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Dazl and *dnd* Identify Both Embryonic and Gonadal Germ Cells in Chinese Hook Snout Carp (*Opsariichthys bidens*)

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Abstract: In recent years, the Chinese hook snout carp (*Opsariichthys bidens*) is becoming increasingly popular due to its high nutritional value and delicious taste. However, anthropogenic influences have caused a rapid reduction in its population. Considering long-term development sustainability, it is essential to conduct research on its reproductive development. In this study, the germ-cell related genes *dazl* (*Obdazl*) and *dnd* (*Obdnd*) were cloned, and their expression patterns were revealed. RT-PCR analysis indicated that *Obdazl* and *Obdnd* were specifically expressed in gonads. In the testis, *Obdazl* and *Obdnd* RNAs have a similar expression pattern: their transcripts were abundant in spermatogonia, gradually decreased, and eventually disappeared during spermatogenesis. Early oocytes in the ovary exhibited strong expression of *Obdazl* and *Obdnd* RNAs and reduced remarkably after peak expression at III oocytes. Surprisingly, *Obdazl* RNA was dynamically found in perinuclear spots that finally aggregated into the Balbiani body (Bb) in the early oocytes. Moreover, *Obdazl* 3′ UTR enabled the reporter gene to be expressed stably in medaka PGCs, suggesting that *dazl* was identified as the germ cell marker in *O. bidens*, which may provide perspectives for further studies on the development of PGCs in the future.

Keywords: *Opsariichthys bidens*; germ cells; PGCs; *dazl*; *dnd*

Key Contribution: (1) *Obdazl* and *Obdnd* were specifically expressed in both male and female gonads of *Opsariichthys bidens*. (2) *Obdnd* and *Obdazl* were enriched in spermatogonia and spermatocytes. (3) *Obdazl* was concentrated in the Balbiani body in ovary of *Opsariichthys bidens*. (4) *gfp-Obdazl* 3′ UTR mRNA could label medaka PGCs.

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1. Introduction

Germ cells are of crucial importance in the capability of sexually reproducing organisms to produce bisexual gametes (sperm and eggs), which ensures the transmission of genetic information. Their progenitor cells, called primordial germ cells (PGCs), in Osteichthyes, become independent at the early stage of embryonic development and perform the functions associated with reproductive development. While the initial site of PGCs generation may vary slightly among species, they ultimately migrate into the gonads [1,2]. The formation, specification, and differentiation of PGCs are different across species, which are regulated by a series of genes related to reproduction. Among these, *dead-end* (*dnd*) plays a critical role in the specification and development of the PGCs, while *dazl* can indirectly regulate the germ cell differentiation, growth, and maturation through the interaction of target mRNAs with proteins [3,4].

Dazl belongs to the DAZ (Deleted in Azoospermia) family, which is the earliest fertility factor found in humans. Within the DAZ family, there are three members: *daz*, *boule*, and

dazl, all of which are necessary regulators for sexual reproduction, especially spermatogenesis [5]. Among them, *boule* is expressed in meiotic cells, widely distributed from invertebrate fruit flies (*Drosophilid*) [6] and nematodes (*Caenorhabditis Elegans*) [7] to mice (*Mus musculus*) [5] and humans [8]. *Daz*, whose deletion may cause severe oligospermia or azoospermia, exists only in primates or higher mammals [7,9,10]. In vertebrates, *dazl* is expressed in primordial germ cells and/or in pre-meiotic and meiotic germ cells [4,11]. In medaka (*Oryzias latipes*), *dazl* is referred to as a gonadal marker and counts in the PGC formation. Knockdown of *dazl* cannot prevent the formation of PGCs, while the injection of *dazl* antibody induces a significant decrease in PGCs, even resulting in the failure of its formation [12]. In mice, it has been found that the protein DAZL can repress and activate mRNA translation during oocyte maturation [13]. Furthermore, overexpression of *dazl* shows testis-biased expression patterns in Hu sheep and can reprogram human Sertoli cells into male germline stem cells, suggesting that *dazl* plays a crucial part in male reproductive development [14,15].

Dnd was first identified in zebrafish as a vital component of germplasm. It is specifically expressed in germ cells, and when knocked down can result in abnormal migration of PGCs [16]. Its homologous genes are widely found in frogs (*Xenopus laevis*) [17], mice [18], chickens (*Gallus gallus*) [19], medaka [20], Chinese sturgeon (*Acipenser sinensis*) [21], Celebes medaka (*Oryzias celebensis*) [22], Atlantic salmon (*Salmo salar*) [23], Starry Flounder (*Platichthys stellatus*) [24], and Spinyhead croaker *(Collichthys lucidus)* [25]. In medaka and Celebes medaka, *dnd* is bisexually and differentially expressed in gonads. In the testis, *dnd* is abundant in spermatogonia and remarkably decreases and even disappears with spermatogenesis, but it is present throughout oogenesis. Moreover, *dnd* is regarded as a critical PGCs specifier, the knockdown of which by morpholino specifically abolishes PGCs, while its overexpression boosts PGCs via the increase in its precursors [20,22,26]. Attributed to the protection of *dnd* in the face of signals leading to somatic cell fate during PGCs migration, PGCs differentiate into gametes during embryogenesis, which ensures the fertility of the organism [27]. Recently, knockout of *dnd* utilizing CRISPR-Cas9 in *S. salar* helped to achieve sterile fish which successfully solve the genetic introgression in aquaculture, suggesting that sterile fish can also function as recipients for germ cell transplantation [3,23,28,29]. The PGCs-depleted gonads, due to the knockdown of *dnd*, benefit the function exploration of PGCs in hermaphroditic fish and eventually give support to the establishment of sex control techniques [30]. Furthermore, recent studies have shown that the protein Dnd1 is able to regulate the function of germ granules [31].

