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Identification of a germ cell marker gene, the *dead end* homologue, in Chinese sturgeon *Acipenser sinensis*

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ABSTRACT

Dead end (*dnd*) encodes an RNA-binding protein that is essential for primordial germ cell (PGC) migration and gametogenesis in vertebrates. In this study, a Chinese sturgeon *Acipenser sinensis dead end* homologue, designated *Asdnd*, was identified and characterized. The full-length cDNA of *Asdnd* was 1630 base pairs (bp) and encoded a peptide of 396 amino acid residues. Multiple sequence alignment showed that *As*Dnd shared six conserved regions of Dnd orthologs, including the RNA recognition motif. Phylogenetic analysis revealed that *As*Dnd was grouped with teleosts. By quantitative real-time PCR analysis, the *Asdnd* transcripts were found to originate from the maternal parent and be specifically expressed in gonads of immature Chinese sturgeons of both sexes. Fluorescent *in situ* hybridization indicated that *Asdnd* transcripts were restricted to germ cells. In the testis, *Asdnd* was abundant in spermatogonia and tended to gradually diminish in late spermatogenic stages, while strong signals were found in primary oocytes, as opposed to oogonia, in the ovary. Zebrafish PGCs were clearly visualized at 24 h post-fertilization by co-injecting RFP-*Asdnd* 3′ UTR and GFP-*nos*3 3′ UTR mRNA, indicating that *dnd* 3′ UTR has a conserved function among teleosts. Therefore, *dnd* could serve as a germ cell marker in Chinese sturgeon.

1. Introduction

Primordial germ cells (PGCs) are the cells that eventually differentiate into gametes, producing spermatozoa in males and eggs in females, which transmit genetic information from one generation to the next (Wylie, 1999). In many animals, PGCs segregate from the soma in early development and migrate to the developing gonad, and the specification and development of PGCs depends on germ plasm, a specialized region of cytoplasm in the embryo (Saffman and Lasko, 1999). *Dead end* (*dnd*) is a germ plasm component in vertebrates that is crucial for zebrafish PGC migration and survival (Weidinger et al., 2003). The gene has been subsequently isolated in diverse vertebrates, including mouse, *Xenopus*, chicken, and medaka (Youngren et al., 2005; Horvay et al., 2006; Aramaki et al., 2007; Liu et al., 2009). In mouse, mutations of the *dnd* gene were shown to cause germ cell loss and increase the incidence of testicular germ cell tumor (Youngren et al., 2005). When the *Xenopus dnd* translation was inhibited, PGCs failed to migrate normally and eventually became undetectable (Horvay et al., 2006). Chicken *dnd* is reported to be specifically expressed in PGCs during embryogenesis and can be used to trace the migration of PGCs (Aramaki et al., 2007).

These results suggest that the role of *dnd* in PGC development is conserved during evolution, but the expression of *dnd* in the adult organism appears to differ with sex. *Xenopus dnd* transcripts are limited to the adult ovary (Horvay et al., 2006). On the contrary, mouse *dnd1* expression is restricted to germ cells of adult testis (Bhattacharya et al., 2007). The adult expression of medaka *dnd* occurs in germ cells of both sexes (Liu et al., 2009). This expression pattern was also found in turbot *Scophthalmus maximus* (Lin et al., 2013). It has been reported that *dnd* could distinguish type A spermatogonia in the Pacific bluefin tuna *Thunnus orientalis*, a basic step in spermatogonial stem cell transplantation (Yazawa et al., 2013).

Research has shown that *dnd* is exclusively expressed in PGCs during embryogenesis, and its 3′-untranslated region (UTR) could play a role in directing PGC-specific expression of the protein (Slanchev et al., 2009). In zebrafish, PGCs could be visualized by injecting artificial mRNA constructed by fusing zebrafish *nanos3* 3′ UTR with a green fluorescent protein (GFP) sequence, namely GFP-*nos*3 3′ UTR (Koprunner et al., 2001). The function of *nanos3* 3′ UTR is shown to be conserved, as PGCs of other teleost species can be observed by injection of GFP-*nos*3 3′ UTR into

Abbreviations: bp, base pairs; PGCs, primordial germ cells; MS-222, 3-aminobenzoic acid ethyl eater methanesulfonate-222; FISH, fluorescent *in situ* hybridization; RACE, rapid amplification of cDNA ends; qRT-PCR, quantitative real time-polymerase chain reaction; hpf, hour post-fertilization; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate buffer solution.

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their embryos (Saito et al., 2006). The mechanism responsible for *nanos3* mRNA stabilization in PGCs was shown related to the interaction of microRNA and Dnd (Mishima et al., 2006; Kedde et al., 2007). However, the use of *dnd* 3′ UTR to identify PGCs of different fish species has not been reported.

Chinese sturgeon *Acipenser sinensis* is a large anadromous bony fish belonging to Acipenseriformes. Populations of Chinese sturgeon have declined dramatically due to over-fishing, construction of hydroelectric dams, pollution, and other anthropogenic factors that destroyed their habitat in the late 20th century (Birstein et al., 1997; Wei et al., 1997). The sturgeon is listed as a Category I protected species in China and classified as critically endangered in the International Union for Conservation of Nature and Natural Resources (IUCN) Red list. Efforts have been made to conserve the species through a program of controlled reproduction which will make a significant contribution to Chinese sturgeon protection (Wei et al., 2013).

