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厦门大学

硕士学位论文

杂色鲍早期发育相关基因的时空表达研究

Spatio-temporal Expressions of Early Development Related Genes of Abalone, *Haliotis diversicolor*

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摘 要

杂色鲍（*Haliotis diversicolor*）作为软体动物腹足类的重要物种，不仅是水产养殖重要经济贝类，而且是研究海洋无脊椎动物幼体发育良好模型，对贝类早期发育机理的阐述具有重要的理论和实践意义。当前借助于新一代高通量测序技术平台，大量海洋贝类基因资源被挖掘出来。这些基因中的大部分却是功能未知的，因此发展和应用新的实验手段系统地分析基因的功能成为当前迫切需要解决的问题。

本实验室先前通过 454 测序和基因表达聚类方法获得了大量的杂色鲍幼体发育相关基因，这些基因的表达具有明确的发育阶段性，即仅在某些发育时期高水平表达。本研究针对其中一批差异表达基因和一系列同源基因进行了功能上的探索：

1、建立杂色鲍幼体原位杂交方法（WMISH）。建立了用于全胚原位杂交的幼体采集、麻醉、固定和保存方法，确保了幼体形态在两年多的保存时间里的完整性与舒展性；建立了 cRNA 探针制备技术，通过 T_7RNA 聚合酶体外转录得到的 6 个基因 cRNA 探针序列具有极高准确度；建立了杂交流程并进行优化，降低了背景信号，获得了较高清晰度的原位杂交照片。经过 6 个基因的 WMISH 实验，表明此方法已经完整建立并具有较高通量。

2、以基因群为单位开展发育特征基因的时空表达模式研究。本研究以基因家族的方式或以时序表达模式的相关性挑选了 4 个特征基因群：*SARP-19* 基因家族、*Vdg-3* 基因家族、与 *CMBL* 基因的时序表达模式相类似的基因群、与 *Fucolectin* 基因的时序表达模式相类似的基因群，并获得了这些基因群共 15 个基因的时空表达模式。S*ARP-19* 和 *Vdg-3* 基因家族大部分成员在附着后幼体的消化腺高水平表达；*CMBL* 基因群多在扭转后面盘时期开始表达，空间上集中于套膜腔、外套膜边缘、顶端感受器官，可能与幼体探索合适附着基、附着变态以及次生壳形成有密切联系；*Fucolectin* 基因群多在感受态期间高水平表达，空间上成局部“点状”模式，这些表达位点鉴定为神经节部位，因此很可能是与神经系统在幼体早期的发育有密切关系。

3、*HdEGF1* 基因的克隆、qPCR 和 WMISH。运用 cDNA 末端快速扩增技术

（RACE）克隆了 *HdEGF1* 基因全长 cDNA 序列。该基因全长 1239bp，含一个 343 个氨基酸残基的 ORF（开放阅读框），推测的蛋白序列包含 3 个 EGF-CA 结构域和 1 个 vWFA 结构域。通过序列相似性比较，我们确定其为新型 EGF1 相关蛋白。荧光定量 PCR（qPCR）结果显示 *HdEGF1* 基因在幼体附着后的表达量比附着前任一时期均高 19 倍以上。全胚原位杂交（WMISH）结果显示 *HdEGF1* 基因的集中表达在变态后幼体后消化道的表皮细胞。*HdEGF1* 基因的这种时空表达模式表明 *HdEGF1* 受严格的时空调控，直接参与了附着后杂色鲍幼体后消化道的生长和细胞分化。

4、CMBL 蛋白的原核表达。根据已获得的杂色鲍 *CMBL* 的 cDNA 序列，成功构建了 pET-CMBL 的重组融合表达质粒，重组质粒在大肠杆菌 BL21（DE3）中经 IPTG 诱导后，所获得的 CMBL 融合蛋白主要以可溶形式存在于菌体中。利用亲和纯化的方法纯化了融合蛋白，并尝试利用肠激酶切除标签。

本研究通过成熟的 WMISH 方法对多个基因群进行了时空表达模式的研究，获得了大量的重要线索，并根据这些线索开展了全长 cDNA 克隆和原核表达工作，为后续更深入的功能研究打下了基础。同时，本文对基因群的研究方法有助于从代谢通路和基因调控网络的视角探索杂色鲍早期发育生物学过程。