Chinese hook snout carp (*Opsariichthys bidens*) is a small, freshwater, economically farmed species with a delicious taste and high nutrition value, widely distributed in China [32]. However, due to the excessive fishing intensity and environmental destruction in recent years, the wild resources of *O. bidens* are very scarce. The conservation of its germplasm resources is an urgent problem to be solved. It is thereby imperative to increase the yield of *O. bidens* and strengthen the research on its artificial reproduction. However, studies on *O. bidens* focused mostly on its morphology, histology, genome assembly, and biological characteristics, suggesting that research on reproductive biology is urgently needed [33–37]. Our research group has recently succeeded in establishing a spermatogonial stem cell line capable of normal long-term culture in *O. bidens* [38]. Furthermore, a series of sex-related genes and sex-biased miRNAs have been revealed by us using RNA-Seq in *O. bidens* [36,39]. In this study, *Obdazl* and *Obdnd* complementary DNA were cloned, and RT-PCR results revealed that both were only expressed in gonads. For further research, to verify its ability to label PGCs, a chimeric mRNA of *gfp* and *O. bidens dazl* 3′ UTR (*gfp-Obdazl* 3′ UTR) was injected into medaka embryos by microinjection. The results revealed that *dazl* was a germline marker, which seemed to be an underlying gene affecting the process of germ cell development in *O. bidens*. In brief, the purpose of this study was to provide a new reference for the study of the potential role of reproductive development-related genes in the development of PGCs in fishes, as well as the exploration of genes for germ cell markers in *O. bidens*.

2. Materials and Methods

2.1. Fish and Sampling

The *O. bidens* used in this experiment were purchased from Shunchang County Zhaoxing Fish Breeding Co., Ltd. (Nanping, China) and subsequently reared in the recirculating aquaculture systems at Shanghai Ocean University (Shanghai, China). During this period, the *O. bidens* culture systems were maintained for stabilization (water temperature 26 ± 2 °C, dissolved oxygen > 6.2 mg/L, pH 7.4 ± 2) and the systems were cleaned regularly.

Three male and female adult *O. bidens* were used for adult tissue sampling. Fish are first anesthetized on the ice, and then several tissues (brain, eye, liver, kidney, gut, ovary, and testis) are dissected form them. At the same time, a pair of *O. bidens* with distinct secondary sex characteristics were selected for artificial insemination to obtain embryos at different developmental stages (2-cell, blastula, gastrula, neurula, 30 hpf, 48 hpf, and fry), and twenty embryos from each period were collected for subsequent experiments.

All experiments were strictly conducted in the direction of the guidelines of the Shanghai Ocean University Committee for Laboratory Animal Research.

2.2. RNA Isolation and cDNA Synthesis

Total RNA was extracted from embryos at different developmental stages and adult tissues using TRizol (Invitrogen, Carlsbad, CA, USA). RNA integrity was checked by 1% agarose gel electrophoresis, stained with ethidium bromide. The RNA quantity was examined by a NanoDrop 2000 c spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the 1 μg of total RNA was used to synthesize the corresponding cDNA by reverse transcription using the M-MLV Reverse Transcription Kit (Takara, Shiga, Japan) and the oligo dT_{18}.

2.3. Molecular Cloning and Analysis of Obdazl and Obdnd Gene

Previously, cDNA fragments corresponding to *Obdazl* and *Obdnd* have been obtained from the gonadal transcriptome that was described previously [35]. Subsequently, two pairs of specific primers were constructed from these fragments, respectively, and full-length cDNA of *Obdazl* and *Obdnd* were identified by the RACEs approach using the BD SMART RACEs kit (Clontech, Beijing, China). Following that, using the Vector NTI Advance 11.5 software, the Dazl and Dnd proteins were individually aligned across species, and the resulting alignment findings were employed in the MEGA 7.0 program and maximum likelihood method with 1000 repeat of bootstrap method analysis, respectively, to produce phylogenetic trees.

