

Identification and sexually dimorphic expression of *vasa* isoforms in Dabry's sturgeon (*Acipenser dabryanus*), and functional analysis of *vasa* 3'-untranslated region

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Abstract Germ cells are set aside from somatic cells early in embryogenesis, and are responsible for transmitting genetic information through generations. *Vasa* is a highly conserved germ cell marker across animal phyla, and widely used to label primordial germ cells. Dabry's sturgeon is a rare and endangered species distributed solely in the Yangtze River basin. Here, seven *vasa* isoforms, named *Advasa1–7*, were isolated and characterized in Dabry's sturgeon. RT-PCR and western blot analyses revealed that *vasa* mRNA and protein were mainly restricted to the testis and ovary, but exhibited sexually dimorphic expression. Cellular and subcellular localization uncovered that *Advasa* mRNA and protein displayed mitotic and meiotic expression in females, and mainly showed mitotic expression in males; surprisingly, they exhibited both cytoplasmic and nuclear expression in the ovarian germ cells, while showing exclusively cytoplasmic expression in the testicular germ cells. By microinjecting chimeric RNA consisting of the red fluorescent protein coding region and the *Advasa* 3'-untranslated region into embryos of Dabry's sturgeon, zebrafish and medaka, we demonstrated that it

had the ability to visualize primordial germ cells (PGCs) in Dabry's sturgeon and zebrafish but not in medaka. It seemed that the machinery of *vasa* 3'UTR RNA localization was conserved between Dabry's sturgeon and ostariophysan, while possibly changed during the divergence of euteleosts and ostariophysan. Finally, Dabry's sturgeon PGCs moved on the yolk ball, and migrated toward the genital ridge via mesenchyme. Taken together, these results provide new information for *vasa* expression pattern and function, and lay a foundation for PGC cryopreservation and conservation of Dabry's sturgeon.

Keywords Dabry's sturgeon · *Vasa* · Dimorphic expression · Primordial germ cells · Visualization

Introduction

In sexually reproducing animals, primordial germ cells (PGCs) are generated apart from the soma in early embryonic development and migrate to the future gonad to give rise to sperm in males and eggs in females (Wylie 1999). The specification and maintenance of PGCs in a variety of organisms rely upon the inheritance of maternal determinants, containing RNAs and proteins, termed germ plasm (Houston and King 2000). One of the best studied components of germ plasm is the gene *vasa*, encoded as an ATP-dependent RNA helicase of the DEAD (Asp-Glu-Ala-Asp)-box family, which was originally identified in *Drosophila* as a maternal-effect gene involved in anterior-posterior patterning and germ cell formation (Hay et al. 1988; Schüpbach and Wieschaus 1986). The *Drosophila vasa* homologues have been isolated from diverse animals ranging from invertebrates such as hydra (Mochizuki et al. 2001) to vertebrates such as fish, including zebrafish (Yoon et al. 1997), rainbow trout (Yoshizaki et al. 2000), tilapia (Kobayashi et al. 2000) and medaka (Shinomiya et al. 2000), *Xenopus* (Komiya et al.

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1994), chicken (Tsunekawa et al. 2000), mouse (Fujiwara et al. 1994), and human (Castrillon et al. 2000). Analysis of these *vasa* homologs has revealed a highly conserved protein sequence and germ cell lineage expression.

The *vasa* gene performs an essential function in germ cell development. Loss-of-function of *vasa* in *Drosophila* resulted in PGC deficiency (Lasko and Ashburner 1990), while female sterility was observed in *vasa* null mutants (Styhler et al. 1998). In mouse, *vasa* mutations displayed serious defects in male germ cell development (Tanaka et al. 2000). In fish, medaka *vasa* was necessary for migration (Li et al. 2009), while zebrafish *vasa* was crucial for germ cell differentiation and maintenance (Hartung et al. 2014). Additionally, works on expression pattern and localization of *vasa* mRNA and protein during embryogenesis, gonadal differentiation and gametogenesis have been investigated in teleosts, such as shiro-uo (Miyake et al. 2006), Atlantic cod (Presslauer et al. 2012), European sea bass (Blazquez et al. 2011), and gibel carp (Xu et al. 2005). Moreover, different *vasa* transcripts have been identified in several fish (Pacchiarini et al. 2013; Ubeda-Manzanaro et al. 2014; Wang et al. 2014), showing sexual dimorphic expression. The *vasa* homologs in fish play important roles in germ cell development, although the expression pattern and function may differ among species.

With the occurrence of a novel technique, localized RNA expression (LRE), the germ cell marker gene, *vasa*, was utilized to label living PGCs (Wolke et al. 2002). Briefly, synthetic chimeric mRNA constructed by fusing the green/red fluorescent protein (GFP/RFP) coding region to *vasa* 3'-untranslated region (3'UTR) can be used to visualize PGCs during embryogenesis, following injection into fertilized eggs. In zebrafish, rainbow trout and Nibe croaker (Yoshizaki et al. 2005), their GFP-*vasa* 3'UTR mRNA successfully traced each other's PGCs. Furthermore, medaka PGCs were visualized by injection of GFP-*vasa* 3'UTR mRNA of tongue sole (Huang et al. 2014). Medaka GFP-*vasa* 3'UTR mRNA, however, failed to tag the PGCs of zebrafish and loach (Saito et al. 2006), indicating that the mechanism of *vasa* 3'UTR RNA localization function varied in teleosts. Visualization of PGCs in vivo with GFP/RFP *vasa* 3'UTR mRNA has been applied to not only study the mechanism of PGC formation and migration but also cryopreserve and transplant PGCs in endangered and commercial fish (Nagasawa et al. 2013; Okutsu et al. 2006; Saito et al. 2006; Yoshizaki et al. 2005).

Dabry's sturgeon, *Acipenser dabryanus*, belongs to the Acipenseriformes, which is one of the most primitive Actinopterygii species. Here, the investigation of the ability of chimeric RNA contained *vasa* 3'UTR from Dabry's sturgeon for labeling PGCs, and even how conserved the ability is among different fish, will facilitate to elucidate the function of *vasa* 3'UTR during evolution. On the other hand, Dabry's sturgeon is a freshwater sturgeon distributed solely in the Yangtze River basin. Its sexual maturity in males is reached at 4–6 years and in

females at 6–8 years (Zhuang et al. 1997). Due to overfishing, habitat degradation and pollution, the wild population of Dabry's sturgeon declined sharply in the late twentieth century (Zhang et al. 2011; Zhuang et al. 1997), and it is presently listed as a Critical Endangered species in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List. Efforts have been taken to protect and rehabilitate this endemic species of the upper Yangtze River since 2000, such as the construction of nature reserves, fishing bans and programmes for releasing propagated fish (Zhang et al. 2011). The situation, however, seems increasingly serious, since, in the last few years, there has been no recruitment of Dabry's sturgeon in the Yangtze River (Wu et al. 2014a). With cryopreservation and transplantation of germ cells applied successfully in fish (Inoue et al. 2012; Kobayashi et al. 2003; Lacerda et al. 2013; Okutsu et al. 2006; Riesco et al. 2012), this provides a powerful tool for conservation of Dabry's sturgeon. Presently, no germ cell marker or PGC visualization and labeling has been reported in this species, although new techniques for labelling sturgeon PGCs using fluorescein isothiocyanate (FITC)-dextran (Saito and Psenicka 2015) is supposed to also be functional in Dabry's sturgeon. In the present study, we identified *vasa* homologues in Dabry's sturgeon, characterized expression pattern of *vasa* isoforms in diverse tissues and embryonic stages, and examined their cellular and subcellular localization during gonad development. Furthermore, chimeric mRNA was injected into the fertilized eggs of Dabry's sturgeon, zebrafish and medaka to validate its ability for labeling PGCs. Additionally, the PGC migration pattern of Dabry's sturgeon was identified.

Materials and methods

Fish and samples

Immature Dabry's sturgeon, *A. dabryanus*, were cultured at the Taihu station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Deep anaesthesia was induced by a 0.05 % solution of 3-aminobenzoic acid ethyl ester methanesulfonate-222 (MS-222) (Sigma, USA). The tissue samples from six 3-year-old Dabry's sturgeons (96.1 ± 8.5 cm in total length and 5.3 ± 0.6 kg in weight), including 3 males and 3 females, were collected within 30 min of exsanguination by tailing, and immediately dipped into liquid nitrogen and stored at -80 °C. All the samples were used for the experiments of this study, containing histology, sequences and expression analysis, in situ hybridization and immunohistochemistry. Fertilized embryos from four pairs of parents were obtained by controlled propagation in May, 2014 and April, 2015, and incubated at 19 ± 1 °C in the hatching tank. These embryos were used for chimeric mRNA injection for labeling PGCs and expression analysis. Unfertilized eggs and fertilized embryos from different stages were identified, collected in

liquid nitrogen and stored at -80°C . The embryonic development stages of Dabry's sturgeon were referenced with previous descriptions (Dettlaff et al. 1993). Zebrafish were kindly provided by Professor Jianfang Gui (Institute of Hydrobiology, Chinese Academy of Sciences), and maintained under an artificial photoperiod of 14 h/10 h light/dark at 28.5°C . Medaka were kindly provided to us by Dr. Mingyou Li (Shanghai Ocean University), and kept at 26°C on a 14 h/10 h light/dark cycle. The experimental procedures were based on the standards of the Chinese Council on Animal Care.

Gonadal histology

The ovary and testis tissues for histological observation were fixed overnight in Bouin's solution at 4°C , and then stored in 70 % ethanol. They were dehydrated in an increasing ethanol series, and then embedded in paraffin. The gonads were cut at $4\ \mu\text{m}$ on a Leica RM2135 Microtome. Subsequently, the sections were stained with hematoxylin and eosin, and checked under a microscope.

Isolation of *vasa* cDNA sequence

Total RNA was extracted by using the RNeasy Plus Mini Kit (Qiagen). The RNA quality and purity were checked by Nanodrop 2000 (Thermo). SMART cDNA was synthesized by the SMARTer™ RACE cDNA Amplification Kit (Clontech). To obtain fragments of the Dabry's sturgeon *vasa* cDNA, RT-PCR was performed with degenerate primers

designed by conserved amino acid sequences (Table 1). Then, a partial fragment of *vasa* homologue was obtained, which was used to design gene-specific primers for 5' and 3' RACE (Table 1). The PCR products were visualized by electrophoresis of ethidium bromide stained agarose gel, cloned into pMD19-T vector (Takara), and sequenced by Sangon Biotech.

