmed that SSH is valid for studies of mechanisms of ovarian development in the swimming crab.

Gene ontology analysis and metabolic pathway annotation

Functional similarities among genes are the strength of the relatedness of gene functions (Chen et al., 2012). Gene ontology was useful for analysis of identified ESTs. In the current study, 5.83% of the differential genes from cellular process is involved in reproduction in reverse subtraction cDNA library, and 5.88% were involved in developmental process in forward library. KEGG annotation indicated that transcription of genes related to nutrition metabolism pathways, such as oxidative phosphorylation, carbon metabolism, fatty acid degradation, protein digestion and absorption, were reduced in the mature ovary, which is consistent with the requirement by the immature ovary of more nutrition for development.

Identification of differentially expressed ESTs

Cholesterol is required for the synthesis of cell membranes and its uptake is mediated by the low-density lipoprotein (LDL) receptor (Goldstein et al., 1979). Fatty acid-binding protein (FABP) is involved in the uptake and intracellular translocation of long-chain fatty acids (Glatz and van der Vusse, 1996). Additionally, FABPs have been reported to govern transcriptional activities of their ligands by targeting them to cognate peroxisome proliferator-activated receptors (PPARs) in the nucleus in a receptor- and ligand-selective manner, thereby enabling PPARs to exert their biological functions (Tan et al., 2002). Peroxisome proliferator-activated receptors regulate transcription of genes involved in glucose uptake, lipid metabolism, and inflammation (Lincoff et al., 2013). The up-regulation of LDL receptor and FABP in the mature ovary may provide materials for the coming embryonic development.

Carboxypeptidases catalyze hydrolysis of C-terminal amino acids from their substrates and are involved in the digestion process (Gomis-Ruth, 2008). Members of the alcohol dehydrogenase (ADH) family catalyze the reversible oxidation of a wide variety of primary and secondary alcohols into corresponding aldehydes and ketones (Dołęga, 2010). Aldehyde dehydrogenases (ALDH) are a group of enzymes that catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes (Lindahl, 1992). Arginine kinase plays a key role in the coupling of energy production and utilization in animals (Ellington, 2001). In the present study, down-regulation of carboxypeptidase, alcohol dehydrogenase class, carboxypeptidase, aldehyde dehydrogenase and arginine kinase transcription, in the mature ovary, is consistent with results of metabolism pathway annotation.

COMM domain-containing protein was demonstrated to associate with with NF-κB and inhibit its transcriptional activity (Burstein et al., 2005). NF-κB was demonstrated to play major role in the activation of numerous genes involved in the function and development of immune system (Baeuerle and Henkel, 1994, Baldwin, 1996). Chymotrypsin-like serine proteinase is involved in immune defense reactions against bacteria in *Drosophila* (de Morais Guedes et al., 2005), and innate immune reactions in *Fenneropenaeus chinensis* (Shi et al., 2008). Alpha 2-macroglobulin (α2-M) is protease inhibitor that can neutralize pathogenic proteases that contribute to pathogen virulence (Lin et al., 2008).

Alpha 2-macroglobulinis an important innate immune element in crustaceans (Rattanachai et al., 2004). Lectins comprise a structurally diverse class of proteins characterized by their ability to bind carbohydrates with considerable specificity and play roles in biological-recognition events (Rini, 1995). The different transcript characterization of immune related genes indicated that the innate immune system was involved in ovarian maturation.

Identification of ovarian development-related ESTs

The development of oocytes is a series of complex cellular events, in which differential genes are temporally and spatially expressed to ensure proper development of oocytes, or to store transcripts and proteins as maternal factors for early embryogenesis (Qiu et al., 2005). Myosin II, expressed in non-muscle tissues, plays a central role in cell adhesion, migration and division; myosin regulatory light chains are essential for maintaining the integrity of myosin II, and are critical for cell structure and dynamics (Park et al., 2011). Polehole-like protein in *Penaeus monodon* was appears to be involved in ovarian development (Klinbunga et al., 2009). The up-regulated expression level of polehole-like protein in mature ovary of *P. trituberculatus* indicated its involvement in the ovarian development of the swimming crab. Histone h3 of *Drosophila* was reported to be necessary for fertility (Sakai et al., 2009), suggesting an important role in gametogenesis (Filipescu et al., 2013). Up-regulated expression level of histone h3 in the mature ovary may be beneficial to the developmental of the ovary. Ovigerous-hair stripping substance (OHSS), released by the embryo upon hatching (Gusev et al., 2004), plays a role in the stripping of the embryo attachment system from the maternal ovigerous hairs following hatching, in preparation for the next clutch of embryos (Saigusa, 1995). Ovary development related protein was firstly cloned in *Eriocheir sinensis* (AEC22817), and was up-regulated in the mature ovary. Peritrophic matrix (or peritrophin) was involved in the digestive process through its role in partitioning of digestive enzymes and semi-digested food between the endo- and ecto-peritrophic spaces (Vuocolo et al., 2001). Peritrophic matrix plays key roles in the intestinal biology of insects, and might protect the midgut epithelium from mechanical damage and insults from pathogens and toxins (Lehane, 1997). In addition, peritrophin was demonstrated to be involved in ovary development of the red swamp crayfish *Procambarus clarkii* (Shui et al., 2012). In the present study, 6 genes involved in ovarian development were found to be up-regulated in mature ovary.