However, Chinese sturgeon is an extremely late and asynchronous sexually maturing species, which makes restoration more difficult. Recent developments in biotechnology, including isolation and transplantation of germ cells from donor into a host species lacking germ cells, for surrogate reproduction, has opened the possibility of efficient and sustainable aquaculture and conservation of endangered species (Yamaha et al., 2007; Xu et al., 2010). Identification and isolation of germ cells are the basic and critical steps in this technology. A recent study demonstrated that sturgeon PGCs can be labeled by injecting GFP-*nos*3 3′ UTR into the vegetal pole of embryos (Saito et al., 2014). In Chinese sturgeon, a *nanos*-related gene (*nanos1*) was isolated and the subcellular localization of *As*Nanos1 protein in the gonads was reported (Ye et al., 2012a,b). Nevertheless, little is known about germ cell specific genes in sturgeon.

We isolated a candidate germ cell marker in Chinese sturgeon, the *dead end* homologue (*Asdnd*), and analyzed its molecular characteristics and tissue expression patterns. Fluorescent *in situ* hybridization demonstrated the cellular distributions of *Asdnd* transcripts in the testis and ovary. In addition, the function of *dnd* 3′ UTR was investigated by co-injecting RFP-*Asdnd* 3′ UTR and GFP-*nos*3 3′ UTR into zebrafish embryos.

2. Materials and methods

2.1. Fish and samples

Chinese sturgeons used in this study were cultured at Taihu station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Fertilized eggs were obtained by controlled propagation in November, 2012. Fish, approximately 3 years old, were deep-anesthetized with 0.05% MS-222 (Sigma, USA). Tissue samples were rapidly collected and immediately dipped into liquid nitrogen and stored at −80 °C for quantitative real-time PCR. Gonad tissue used for fluorescent *in situ* hybridization was surgically obtained from a 10 year old male and a 4.5 year old female Chinese sturgeon. The gonad development stages were classified as described (Chen et al., 2006). The testis was at stage III, containing many seminal vesicles and spermatocytes and few spermatids and spermatogonia. The ovary was at stage II and mainly composed of oocytes with a few dispersed oogonia. The zebrafish embryos used for injection were provided by State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences. The experimental procedures were conducted in compliance with standards of the Chinese Council on Animal Care.

2.2. Total RNA extraction and SMART cDNA synthesis

Total RNA was extracted from the ovary of a 3 year old Chinese sturgeon with the SV total RNA isolation system (Promega, USA). The RNA quality and purity were evaluated by Nanodrop 2000 (Thermo Scientific, USA). The SMART cDNA library was synthesized from 1 μg of ovarian total RNA by using Super SMART™ PCR cDNA Synthesis Kit (Clontech, USA) according to the manufacturer's instructions.

2.3. Isolation of Asdnd cDNA sequence

The full-length *Asdnd* cDNA was amplified by 5′ and 3′ rapid amplification of cDNA ends (RACE). First, a cDNA fragment of ~200 bp was amplified from the ovarian cDNA library with degenerate primers designed using the highly conserved amino acid sequences. This fragment-deduced amino acid sequence was most similar to Dnd protein and was used to design gene-specific primers. Following instructions for the SMARTer RACE cDNA Amplification Kit (Clontech, USA), 5′ RACE was performed with the gene-specific primer (*Asdnd*-5′-R1 and *Asdnd*-5′-R2) and in combination with a 5′ PCR anchor primer (5′-AP, Table 1). The 3′-end of *Asdnd* cDNA was amplified using gene-specific primers (*Asdnd*-3′-F1 and *Asdnd*-3′-F2) and a PCR anchor primer corresponding to the terminal anchor sequence of the cDNA (3′-AP, Table 1). All PCRs were performed on a PTC-200 thermal cycler (Bio-Rad, USA). Denaturation at 94 °C for 3 min was followed by 35 cycles of amplification at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by an additional extension at 72 °C for 10 min. The RACE products were electrophoresed on a 1.2% agarose gel and cloned into pMD18-T vector (Takara, Japan) after purification. Finally, the full length cDNA of *Asdnd* was validated by sequencing.

2.4. Sequence analysis

The cDNA and deduced amino acid sequences were analyzed by the BLAST program (NCBI, http://www.blast.ncbi.nim.nih.gov/Blast.cgi). Multiple amino acid sequence alignments were run on the Vector NTI Suite 8. A phylogenetic tree was constructed using the neighbor-joining method on Mega 5.1.

2.5. Quantitative real-time PCR

For quantitative real-time PCR, total RNA extraction was performed as described from embryos at different stages of development (unfertilized egg, morula, blastula, gastrula, neurula, rudiment of heart, heartbeat, hatching) and from different tissues (liver, spleen, kidney, heart, testis, ovary, muscle, intestine, brain) of 3 year old Chinese sturgeons. Total RNA of each sample was reverse transcribed with

Table 1
Primers used in this study.

Name	Sequence (5′–3′)	Purpose
Degenerate_forward	CCCAGGTGAACGGCCA GMGNAARTAYGG	Partial
Degenerate_reverse	CCCGGTTCTGGCCGSWRAARTTCAT	
Asdnd-3′-F1	GGTGAACGGCCAGAGAAAATACGG	3′ RACE
Asdnd-3′-F2	CCCACCAGGTTGGACTGCATTC	
Asdnd-5′-R1	GCCTGAACTCATAGAGCACGCCCG	5′ RACE
Asdnd-5′-R2	CTGCTGGAACAAGGGGATGAGGCT	
3′-AP	GGTATCAACGCAGAGTACTT	RACE
5′-AP	ATCAACGCAGAGTACGCGGG	
Asdnd-RT-F	AACGGGTGCTGCTCCTCGATG	Quantitative real-time
Asdnd-RT-R	AGCGTGATGCGACACATACTGC	PCR
Asβ-actin-F	CCTTCTTGGGTATGGAATCTTGC	Control
Asβ-actin-R	CAGAGTATTTACGCTCAGGTGGG	
Asdnd-probe-F	GAACTTCAGCGGGCAGAATC	Probe
Asdnd-probe-R	TTATTACAAAAACTGAACATGC	
*Eco*RI-*Asdnd* 3′ UTR-F	GAATTCTTCATTATATCCTTAAAGCC AAC	Plasmid
*Xho*I-*Asdnd* 3′ UTR-R	CTCGAGTGTACTTACAAAAACTGA ACATGC	
*Bam*HI-RFP-F	GGATCCATGGTGTCTAAGGGCGAA GAG	Plasmid
*Eco*RI-RFP-R	GAATTCTCAATTAAGTTTGTGCCC CAGT	