Sequence analysis

The cDNA and deduced amino acid sequences were identified by the BLAST program at the NCBI web server. Amino acid sequence identity was calculated by CLUSTAL W. Multiple sequence alignments were performed with the CLUSTAL X program (v.2.1), with the printing output shaded by BOXSHADE 3.21. In addition, maximum likelihood analyses were performed using Mega 5.2 software with a bootstrap of 1,000 replicates.

Spatial and temporal expression analyses of *vasa* isoforms

Total RNA from different tissues and embryo stages was reverse transcribed with the PrimeScript RT Reagent Kit With gDNA Eraser (Takara). Spatial and temporal expression patterns of *vasa* isoforms were analyzed by RT-PCR. The primer pairs, *vasa*-RTF and *vasa*-RTR (Table 1), were designed to distinguish different *Adv* transcripts. Amplification reactions were displayed in a volume of $25\ \mu\text{L}$ containing $1\ \mu\text{L}$ cDNA as template DNA, $0.5\ \mu\text{M}$ of each primer, $0.5\ \text{U}$ Taq

Table 1 Sequences of the primers used for PCR

Primer name	Sequence (5' to 3')	Purpose
Degenerate-F	AAGCCNACHCCWGTNCAGAADYA	Partial
Degenerate-R	CCDATRCGRTGRACRTABTCRTC	
V-F1	AGACTACAGGCACGGAACGCACAATG	3' RACE
V-F2	AGAGGGAAAGGGAGCAAGCACTTGGA	
V-R1	ATCCAACATCCGATCTGCTTCATCCAG	5' RACE
V-R2	AACTATTGGAATGCCATGCTTCTGAAC	
<i>Vasa</i> -RT-F	TGGACAACCTTTGGCAAGAAAGAAG	RT-PCR
<i>Vasa</i> -RT-R	ACCTGCATTTTCACCACCTTCATC	
β -actin-RT-F	CCTTCTTGGGTATGGAATCTTGC	
β -actin-RT-R	CAGAGTATTTACGCTCAGGTGGG	
<i>Vasa</i> -probe-F	GCTCCACTGGCAATAATGAC	Probe
<i>Vasa</i> -probe-R	CTGAACAATGAATGCTTACTTTA	
<i>Vasa</i> -3'-UTR-F	<u>GAATTCTTTCC</u> TTTACAAGAACTCAACTCT	<i>Vasa</i> 3'-UTR
<i>Vasa</i> -3'-UTR-R	<u>CTCGAGCTGAACAATGAATGCTTACTTTA</u>	
RFP-F	<u>GGATCC</u> ATGGTGTCTAAGGGCGAAGAG	RFP
RFP-R	<u>GAATTC</u> TAATTAAGTTTGTGCCCCAGT	
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAG	RACE
NUP	and CTAATACGACTCACTATAGGGC AAGCAGTGGTATCAACGCAGAGT	

The bases with underlining are restriction enzyme cutting sites

Vasa-1	1	MADEDWDEVTSSALSFPPNKNIMDNFGKKEDEKFSNTFTAGGFSSYQPFKPQGAAFHRMQSYNDGWCTSVAAQQHQDGNENSSWNNNN
Vasa-2	1	MADEDWDEVTSSALSFPPNKNIMDNFGKKEDEKFSNTFTAGGFSSYQPFKPQGAAFHRMQSYNDGWCTSVAAQQHQDGNENSSWNNNN
Vasa-3	1	MADEDWDEVTSSALSFPPNKNIMDNFGKKEDEKFSNTFTAG-----NENSSWNNNN
Vasa-4	1	MADEDWDEVTSSALSFPPNKNIMDNFGKKEDEKFSNTFTAG-----NENSSWNNNN
Vasa-5	1	MADEDWDEVTSSALSFPPNKNIMDNFGKKEDEKFSNTFTAG-----NENSSWNNNN
Vasa-6	1	MADEDWDEVTSSALSFPPNKNIMDNFGKKEDEKFSNTFTAGGFSSYQPFKPQGAAFHRMQSYNDGWCTSVAAQQHQDGNENSSWNNNN
Vasa-7	1	MADEDWDEVTSSALSFPPNKNIMDNFGKKEDEKFSNTFTAG-----NENSSWNNNN
Consensus	1	*****
Vasa-1	91	EKSSGFGRGGFGNQRGADYQNGGGSFGNRGGGFRGGSSFESGGGRREGGRGFRGGRGGFQQDNGDNDFGSSGGFGRGGFGRSRGDD
Vasa-2	91	EKSSGFGRGGFGNQRGADYQNGGGSFGNRGGGFRGGSSFESGGGRREGGRGFRGGRGGFQQDNGDNDFGSSGGFGRGGFGRSRGDD
Vasa-3	53	EKSSGFGRGGFGNQRGADYQNGGGSFGNRGGGFRGGSSFESGGGRREGGRGFRGGRGGFQQDNGDNDFGSSGGFGRGGFGRSRGDD
Vasa-4	53	EKSSGFGRGGFGNQRGADYQNGGGSFGNRGGGFRGGSSFESGGGRREGGRGFRGGRGGFQQDNGDNDFGSSGGFGRGGFGRSRGDD
Vasa-5	43	-----ADYQNGGGSFGNRGGGFRGGSSFESGGGRREGGRGFRGGRGGFQQDNGDNDFGSSGGFGRGGFGRSRGDD
Vasa-6	91	EKSSGFGRGGFGNQRG-----DD
Vasa-7	43	-----ADYQNGGGSFGNRGGGFRGGSSFESGGGRREGGRGFRGGRGGFQQDNGDNDFGSSGGFGRGGFGRSRGDD
Consensus	91	*****
Vasa-1	181	SGEGGRGFRGGRGGYKGRDEEVLSEKAPSRFAREDEGGENAGPKVIYVPPPPPEEEDTIFAHYETGINFNKYDDILVDVSGNAPL
Vasa-2	181	SGEGGRGFRGGRGGYKGRDEEVLSEKAPSRFAREDEGGENAGPKVIYVPPPPPEEEDTIFAHYETGINFNKYDDILVDVSGNAPL
Vasa-3	143	SGEGGRGFRGGRGGYKGRDEEVLSEKAPSRFAREDEGGENAGPKVIYVPPPPPEEEDTIFAHYETGINFNKYDDILVDVSGNAPL
Vasa-4	143	SGEGGRGFRGGRGGYKGRDEEVLSEKAPSRFAREDEGGENAGPKVIYVPPPPPEEEDTIFAHYETGINFNKYDDILVDVSGNAPL
Vasa-5	116	SGEGGRGFRGGRGGYKGRDEEVLSEKAPSRFAREDEGGENAGPKVIYVPPPPPEEEDTIFAHYETGINFNKYDDILVDVSGNAPL
Vasa-6	110	SGEGGRGFRGGRGGYKGRDEEVLSEKAPSRFAREDEGGENAGPKVIYVPPPPPEEEDTIFAHYETGINFNKYDDILVDVSGNAPL
Vasa-7	116	SGEGGRGFRGGRGGYKGRDEEVLSEKAPSRFAREDEGGENAGPKVIYVPPPPPEEEDTIFAHYETGINFNKYDDILVDVSGNAPL
Consensus	181	*****
Vasa-1	271	AIMTFDEAGLCDTLNKNISKSGYVKPTPVQKHGIPVLGGCDLMACQGTSGKTAFFLLPILQLMKDGAASKFSELQEPEAIIIVAPTR
Vasa-2	256	AIMTFDEAGLCDTLNKNISKSGYVKPTPVQKHGIPVLGGCDLMACQGTSGKTAFFLLPILQLMKDGAASKFSELQEPEAIIIVAPTR
Vasa-3	233	AIMTFDEAGLCDTLNKNISKSGYVKPTPVQKHGIPVLGGCDLMACQGTSGKTAFFLLPILQLMKDGAASKFSELQEPEAIIIVAPTR
Vasa-4	218	AIMTFDEAGLCDTLNKNISKSGYVKPTPVQKHGIPVLGGCDLMACQGTSGKTAFFLLPILQLMKDGAASKFSELQEPEAIIIVAPTR
Vasa-5	206	AIMTFDEAGLCDTLNKNISKSGYVKPTPVQKHGIPVLGGCDLMACQGTSGKTAFFLLPILQLMKDGAASKFSELQEPEAIIIVAPTR
Vasa-6	200	AIMTFDEAGLCDTLNKNISKSGYVKPTPVQKHGIPVLGGCDLMACQGTSGKTAFFLLPILQLMKDGAASKFSELQEPEAIIIVAPTR
Vasa-7	191	AIMTFDEAGLCDTLNKNISKSGYVKPTPVQKHGIPVLGGCDLMACQGTSGKTAFFLLPILQLMKDGAASKFSELQEPEAIIIVAPTR
Consensus	271	*****
Vasa-1	361	ELINQIYLEARKFAYGTCVRPVVVYGGISTGYTIREVLKGCNVLCGTPGRLMDIIGRGKVLRLRRLVLEADRMLDMGFEPERMRLVA
Vasa-2	346	ELINQIYLEARKFAYGTCVRPVVVYGGISTGYTIREVLKGCNVLCGTPGRLMDIIGRGKVLRLRRLVLEADRMLDMGFEPERMRLVA
Vasa-3	323	ELINQIYLEARKFAYGTCVRPVVVYGGISTGYTIREVLKGCNVLCGTPGRLMDIIGRGKVLRLRRLVLEADRMLDMGFEPERMRLVA
Vasa-4	308	ELINQIYLEARKFAYGTCVRPVVVYGGISTGYTIREVLKGCNVLCGTPGRLMDIIGRGKVLRLRRLVLEADRMLDMGFEPERMRLVA
Vasa-5	296	ELINQIYLEARKFAYGTCVRPVVVYGGISTGYTIREVLKGCNVLCGTPGRLMDIIGRGKVLRLRRLVLEADRMLDMGFEPERMRLVA
Vasa-6	290	ELINQIYLEARKFAYGTCVRPVVVYGGISTGYTIREVLKGCNVLCGTPGRLMDIIGRGKVLRLRRLVLEADRMLDMGFEPERMRLVA
Vasa-7	281	ELINQIYLEARKFAYGTCVRPVVVYGGISTGYTIREVLKGCNVLCGTPGRLMDIIGRGKVLRLRRLVLEADRMLDMGFEPERMRLVA
Consensus	361	*****
Vasa-1	451	SPGMPNNEERQTLFSAATYPEDIQKLAADFLKPGYIFLAVGQVGGACSDVEQNVLQVTQYSKRDKLVEMLQTTGTERTMVFVETKRKADF
Vasa-2	436	SPGMPNNEERQTLFSAATYPEDIQKLAADFLKPGYIFLAVGQVGGACSDVEQNVLQVTQYSKRDKLVEMLQTTGTERTMVFVETKRKADF
Vasa-3	413	SPGMPNNEERQTLFSAATYPEDIQKLAADFLKPGYIFLAVGQVGGACSDVEQNVLQVTQYSKRDKLVEMLQTTGTERTMVFVETKRKADF
Vasa-4	398	SPGMPNNEERQTLFSAATYPEDIQKLAADFLKPGYIFLAVGQVGGACSDVEQNVLQVTQYSKRDKLVEMLQTTGTERTMVFVETKRKADF
Vasa-5	386	SPGMPNNEERQTLFSAATYPEDIQKLAADFLKPGYIFLAVGQVGGACSDVEQNVLQVTQYSKRDKLVEMLQTTGTERTMVFVETKRKADF
Vasa-6	380	SPGMPNNEERQTLFSAATYPEDIQKLAADFLKPGYIFLAVGQVGGACSDVEQNVLQVTQYSKRDKLVEMLQTTGTERTMVFVETKRKADF
Vasa-7	371	SPGMPNNEERQTLFSAATYPEDIQKLAADFLKPGYIFLAVGQVGGACSDVEQNVLQVTQYSKRDKLVEMLQTTGTERTMVFVETKRKADF
Consensus	451	*****
Vasa-1	541	IATFLCQEKIPTTSIHGDREQREREQALGDFRTGKCPVLVATSVAAARGLDIEHVQHVVNFDLPSSIDEYVHRIIGRTGRCGNTGKAISFFD
Vasa-2	526	IATFLCQEKIPTTSIHGDREQREREQALGDFRTGKCPVLVATSVAAARGLDIEHVQHVVNFDLPSSIDEYVHRIIGRTGRCGNTGKAISFFD
Vasa-3	503	IATFLCQEKIPTTSIHGDREQREREQALGDFRTGKCPVLVATSVAAARGLDIEHVQHVVNFDLPSSIDEYVHRIIGRTGRCGNTGKAISFFD
Vasa-4	488	IATFLCQEKIPTTSIHGDREQREREQALGDFRTGKCPVLVATSVAAARGLDIEHVQHVVNFDLPSSIDEYVHRIIGRTGRCGNTGKAISFFD
Vasa-5	476	IATFLCQEKIPTTSIHGDREQREREQALGDFRTGKCPVLVATSVAAARGLDIEHVQHVVNFDLPSSIDEYVHRIIGRTGRCGNTGKAISFFD
Vasa-6	470	IATFLCQEKIPTTSIHGDREQREREQALGDFRTGKCPVLVATSVAAARGLDIEHVQHVVNFDLPSSIDEYVHRIIGRTGRCGNTGKAISFFD
Vasa-7	461	IATFLCQEKIPTTSIHGDREQREREQALGDFRTGKCPVLVATSVAAARGLDIEHVQHVVNFDLPSSIDEYVHRIIGRTGRCGNTGKAISFFD
Consensus	541	*****
Vasa-1	631	PESDTPPIARSLVKVLSDAKQEVPAWLEEVAFSAHGTTFGNPRGNVAFVSDSRKGGPGRNRSFGNRESSQPAIAVDEDADWE
Vasa-2	616	PESDTPPIARSLVKVLSDAKQEVPAWLEEVAFSAHGTTFGNPRGNVAFVSDSRKGGPGRNRSFGNRESSQPAIAVDEDADWE
Vasa-3	593	PESDTPPIARSLVKVLSDAKQEVPAWLEEVAFSAHGTTFGNPRGNVAFVSDSRKGGPGRNRSFGNRESSQPAIAVDEDADWE
Vasa-4	578	PESDTPPIARSLVKVLSDAKQEVPAWLEEVAFSAHGTTFGNPRGNVAFVSDSRKGGPGRNRSFGNRESSQPAIAVDEDADWE
Vasa-5	566	PESDTPPIARSLVKVLSDAKQEVPAWLEEVAFSAHGTTFGNPRGNVAFVSDSRKGGPGRNRSFGNRESSQPAIAVDEDADWE
Vasa-6	560	PESDTPPIARSLVKVLSDAKQEVPAWLEEVAFSAHGTTFGNPRGNVAFVSDSRKGGPGRNRSFGNRESSQPAIAVDEDADWE
Vasa-7	551	PESDTPPIARSLVKVLSDAKQEVPAWLEEVAFSAHGTTFGNPRGNVAFVSDSRKGGPGRNRSFGNRESSQPAIAVDEDADWE
Consensus	631	*****