2.4. RT-PCR

To identify the expression of *Obdazl* and *Obdnd* in embryos and adult tissues, two sets of primers (Table 1) were constructed, as well as primers for *β-actin* as an internal reference (Table 1). Each PCR was performed in a reaction system with a volume of 25 μL for a total of 35 cycles, consisting of a denaturation step at 95 °C for 10 s, an annealing step at 58 °C for 10 s, and then an extension step at 72 °C for 1 min; these three steps are included in each cycle. The resultant products of PCR were separated using an agarose gel with a concentration of 1.5%, stained using ethidium bromide, and photographed using a bio-imaging system (Bio-Rad, Hercules, CA, USA).

Table 1. Sequences of the relevant primers used during the experiments.

Primer	Sequence (5′ to 3′ Direction)	Purpose
Obdazl-5′-R1	GGCAAGATGACGCCCAACACACT	5′ RACE
Obdazl-5′-NR1	ACTCAAACTGGGACCTGCCATCAT	
Obdazl-3′-F1	GTGAAAATCATCACTTATCGCGTT	3′ RACE
Obdazl-3′-NF1	CCTGCAGGATTTATGGTCCCACCGAT	

Table 1. *Cont.*

Primer	Sequence (5′ to 3′ Direction)	Purpose
Obdazl full F	ATGTATCAGGGCGTTAAGTTACC	CDS cloning
Obdazl full R	GACTCTGTTAACTCTTCTGTAG	
Obdazl 3′ UTR F	ctcgagTAGCTAACCCTTCATCCTTTTTTT	mRNA
Obdazl 3′ UTR R	ggtacctctagaGCAGTTAAAAGGTGTAAATA	
Obdnd 5′ R1	ATGGCGTGCGCTCAGACCGGC	5′ RACE
Obdnd 5′ NR1	ATCCTGCAGCAGCTGATGACAG	
Obdnd 3′ F1	CGAAGCCCATGTCCAACATGCG	3′ RACE
Obdnd 3′ NF1	CGTCCAGGACCAAGTGGCGAAC	
Obdnd full F	ATGGACGATTGGGAGGAGG	CDS cloning
Obdnd full R	CTCCCAGTTCTCCTCCTCGTCAG	
β–actin F	TTCAACAGCCCTGCCATGTA	Internal control
β–actin R	CCTCCAATCCAGACAGTAT	

2.5. RNA Synthesis and In Situ Hybridization

The *O. bidens* testis and ovary were embedded in Optimal Cutting Temperature (O.C.T Sakura, Torrance, CA, USA) after fixation in 4% paraformaldehyde (PFA) and graded dehydration with RNase-free sucrose. After embedding, the tissue was sectioned at the thickness of 6 µm using a freezing microtome Leica CM1950 (Leica, Wetzlar, Germany). The subcellular localization of *Obdazl* and *Obdnd* RNA was investigated by using both whole-mount in situ hybridization (WISH) and gonadal section in situ hybridization (SISH). Furthermore, the expression of *Obdazl* together with *Obdnd* in the ovary was also distinguished by dual fluorescence in situ hybridization (FISH). WISH, SISH, and FISH were carried out as previously described [40,41]. Briefly, two plasmids (pT-*Obdazl* and pT-*Obdnd*) were obtained by inserting the open reading frames (ORF) of *Obdazl* and *Obdnd* into the pGEM-T vector species (Promega, Fitchburg, WI, USA). The SP6/T7 promoter was employed to generate the sense and antisense probes, which were tagged with the FITC labeling kit (Roche, Basel, Switzerland) and Digoxigenin (DIG) labeling kit (Roche).

2.6. Preparation of Chimeric mRNAs and Microinjection

Construction of the pCSpf*Obdazl* 3′ UTR (*gfp-Obdazl* 3′ UTR) recombinant plasmid was completed after the *Clvasa* 3′ UTR in pCSpf*Clvasa* 3′ UTR (*gfp-Clvasa* 3′ UTR) had been replaced with the *Obdazl* 3′ UTR (amplified by the primer *Obdazl* 3′ UTR F&R) [42]. Capped mRNAs for microinjection were created from the fully linearized *gfp-Obdazl* 3′ UTR and *rfp-Drnos1* 3′ UTR by using the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA). The plasmid pCSch*Drnos1* 3′ UTR has been previously described [22]. Co-injection of the two chimeric mRNAs into one-cell-stage medaka embryos at a dose of 100 ng/µL resulted in the observation of PGCs.

2.7. Microscopy

The Ni-E microscope with a Nikon Ds-Ri2 camera (Nikon, Tokyo, Japan) was utilized in order to carry out microscopy and photography, as previously explained [43].

3. Results

3.1. Cloning and Characterization of Obdazl and Obdnd

Employing the RACEs method, the full-length cDNA sequences of *Obdazl* and *Obdnd* were cloned. A 1548 bp *Obdazl* cDNA was obtained, containing a 690 bp coding sequence (CDS), which encoded 229 amino acids and 3′ UTR 858 bp. It contained a DAZ domain and a conserved RNA recognition domain (RRM) (Supplementary Figure S1). The full-length *Obdnd* cDNA was 1592 bp in size, including 12 bp 5′ UTR, 1107 bp CDS encoding 368 amino acids, and 473 bp 3′ UTR, which included RNA recognition motif (RRM) as well as the domains, N-terminal region (NR), and C-terminal regions (CR1–4) (Supplementary Figure S2).