PrimeScript RT reagent Kit With gDNA Eraser (Takara, Japan) as described (Ye et al., 2012a,b). A pair of primers *Asdnd*-RT-F and *Asdnd*-RT-R (Table 1) producing a 150 bp fragment was designed according to the cDNA sequence of *Asdnd*. Embryo and tissue expression of *Asdnd* were analyzed using quantitative real-time PCR performed on DNA Engine Chromo 4 real-time system (Bio-Rad, USA) with SYBR green real-time PCR master mix (Bio-Rad, USA) according to the manufacturer's protocol. As an internal control, *β-actin* from Chinese sturgeon was amplified by the primers *Asβ-actin*-F and *Asβ-actin*-R (Table 1). Each sample was run in triplicate along with the *Asβ-actin*. The relative expression level of *Asdnd* was calculated by the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008).

2.6. RNA probe synthesis and fluorescent in situ hybridization

We designed a pair of primers (Table 1) used for probe synthesis. A fragment of *Asdnd* cDNA including 3′ UTR was obtained by PCR and inserted into pGEM-T Easy Vector (Promega, USA) and sequenced. The recombinant plasmid was linearized for the synthesis of anti-sense or sense probe with T7 or SP6 polymerase by using the FITC RNA Labeling Kit (Roche, Switzerland). The probes were treated with RNase-free TURBO DNase (Ambion, USA). The Chinese sturgeon gonads used for fluorescent *in situ* hybridization were immediately fixed in 4% paraformaldehyde in PBS (pH = 7.4) at 4 °C overnight. Dehydration for storing and rehydration prior to sectioning were carried as described (Ye et al., 2012a,b). The samples were subsequently embedded in Optimal Cutting Temperature (O.C.T., Sakure) and sectioned at 8 μm with freezing microtome (Leica, Germany). Fluorescent *in situ* hybridization on sections was performed as described (Li et al., 2011). Nuclear staining was done by using DAPI for 10 min at room temperature, and the slides were mounted for microscopy after washed in PBS. Microscopy and photography were performed on a confocal microscope (Zeiss, Germany).

2.7. Plasmids and microinjection

The recombinant plasmid of RFP-*Asdnd* 3′ UTR was obtained by inserting fragments (RFP-coding sequence and *Asdnd* 3′ UTR sequence) into the pCS^{2+} vector (Table 1). Capped sense mRNA of RFP-*Asdnd* 3′ UTR was synthesized *in vitro* using the mMessage Machine kit (Ambion, USA) and stored at −80 °C before use. Zebrafish embryos at 1-cell stage were injected with RFP-*Asdnd* 3′ UTR and GFP-*nos*3 3′ UTR mRNA as described (Koprunner et al., 2001). The injected embryos were monitored and photographed by fluorescent stereomicroscopy (Leica, Germany).

3. Results

3.1. Cloning and characterization of Asdnd

We cloned the full-length cDNA of *Asdnd* from the ovary of Chinese sturgeon using degenerate primers and RACE strategy (accession no. KM655832). A cDNA fragment was obtained using degenerate primers that were designed on the basis of two conserved regions by alignment of Dnd proteins. RACE strategy was subsequently performed to produce the full-length cDNA of *Asdnd*. It was 1630 bp in total length and contained a 64 bp 5′ UTR, an open reading frame (ORF) of 1191 bp for 396 amino acids (aa), and a 375 bp 3′ UTR containing a presumptive miR-430 target site (Fig. 1).

The predicted *As*Dnd possessed an RNA recognition motif (RRM), characteristic domain of *dnd* homologues, which showed high identities across species. A multiple Dnd protein sequence alignment was performed, including fish and a representative of tetrapod (Fig. 2A). In addition to the typical RRM, the Dnd proteins were shown to possess five conserved motifs including an N-terminal (NR) region and four C-terminal regions (CR1–4). The Dnd amino acid sequence identity between Chinese sturgeon and other vertebrate species was 36.0–44.3%. A partial 3′ UTR alignment of *Asdnd*, *Xenopus dead end* and zebrafish *nanos1* was analyzed, revealing a miR-430 binding consensus sequence (Fig. 2B). A molecular phylogenetic tree of Dnd was constructed by the neighbor-joining method. Dnd proteins formed two distinct clades, the fish clade and the tetrapod clade (Fig. 3). Obviously, the Chinese sturgeon Dnd homologue clustered to the fish clade, but it was separate from all other fishes.

3.2. Expression of Asdnd during embryogenesis

In many organisms, including zebrafish and medaka, *dnd* RNA is maternally inherited. In this study, the expression of *Asdnd* RNA during embryogenesis, found in unfertilized eggs, morula, blastula, gastrula, neurula, rudimentary heart, heartbeat, and at hatching, was analyzed by quantitative real-time PCR. The *Asdnd* transcripts were present at high levels in early developing stages through gastrula, with an apparent subsequent decrease (Fig. 4A). The highest expression level was detected in the unfertilized eggs, evidence that the *Asdnd* was provided by maternal parent.