◀ **Fig. 1** Multiple deduced amino acid sequence alignments of Vasa isoforms of Dabry's sturgeon (*Acipenser dabryanus*). Multiple alignments are displayed with the Clustal X 2.1 and refined using BOXSHADE 3.21. Identical residues are indicated by asterisks. Arginine-glycine-glycine (RGG) and arginine-glycine (RG) repeats are in red and blue, and marked with *underlining* and *dashed lines*, respectively. The amino acids for the polyclonal antibody are shaded in yellow

polymerase, 0.1 μ M of each dNTP, and 10 \times buffer for Taq polymerase. Each PCR cycle included denaturation at 94 $^{\circ}$ C for 30 s, annealing at 54 $^{\circ}$ C for 30 s, and extension at 72 $^{\circ}$ C for 20 s, with 35 cycles. The β -actin was amplified with 24 cycles as the internal control to calibrate the expression of *Advasa* isoforms. To discriminate the *Advasa4* and *Advasa5* in the ovary, *Advasa* was amplified with 32 cycles. The products were separated by 12 % polyacrylamide gels, and stained with silver.

Fluorescent in situ hybridization

Part of *Advasa* cDNA including the 3'UTR was acquired by PCR, and inserted into pGEM-T Easy Vector (Promega) and sequenced (Table 1). The plasmid was linearized for the synthesis of anti-sense and sense probes with T7 or SP6 polymerase by in vitro transcription (DIG RNA Labeling Kit, Roche). Fluorescent in situ hybridization was performed according to Li et al. (2011).

Antibody production

The polyclonal antibody specific to Dabry's sturgeon Vasa was prepared against the peptide of 14 amino acids in the N-terminus (NIMDNFGKKEDFKS). The peptide was conjugated to the KLH peptide, and then used to immunize a rabbit, with the polyclonal antibody harvested after 6 weeks (GenScript).

Western blot

Western blot detection was carried out as per the previous reports using Dabry's sturgeon Vasa-specific polyclonal antibody (Ye et al. 2012). The blot was detected by horseradish peroxidase (HRP) staining with Luminata Crescendo Western HRP substrate.

Fluorescent immunohistochemistry

Fluorescent immunohistochemistry on sections were performed as described previously with some differences (Ye et al. 2012). Sections were conducted with both anti-Vasa antibody (1:400 dilution) and anti-proliferating cell nuclear antigen (PCNA) antibody (1:200 dilution; Abcam). Fluorescent secondary antibodies against mouse and rabbit used for detection were TRITC (1:150 dilution) and FITC (1:150 dilution), respectively.

Preparation of chimeric mRNA and microinjection

The recombinant plasmid of RFP-*Advasa* 3'-UTR was constructed by inserting the RFP-coding region and *Advasa* 3'-UTR into the vector of pCS²⁺ (Table 1). The plasmid of GFP-*Drnanos3* 3'-UTR was constructed according to a previous description (Koprunner et al. 2001), and GFP-*Olvasa* 3'-UTR plasmid was kindly provided by Professor Mingyou Li, Shanghai Ocean University. Capped sense mRNA was synthesized in vitro by using the mMessage Machine SP6 kit (Ambion). GFP-*Drnanos3* 3'UTR and RFP-*Advasa* 3'UTR mRNA were co-injected into the vegetal pole of the 1- to 4-cell stage embryos of sturgeon (Saito et al. 2014), and the animal pole of zebrafish embryos at the 1- to 2-cell stage. Moreover, the GFP-*Olvasa* 3'-UTR and RFP-*Advasa* 3'UTR mRNA were also co-injected into the animal pole of medaka embryo at the 1- to 2-cell stage. The injected embryos were observed and photographed under a fluorescent microscope.

Microscopy and photography

Cross-sections after fluorescent in situ hybridization and histology were observed and photographed under a Leica upright microscope (DM5000B) with a Leica DFC365FX digital camera. Slides after fluorescent immunohistochemical staining were examined by a Confocal Microscope (Zeiss LSM710). The embryos were monitored and photographed under fluorescent stereomicroscopy (Leica M205FA) with a Leica DFC550 digital camera.

Results

Characterization of *vasa* isoforms in Dabry's sturgeon

Using degenerate primers based on the conserved regions of vertebrate Vasa proteins, a partial 959-bp cDNA fragment of *vasa* homologue, *Advasa*, in Dabry's sturgeon was obtained. Following the fragment sequence, the full-length cDNA of *Advasa* was isolated by RACE. Seven types of 5' extremities, however, were identified from 5' RACE products. Subsequently, the seven isoforms of *Advasa*, named *Advasa1*–7, were assembled. The complete cDNA sequence of *Advasa1* (GenBank: KT781113) was 3060 base pairs (bp) in length, contained a 122-bp 5'-untranslated region (UTR), a 2130-bp open reading frame (ORF) for 709 amino acids (aa), and a 808-bp 3'-UTR. The full-length *Advasa2*–7 (GenBank: KT781114, KT781115, KT781116, KT781117, KT781118 and KT781119) were 3015, 2946, 2901, 2865, 2847, and 2820 bp, respectively, which encoded 694, 671, 656, 644, 638 and 629 aa, respectively.

