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Expression pattern of *vasa* in gonads of sea cucumber *Apostichopus japonicus* during gametogenesis and reproductive cycle

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ABSTRACT

The *vasa* gene is a reliable germline marker to study the origin and development of germ cells and gonads, although the gene product (mRNA or protein) varies between different species. However, there has been little study on *vasa* genes in holothuroids to date. Here we determined the expression characteristics of the *Apostichopus japonicus vasa* gene (*Aj-vasa*) during gametogenesis in the ovary and testis using *in situ* hybridization and immunohistochemistry. During oogenesis, the expression pattern of *Aj-vasa* coincided at the mRNA and protein levels. Intensive signals in oogonia decreased gradually with the development of oocytes. Interestingly, the pattern was different during spermatogenesis. The *Aj-vasa* mRNA level was the highest in spermatogonia, reduced in spermatocytes, low in spermatids and absent in spermatozoa, but the Aj-VASA protein was restricted to spermatogonia and early spermatocytes. These expression characteristics of *Aj-vasa* persisted in both male and female gonads throughout the reproductive cycle. Our findings show that *Aj-vasa* mRNA is a good marker for studying the origin and migration of germline cells; moreover, Aj-VASA is a useful tool to identify spermatogonia in *A. japonicus*. Our findings indicate that *Aj-vasa* is vital in the development and differentiation of germ cells.

The *vasa* gene was first identified in *Drosophila*, where it is restricted to germ cells during embryogenesis (Schüpbach and Wieschaus, 1986). It is a valuable marker for tracing the origin of PGCs in zebrafish (Yoon et al., 1997) and has been used widely as a credible germline marker molecule in about 20 vertebrate and invertebrate organisms (Gustafson and Wessel, 2010; Wang et al., 2012a,b; Xu et al., 2005). It has been cloned from more than 70 species among the cnidaria, tunicates, nematodes, platyhelminths, molluscs, annelids, crustaceans, insects, echinoderms and vertebrates (Gustafson and Wessel, 2010; Wang et al., 2012a,b), but its expression characteristics vary between species. In *Drosophila*, the VASA protein is initially localized in the polar granules of the posterior pole as soon as it becomes detectable in the oocyte, and *vasa* transcripts are maintained at a uniform distribution up to the early embryo stage. Thus VASA, but not *vasa* mRNA, has been confirmed as a germline marker in *Drosophila*, with a specific location in germ cells (Hay et al., 1988a; Liang et al., 1994). Similarly, VASA as a germline marker has also been verified in the amphibian *Xenopus laevis* (Komiya et al., 1994; Ikenishi and Tanaka, 1997), the mouse *Mus musculus* (Toyooka et al., 2000), the chicken *Gallus domesticus* (Tsunekawa et al., 2000) and the human *Homo sapiens* (Castrillon et al., 2000; Anderson et al., 2007; Albamonte et al., 2008). By contrast, *vasa* mRNA is a better germline marker in some species such as the zebrafish *Danio rerio* (Yoon et al., 1997; Olsen et al., 1997; Knaut et al., 2000), trout *Oncorhynchus mykiss* (Yoshizaki et al., 2000), tilapia *Oreochromis niloticus* (Kobayashi et al., 2000), medaka *Oryzias latipes* (Shinomiya et al., 2002), oyster *Crassostrea gigas* (Fabioux et al., 2004) and silkworm *Bombyx mori* (Nakao, 1999). In zebrafish oogenesis, the VASA protein is lost after germinal vesicle breakdown while the *vasa* mRNA remains in the cortex. Furthermore, the maternal *vasa* mRNA is restricted to the animal pole of the first two cleavage furrows in 4-cell embryos and is located in the four cells that are the precursors of zebrafish PGCs at the blastula stage (Knaut et al., 2000).

The expression level of *vasa* varies between the ovary and testis, and the location of *vasa* also varies at mRNA and protein levels. In human gametogenesis, the expression of *vasa* mRNA is less marked in the fetal testis than in the fetal ovary (Anderson et al., 2007). In the gibel carp *Carassius auratus gibelio* both *vasa* mRNA and VASA persist throughout oogenesis with dynamic expression from

Abbreviations: Aj-vasa, Apostichopus japonicus vasa gene; Aj-VASA, protein of *Aj-vasa*; PGCs, primordial germ cells; sqRT-PCR, semi-quantitative reverse transcription and polymerase chain reaction; H&E, hematoxylin and eosin; ISH, *in situ* hybridization; IHC, immunohistochemistry.

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oogonia to maturing oocytes. In contrast, *vasa* expression products are detectable in spermatogonia and spermatocytes, but disappear in spermatids and spermatozoa (Xu et al., 2005). There are also differential and dynamic expression patterns of *vasa* between the ovary and testis is also discovered in the mouse (Toyooka et al., 2000), tilapia (Kobayashi et al., 2000), and catfish *Clarias gariepinus* (Raghuveer and Senthilkumaran, 2010).

Although *vasa* has been proven to be a germline marker in many animals, recent studies indicate that *vasa* is also expressed in multipotent stem cells in the flatworm *Macrostomum lignano* and cnidarian *Hydractinia echinata* (Pfister et al., 2008; Rebscher et al., 2008). However, RNA interference (RNAi) knockdown of *vasa* expression had no effect on the somatic neoblasts in *M. lignano* (Pfister et al., 2008). The function of VASA in somatic cells is unknown in most animals and needs to be studied further.

The sea cucumber *Apostichopus japonicus*, a species of the Holothuroldea in the phylum Echinodermata is an important commercial species in China. There have been numerous studies on its reproductive biology, such as morphology of the reproductive system, reproductive behavior and larval development (Sui et al., 1985; Pang et al., 2006; Kato et al., 2009). However, basic knowledge of the molecular biology of gametogenesis and gonadogenesis is limited in this species. In this study, we examined *vasa* expression in the gonads of *A. japonicus* during gametogenesis at the mRNA and protein levels. These data will provide a basic understanding of the expression of *vasa* in the development of *A. japonicus* germ cells.

