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Expression patterns of *wnt8* orthologs in two sand dollar species with different developmental modes

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ABSTRACT

Two *wnt8* orthologs, *Smwnt8* and *Pjwnt8*, were isolated from an indirect developing sand dollar, *Scaphechinus mirabilis*, and a direct developing sand dollar, *Peronella japonica*, respectively. The expression patterns of two genes during early development were examined by whole mount *in situ* hybridization. The expression of *Smwnt8* was initiated in the micromeres at the late 16-cell stage and expanded at the 64-cell stage to the whole vegetal hemisphere, including the presumptive endomesodermal regions. The timing of the initiation of *Pjwnt8* transcription in the presumptive endomesoderm region was delayed by 2–3 cell cycles compared to that of *Smwnt8*. The delay, or molecular heterochrony, of *Pjwnt8* transcription strongly suggests the existence of a substantial evolutionary change in the early endomesodermal specification of *P. japonica*. In addition to the endomesodermal expression during early embryogenesis, bilateral expressions were observed commonly in the ectoderm of two sand dollar species during larval stages.

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1. Results and discussion

The *wnt8* gene is a developmental regulatory gene encoding a wnt-type signaling molecule. In sea urchin development, *wnt8* is responsible for the early part of endomesodermal specification. *Spwnt8*, a *wnt8* ortholog in an indirect developing regular sea urchin, *Strongylocentrotus purpuratus*, has been well studied for its developmental expression, function in early endomesoderm specification, and transcriptional control (Wikramanayake et al., 2004; Minokawa et al., 2005). The expression of *Spwnt8* starts at the late 16-cell stage in micromeres. The nuclearlization of β-catenin occurs at the same time and in the same location (Logan et al., 1999; Weitzel et al., 2004). According to the cis-regulatory analysis of *Spwnt8*, the complex of TCF/β-catenin has been suggested as one of the positive regulators for *Spwnt8* (Minokawa et al., 2005). A portion of the regulatory network, consisting of *Spwnt8*, TCF/β-catenin and *Spblimp1* initiates and stabilizes the early stage of endomesoderm specification (Smith et al., 2007, 2008).

We are interested in the evolution of regulatory mechanisms of *wnt8* in echinoids. The purpose of the present study was twofold. The first objective was to investigate whether the expression patterns and underlying regulatory mechanisms for *wnt8* genes have changed during echinoid evolution. Diversification of echinoids occurred at around 200 million years ago, and the lineages for Camarodonta (regular sea urchins) and Clypeasteroida (sand dollars) separated at that point (Smith, 1984). Early endomesoderm specification and its gene regulatory networks have been studied extensively for the indirect developing regular sea urchins (Reviewed by Davidson et al., 2002a,b). On the other hand, studies of the endomesoderm specification of the sand dollars have focused mainly on embryological points of view (Kominami and Masui, 1996; Minokawa and Amemiya, 1999; Ishizuka et al., 2001). Our knowledge of regulatory genes in sand dollars is limited for genes that are expressed in the relatively later stages of embryogenesis (for *Brachyury*, Hibino et al., 2004; for *Pitx*, Hibino et al., 2006), and no study has examined the genes responsible for the early phase of endomesoderm specification, including *wnt8*, *tcf*, and *blimp1*. One of the subjects of the present study is *Smwnt8*, a *wnt8* ortholog in an indirect developing sand dollar, *Scaphechinus mirabilis*. Comparison of expression patterns of *wnt8* orthologs between the indirect developing sand dollars and sea urchins will demonstrate whether the regulatory mechanism of *wnt8* changed during echinoid divergence.

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The second objective of the present study was to reveal whether the expression pattern of *wnt8* changed during the evolution of ontogeny from indirect to direct development. In the echinoids, the ancestral mode of larval development is indirect development through the pluteus stage (Strathmann, 1978). Direct development is considered as a derived form of ontogeny (Raff, 1987). Unlike indirect developing species, in direct developing species there is no differentiation of the larval digestive tract, a derivative of the endomesoderm. Therefore, the developmental mechanisms responsible for the endomesoderm specification and following archenteron formation must have been modified during the evolution of direct development. Kauffman and Raff (2003) elucidated by RT-PCR that the expression of *Hewnt8*, an ortholog of *wnt8* in a direct developing regular sea urchin, *Heliocidaris erythrogramma*, starts at the 64-cell stage rather than at the 16-cell stage, implicating the existence of some modification in endomesoderm specification and regulation of *wnt8* in direct developing species. Their study, however, did not provide any information for the spatial expression patterns of *Hewnt8* at the 64-cell stage. In the present study, we examined the spatial and temporal expression patterns of *Pjwnt8*, a *wnt8* ortholog in another direct developing echinoid, a sand dollar *Peronella japonica*. This species is known as an exceptional direct developer that forms micromeres at the 16-cell stage (Amemiya and Arakawa, 1996). The present study demonstrates that the timing of the transcription initiation of *Pjwnt8* is also delayed, compared to that of the *wnt8* genes of indirect developing species. However, the location of the initiation is similar, in the vegetal pole region occupied by the micromere-descendant cells. The delay, or molecular heterochrony, of *Pjwnt8* transcription strongly suggests the occurrence of a change in the early endomesodermal specification mechanism during evolution to direct development.