The resulting ObDazl protein was found to have the greatest degree of similarity to the Dazl ortholog in *Danio rerio* (90.83%) according to the results of multiple amino acid alignments, and the homology with that in humans was 28.89% (Supplementary Figure S3). ObDnd protein also had high homology with Dnd protein in *Danio rerio*, but its identity was only 60.83% (Supplementary Figure S4), which was significantly lower than that with ObDazl. ObDazl and ObDnd proteins were identified in two separate clades in fish and other vertebrates, according to phylogenetic tree analysis (Figure 1).

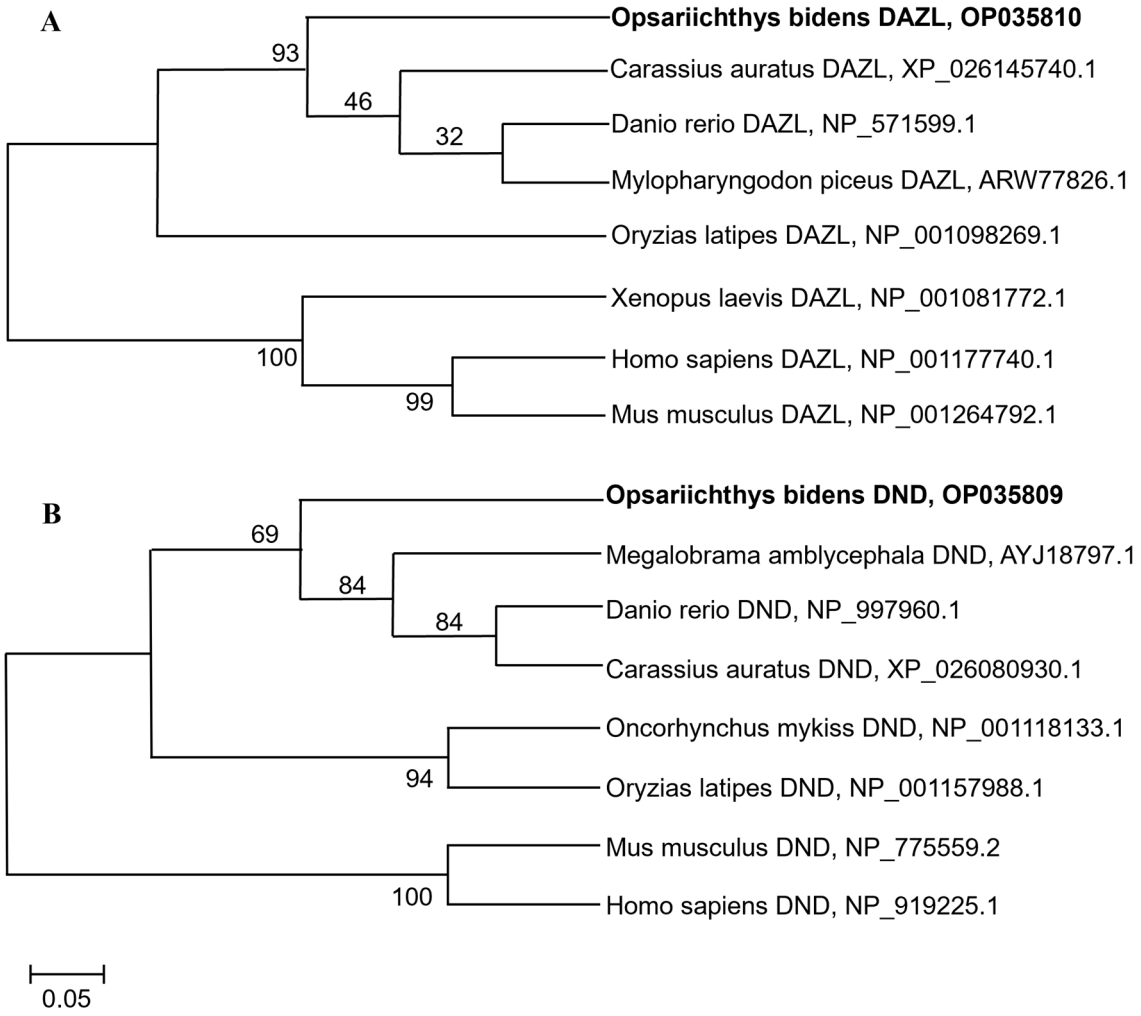

Figure 1. Phylogenetic tree of Dazl and Dnd proteins in different species. (**A**) Phylogenetic tree of Dazl proteins. (**B**) Phylogenetic tree of Dnd proteins. The sequences of Dazl and Dnd homologs were all obtained through NCBI. The bootstrap values are presented. The species name is followed by the accession number.