Alignments of *AdVasa* isoforms revealed that the sequence difference was only located in the N-terminus (Fig. 1), with

some deletion in the N-terminal region referenced with *AdVasa1*. Furthermore, comparison of *AdVasa1* with several model organisms disclosed that there were eight highly conserved motifs of the DEAD-box protein family in *AdVasa1* protein sequence (Fig. S1), involving ATPase binding and RNA unwinding activity. Additionally, there were 5 arginine-glycine (RG) and 7 arginine-glycine-glycine (RGG) repeats in the N-terminus of *AdVasa1* (Fig. 1), functioning in RNA binding and subcellular protein localization (Liang et al. 1994; Wolke et al. 2002). The predicted *AdVasa1* protein shared 68.5 and 65.3 % identity with that of zebrafish and medaka, and was 52.5, 49.6, 57.5 and 58.6 % identical to the *Vasa* of frog, chicken, mouse and human, respectively (Fig. S1). Phylogenetic analysis revealed that *AdVasa1* belonged to *Vasa*, but not its related family members, PL10 and P68. Moreover, *AdVasa1* fell into the cluster of teleost *Vasa* (Fig. 2).

Embryonic and tissue expression of *Advassa* isoforms transcripts

The embryonic and tissue distribution of *Advassa* isoforms was detected by RT-PCR. During embryogenesis, all transcripts except for *Advassa4* were highly expressed

from unfertilized eggs to gastrula stage, dramatically declined and persisted at a reduced and detectable level from neurula to heartbeat stage, and were hardly observed in hatching larvae (Fig. 3a). Moreover, the expression levels of *Advassa1–3* and *Advassa5* were much higher than for *Advassa6* and *Advassa7* in early stages (Fig. 3a). Since fish zygotic transcription initially occurs after midblastula transition, the *Advassa* isoforms except for *Advassa4* were maternal deposits.

The expression pattern of *Advassa* isoforms in somatic tissues, including liver, intestines, spleen, kidney, heart, muscle, brain, testis and ovary, was investigated. As shown in Fig. 3b, *Advassa* isoforms were mainly restricted to the gonads. The lower levels of *Advassa* isoforms were found in the liver, intestines, spleen, kidney, heart and brain, but were hardly observed in the muscle. In detail, all *Advassa* isoforms were expressed in liver and ovary. The band of *Advassa4* and *Advassa5* merged in the ovary, due to their high expression, but it is obviously two bands in Fig. 3c. In intestines and testis, *Advassa3*, *Advassa5* and *Advassa6* were detected. In kidney and brain, *Advassa2*, *Advassa3* and *Advassa5* were observed. In spleen, *Advassa1* and *Advassa3* were found. In heart,

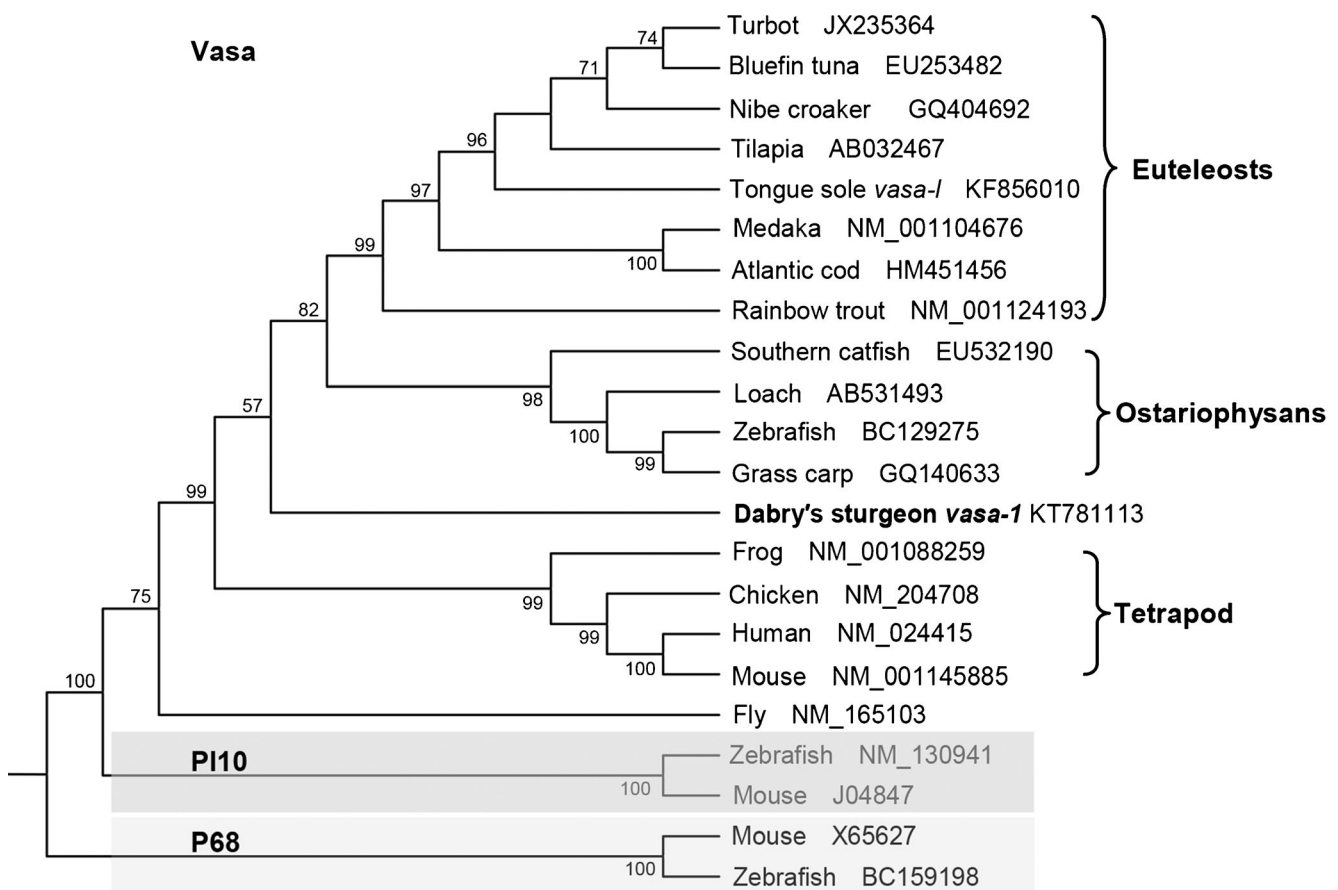


Fig. 2 Phylogenetic tree of *Vasa* proteins. Phylogenetic relationship of *Vasa* is analyzed with Mega 5.2 by bootstrap analysis using maximum

likelihood (1000 replicates). Numbers near the nodes represent bootstrap values. The accession numbers are exhibited after the species

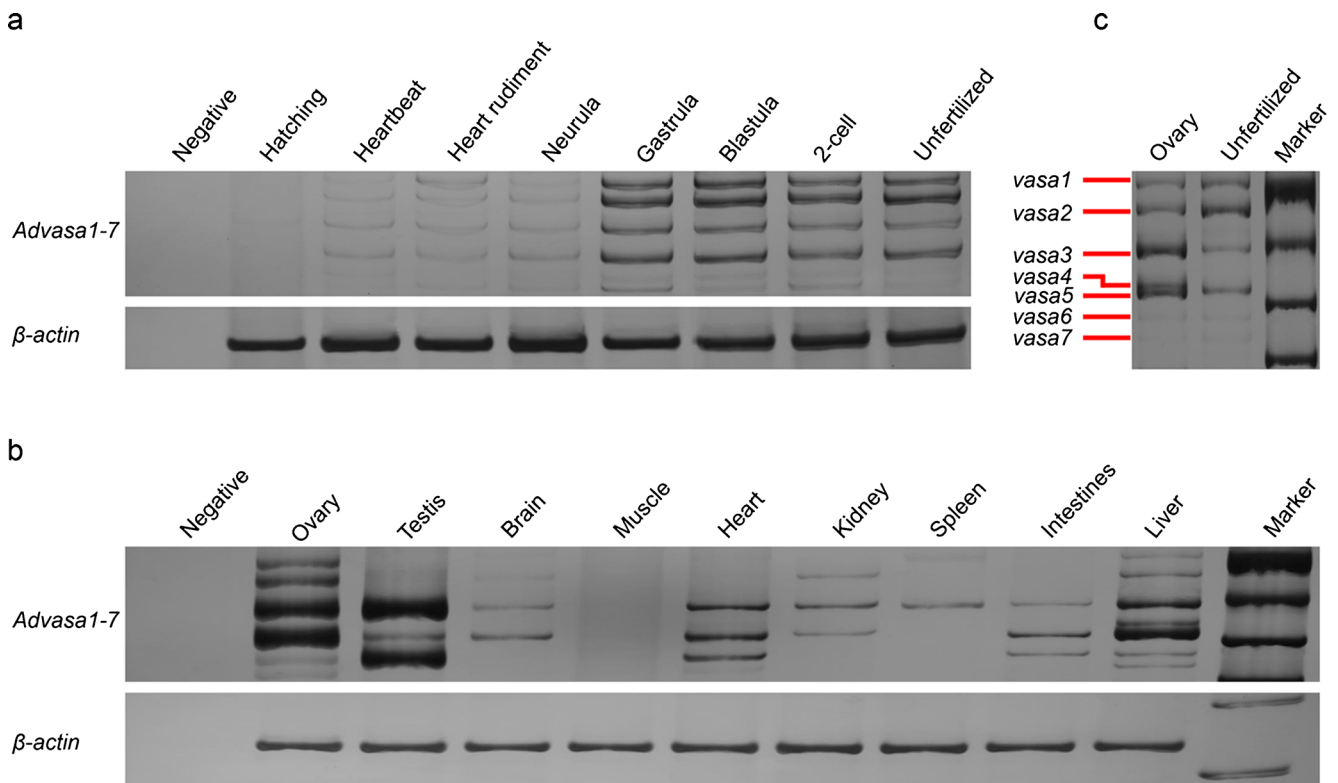


Fig. 3 Spatial and temporal expression analyses of *Advasa* isoforms detected by RT-PCR. **a** Different development stages of embryos. **b** Different tissues. **c** Comparison analysis of the expression pattern of ovary and unfertilized embryos. *β-actin* served as RT-PCR control

Advasa3, *Advasa5*, *Advasa6* and *Advasa7* were identified.

Tissue distribution of *AdVasa* proteins

To examine Vasa protein expression in Dabry’s sturgeon, a polyclonal antibody against *AdVasa* N-terminus at 23–36 was obtained, which was not able to distinguish different *AdVasa* isoforms. The specificity of the anti-*AdVasa* antibody was confirmed by comparison with the pre-immune serum and pre-adsorbed antibody (Fig. S2). Immunoblot analysis of tissues revealed that several bands of the expected size (approximately 67.9–76.7 kDa) were detected in the ovary, but only one band was found in the testis, and was not observed in other somatic tissues (Fig. 4).