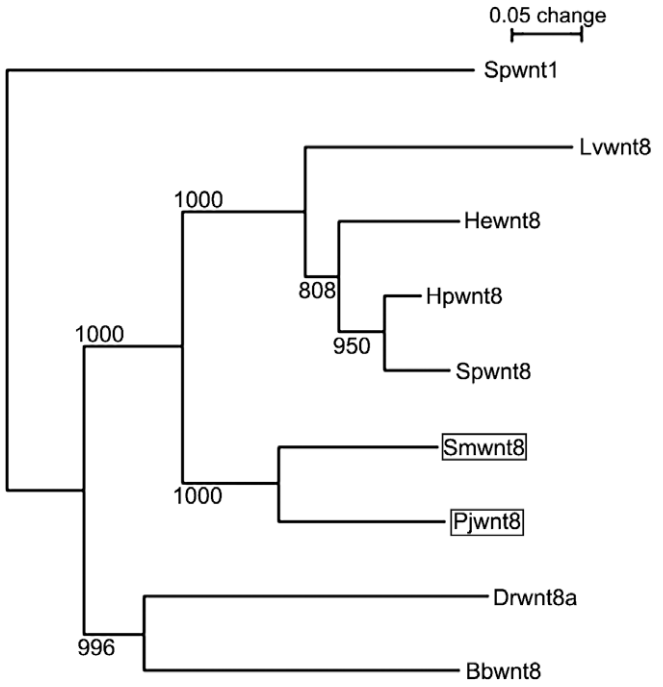

Fig. 1. Molecular phylogenic relationship among *Smwnt8*, *Pjwnt8*, and other *wnt* genes. Tree topology was determined using a neighbor-joining analysis based on the full deduced amino acid sequences. The lengths of the branches are drawn to the scale indicated. Numbers indicate the times that the node was supported in 1000 bootstrap replications. *Spwnt1*, *S. purpuratus wnt1; Hpwnt8, H. pulcherrimus wnt8*; *Spwnt8*, *S. purpuratus wnt8*; *Hewnt8, H. erythrogramma wnt8*; *Lvwnt8, L. variegatus wnt8*; *Drwnt8a, D. rerio wnt8a, and Bbwnt8, B. belcheri wnt8*.

1.1. Characterization of the wnt8 orthologs from sand dollars

We isolated degenerate and RACE PCR products (both 3′ and 5′) corresponding to the *wnt8* ortholog of *S. mirabilis*. Assembly of the isolated fragments revealed a stretch of cDNA of 2174 bp (without poly A) that contained a putative full open reading frame sequence (1107 bp) corresponding to 369 amino acids (AA). We designated this combined cDNA as *Smwnt8* (NCBI GenBank Accession No. EU434306). Similar PCR-based cloning and sequence assembly were performed to obtain a putative cDNA sequence for *Pjwnt8*, a *wnt8* ortholog of *P. japonica* (NCBI GenBank Accession No. EU434305). The length of *Pjwnt8* was 1954 bp (without poly A) and the putative full open reading frame sequence was 1116 bp (372 AA) in length.

A phylogenic analysis was performed using a neighbor-joining method for the deduced amino acid sequences of full coding sequences (Fig. 1). The phylogenic tree revealed that Clypeasteroida *wnt8* genes were monophyletic with a significant bootstrap value and constructed a sister group of Camarodonta *wnt8*, whereas chordate *wnt8* belonged to another cluster (Fig. 1). This analysis strongly suggests that both *Spwnt8* and *Pjwnt8* are orthologs of *wnt8* in sand dollars.

1.2. Expression pattern of Smwnt8

The expression pattern of *Smwnt8* mRNA was examined by whole mount *in situ* hybridization (WMISH). Two different probes were used (*Smwnt8*-A and -B; Fig. 2A). Both probes yielded identical staining patterns (data not shown). The strongest signals were obtained when the two probes were mixed. Therefore, we used the mixed probes for evaluation of expression patterns. No expression was observed until the early 16-cell stage (Fig. 2B). The expression was first confirmed in the fourth cleavage micromeres at the late 16-cell stage and became obvious at the 28-cell stage when the neighboring macromeres completed the fifth cleavage (Fig. 2C). At the 32-cell stage, expression was observed in both large- and small-micromeres (Fig. 2D). At the 64-cell stage, the expression started in both veg1 and veg2 cells simultaneously (Fig. 2E). As a result, the whole vegetal hemisphere expressed *Smwnt8*. At the early blastula stage, expression in the vegetal pole region was diminished and the expression pattern became a subequatorial "torus" (Fig. 2F). At the mesenchyme blastula stage, expression was observed in the region containing equatorial mesomere-descendants and veg1 (Fig. 2G). At the prism stage, the expression was restricted to two bilateral patches in the ectoderm (Fig. 2H). These *wnt8*-positive regions corresponded to the area between the apical plate and the presumptive regions for postoral arms. At the early pluteus stage, two bilateral wedge-shaped regions were observed just beneath the apical plate region (Figs. 2I and J). No signal was detected when the sense-strand probes were used (data not shown).

1.3. Expression pattern of Pjwnt8

The expression pattern of *Pjwnt8* was examined by WMISH. Three different probes were used (*Pjwnt8*-A, -B, and -UTR; Fig. 3A). The three probes yielded essentially identical staining patterns (data not shown). The strongest signals were obtained when the *Pjwnt8*-A and -B probes were mixed. Therefore, we used the mixed probes for subsequent examinations. No obvious expression was observed until the 32-cell stage, the fifth cleavage (Fig. 3B and Table 1). The timing of the *Pjwnt8* expression varied slightly among embryos. Approximately 30% of the embryos exhibited expression of *Pjwnt8* at the 64-cell stage (the sixth cleavage) in the micromere-descendants (Fig. 3C and Table 1). About 90% of the embryos expressed *Pjwnt8* in micromere-descendant cells at the 128- to

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