3.2. Germ Cell-Specific Expression of Obdazl and Obdnd RNAs

To explore the expression of *Obdazl* and *Obdnd*, RT-PCR was conducted. In adult tissues, *Obdazl* and *Obdnd* were strongly expressed in the ovary and testis, while neither was detected in body tissues including the brain, eye, liver, kidney, and gut (Figures 2A and 3A). In embryos, *Obdazl* was strongly expressed in the 2-cell stage and expressed constantly, while the expression of *Obdnd* gradually weakened during embryogenesis (Figures 2B and 3B).

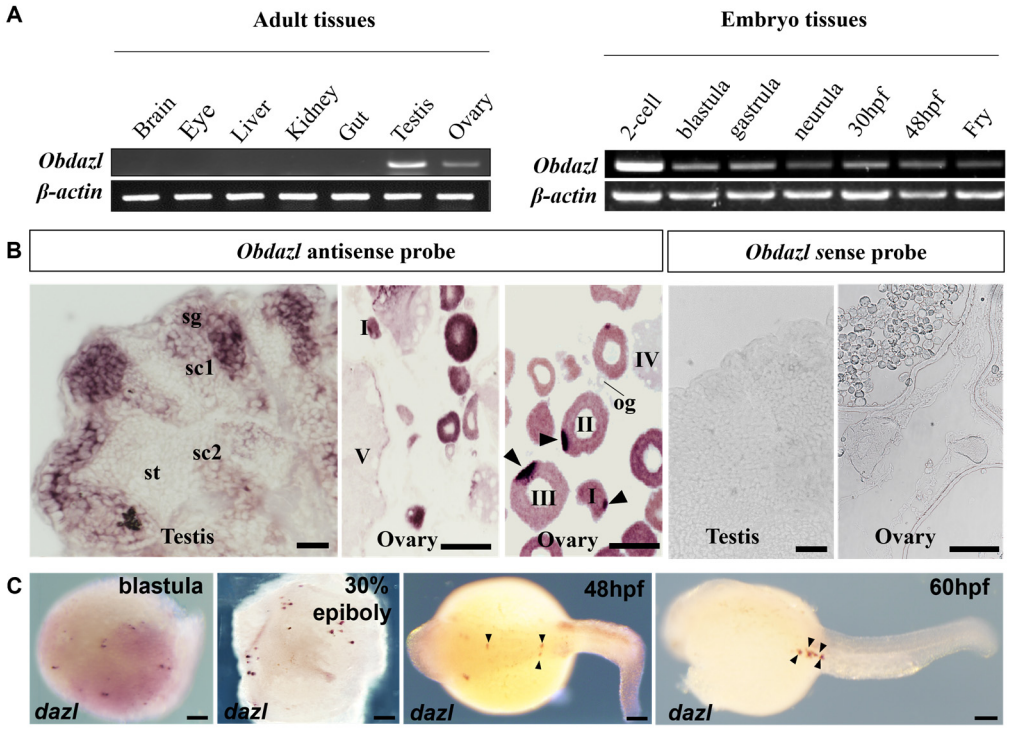

Figure 2. Expression of *Obdazl* in different tissues and embryos. (**A**) RT-PCR analysis of *Obdazl* in adult tissues and developmental embryos. *β-actin* served as an internal control. (**B**) SISH on the testis and ovary by using *Obdazl* antisense probe and *Obdazl* sense probe. sg, spermatogonia; sc1 and sc2, primary and secondary spermatocytes; st, spermatids; og, oogonia; I–V, stages of oocytes. (**C**) WISH on developmental embryos by using *Obdazl* antisense probe. The arrow pointed to the PGC. Scale bar, 200 μm.