3.3. Tissue distribution of Asdnd RNA

Tissue distribution of *dnd* RNA in immature Chinese sturgeon was examined by quantitative real-time PCR. The *Asdnd* transcripts were detected only in gonad and were absent in somatic and brain tissue (Fig. 4B). In addition, its expression level in ovary was 2.23-fold that in testis.

3.4. Germ cell-specific expression of Asdnd RNA

Fluorescent *in situ* hybridization was performed to examine the *Asdnd* cellular distribution in testis and ovary. The *Asdnd* transcripts were found only in germ cells and were absent in surrounding somatic cells (Fig. 5). In this study, the testis of adult Chinese sturgeon was at stage III and contained germ cells at varying stages of spermtogenesis. Hybridization signals were abundant in spermatogonia and primary spermatocytes, decreased in secondary spermatocytes and spermatids, and weak in elongating spermatids (Fig. 5A, B, and C). The ovarian tissue examined was at stage II and did not undergo meiosis. At this stage, the ovary was mainly composed of primary oocytes and a small number of oogonia. In contrast to testis, the *Asdnd* transcripts were weak in the cytoplasm of oogonia, but strong in that of primary oocytes (Fig. 5D, E, and F). Therefore, the *Asdnd* RNA expression was germ cell specific in both sexes of Chinese sturgeon. No signals were found in testis or ovary with the *Asdnd* sense RNA probe (Fig. S1).

3.5. Visualization of zebrafish PGCs by microinjection

To determine whether the *Asdnd* 3′ UTR could identify PGCs, we co-injected RFP-*Asdnd* 3′ UTR and GFP-*nos*3 3′ UTR mRNA into the zebrafish embryos. The latter is known to visualize PGCs effectively in teleosts (Saito et al., 2006). In early embryonic development, RFP was expressed uniformly, similar to GFP (data not shown). Specific RFP expression was observed in a limited number of cells at the somitogenesis stage and became more pronounced at 24 hpf (Fig. 6B and F), we observed GFP signal at the same position (Fig. 6C and G). Cells that showed stronger fluorescence appeared in the majority of the injected embryos. Thus, we conclude that *Asdnd* 3′ UTR could identify PGCs in zebrafish.

4. Discussion

In this study, we identified a *dead end* homologue in Chinese sturgeon as a germ cell marker. In addition, embryonic and tissue-specific expression was detected by quantitative real-time PCR. Gonad *Asdnd* RNA expression was analyzed by fluorescent *in situ* hybridization,