1. Results

1.1. Specific expression of vasa mRNA in gonads

Results from sqRT–PCR showed that *Aj-vasa* mRNA was expressed specifically in the ovary and testis of adult *A. japonicus*; however, no *vasa* mRNA appeared in any of the somatic organs examined, namely the respiratory tree and intestine (Fig. 1).

1.2. Germ cell-specific location of Aj-vasa mRNA and Aj-VASA protein during gametogenesis

Gonads of *A. japonicus* are elongate, slender, branching tube-like structures. Germ cells in the growing stage ovary are composed of aggregated oogonia with a high nucleus-to-cytoplasm ratio; 5–8 μm in diameter and primary oocytes 15–100 μm in diameter; primary oocytes are 110–130 μm in diameter when mature. The testis at this stage contains spermatogonia with a high nucleus-to-cytoplasm ratio measuring 5–7 μm in diameter with clear nuclear material. It also contains spermatocytes with a dense nucleus 4–6 μm in diameter; round spermatids with no flagellum 3–4 μm in diameter; and spermatozoa with elliptical heads 2.5–3 μm long and an acidophilic flagellum. These elements are arranged from the periphery to the center of the seminiferous tubule, respectively (Sui et al., 1985; Pang et al., 2006) (Fig. 2A and B).

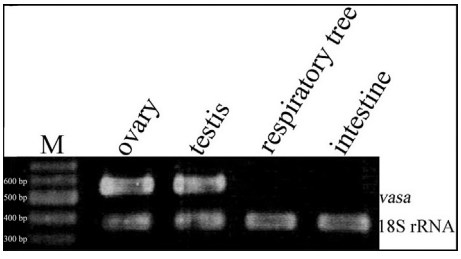

Fig. 1. Spatial expression of *Aj-vasa* mRNA in adult *A. japonicus* tissues analyzed by sqRT-PCR.

The expression and distribution of *Aj-vasa* mRNA and Aj-VASA during oogenesis and spermatogenesis were detected using ISH and IHC. In the ovary, both mRNA and Aj-VASA were present in germ cells throughout oogenesis. The *Aj-vasa* mRNA signal was most intense in the cytoplasm of oogonia and early primary oocytes, and declined with oocyte development (Fig. 2C). The locations of Aj-VASA and changes in its signal intensity in the ovary were similar to those of *Aj-vasa* mRNA (Fig. 2E and G). No signal was observed in somatic cells of the ovary (Fig. 2G). In the testis, *Aj-vasa* mRNA was expressed in the cytoplasm of spermatogonia, spermatocytes and spermatids. The signal intensity was the highest in spermatogonia, decreased gradually with spermatocyte development, was weak in spermatid and absent from the mature spermatozoa (Fig. 2D). The expression locations differed between *Aj-vasa* mRNA and Aj-VASA during spermatogenesis, in that Aj-VASA was only located in the cytoplasm of spermatogonia and early spermatocytes, and not present in late spermatocytes, spermatids or spermatozoa (Fig. 2F and H). No expression was found in somatic cells of the testis (Fig. 2H).

1.3. Expression of Aj-VASA in gonads during the reproductive cycle

An abundance of Aj-VASA was located in oogonia and oocytes of the ovary at the growing stage (Fig. 3A and C). It declined in developing oocytes in the ovary at the growing stage. The expression of Aj-VASA was weak in oocytes of the mature ovary and in residual oocytes of the ovary at the post-spawning stage (Fig. 3E and G). In the testis, Aj-VASA expression was concentrated in spermatogonia and early spermatocytes during the early growing stage, and restricted to spermatogonia at the later growing and mature stages (Fig. 3D and F). It was absent from the testis at the post-spawning stage (Fig. 3H).

2. Discussion

2.1. Expression of the vasa gene and potential application as a marker for A. japonicus germ cells

The *vasa* gene has been reported as a molecular marker of germline in many invertebrates such as the Chinese shrimp *Fenneropenaeus chinensis* (Zhou et al., 2010; Feng et al., 2011), oyster *C. gigas* (Fabioux et al., 2004) and Chinese mitten crab *Eriocheir sinensis* (Wang et al., 2012a). Like most species, the expression of *Aj-vasa* detected by sqRT–PCR was also specific for the *A. japonicus* gonads (Fig. 1). Furthermore, ISH and IHC revealed that both *Aj-vasa* mRNA and Aj-VASA were restricted to the germ cells, and no signal was detected in somatic cells of the ovary or testis. Thus the expression pattern of *Aj-vasa* in the germ cells of *A. japonicus* was similar to that of equivalent genes in other organisms examined to date.

The VASA protein, but not *vasa* mRNA, has been demonstrated to be a germ cell marker in species such as *Drosophila* and *Xenopus* with an initial restriction to germline cells (Hay et al.,1988b; Komiya et al., 1994). However, in the zebrafish, *vasa* mRNA is more specific as a germline marker than VASA protein during gametogenesis and embryogenesis (Knaut et al., 2000; Braat et al., 2000). Here we found that *Aj-vasa* mRNA was expressed in a wider range of germ cells including oogonia, oocytes, spermatogonia, spermatocytes and spermatids, but was absent from somatic cells of the gonads. This suggests that the *Aj-vasa* mRNA might be a better germ cell marker than Aj-VASA to study the origination and migration of PGCs and differentiation of germ cells in *A. japonicus*.

In the male specimens, Aj-VASA expression was restricted to spermatogonia and few early spermatocytes. These distribution