Vitellogenin (Vg) is the precursor of vitellin, the major yolk protein stored in crustacean oocytes that provides free amino acids, lipids, carbohydrates, carotenoids, and minerals to the developing embryo and larva (Byrne et al., 1989). It is mainly expressed in the hepatopancreas, and trace amounts of transcript have been found in the ovary during vitellogenesis in *P. trituberculatus* (Byrne et al., 1989). Increased vitellogenin mRNA in the mature ovary of *P. trituberculatus* might be useful for ovary development.

Vitelline membrane outer layer protein is the major component of the outer layer of vitelline membrane (Kido et al., 1992). Decreased transcription of this protein in the mature ovary may result from consumption of mRNAs for protein synthesis, consistent with the phenomenon that more eggs are found in mature than immature ovaries.

In conclusion, SSH libraries were constructed from mature and immature ovaries of *P. trituberculatus*. Four annotated pathways were suggested to be involved in ovarian development. Gene ontology analysis indicated that in the reverse subtraction cDNA library, 5.83% of the differential expressed genes in cellular processes involved in reproduction, and 5.88% in the forward library

were involved in developmental processes. Six genes (myosin II expressed, polehole-like protein, histone h3, ovigerous-hair stripping substance (OHSS), peritrophin 48 and ovary development related protein) were up-regulated in mature ovary. This study provides new insights for further studies onovarian development in the swimming crab.

Conflicts of interest

The authors declared that they have no conflicts of interest to this work.

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REFERENCES

Baeuerle PA and Henkel T (1994). Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.* 12: 141-179.

Baldwin AS Jr (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14: 649-683.

Byrne BM, Gruber M and Ab G (1989). The evolution of egg yolk proteins. *Prog. Biophys. Mol. Biol.* 53: 33-69.

Chen X, Yang R, Xu J, Ma H, et al. (2012). A sensitive method for computing GO-based functional similarities among genes with 'shallow annotation'. *Gene* 509: 131-135.

de Morais Guedes S, Vitorino R, Domingues R, Tomer K, et al. (2005). Proteomics of immune-challenged *Drosophila melanogaster* larvae hemolymph. *Biochem. Biophys. Res. Commun.* 328: 106-115.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, et al. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. U. S. A.* 93: 6025-6030.

Dołęga A (2010). Alcohol dehydrogenase and its simple inorganic models. *Coord. Chem. Rev.* 254: 916-937.

Duguid JR and Dinauer MC (1990). Library subtraction of *in vitro* cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucleic Acids Res.* 18: 2789-2792.

Ellington WR (2001). Evolution and physiological roles of phosphagen systems. *Annu. Rev. Physiol.* 63: 289-325.

Elner RW and Beninger PG (1992). The reproductive biology of snow crab, *Chionoecetes opilio*: a synthesis of recent contributions. *Am. Zool.* 32: 524-533.

Filipescu D, Szenker E and Almouzni G (2013). Developmental roles of histone H3 variants and their chaperones. *Trends Genet.* 29:630-640.

Goldstein JL, Anderson RG and Brown MS (1979). Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature* 279: 679-685.

Gomis-Ruth FX (2008). Structure and mechanism of metallocarboxypeptidases. *Crit. Rev. Biochem. Mol. Biol.* 43: 319-345.

Glatz JF and van der Vusse GJ (1996). Cellular fatty acid-binding proteins: their function and physiological significance. *Prog. Lipid Res.* 35: 243-282.

Gusev O, Ikeda H, Okochi T, Lee JM, et al. (2004). Purification and cDNA cloning of the ovigerous-hair stripping substance (OHSS) contained in the hatch water of an estuarine crab *Sesarma haematocheir. J. Exp. Biol.* 207: 621-632.

Kido S, Morimoto A, Kim F and Doi Y (1992). Isolation of a novel protein from the outer layer of the vitelline membrane. *Biochem. J.* 286: 17-22.