Differential germ cell-specific expression of *Advasa* RNA in gonad

The localization of *Advasa* in gonad was analyzed by fluorescent in situ hybridization (FISH) using a sense or antisense RNA probe of *Advasa*, and the antisense probe was not identified in each *Advasa* transcript. In 3-year-old Dabry’s sturgeon, the immature ovary was comprised of follicle cells, a number of oogonia and numerous primary oocytes (Fig. 5a, c) (Doroshov et al. 1997; Flynn and Benfey 2007). As shown in Fig. 6a, *Advasa* RNAs were found only in germ cells but were absent in surrounding somatic cells. The signal was strong in stage I–II oocytes, and seemed predominantly uniform in the cytoplasm and faint in the nucleus. At higher magnification (Fig. 6d), *vasa* and DAPI staining identified two types of oogonia: one exhibiting weak *vasa* staining and intense nuclear staining, called undifferentiated oogonia (og1), and the other displaying

Fig. 4 Western blot detection of *AdVasa* protein in different tissues. *β-Actin* was used as the internal control

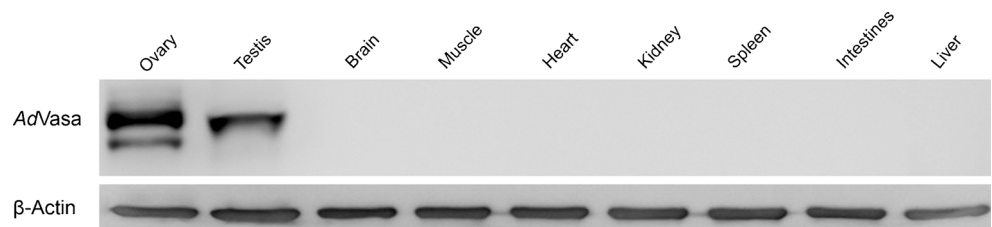
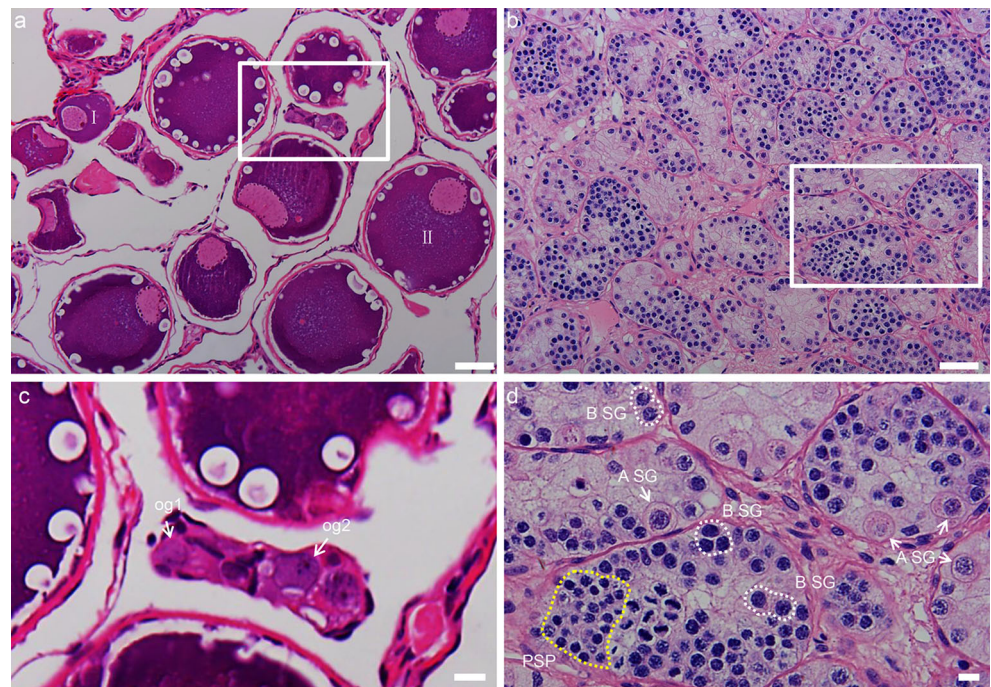


Fig. 5 Histological sections of 3-year-old Dabry's sturgeon gonads stained with hematoxylin and eosin. **c, d** Higher magnifications of the boxed areas in **(a)** and **(b)**, respectively. *og1* undifferentiated oogonia; *og2* differentiating oogonia; *A SG* primary A spermatogonia; *B SG* transitional spermatogonia; *psp* primary spermatocytes. Bars **(a, b)** 50 μm , **(c, d)** 10 μm



strong *vasa* staining and moderate nuclear staining, called differentiating oogonia (*og2*). In particular, the *vasa* signal was

weakly detectable in undifferentiated oogonia, forming small speckles surrounding the nucleus, while in differentiating

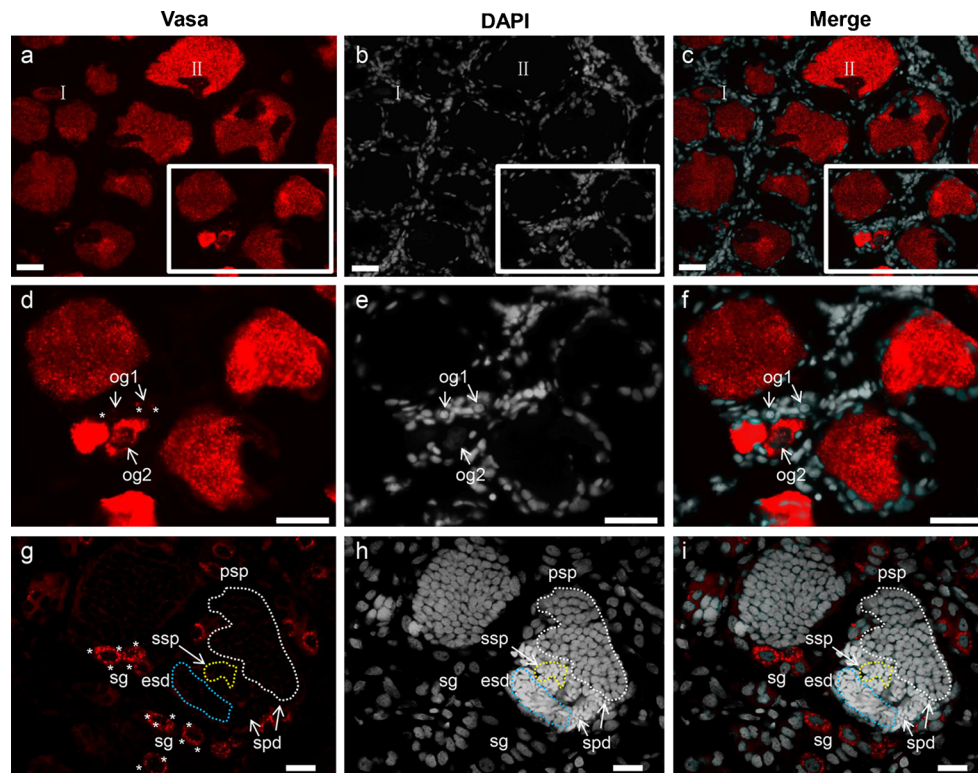


Fig. 6 Expression of Dabry's sturgeon *vasa* mRNA in the ovary and testis. The cryosections were hybridized to the *Advasa*-specific antisense and sense RNA probes, respectively. Fluorescent in situ hybridization was done by using the TSA™ Plus TMR/Fluorescein System. Nuclei were stained with DAPI **(b, e and h)**. **d–f** Higher magnifications of the boxed areas in **(a–c)**, respectively. The *Advasa* signal

formed a few speckles indicated by asterisks in the cytoplasm of undifferentiated oogonia **(d)** and spermatogonia **(g)**. Merge of *Advasa* signal and DAPI staining **(c, f and i)**. Roman numerals indicate stages of oocytes. *og1* undifferentiated oogonia; *og2* differentiating oogonia; *sg* spermatogonia; *psp* primary spermatocytes; *ssp* secondary spermatocytes; *spd* spermatid; *esd* elongated spermatid. Bars **(a–f)** 50 μm , **(g–i)** 20 μm

oogonia, *Advava* transcripts were faint in the nucleus and abundant in the cytoplasm. No reproducible staining could be detected by the sense probe (Fig. S3a).

The testes with many seminiferous cysts contained spermatogonia and spermatogenic cells (Fig. 5b, d) (Campbell et al. 2003; Doroshov et al. 1997; Flynn and Benfey 2007). The expression of *Advava* RNAs was restricted to germ cells, with the signal being strong in spermatogonia with shaping particles in cytoplasm, very weak in spermatocytes, and barely detectable in spermatid and elongated spermatid (Fig. 6g). Especially, the *Advava* signal was distributed as small particles in the cytoplasm of spermatogonia. Sense probes did not show reproducible staining (Fig. S3d).

Differential germ cell-specific expression of *AdVasa* proteins in gonad

We further took immunofluorescence co-localization of proliferating cell nuclear antigen (PCNA) and *AdVasa* to investigate its expression and subcellular localization in gonads. In the ovary, *AdVasa* proteins were limited to germ cells (Fig. 7a), and *AdVasa*-positive cells simultaneously displayed strong PCNA signals in the nucleus (Fig. 7b). Surprisingly, the germinal vesicle of primary oocytes was also stained with PCNA. Moreover, *AdVasa* was present not only in the cytoplasm but also in the nucleus. A closer inspection uncovered that the *AdVasa* signal was faint in both cytoplasm and nucleus of undifferentiated oogonia (og1), and strong in the cytoplasm and weak in the nucleus of differentiating oogonia (og2; Fig. 7e). In particular, *AdVasa* proteins were abundant in both the ooplasm and nucleus of stage I–II oocytes. Furthermore, the expression of *AdVasa* in the cytoplasm of stage I oocytes was higher than that in the nucleus. The pre-immune serums and pre-adsorbed antibody did not have reproducible staining (Fig. S4).