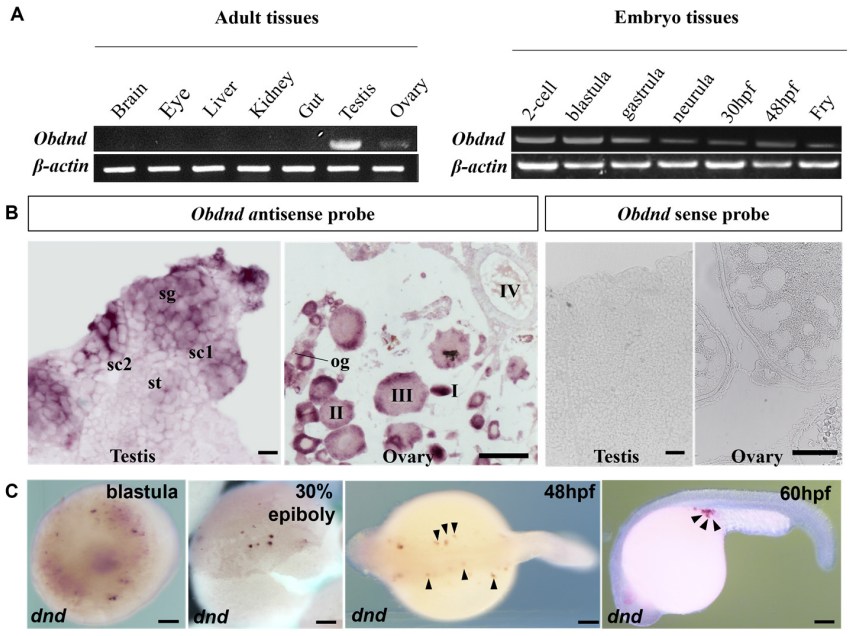

Figure 3. Expression of *Obdnd* in different tissues and embryos. (**A**) RT-PCR analysis of *Obdnd* in adult tissues and developmental embryos. *β-actin* served as an internal control. (**B**) SISH on the testis and ovary by using *Obdnd* antisense probe and *Obdnd* antisense probe. sg, spermatogonia; sc1 and sc2, primary and secondary spermatocytes; st, spermatids; og, oogonia; I–IV, stages of oocytes. (**C**) WISH on developmental embryos by using *Obdnd* antisense probe. The arrow pointed to the PGC. Scale bar, 200 μm.

Furthermore, SISH and WISH were carried out to detect the temporospatial expression of *Obdazl* and *Obdnd* RNAs in gonads. SISH revealed that *Obdazl* and *Obdnd* RNAs were examined only in germ cells but were not detectable in somatic cells. (Figures 2B and 3B), which was consistent with the results of RT-PCR (Figures 2A and 3A). The expression of the *Obdazl* and *Obdnd* RNAs follows a similar pattern during spermatogenesis. Both *Obdazl* and *Obdnd* RNA transcripts were abundant in spermatogonia and remarkably decreased in primary and secondary spermatocytes, with no signals detectable in spermatids (Figures 2B and 3B). In contrast, sense probes as a negative control did not produce any signal above background in the gonads (Figures 2B and 3B).

Whereas the expression patterns of *Obdazl* and *Obdnd* RNAs in the ovary are not entirely consistent, it is difficult to detect the signals of *Obdazl* and *Obdnd* RNAs in oogonia. *Obdnd* RNA was expressed strongly in oocytes in their early stages I-III, although this level of expression steadily decreased as oogenesis continued (Figure 3B). In an analogous manner, *Obdazl* RNA was able to be easily found in the cytoplasm around the nucleus of stage I-III oocytes, but its presence was noticeably diminished in stage IV-V oocytes (Figure 2B). Moreover, *Obdazl* RNA was dynamically found in which perinuclear spots aggregated into the Balbiani body (Bb) in the early oocytes (Figure 2B). To properly validate the distribution of *Obdazl* and *Obdnd*, FISH was carried out to evaluate their differential expression in the ovary. Intriguingly, *Obdnd* was not detected in Bb but was present in large quantities in the ooplasm (Figure 4B). It was in stark contrast to *Obdazl*, which was concentrated in Bb rather than the ooplasm (Figure 4A). The dual colocalization of FISH validated the different expression patterns between *Obdazl* and *Obdnd* in Bb subcellular localization (Figure 4C,D). Again, in contrast to the negative control, sense probes provided no specific signal in gonads (Supplementary Figure S5).

