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Involvement of clustered oyster *Wnt* genes in gut formation*

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Abstract Genes encoding Wnt ligands play important roles in organ development. The *Wnt10-Wnt6-Wnt1-Wnt9* cluster widely presents in many metazoan genomes, indicating the importance of gene arrangement. Hypothesis has been proposed that they may be coordinately regulated. However, few expression correlations were identified in model animals. We analyzed the tissue expression pattern of clustered oyster *Wnt10*, *Wnt6*, *Wnt1*, and *Wnt9a* genes in this study. The results indicated the highest expression level in adult gut system of these clustered *Wnt* genes, except for *Wnt6*, which had highest expression in mantle. Further whole-mount immunofluorescence assay indicated that Wnt6 protein was restricted to gut region in oyster larvae. These results suggest the possible important role of the *Wnt10-Wnt6-Wnt1-Wnt9* cluster in oyster gut formation.

Keyword: gene cluster; tissue distribution; digestive gland; immunofluorescence; *Crassostrea gigas*

1 INTRODUCTION

Organ development involves a series of complex but ordered cellular processes including cell proliferation, differentiation, and migration, under robust and precise management by gene regulatory networks (Nelson and Nusse, 2004; Ryan and Baxevanis, 2007; Nusse, 2008; Saito-Diaz et al., 2013). Genes encoding Wnt ligands, which have important roles during the complicated developmental processes, are restricted to multicellular animals (Nusse and Varmus, 1992; Logan and Nusse, 2004). In sponge, three *Wnt* genes were reported to expressed from the earliest stages of *Amphimedon* embryonic development in highly dynamic patterns and involved in the patterning of a sub-community of cells that form a simple tissue-like structure, the pigment ring (Adamska et al., 2007, 2010). In cnidarians, the expression patterns of sea anemone *Wnt* genes during embryogenesis indicated crucial function in the diversification of eumetazoan body plans (Kusserow et al., 2005). In bilaterians, the *Wnt* genes were reported to relate to the segmental and parasegmental regulation in annelids and arthropods (Janssen et al., 2010; Hayden et al., 2015). Gene function studies also showed that some *Wnt* genes in the *Tribolium* display multiple overlapping expression patterns, suggesting that they may be functionally redundant in segmentation, brain, appendage and hindgut development (Bolognesi et al., 2008). In *Caenorhabditis elegans* embryo, the *Wnt* member *mom-2* was reported to be required in the signaling

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LIU Jun and XU Fei contributed equally to this work.

Table 1 qRT-PCR (qPCR) and in situ hybridization (ISH) primers used in this study

Primer ID	Usage	Sequence
CgiWnt1.F	qPCR	GAAGGGTGACCGAAAGAGATATAA
CgiWnt1.R	qPCR	CGACAGTAATGTCACCAATGAAAG
CgiWnt6.F	qPCR	CTGGACGATCGGCTGTTAAG
CgiWnt6.R	qPCR	GGCATCTTTCTCCAACAGGT
CgiWnt9a.F	qPCR	GTCAGGTTAATGCGCGAAAC
CgiWnt9b.R	qPCR	CAGACGATAAGTTCTGAGGGATG
CgiWnt10.F	qPCR	GGATGCAGTCACAACGTAGA
CgiWnt10.R	qPCR	GCTCGGTTGTTGTGTAAGTTAAT
CgiWnt6-F_ish	ISH	GAGGCTACAAATCGGAGAAAG
CgiWnt6-R_ish	ISH	GTATAAAGTGCCCTTGTGGTAG