In the testis, *AdVasa* proteins were also detected exclusively in germ cells but not found in somatic cells (Fig. 7i), and spermatogonia exhibited strong and condensed PCNA staining. The *Vasa* signal was peaked in spermatogonia, slightly decreased in the primary spermatocytes, faint in secondary spermatocytes, and barely detectable in spermatid and elongated spermatid, where it was only detected in the cytoplasm. Noticeably, *AdVasa* in spermatogonia and primary spermatocytes concentrated into speckles in the cytoplasm (Fig. 7i).

Visualization PGCs by co-microinjection mRNA

To determine the competence of labeling Dabry's sturgeon PGCs, GFP-*Drnanos3* 3'UTR and RFP-*Advava* 3'UTR mRNA were co-injected into the vegetal pole of 1- to 4-cell stage embryos. Though weak green fluorescence was observed throughout embryos before the neural tube closed (stage 23), strong GFP-positive cells demonstrated antecedently as PGCs

was distributed around the tail bud on the yolk ball (Saito et al. 2014) (Fig. 8a'; Table 2). Meanwhile, the red fluorescence displayed nearly the same expression pattern as the green fluorescence (Fig. 8a''), suggesting that RFP-*Advava* 3'UTR mRNA had the ability to tag the PGCs of Dabry's sturgeon. With embryos, during the development to tail rudiment separating stage (stage 26), PGCs migrated dorsally and separated into two clusters, at the left and right sides of the embryonic body (Fig. 8b–b'''). In some cases, PGCs were just split into one side (data not shown). At the heart rudiment presented as the long tube stage (stage 28), PGCs kept on moving dorsally, and were much closer to the embryonic body (Fig. 8c–c'''). In addition, the fluorescence of PGCs was much more intense than earlier. At the hatching stage (stage 35), PGCs aligned at the dorsal of the peritoneal cavity where the gonad would form (Fig. 8d–d'''). In summary, Dabry's sturgeon PGCs could be visualized and traced by microinjection of RFP-*Advava* 3'UTR mRNA during embryogenesis.

Since PGCs can be labeled by injecting artificial mRNAs consisting of the GFP/RFP fused to *vasa* 3'UTR from fish in some species, it is significant to address how widely the function of *vasa* 3'UTR sequence is conserved among fish. In addition, it is known that sturgeon is a basal actinopterygian fish. Consequently, RFP-*Advava* 3'UTR mRNA was injected into zebrafish and medaka embryos. In zebrafish, GFP-*Drnanos3* 3'UTR mRNA was used as a positive control to indicate PGCs. As shown in Fig. 9 and Table 2, at 26 h post-fertilization, PGCs with green fluorescence also exhibited intense red fluorescence, suggesting the ability of RFP-*Advava* 3'UTR mRNA to label zebrafish PGCs, though a high level of RFP expression was found in the embryonic body. On the other hand, RFP-*Advava* 3'UTR and GFP-*Olvasa* 3'UTR mRNA was co-injected into medaka embryos. However, medaka PGCs with green fluorescence were not observed with red fluorescence (Fig. 10; Table 2), demonstrating that RFP-*Advava* 3'UTR mRNA had no competence to mark medaka PGCs.

Discussion

This study has reported the isolation and characterization of seven isoforms of *vasa* homologue in Dabry's sturgeon. Multiple alignments revealed that all deduced amino acid sequences of *vasa* transcripts possessed the conserved characteristics of *Vasa* homologs, and shared high identity with *Vasa* protein from other species. Phylogenetic analysis showed that *AdVasa1* clustered in a teleost *Vasa* clade. This was the first time that the *Vasa* sequence in sturgeon has been obtained, displaying germ cell-specific expression in the gonads. Together, these sequences supported that *vasa* isoforms isolated in Dabry's sturgeon was a true *vasa* homolog, and its protein was conserved during evolution.

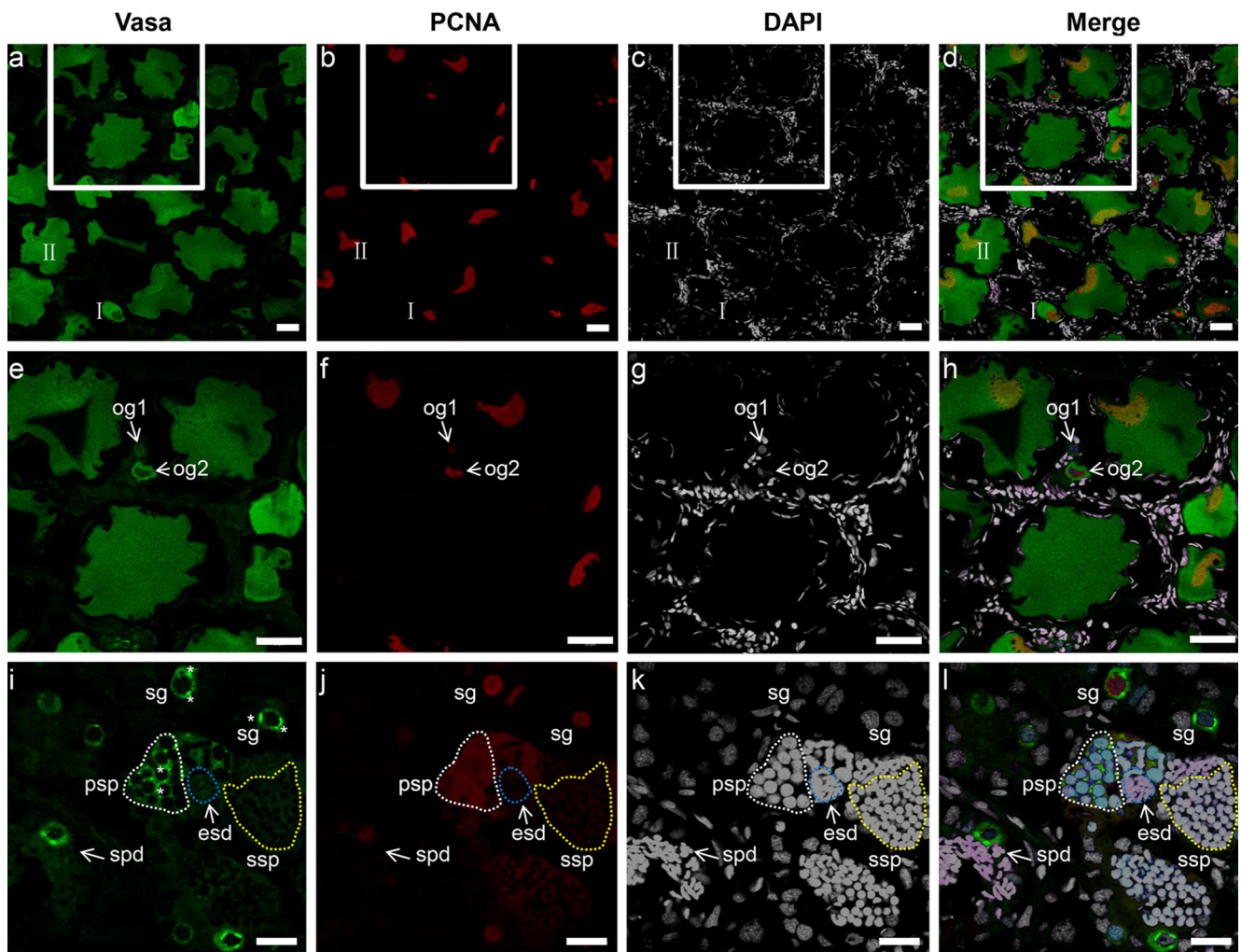


Fig. 7 Immunofluorescence co-localization of Dabry's sturgeon Vasa and PCNA in the gonad. The sections of ovary and testis were stained *green* for Vasa protein, *red* for PCNA protein and *blue* for DAPI. The ovary of 3-year-old Dabry's sturgeon stained by the anti-Vasa and anti-PCNA antibodies, respectively (**a**, **b**). Nuclei were stained by DAPI (**c**). Merge of *AdVasa* signal, PCNA signal and DAPI staining (**d**, **h**, **l**). **e–h** Higher magnification of the boxed areas in (**a–d**), respectively. The testis of Dabry's sturgeon stained by the anti-*AdVasa* and anti-PCNA antibodies,

respectively (**i**, **j**). Nuclei were stained with DAPI (**k**). The signal of *AdVasa* in spermatogonia and primary spermatocytes concentrated into speckles (*) in cytoplasm (**i**). *Roman numerals* indicate stages of oocytes. *og1* undifferentiated oogonia; *og2* differentiating oogonia; *sg* spermatogonia; *psp* primary spermatocytes; *ssp* secondary spermatocytes; *spd* spermatid; *esd* elongated spermatid. Bars (**a–h**) 50 μ m, (**i–l**) 20 μ m

Different isoforms of Vasa homologues have also been identified in other fish. Two *vasa* variants were found in tilapia (Kobayashi et al. 2002), zebrafish (Krovel and Olsen 2004) and the rare minnow (Cao et al. 2012), while more than two transcripts were characterized in Senegalese sole (Pacchiarini et al. 2013), Japanese flounder (Wu et al. 2014b), tongue sole (Wang et al. 2014) and Lusitanian toadfish (Ubeda-Manzanaro et al. 2014). Most isoforms from each species displayed slight sequence differences at the N-terminus. Similarly, the seven variants detected in the present study still varied only in the N-terminal region which was important for the function of post-translational modification and subcellular localization of Vasa (Liang et al. 1994), and even for *vasa* expression (Wolke et al. 2002). Subsequently, it was

reasonable to question how these isoforms generated in each species. In zebrafish (Yoon et al. 1997), tilapia (Kobayashi et al. 2002) and tongue sole (Wang et al. 2014), only one *vasa* gene locus was found in their genome, suggesting that their different transcripts were derived from alternative splicing. Thus, further study is required to discover how many *vasa* loci are in Dabry's sturgeon genome, facilitating the investigation of different *Advasa* variants.