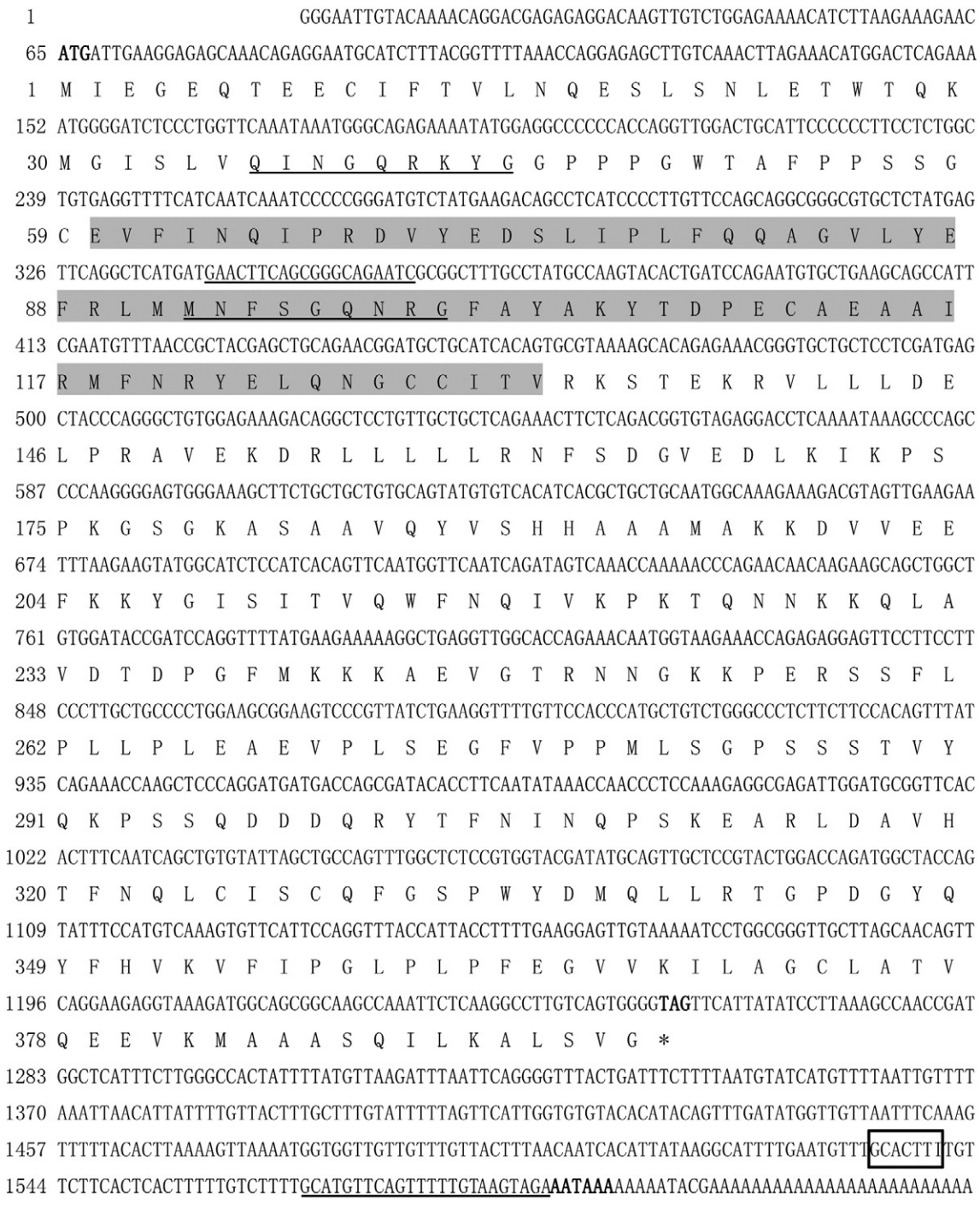

Fig. 1. Nucleotide and deduced amino acid sequences of Chinese sturgeon *dnd*. Initiation codon, termination codon, and putative polyadenylation signal are in bold. Underlined sequences are amino acids used for degenerate primers and nucleotide sequences used for RNA probe preparation. The RNA recognition motif is shaded gray. The presumptive micro-RNA binding sequence is boxed.

which, for the first time, revealed the expression pattern of the *dnd* gene in sturgeon. Co-injection of RFP-*Asdnd* 3′ UTR and GFP-*nos*3 3′ UTR mRNA indicated that *Asdnd* 3′ UTR was able to identify zebrafish PGCs.

A. sinensis is a functional tetraploid species, thus more than one isoform of a specific gene should be present, possibly the result of genome polyploidization (Zhang et al., 1999). However, in our study, only one *Asdnd* gene was identified by multiple times of PCR cloning and sequencing with a pair of primers covering the full-length cDNA of *Asdnd*. Besides, only one *Asdnd* gene was found in the Chinese sturgeon gonad transcriptome constructed by Illumina sequencing (unpublished data). It is worth the effort to explore further whether more *dnd* isoforms were present in sturgeons. *As*Dnd showed high sequence identity to other Dnd proteins. It possessed the characteristic RRM, which is the main functional domain and one of the most abundant protein domains in eukaryotes (Maris et al., 2005). Four conserved regions (CR1–4) were found in the C terminus of *As*Dnd. In zebrafish, Dnd was demonstrated to possess ATPase activity that was required for normal PGC development and survival (Liu and Collodi, 2010). The ATPase domain is located in the last 91 aa of C terminus covering the conserved regions of CR3 and CR4. In other words, CR3 and CR4 are closely associated with the core element of zebrafish Dnd ATPase. Thus, in Chinese sturgeon, CR3 and CR4 of *As*Dnd may play an important role in its proper