process from P2 to EMS at the four-cell stage (Thorpe et al., 1997; Wodarz and Nusse, 1998). And in vertebrate, *Wnt* genes were reported to involve in the embryonic cell migration, gastrulation and organogenesis (Ulrich et al., 2003; Shimizu et al., 2005; Gessert and Kuhl, 2010). Besides, studies have found that some *Wnt* genes are present in clusters on the chromosomes of various animals (Nusse, 2001; Cho et al., 2010), and that the extracellular proteins they encoded have crucial conserved roles in signal transduction regulatory processes, including embryonic induction, generation of cell polarity, and the specification of cell fate (Nusse and Varmus, 1992; Hobmayer et al., 2000; Miller, 2002; Eisenmann, 2005; Bodine, 2008). These findings indicate that *Wnt* genes are likely to have evolutionary trajectories similar to those of *Hox* and *ParaHox* genes; i.e., multiple of these genes arise as a result of tandem duplication events and are subsequently transposed away from the expanding cluster (Brooke et al., 1998), and may be coordinately regulated (Nusse, 2001) to conduct similar functions.

Many important biological functions have been reported in model animals on genes belonging to the *Wnt10-Wnt6-Wnt1-Wnt9* cluster. However, few evidences were found on the cooperation of these genes. In contrast, the expression pattern of these genes were reported to be different in *Drosophila* (Murat et al., 2010) and other model animals (Holstein, 2012). Here, we characterized the functional correlation of clustered *Wnt* genes in oyster by comparing their tissue expression patterns and we inferred the possible involvement of genes of the *Wnt10-Wnt6-Wnt1-Wnt9* cluster in gut formation.

2 MATERIAL AND METHOD

2.1 Animal materials and ethics statement

All of the Pacific oyster (*C. gigas*) specimens used in this study were collected from Qingdao, Shandong, China, and acclimatized in seawater at 22°C for one week before use. Fresh oyster tissues for RNA isolation were isolated, those from three animals mixed, and then frozen immediately and stored in liquid nitrogen. Around 1×10^5 larvae for each developmental stage of *C. gigas* were collected and directly fixed in fresh 4% paraformaldehyde in 0.01 mol/L phosphate-buffered saline (PBS) for 2 h at 4°C and washed three times (15 min each) with cold PBS. Starting from the D-shaped larvae stage, samples were relaxed by gradual addition of 7.5% $MgCl_2$ prior to fixation. All larvae samples were stored in 75% methanol at -20°C.

2.2 RNA isolation, cDNA synthesis, and quantitative reverse transcription (qRT-)PCR

RNA isolation and cDNA synthesis were conducted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and a PrimeScript RT reagent kit (TaKaRa, Shiga, Japan), respectively, according to the manufacturers' instructions. cDNA of each tissue were mixed from three individuals and was treated as one sample. qRT-PCR of the *C. gigas Wnt* genes was conducted on ABI 7500 Fast qPCR systems (Applied Biosystems, Foster City, CA, USA), as previously described (Qu et al., 2014). Three technical replicates were conducted on each tissue sample, with *EF-1α* gene as an internal control (Du et al., 2013). The expression level and confidence interval were calculated with 7500 software v2.0.6 (Applied Biosystems) where $2^{-\Delta\Delta Ct}$ method was used (Livak and Schmittgen, 2001). All primers used for qRT-PCR are listed in Table 1.

2.3 Cloning and in situ hybridization

Forward and reverse primers used for amplification of the oyster *CgiWnt6* gene are detailed in Table 1. Amplified fragments were cloned into the pGEM-T Easy vector (Promega) and verified by sequencing. For in situ hybridization, digoxigenin-labeled probes were synthesized from cloned fragments in both sense and antisense directions. The in situ hybridization protocol was adapted from that previously described for use in *P. vulgata* embryos (Bao et al., 2017). Sense and antisense probes were analyzed in parallel.