In teleosts, the *vasa* gene is maternally inherited and mainly expressed in the germ line. The embryonic expression of *Advasa* isoforms except for *Advasa4* indicated that they were maternally supplied, since they were sustained in abundance from unfertilized eggs to the gastrula stage. A similar expression pattern was also observed in other fish. The *Advasa4* was

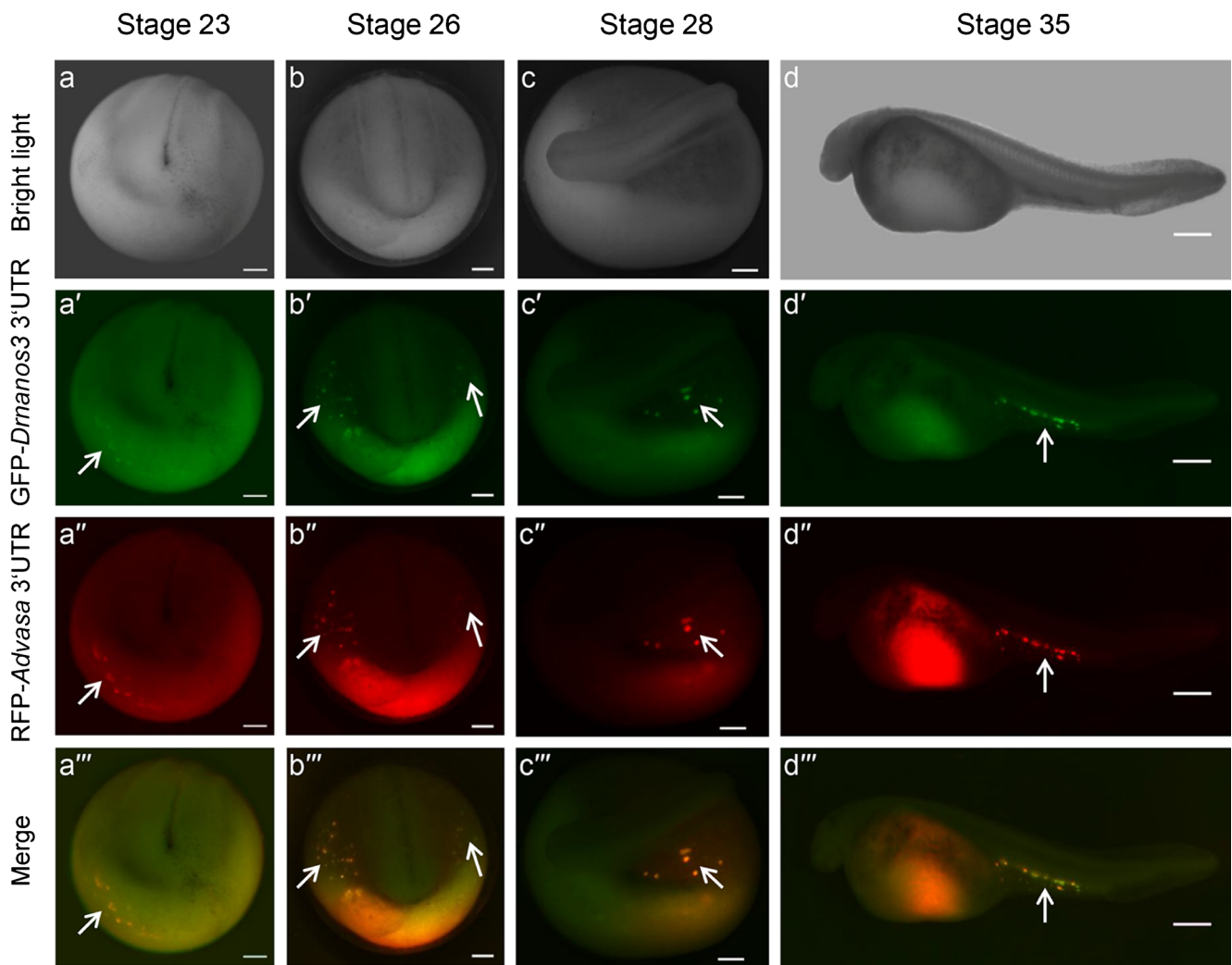


Fig. 8 Migration of labeled PGCs in Dabry’s sturgeon embryo. The GFP-*Drnanos3* 3’UTR and RFP-*Advava* 3’UTR mRNA were co-injected into the vegetal pole of 1- to 4-cell stage embryos of sturgeon. **a** Neural tube closed stage (stage 23). **b** Tail rudiment separating stage (stage 26). **c** Heart rudiment presented as long tube stage (stage 28). **d** Hatching stage (stage 35). Bars (a–c’’) 500 μm, (d–d’’) 1 mm

not detected during embryogenesis, but found in the ovary of immature individuals. This also happened in Senegalese sole,

which has four *vasa* isoforms, *Ssvasa1–4* (Pacchiarini et al. 2013). *Ssvasa1* and *Ssvasa2* were maternal deposits, while

Table 2 Number of embryos with fluorescence-labeled PGCs after co-injection of GFP-*Drnanos3* 3’UTR and RFP-*Advava* 3’UTR mRNA or GFP-*Olvvasa* 3’-UTR and RFP-*Advava* mRNA into the 1- to 4-cell stage embryos

Species	Experimental group	Number of embryos	Number of embryos developed normally (%)	Number of embryos with labeled PGC (%)	
				GFP	RFP
Sturgeon	injected	135	94 (69.6)	74 (78.7)	74 (78.7)
	control	92	75 (81.5)		
Zebrafish	injected	180	168 (93.3)	161 (95.8)	161 (95.8)
	control	120	113 (94.2)		
Medaka	injected	96	88 (91.7)	84 (95.5)	0 (0)
	control	90	83 (92.2)		

The data were summarized after three experiments. The fluorescence-labeled PGCs of Dabry’s sturgeon, zebrafish and medaka embryos were examined at tail rudiment separating stage, 26 h post-fertilization and 32 somite stage, respectively. The PGCs of Dabry’s sturgeon and zebrafish displayed both green and red fluorescence, while medaka PGCs exhibited only green fluorescence

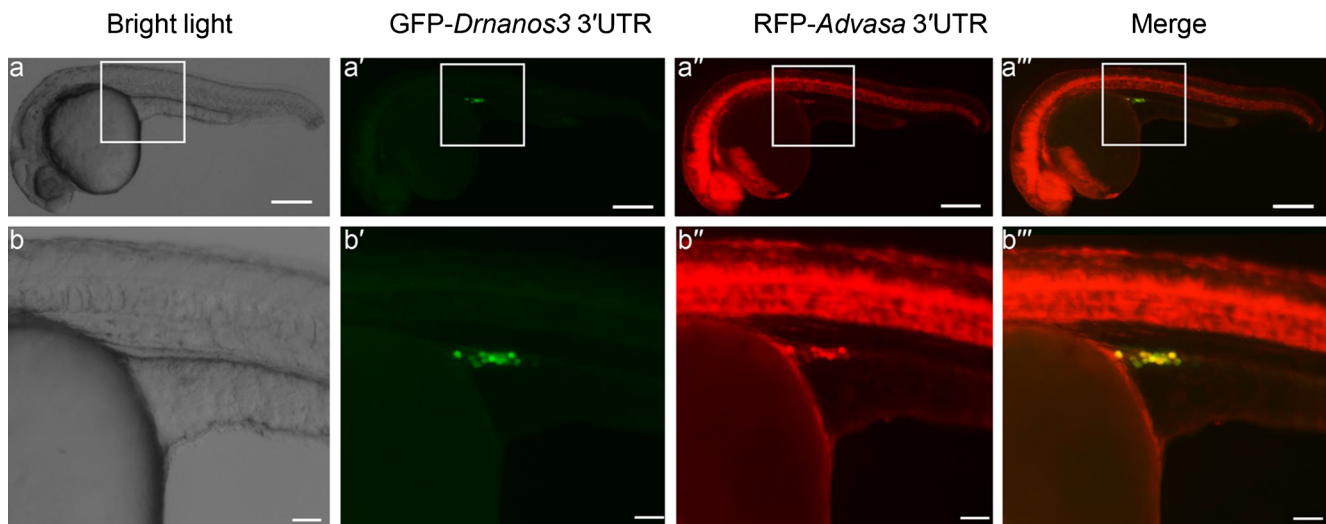


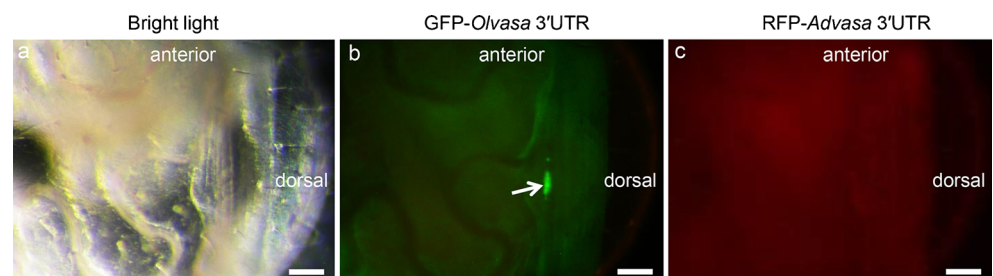
Fig. 9 The visualization of zebrafish PGCs by co-injection GFP-*Drnanos3* 3'UTR and RFP-*Advasa* 3'UTR mRNA at 26 h post-fertilization. **b–b'''** Higher magnification of the boxed areas in (a–a'''), respectively. Bars (a–a''') 250 μ m, (b–b''') 50 μ m

Svasa3 and *Svasa4* started to express in juveniles, indicating their function in PGCs and germinal cell proliferation. On the other hand, tissue distribution analysis in immature Dabry's sturgeons revealed that *Advasa* transcripts were predominantly found in the ovary and testis, and also detected in the liver, intestines, spleen, kidney, heart and brain. Low expression levels of *vasa* in the extragonadal tissues was described in rainbow trout (Yoshizaki et al. 2000), European sea bass (Blazquez et al. 2011), Senegalese sole (Pacchiarini et al. 2013), tongue sole (Wang et al. 2014) and Lusitanian toadfish (Ubeda-Manzanaro et al. 2014). This was ascribed to Vasa helicase activity referring to translational regulation of mRNAs involved in the specification and differentiation of other tissue-specific cell types (Ikenishi and Tanaka 2000). At the protein level, several *AdVasa* isoforms were present in the ovary, but only one *AdVasa* isoform was observed in the testis. However, it was possible that there were several *AdVasa* isoforms in both ovary and testis, due to the small difference of molecular weight among each isoform. This also occurred in Siberian sturgeon (Psenicka, et al. 2015), where two Vasa isoforms were found in the ovary, and one was in the testis. The inconsistency of *Advasa* expression in gonads at RNA and protein level was possible because not all *AdVasa* isoforms were translated in the ovary and testis, showing the

post-transcriptional regulation of *Advasa*. However, no *AdVasa* was discovered in the somatic tissues, which was not in agreement with its mRNA expression. The potential reason was that, compared with *AdVasa* expression in gonads, its amount in somatic tissues was too small to detect, or that *AdVasa* isoforms were not translated in somatic tissues. Additionally, sexual dimorphic expression of *vasa* variants has also been reported. Zebrafish had long and short isoforms of *vasa* in the ovary, and only short transcript in the testis (Krovel and Olsen 2004), while the rare minnow had long and short variants of *vasa* in the testis, and only a long variant in the ovary (Cao et al. 2012). In tilapia, *vas* and *vas-s* variants were both detected in the ovary and the testis, with *vas* mainly expressed in the testis and *vas-s* abundant in the ovary (Kobayashi et al. 2002). There were ten *vasa* isoforms, *vasa A–J*, in Japanese flounder, and all of them were found in the testis and only *vasa A*, *vasa B* and *vasa D* were observed in the ovary (Wu et al. 2014b). In contrast, all seven *vasa* variants of Dabry's sturgeon were present in the ovary, while merely *Advasa3*, *Advasa5* and *Advasa6* were identified in the testis. In summary, *Advasa* variants displayed differential expression in the gonads.