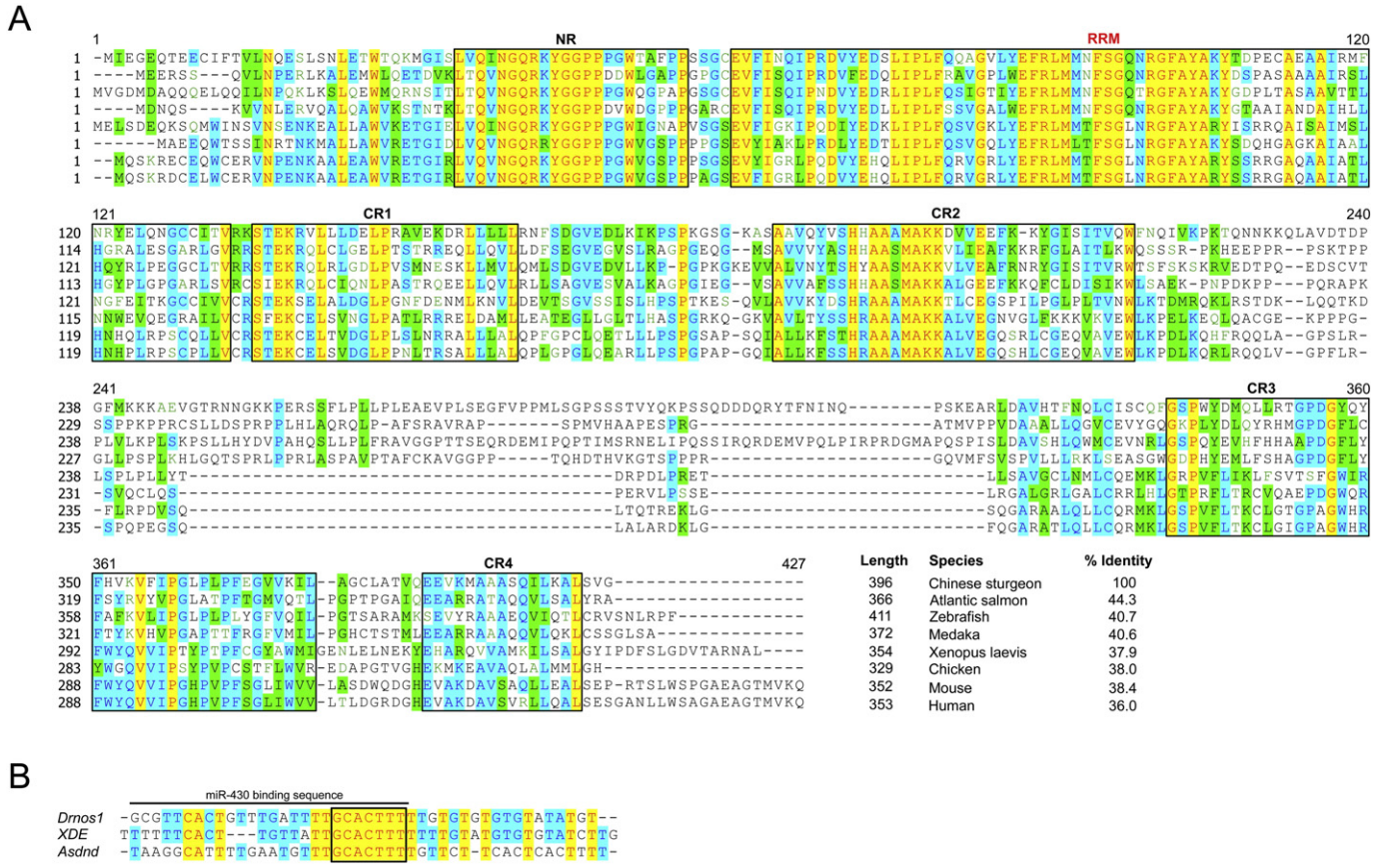

Fig. 2. (A) Multiple amino acid sequence alignment of *As*Dnd and other vertebrate Dnd proteins. (B) Partial alignment of *Asdnd* 3′ UTR with that of zebrafish *nanos1* and *Xenopus dead end*. All the other sequences of Dnd homologues were obtained from NCBI. Multiple alignment was carried out with AlignX. The conserved motifs are indicated in the frame. NR, N-terminal region; RRM, RNA recognition motif; CR1–4, C-terminal regions. See Fig. 3 for accession numbers. MiR-430 target motif is shown in the box.

function during PGC development. Acipenseriformes occupy a special place in the classification and evolution of fishes and are known as an out-group of teleosts (Bemis et al., 1997). Based on phylogenetic analysis of the *dnd* gene, two distinct clades were found to be formed in vertebrates, associated with the separation of fish and tetrapod lineages. The Chinese sturgeon Dnd homologue belongs to the fish clades, but is separate from other teleosts. The conservation of *As*Dnd might suggest a similar function in other species.

Previous studies have shown that *dnd* mRNA is supplied maternally. In medaka, *dnd* RNA is present at high levels until the morula and apparently decreases thereafter (Liu et al., 2009). A similar pattern of *dnd* mRNA has been reported in turbot and Atlantic salmon *Salmo salar* (Lin et al., 2013; Nagasawa et al., 2013). In the present study, quantitative real-time PCR showed that *Asdnd* mRNA was also maternally expressed. A high level of *Asdnd* transcripts was detected in the unfertilized eggs, declining to a low level post-gastrula. Thus, the embryonic expression of *Asdnd* mRNA is consistent with that of other fish species.

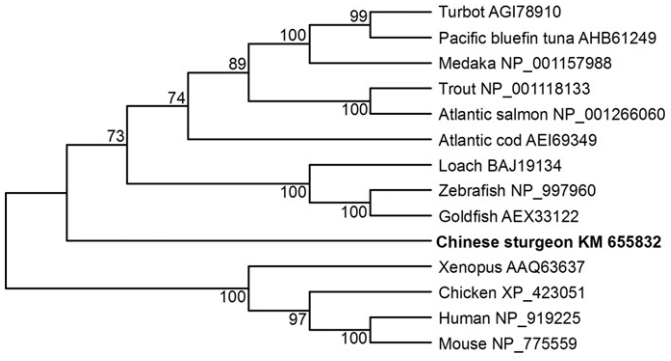

Fig. 3. Phylogenetic tree of Dnd proteins. The phylogenetic relationship of Dnd proteins was analyzed by Mega 5.1 with the neighbor-joining method (1000 replicates). Bootstrap values are given. Species names are followed by sequence accession numbers.

The expression pattern of *dnd* in gonads appears to be dependent on the sex in some tetrapods including *Xenopus* and mouse (Bhattacharya et al., 2007). However, *dnd* is expressed in both sexes of chicken, medaka, Atlantic salmon, Pacific bluefin tuna, and turbot (Aramaki et al., 2007; Liu et al., 2009; Lin et al., 2013; Nagasawa et al., 2013; Yazawa et al., 2013). In the present study, *Asdnd* transcripts were found in both testis and ovary. Strong *Asdnd* signals were observed in spermatogonia and primary spermatocytes, while the fluorescent *in situ* hybridization signal weakened at the late stages of spermatogenesis. Similar results were found in medaka and turbot (Lin et al., 2013), suggesting that the *dnd* gene may play an important role in spermatogenesis. *Asdnd* transcripts were weakly detected in oogonia and abundant in primary oocyte. In medaka, the strongest *Odnd* signals were also observed in early oocytes, as opposed to oogonia, in slide samples (Liu et al., 2009). We concluded that *Asdnd* RNA is exclusively expressed germ cells in both male and female Chinese sturgeons.