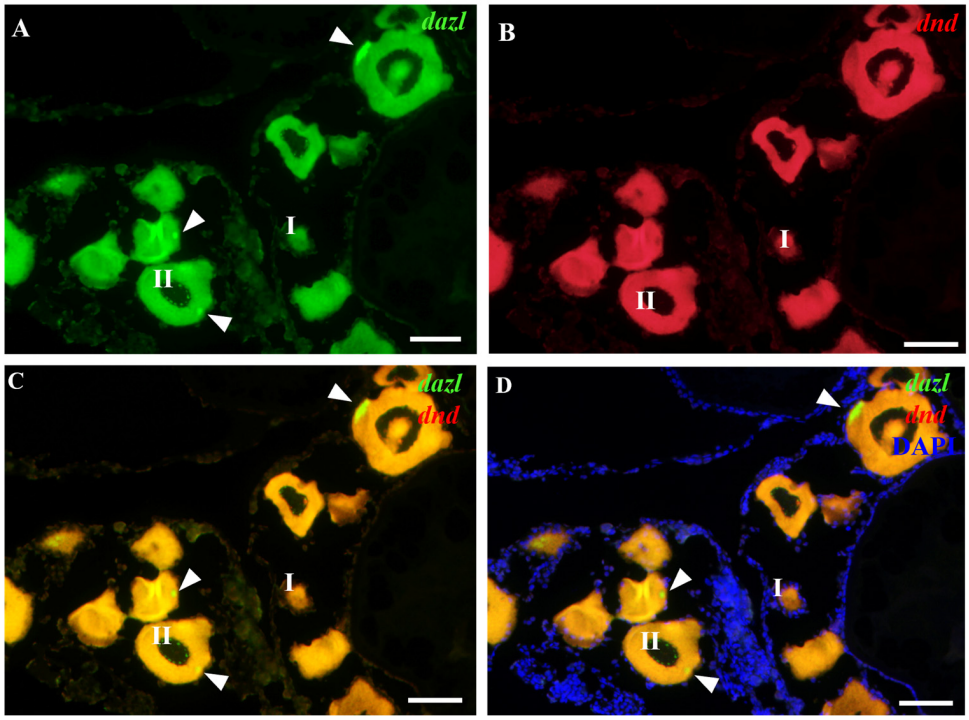

Figure 4. Localization of *Obdnd* and *Obdazl* RNAs in adult ovary. (**A**,**B**) In FISH, *Obdazl* RNA was labeled by green, *Obdnd* RNA was red, and nuclei were labeled by the blue color of DAPI. (**C**) Merges of *Obdazl* with *Obdnd*. (**D**) Merges of *Obdazl* with *Obdnd* and DAPI. *Obdazl* concentrates in the Balbiani body, whereas *Obdnd* is diffuse in the ooplasm and essentially absent in the Balbiani body. I and II, different stages of the oocyte. The arrow pointed to the Balbiani body. Scale bar, 200 μm.

During embryogenesis, the maternal *Obdazl* and *Obdnd* RNAs were highly expressed at the cleavage stage (Figures 2A and 3A). During the blastula stage, *Obdazl* and *Obdnd*

RNAs concentrated in a few conspicuous particles referred to as presumptive PGCs that could differentiate into PGCs as follows (Figures 2C and 3C). *Obdazl* and *Obdnd* RNAs were found to be confined in PGCs and bilaterally orientated along the axis as embryogenesis progressed, eventually located in germinal primordia.

3.3. Obdazl 3′ UTR Enables GFP to Express in the PGCs of Medaka Stably

Investigations of PGCs visualization revealed that the 3′ UTR of *nanos1* in *Danio rerio* as a protector of certain proteins was conserved across species, which ensured the stabilization of these RNAs in PGCs [44]. To make clear whether *Obdazl* 3′ UTR played the same role as that in *Danio rerio*, the mRNA of *gfp-Obdazl* 3′ UTR was microinjected into medaka embryos combined with *rfp-Drnos1* 3′ UTR mRNA, which proved to be effective on the visualization of PGCs (Figure 5). The GFP signaling granules were dispersed throughout the cell during the early stages of embryogenesis. During somitogenesis, cells displaying GFP signaling aggregate dorsally along the trunk on both sides of the embryoid body (Figure 5A–D). These cells made their way finally to the genital ridge by moving along the body of the embryo, where germ cells and mesoderm merged to produce gonads. (Figure 5F,J). Meanwhile, the RFP signal showed a comparable expression pattern to GFP (Figure 5G,K), indicating *gfp-Obdazl* 3′ UTR mRNA might be applied to mark medaka PGCs. (Figure 5H,L).