The germ cell-specific expression of the *vasa* gene is usually conserved in teleosts. In many fish ovaries, the *vasa* signal

Fig. 10 The visualization of medaka PGCs by co-injection GFP-*Olnos3* 3'UTR and RFP-*Advasa* 3'UTR mRNA at 32 somite stage. Bars 100 μ m



was strong in oogonia and early vitellogenic oocytes, and decreased with oocyte development (Braat et al. 1999; Kobayashi et al. 2000; Lin et al. 2012b; Shinomiya et al. 2000; Xu et al. 2014). In the present study, the expression of Dabry's sturgeon *vasa* was investigated in the early developing oocytes. The signal of *Advava* mRNA and protein was faint in undifferentiated oogonia (og1), drastically increased in differentiating oogonia (og2), and intense in primary oocytes, similar to some extent to that of gibel carp (Xu et al. 2005) and Asian seabass (Xu et al. 2014). In gibel carp, oogonia exhibited a low level of *vasa* mRNA but a high level of its protein, suggesting the differentially translational regulation of *vasa* in self-renewing germ stem cells, where a similar case was happening in primary spermatocytes of Dabry's sturgeon. A significant observation described in this study was that there was both cytoplasmic and nuclear expression of *Advava* mRNA and protein in differentiating oogonia and primary oocytes. Presently, the nuclear expression of *vasa* in teleosts was only identified in the oogonia of Asian seabass (Xu et al. 2014). Three types of oogonia, og1, og2 and og3, were isolated in Asian seabass, showing cytoplasmic and nuclear expression in og1 and chiefly cytoplasmic expression in og2 and og3. The nuclear expression of *vasa* was possibly associated with the mechanism of intron retention (Xu et al. 2014) or the function of DEAD-box proteins in pre-mRNA splicing and nuclear export (Linder 2006). Moreover, there was also another difference of *vasa* expression in ovary between Dabry's sturgeon and many other fish. The *Advava* mRNA and protein were distributed evenly in the oogonia and stage I–II oocytes, while those of other teleosts were generally spread uniformly in the oogonia or in the oogonia and stage I oocytes, and were concentrated in the perinuclear area or patches in stage I, stage II and even stage III oocytes (Cao et al. 2012; Kobayashi et al. 2000; Lin et al. 2012b; Xu et al. 2005, 2014). This may be attributed to the cytoplasm dispersion of the sturgeon mitochondrial cloud in early developing oocytes (Ye et al. 2015). Nevertheless, we cannot exclude the possibility that *Advava* has that characteristic distribution in late oocytes. In addition, Siberian sturgeon Vasa expression in early gonads was detected by human Vasa-antibody (Rzepakowska and Ostaszewska 2014), which aggregated as particles in the cytoplasm of oogonia, condensed in the perinuclear region of late pachytene oocytes, and then both dispersed in the cytoplasm and concentrated in the perinuclear region of stage II oocytes. The expression difference of Vasa in the ovary between Dabry's and Siberian sturgeon may be attributed to the sensitivity of the method used and species specificity, where the human Vasa-antibody applied in the Siberian sturgeon had no positive reaction in Dabry's sturgeon (data not shown). Interestingly, both Dabry's and Siberian sturgeon primary oocytes were observed with strong PCNA staining (Rzepakowska and Ostaszewska 2014). Similar results were reported in zebrafish, explained by the high synthesis of

rDNA in primary oocytes (Korfsmeier 2002). On the other hand, in the testis of most fish (Kobayashi et al. 2000; Lin et al. 2012b; Nagasawa et al. 2009; Rzepakowska and Ostaszewska 2014; Shinomiya et al. 2000; Xu et al. 2005, 2014), the *vasa* signal was strong in the spermatogonia, decreased in the spermatocytes, and was hardly detectable in spermatid and elongated spermatid. Such expression patterns were also found in Dabry's sturgeon at the RNA and protein level. Remarkably, *Advava* mRNA and protein concentrated into speckles, like the mitochondrial cloud (MC) structure, in the cytoplasm of both spermatogonia and primary spermatocytes. Likewise, Siberian sturgeon Vasa also formed aggregations in the cytoplasm of spermatogonia (Rzepakowska and Ostaszewska 2014), and nanogold immunoelectron microscopy revealed that medaka Vasa co-localized with MC in spermatogonia, and intermixed with MC in primary spermatocytes (Yuan et al. 2014), suggesting that the speckles in the spermatogonia and primary spermatocytes of sturgeon were more likely with the MC. Surprisingly, Dabry's sturgeon *vasa* mRNA and protein was exclusively localized in the cytoplasm of spermatogenic germ cells, not like its expression in the ovary, which will require further analysis in future. In brief, *Advava* mRNA and protein exhibited mitotic and meiotic expression in females, and mainly displayed mitotic expression in males. Moreover, they were present in both the cytoplasm and nucleus of the ovarian germ cells, but only in the cytoplasm of testicular germ cells. Additionally, a specific Vasa antibody for Dabry's sturgeon germ cells was obtained, which would be a useful marker to perform the preparation and purification of donor spermatogonia or oogonia for germ cell transplantation or cryopreservation.

It has been reported that *vasa* 3'UTR plays an important role in stabilizing its RNA in PGCs but not in soma (Wolke et al. 2002). Moreover, this function was conserved across fish species, resulting in it being applied to visualizing PGCs with a GFP construct using *vasa* 3'UTR from highly diverged taxonomic groups. For example, in zebrafish, rainbow trout and Nibe croaker, chimeric RNAs constructed by fusing GFP to their *vasa* 3'UTR, successfully labeling each other PGCs (Yoshizaki et al. 2005). Furthermore, medaka PGCs were visualized by GFP-*vasa* 3'UTR mRNA of tongue sole (Huang et al. 2014) and red seabream (Lin et al. 2012a). Our results also suggested that Dabry's sturgeon *vasa* 3'UTR could stabilize the RFP RNA in PGCs, and that the mechanism of *vasa* 3'UTR RNA localization function was conserved between Dabry's sturgeon and zebrafish, where their relationship was very remote. However, not all fish PGCs could be labeled by chimeric GRP RNA containing *vasa* 3'UTR from other fish. For instance, medaka GFP-*vasa* 3'UTR mRNA could not identify PGCs of zebrafish and loach (Saito et al. 2006), though it was able to visualize the medaka PGCs. Previous research has demonstrated that

the loss or variation of *vasa* 3'UTR RNA localization in euteleosts occurred after the euteleosts (including medaka, tongue sole, trout, etc.) and ostariophysan (including zebrafish, etc.) diverged (Knaut et al. 2002). In this study, GFP-*Advasa* 3'UTR mRNA failed to tag PGCs of medaka, indicating the differential machinery of *vasa* 3'UTR RNA localization between these two species. These, together with the visualization of medaka PGCs by GFP chimeric mRNA containing tongue sole or red seabream *vasa* 3'UTR, and the visualization of zebrafish PGCs by trout or Nibe croaker GFP-*vasa* 3'UTR mRNA, suggest that the mechanism of *vasa* 3'UTR RNA localization in euteleosts is complicated, and most likely to vary during the divergence of euteleosts and ostariophysan, which requires further study in the future.

In our study, the PGC migration pattern of Dabry's sturgeon was revealed by injecting its RFP-*Advasa* 3'UTR mRNA into embryos, similar to that of sturgeon injected with GFP-*Drnanos* 3'UTR mRNA (Saito et al. 2014), demonstrating that RFP-*Advasa* 3'UTR mRNA is able to label and trace PGCs in Dabry's sturgeon. In detail, sturgeon PGCs were first identified near the tail bud on the yolk ball, then migrated dorsally and separated into two clusters, at the left and right sides of the embryonic body. Sometimes, PGCs concentrated at just one side, possibly due to the uneven dispersion of mRNA after injection into the embryos. Subsequently, PGCs moved to the genital ridge by way of the mesenchyme. Such a migration pattern is similar to that of numerous teleosts (Saito et al. 2006). Moreover, a PGC transplantation assay between sturgeon and goldfish proved that PGC migration machinery is conserved between these two distantly related species (Saito et al. 2014). The ability of chimeric mRNA to label and trace PGCs in Dabry's sturgeon is extremely useful for PGC cryopreservation and the conservation of endangered species by germ cell transplantation technologies (Okutsu et al. 2006; Psenicka et al. 2015; Saito and Psenicka 2015; Yoshizaki et al. 2005).

In conclusion, the present study identified *vasa* isoforms in the Dabry's sturgeon. The *Advasa* transcripts except for *Advasa4* were maternal deposits, and their mRNA and protein were mainly expressed in the gonad of both sexes, displaying sexually dimorphic expression. Further, *Advasa* mRNA and protein showed mitotic and meiotic female expression, and mainly displayed mitotic expression in males; they exhibited both cytoplasmic and nuclear expression in the ovary, but exclusively cytoplasmic expression in the testis. Moreover, the chimeric RFP mRNA containing *Advasa* 3'UTR has the ability to label PGCs in Dabry's sturgeon and zebrafish, but not in medaka. Additionally, the PGC migration pathway of Dabry's sturgeon was identified. In summary, these results provide especially useful information for *vasa* function and conservation of Dabry's sturgeon by germline engineering.

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