2.4 Western blotting and whole-mount immunofluorescence assays

To verify protein function, a polyclonal antibody, anti-CgiWnt6, was produced by Abmart, Inc. (Shanghai, China) for use in western blotting and whole-mount immunofluorescence assays. An antigen peptide (PDSRFCRRNRKWGS) was selected and produced by chemosynthesis for rabbit polyclonal antibody production. Western blotting was conducted to validate the antibody as previously described (Huang et al., 2015). Whole-mount immunofluorescence assays were conducted according to previously reported methods used in mussels (Voronezhskaya et al., 2008; Dyachuk and Odintsova, 2009) and scallops (Yue et al., 2013), with some modifications. Briefly, oyster larvae samples stored in 75% methanol were successively rinsed in 75%, 50%, and 25% methanol/PBST (0.1% Tween-20 in 0.01 mol/L PBS) for 10 min; larvae with shells (after trochophore) were decalcified with 5% EDTA solution for 30 min. After incubation in 0.05% trypsin solution for 15–20 min, larvae samples were blocked overnight in block buffer (10% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100, 0.05% Tween-20, and 0.05% sodium azide in 0.01 mol/L PBS), and then incubated with the polyclonal antibody, anti-CgiWnt6, as primary antibody (1:100 in block buffer) at 4°C for 3–4 days. These samples were then incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:600 in block buffer, Invitrogen, CA) at room temperature with shaking for 1 day and mounted in 60% glycerol in PBS. A 5×15 min wash procedure was performed after each incubation with primary antibody and block buffer. All samples were examined as whole-mount specimens using the Zeiss Laser-Scanning Confocal Microscopy System LSM 710 (Zeiss, Germany).

3 RESULT AND DISCUSSION

3.1 Tissue expression pattern of the clustered oyster *Wnt* genes

The oyster *Wnt10-Wnt6-Wnt1-Wnt9* cluster has been reported previously (Takeuchi et al., 2016). However, there are two members of *Wnt9* subfamily in oyster genome. We name the clustered one as *Wnt9a* in this study. Previous reports indicated that these genes mainly showed high expression level in gill, mantle and digestive gland (Zhang et al., 2012). To further study the gene expression pattern in fine scale, we dissected the digestive gland into different gastro-intestinal tracts (gut system) including stomach, intestine and hepatopancreas, besides other main organs. The results indicated that *Wnt1*, expressed the highest in hepatopancreas, while *Wnt9a* and *Wnt10* in stomach. *Wnt6* also had high expression level in stomach and intestine, although the highest level was in mantle (Fig.1).

The genes of the *Wnt10-Wnt6-Wnt1-Wnt9* cluster seem to be commonly involved in gut formation. Overexpression of *Wnt1* in mouse extended stomach-type differentiation in the foregut, while misexpression of *Wnt1* in the developing epithelia of the stomach resulted in a posterior shift in the gastric epithelia into the duodenum (Heller et al., 2002). *Wnt1* signaling reportedly drives intestinal stem cell hyperproliferation and is required for regeneration of the adult *Drosophila* midgut (Cordero et al., 2014; Zhai et al., 2015). *Wnt6* and *Wnt9b* are highly expressed in mouse intestinal crypt epithelial cells, hinting to their possible proliferation-driving function in the epithelial progenitor cells lying above the Paneth cells (Gregorieff et al., 2005). *Wnt6* expression is restricted to the developing foregut and midgut in *Drosophila* embryos from stage 13, although only weak expression was detected (Janson et al., 2001). Besides the well-studied functions of *Drosophila Wnt9* in ovarian morphogenesis (Cohen et al., 2002) and target specificity of synapses (Inaki et al., 2007), *Wnt9* was also detected in the gut, suggesting its possible involvement in gut morphogenesis (Graba et al., 1995). In *C. elegans*, loss of Ce*Wnt10*/*Lin-44* causes defects in the polarization and migration of endodermal precursors (Hartin et al., 2015) and in *D. melanogaster*, *DmWnt10* is expressed in the embryonic mesoderm, central nervous system, and gut (Janson et al., 2001).