关键词：杂色鲍；早期发育；全胚原位杂交；原核表达；基因功能

Abstract

The abalone *Haliotis diversicolor*, being an important gastropod, is not only a major species in aquaculture, but also a good model to study the development of marine invertebrate. It is theoretically and practically essential to illuminate the developmental mechanisms in shellfish. Lots of gene resources from marine shellfish have been excavated using the new generation high-throughput sequencing technology, many of them are new genes which functions were not confirmed. Therefore, it is urgent to systematically analyze gene functions with the application of new experimental techniques.

We previously have obtained macro larval development related genes from *H.diversicolor* through the 454 sequencing technology and the cluster genes expression pattern analysis, these gene expressions have the developmental phase specificity, eg, they expressed highly in one or a few of special developtmental stages. In this study, we screened some differentially expression genes and a series of homologous genes for their spatio-temporal expression patterns:

1. The whole mount in situ hybridization (WMISH): we have established these methods for the collection, anesthesia, fixed and preservation of larvae samples, the larvae can be preserved completely and outstretchedly for two years, and also we set up the method of synthesis of the cRNA probes, which obtained by in vitro transcription of the T_7 RNA polymerase and show high specificity and accuracy. Through WMISH experiments of six genes, the WMISH technique have thus been established.The technique was further optimized to reduce the background signal and improve efficiency for obtaining the result pictures with high definition.

2. We screened differentially expressed gene cluster and analysed the temporal and spatial expression patterns by WMISH experiments. We chose four gene chluters based on the gene family or spatio-temporal expression patterns: *SARP-19* gene families, *Vdg-3* gene families, the genes which expression patterns are similar with *CMBL* or *Fucolectin*, and obtained the spatio-temporal expression patterns of these 21

genes. The genes in *SARP-19* and *Vdg-3* gene families express highly in the larval digestive gland. *CMBL* gene cluster begin to express in the velum larvae after torsion period and focus in mantle cavity, mantle edge, apical sensory organ. These genes may be associated with larva exploring suitable substrata in the process of settlement and metamorphosis and secondary shell formation; *Fucolectin* gene cluster mainly express in the larval ganglion druing the compotent larval stage with "dot-like" expression pattern, which is probably related to the early larval nervous system development.

3. Cloning, expression and function analysis of *HdEGF1*. We obtained the full-length cDNA sequence through the technology of Rapid Amplification of cDNA Ends (RACE). This cDNA encodes a novel epidermal growth factor (EGF) related protein with 343 amino acid residues that contains three calcium-binding EGF-like domains (EGF-CA) and a Von Wille brand factor type A domain (vWFA). Using quantitative PCR (qPCR) approach, the expression of the *HdEGF1* gene was found to be 19-fold higher at metamorphosis stage than at any pre-settlement stages. In addition, the result of whole mount in situ hybridization (WMISH) indicated that the mRNAs of the *HdEGF1* gene were found to be accumulated in the epidermal cells of the primitive hindgut of postlarvae. The differences of *HdEGF1* gene expression patterns before and after the settlement indicate that the development of the digestive tract downstream tissues is subjected to the strict regulation of settlement and metamorphosis. In conclusions, the *HdEGF1* gene may play central roles in the development of lower digestive tract.

4. The prokaryotic expression of *CMBL*, laying the foundation of studying for gene function from metabolomics. Based on available CMBL cDNA sequence, we successfully constructed pET-CMBL recombinant plasmid. CMBL soluble fusion protein has been highly expressed in *E. coli* BL21 (DE3) after induction by IPTG. The fusion protein was purified by affinity chromatography and tag was removd using enterokinase.

From the spatio-temporal expression patterns of the four gene clusters by WMISH, many important clues were obtained. These results lay the root for the

searches of the gene functions. And the methods setuped by this study on the gene clusters was helpful for exploring the metabolic pathways and gene regulatory networks during early development of *H.diversicolor.*

Key words: early development; whole mount in situ hybridization; prokaryotic expression; gene function