Klinbunga S, Sittikankaew K, Yuvanatemiya V, Preechaphol R, et al. (2009). Molecular cloning and expression analysis of ovary-specific transcript 1 (Pm-OSTI) of the giant tiger shrimp, *Penaeus monodon. Zool. Sci.* 26: 783-790.

Lehane MJ (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol.* 42: 525-550.

Leelatanawit R, Sittikankeaw K, Yocawibun P, Klinbunga S, et al. (2009). Identification, characterization and expression of sex-related genes in testes of the giant tiger shrimp *Penaeus monodon*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol*. 152: 66-76.

Lin YC, Vaseeharan B and Chen JC (2008). Molecular cloning and phylogenetic analysison alpha2-macroglobulin (alpha2-M) of white shrimp *Litopenaeus vannamei*. *Dev. Comp. Immunol.* 32: 317-329.

Lincoff AM, Tardif JC, Neal B, Nicholls SJ, et al. (2013). Evaluation of the dual peroxisome proliferator-activated receptor α/γ agonist aleglitazar to reduce cardiovascular events in patients with acute coronary syndrome and type 2 diabetes mellitus: Rationale and design of the AleCardio trial. *Am. Heart J.* 166: 429-434.

Lindahl R (1992). Aldehyde dehydrogenases and their role in carcinogenesis. *Crit. Rev. Biochem. Mol. Biol.* 27: 283-335.

Liu Y, Cui Z, Luan W, Song C, et al. (2011). Three isoforms of anti-lipopolysaccharide factor identified from eyestalk cDNA library of swimming crab *Portunus trituberculatus*. *Fish Shellfish Immunol.* 30: 583-591.

Park I, Han C, Jin S, Lee B, et al. (2011). Myosin regulatory light chains are required to maintain the stability of myosin II and cellular integrity. *Biochem. J.* 434: 171-180.

Qiu GF, Yamano K and Unuma T (2005). Cathepsin C transcripts are differentially expressed in the final stages of oocyte maturation in kuruma prawn *Marsupenaeus japonicus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 140: 171-181.

Rattanachai A, Hirono I, Ohira T, Takahashi Y, et al. (2004). Molecular cloning and expression analysis of alpha 2-macroglobulin in the kuruma shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol.* 16: 599-611.

Rini JM (1995). Lectin structure. *Annu. Rev. Biophys. Biomol. Struct.* 24: 551-577.

Sakai A, Schwartz BE, Goldstein S and Ahmad K (2009). Transcriptional and developmental functions of the H3.3 histone variant in *Drosophila*. *Curr. Biol.* 19: 1816-1820.

Saigusa M (1995). Bioassay and preliminary characterization of ovigerous-hair stripping substance (OHSS) in hatch water of crab larvae. *Biol. Bull.* 189: 175-184.

Shi XZ, Zhao XF and Wang JX (2008). Molecular cloning and expression analysis of chymotrypsin-like serine protease from the Chinese shrimp, *Fenneropenaeus chinensis*. *Fish Shellfish Immunol.* 25: 589-597.

Shui Y, Guan ZB, Xu ZH, Zhao CY, et al. (2012). Proteomic identification of proteins relevant to ovarian development in the red swamp crayfish *Procambarus clarkii*. *Aquaculture* 370-371: 14-18.

Tan NS, Shaw NS, Vinckenbosch N, Liu P, et al. (2002). Selective cooperation between fatty acid binding proteins and peroxisomeproliferator-activated receptors in regulating transcription. *Mol. Cell. Biol.* 22: 5114-5127.

Vuocolo T, Eisemann CH, Pearson RD, Willadsen P, et al. (2001). Identification and molecular characterisation of a peritrophin gene, peritrophin-48, from the myiasis fly *Chrysomya bezziana*. *Insect Biochem. Mol. Biol.* 31: 919-932.

Wu XG, Yao GG, Yang XZ, Cheng YX, et al. (2007). A study on the ovarian development of *Portunus trituberculatus* in East China Sea during the first reproductive cycle. *Acta. Oceanol. Sin.* 29: 120-127.

Xie Y, Li F, Wang B, Li S, et al. (2010). Screening of genes related to ovarian development in Chinese shrimp *Fenneropenaeus chinensis* by suppression subtractive hybridization. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 5: 98-104.

Zhang Z, Wang Y, Jiang Y, Lin P, et al. (2007). Ribosomal protein L24 is differentially expressed in ovary and testis of the marine shrimp *Marsupenaeus japonicus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 147: 466-474.

Zou Z, Zhang Z, Wang Y, Han K, et al. (2011). EST analysis on the gonad development related organs and microarray screen for differentially expressed genes in mature ovary and testis of *Scylla paramamosain*. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 6: 150-157.