3.2 Involvement of *Wnt6* in oyster gut formation

Few functional studies of the *Wnt10-Wnt6-Wnt1-Wnt9* cluster have been conducted in non-model animals. Even in model animals, the precise function of *Wnt6*, especially in the gut, is yet to be studied (Murat et al., 2010; Doumpas et al., 2013). By analyzing *Wnt* gene expression patterns in oyster organs, we found that all four genes are highly expressed in the gut system, and *Wnt6* also showed high expression levels in the stomach and intestine (Fig.1), suggesting their involvement in the gut formation. Besides its similar expression pattern in the guts of various animals, *Wnt6* also shows high gene structure conservation across Bilateria. A typical

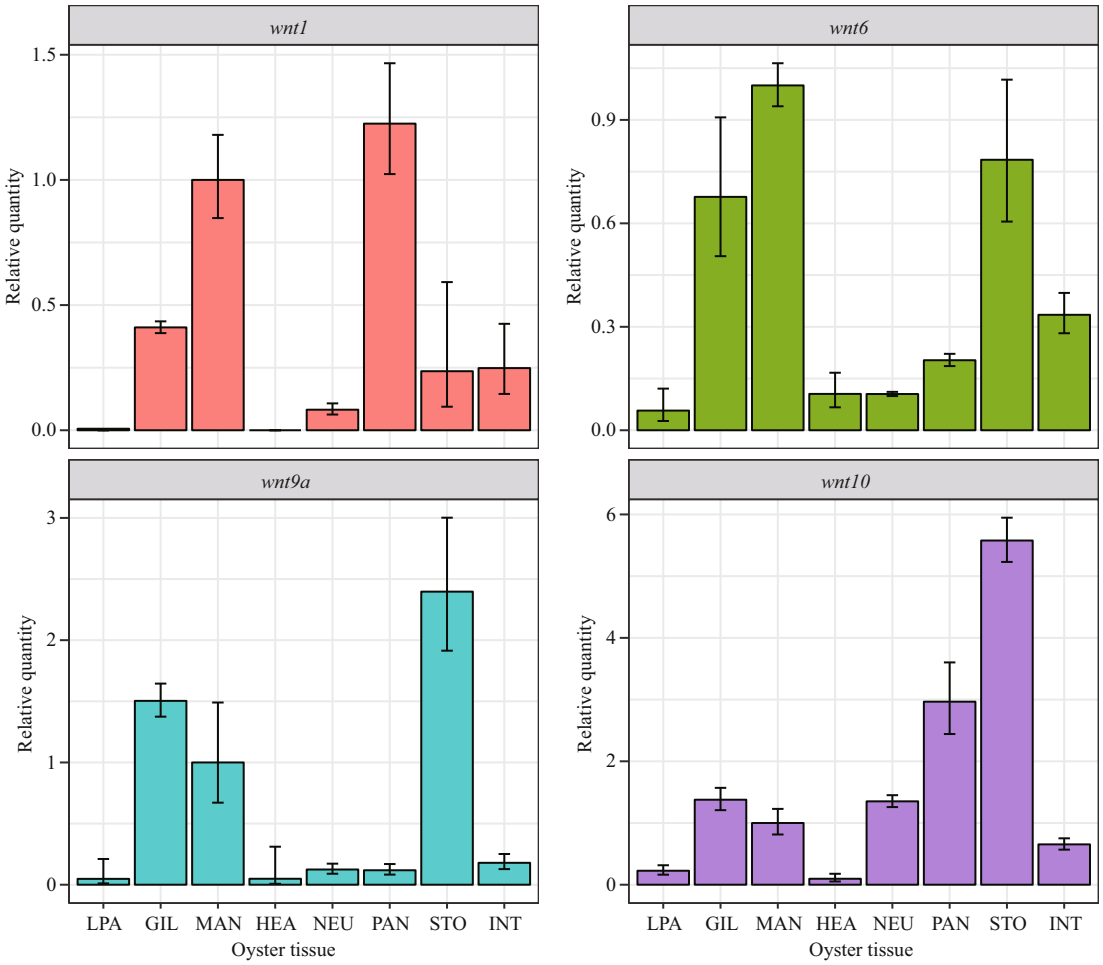

Fig.1 Expression patterns of clustered *Wnt* genes in oyster tissues