Several genes associated with PGC development are conserved across fish species, even in those that are distantly related (Saito et al., 2006, 2011). In this study, zebrafish PGCs were clearly visualized by co-injecting RFP-*Asdnd* 3′ UTR and GFP-*nos*3 3′ UTR mRNA. Because of their special position in phylogeny, the origin and migration of sturgeon PGCs have interested many scientists. Using GFP-*nos*3 3′ UTR mRNA to label sturgeon PGCs, resent research has revealed that the mode of PGC specification in sturgeon is similar to that of anurans, whereas the migration pattern resembles that of teleosts (Saito et al., 2014). We observed both RFP and GFP signal cells as tight clusters located proximal

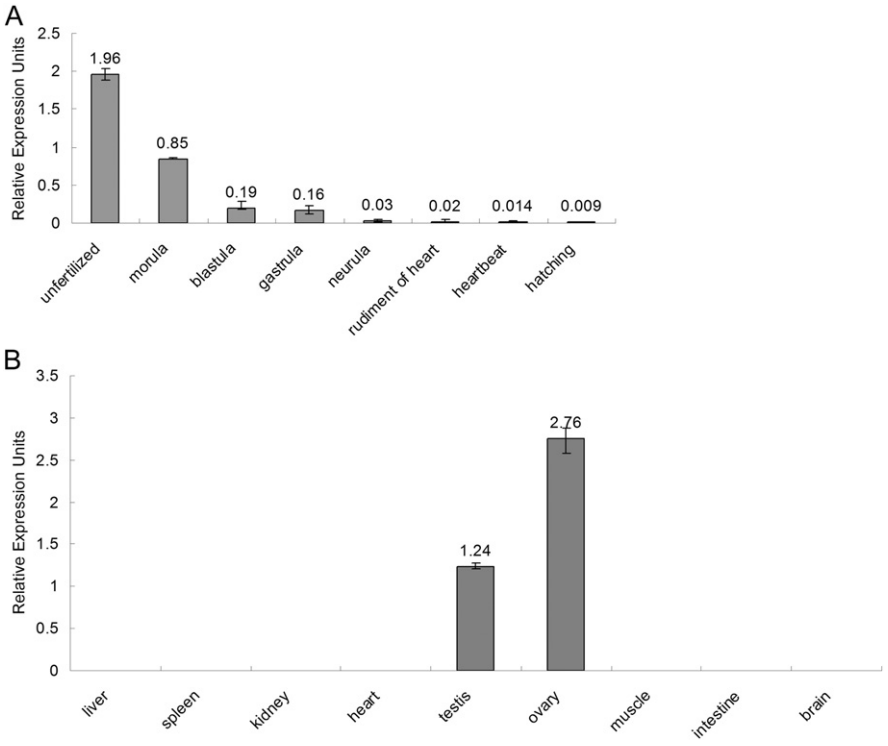

Fig. 4. Quantitative real-time PCR of dnd expression in Chinese sturgeon. (A) Developing embryos. (B) Tissues. The relative expression units are shown at the top of each column. The data were normalized to *β-actin* mRNA and represented mean ± SD of three separate experiments.

to the junction of the yolk ball and yolk tube after 24 hpf (Fig. 6). Therefore, *Asdnd* could be used as a germ cell maker in sturgeons for further study of PGCs.

Similar to zebrafish *nanos1*, the 3′ UTR of *Asdnd* was stabilized in zebrafish PGCs (Fig. 6), suggesting the conserved mechanism for maintenance of *Asdnd* 3′ UTR in zebrafish germ cells. It has been reported that

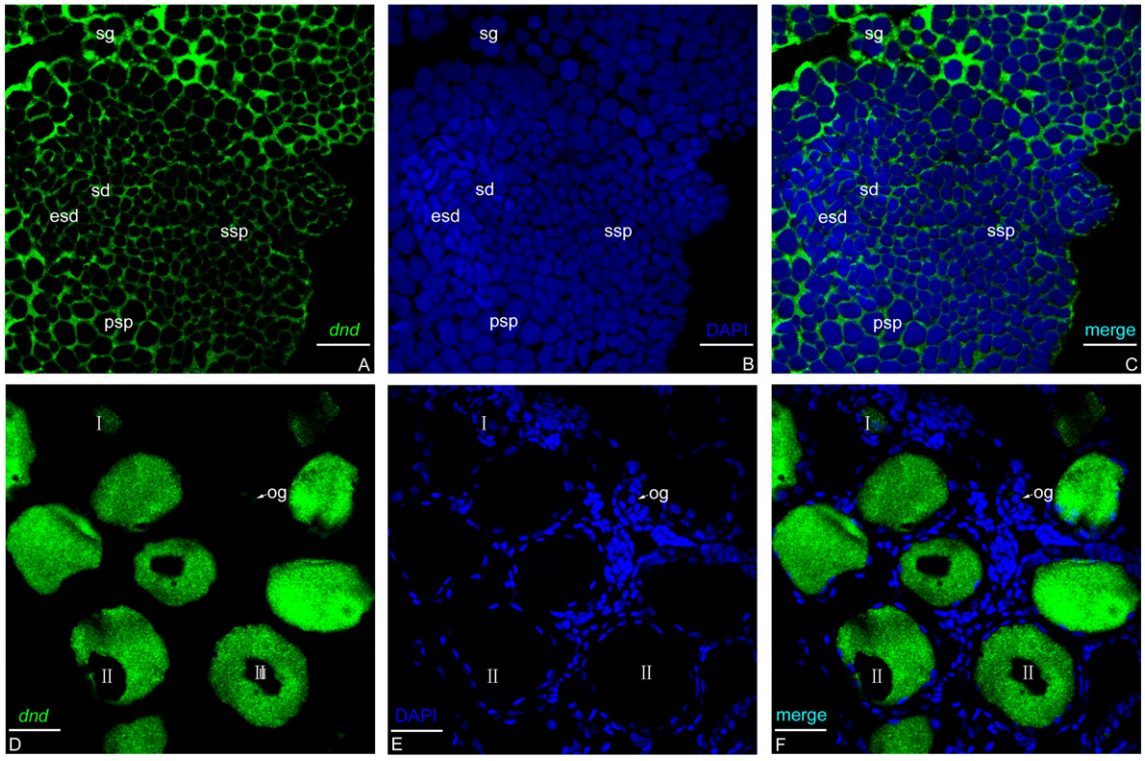

Fig. 5. *Asdnd* RNA expression in the testis and ovary by fluorescent *in situ* hybridization. The cryosections of testis and ovary were hybridized with antisense *Asdnd* RNA probes by fluorescent procedure (green). Nuclei were stained blue with DAPI. Merge of the *Asdnd* signal and DAPI staining are shown in (C) and (F). sg, spermatogonia; psp, primary spermatocyte; ssp, secondary spermatocyte; sd, spermatid; esd, elongating spermatid; og, oogonia; Roman numerals, stages of oocytes; scale bars, 20 μm in (A–C) and 50 μm in (D–F).