The *x*-axis shows the 8 oyster tissues and *y*-axis indicates the relative expression levels compared to a reference sample (MAN). Tissues include: LPA: labial palps; GIL: gill; MAN: mantle; HEA: heart; NEU: neuron; PAN: hepatopancreas; STO: stomach; INT: intestine. The *EF-1α* gene was used as an internal control, and Mantle as reference tissue.

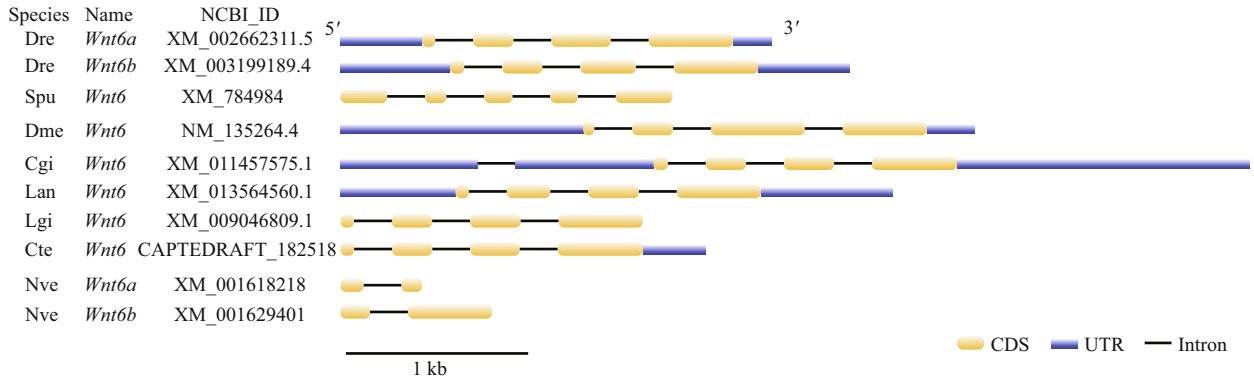

Fig.2 Conserved gene structure (exons and introns) of *Wnt6* genes in different species

four-coding-exons structure can be found in Deuterostomia (*Danio rerio* and *Strongylocentrotus purpuratus*), Ecdysozoa (*D. melanogaster*), and Lophotrocozoa (*Lottia gigantea*, *C. gigas*, *Lingula anatina*, and *Capitella teleta*), with a similar exon length distribution (Fig.2).

To confirm *Wnt6* function in oyster gut formation, we conducted whole-mount in situ hybridization and immunofluorescence assays in larvae. The oyster *CgiWnt6* transcripts were detected in the endoderm as early as at the gastrula stage (Fig.3, c3). CgiWnt6 protein was detected along the outer layer of the