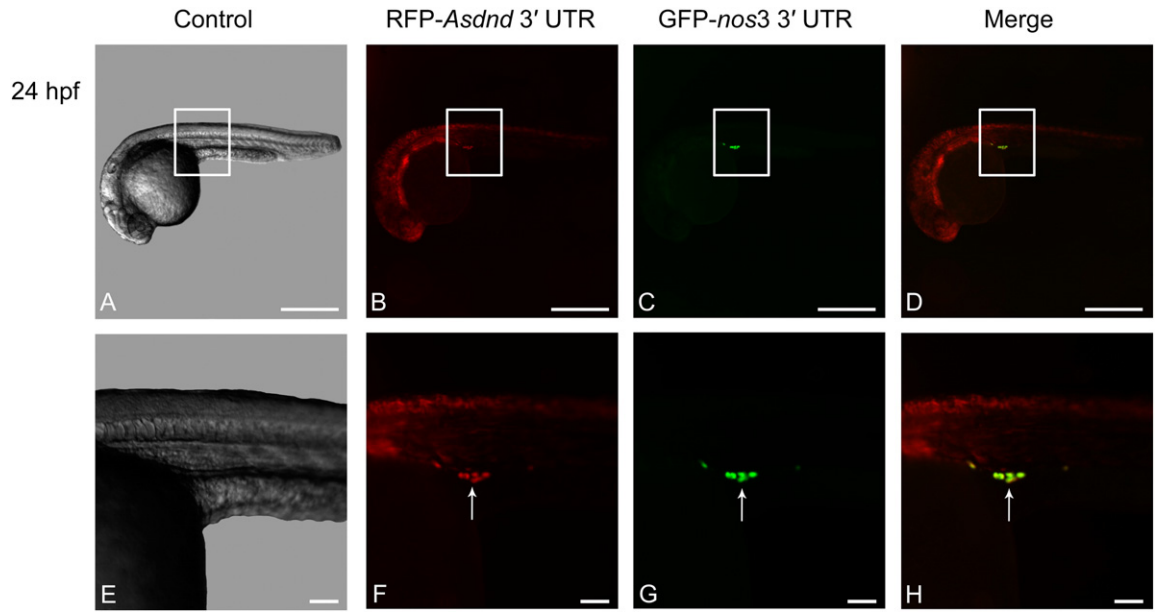

Fig. 6. The visualization of PGCs by co-injection of RFP-*Asdnd* 3′ UTR and GFP-*nos*3 3′ UTR mRNA into zebrafish embryos. (E–H) are magnification of the boxes in (A–D), respectively. Arrows indicate PGCs. Scale bars, 500 μm in (A–D) and 50 μm in (E–H).

the 3′ UTR of zebrafish *nanos1* is subjected to degradation in somatic cells but stabilized in PGCs by interaction with the micro RNA, miR-430 (Giraldez, 2006; Mishima et al., 2006). Furthermore, zebrafish Dnd was revealed to be involved in the mechanism through binding to the 3′ UTR of *nanos1* to prevent miR-430-mediated RNA suppression (Kedde et al., 2007). In addition, zebrafish Dnd ATPase plays a role in protecting *nanos1* and *TDRD7* transcripts (Liu and Collodi, 2010). In previous studies, injection of GFP-*dnd* 3′ UTR showed zebrafish PGCs to specifically express GFP, but zebrafish *dnd* 3′ UTR was shown to lack a miR-430 binding sequence (GCACUUU), which suggested that the mechanisms were independent of miR-430 regulation (Mishima et al., 2006; Slanchev et al., 2009). However, unlike zebrafish *dnd*, *Asdnd* 3′ UTR was found to contain a putative miR-430 binding site (Figs. 1, 2B), which was presumably responsible for the mechanism that eliminated the *Asdnd* transcript from somatic cells. In *Xenopus*, it has been reported that the vegetal localization elements (LE) of *Xenopus dead end* (*XDE*) 3′ UTR included a perfect match with the miR-430 target motif, and further research revealed that *XDE-LE* mRNA appeared to be targeted by *Xenopus* miR-18 for germ cell specific stabilization and somatic clearance (Koebernick et al., 2010). Thus, *Asdnd* could depend on a microRNA-mediated mechanism, as does zebrafish *nanos1* and *Xenopus dnd*.

In conclusion, this study identified a *dead end* homologue in Chinese sturgeon (*Asdnd*) and revealed that *Asdnd* was maternally expressed and restricted to the male and female gonads. Fluorescent *in situ* hybridization showed that *Asdnd* RNA expression was germ cell specific in both sexes of Chinese sturgeon. Zebrafish PGCs were visualized by injection with RFP-*Asdnd* 3′ UTR mRNA. Our results provided a useful marker for the further research in sturgeon germ cells, which will be valuable for biotechnological approaches to reproduction.

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