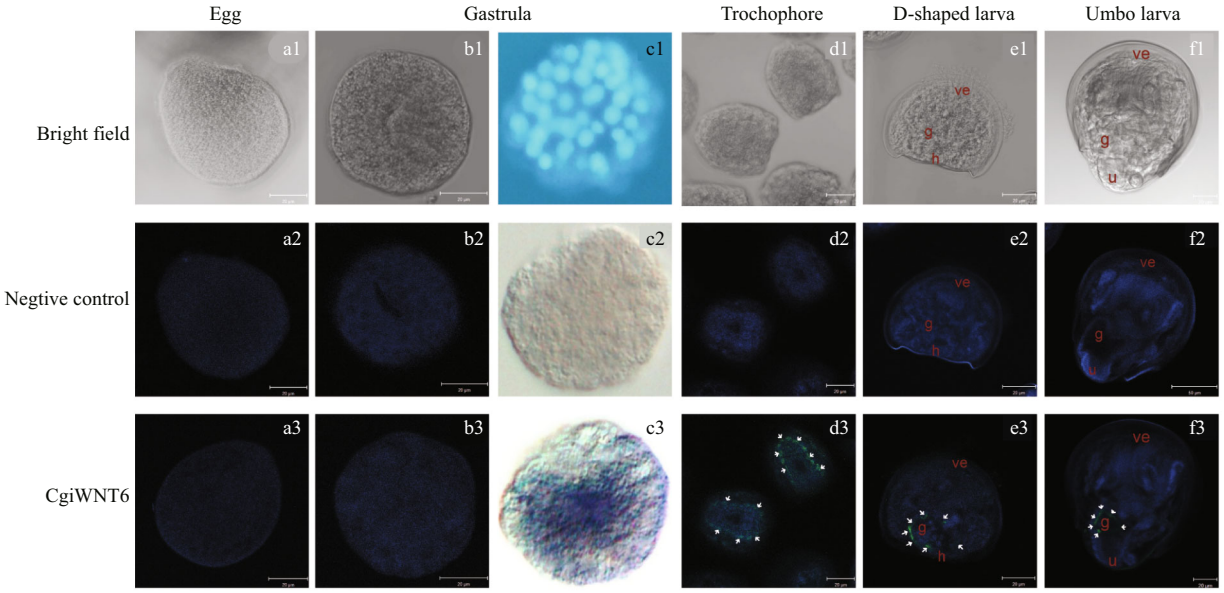

Fig.3 *CgiWnt6* whole-mount in situ hybridization and immunofluorescence assay results

The immunofluorescence assay was conducted for multiple developmental stages including the egg, gastrula, trochophore, D-shaped larvae, and umbo larvae, and the results are shown in panels a, b, d, e, f, respectively. Photos in the first row show bright-field microscopy results. Results for the negative control are shown in the second row, while results for tissues stained with CgiWnt6 antibody are shown in the third row. Whole-mount in situ hybridization was conducted only in the gastrula stage, and the results are shown in c1–c3. c1 shows cell nuclei in the embryo after staining with 4',6-diamidino-2-phenylindole (DAPI), which aids in determining the cell number and staging. c2 shows an embryo treated with sense *CgiWnt6* probe, while c3 shows an embryo treated with antisense probe. The immunoreactive area is marked with an arrow. ve: velum; g: gut; h: hinge; u: umbo. Bar=20 μm.

endoderm in a gut-like pattern at the trochophore stage (Fig.3, d3). The same observation has also been reported in sea urchin, where Wnt6 is one of key factors for activation of the entire endomesoderm gene regulatory network (Croce et al., 2011). Furthermore, CgiWnt6 marked the location of the stomach in the veliger (Fig.3, e3, f3), consistent with a report in the millipede, *Glomeris marginata*, where its *Wnt6* expression was observed in the gut during late embryogenesis (Janssen et al., 2010). In the sea cucumber, *Apostichopus japonicus*, *Wnt6* has been suggested to induce intestine regeneration (Sun et al., 2013). These findings, together with the conserved *Wnt10-Wnt6-Wnt1-Wnt9* cluster arrangement in animals, suggest a common involvement of the clustered *Wnt* genes in animal gut formation.

4 CONCLUSION

The *Wnt10-Wnt6-Wnt1-Wnt9* cluster is widely distributed in the Eumetazoa. Expression correlation of this cluster in oyster suggests they may be coordinately regulated. Further, we report that *Wnt6* likely has an important role in oyster gut formation on the basis of qRT-PCR studies of different organs, in situ hybridization, and whole-mount immunofluorescence assays of embryos. These results indicate that the *Wnt10-Wnt6-Wnt1-Wnt9* cluster possibly plays important roles in oyster gut system formation.

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