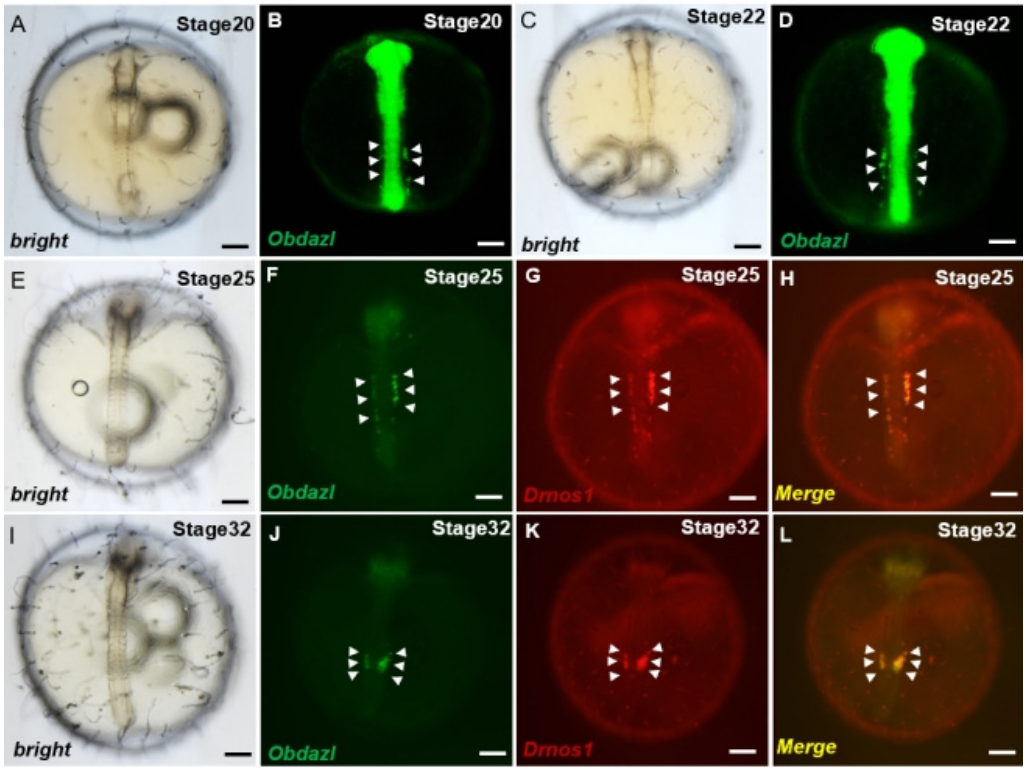

Figure 5. Visualization of medaka PGCs by *Obdazl* 3′ UTR. (**A**–**L**) During development, PGCs were observed by co-injection of *gfp-Obdazl* 3′ UTR mRNA and *rfp-Drnos1* 3′ UTR mRNA. The merged images are presented on the right (**H**,**L**). The injected mRNA concentrations were all 100 ng/μL. The arrow pointed to the PGC. Scale bar, 200 μm.

4. Discussion

In this study, *Obdazl* and *Obdnd* in full-length were cloned to investigate their differential expression in adult tissues and developmental embryos at various stages. *Obdazl* and *Obdnd* RNA transcripts analyzed by ISH appeared to be abundant in the spermatogonia and the oocytes at the early stages. *Obdazl* RNA was concentrated in the Bb, while *Obdnd* RNA was found in the ooplasm. In embryogenesis, *Obdazl* and *Obdnd* RNAs gathered in

presumptive PGCs and eventually migrated into germinal primordia. Furthermore, the *Obdazl* 3′ UTR enabled the visualization of medaka PGCs migration, confirming its role as the germline marker of *O. bidens*.

Multiple alignments of Dazl and Dnd proteins manifest that ObDazl and ObDnd are both RNA-binding proteins with conserved RRM domains, indicating that their functions may be similar across species, complying with what has been proven in *O. latipes* [45], *Carassius gibelio* [46], *O. celebensis* [22] and *C. nasus* [47]. Moreover, Dnd RRM functions as an indispensable indicator for subcellular localization. The RRM mutation induces the failure of Dnd translocation within the germ cell [48]. As for the function of Dazl RRM, it can bind the 3′ UTR of target mRNAs, acting as acceptors as well as activators of their translation [4]. RT-PCR revealed that *Obdazl* and *Obdnd* were both specifically expressed in gonads. During spermatogenesis, *Obdazl* and *Obdnd* were abundant in mitotic spermatogonia and decreased as meiosis progressed. The phenomenon shows a high similarity to the expression pattern in medaka [20,45], rainbow trout (*Oncorhynchus mykiss*) [49], and turbot (*Scophthalmus maximus*) [50]. On the contrary, *dazl* is hardly expressed in spermatogonia and increases progressively during spermatogenesis in Nile tilapia (*Oreochromis niloticus*) [51]. Nevertheless, the expressions of *Obdazl* and *Obdnd* existed throughout the oogenesis. Their signals could be easily detected at early oocytes and reduced gradually after peak expression at III oocytes, which was in line with *dnd* expression in *O. celebensis* [22], large yellow croaker (*Larimichthys crocea*) and yellow drum (*Nibea albiflora*) [52]. Strikingly, while *Obdnd* was abundant in the ooplasm, *Obdazl* was concentrated in Bb, which acted as a conserved oocyte aggregate in favor of polarized cell formation and was discretely distributed in the cytoplasm [53]. In gibel carp, *dazl* is located in Bb and facilitates the formation of PGCs [54]. *Dazl* of the rainbow trout is also concentrated in Bb at the early oocyte stage [49]. However, in the Asian seabass, *dazl* is concentrated in particles around the nucleus that develops into Bb later in oocytes [55]. Furthermore, further research on Bb function has been carried out in most vertebrates, spreading from mice and frogs to zebrafish. In frogs and zebrafish oocytes, Bb plays a crucial role in the localization of germplasm, which is of great importance for the maternally inherited specification of germ cells [53]. The differences in the expression patterns of *Obdazl* and *Obdnd* in the ovary indicated that they may make a difference as distinct factors during oogenesis.

The emergence of PGCs visualization technology has provided a support for further exploration on the functions of genes related to reproductive development. It has been reported that the synthesis of *gfp* with *vasa* or *nanos1* 3′ UTR can stably express in PGCs [44]. In particular, *Danio rerio nanos1* 3′ UTR can protect the expression of reporter gene and can even effectively mark the PGCs in most of the fish that have been studied [21,26,40,56]. In our study, *Obdazl 3′* UTR was selected to combine with the reporter gene to visualize the PGCs. The fact that *gfp-Obdazl* 3′ UTR mRNA can label medaka PGCs gains support for *dazl* as a germline marker highly conserved between *O. bidens* and medaka. Meanwhile, this result can also provide a new angle for the further exploration of the germ cell marker genes in *O. bidens*, as well as the developmental process of PGCs.

5. Conclusions

In summary, *Obdazl* and *Obdnd* show differential and bisexual expression patterns during gametogenesis, suggesting that they fulfill their own part and function in diverse roles. Furthermore, *dazl* is identified as the germ cell marker of *O. bidens*, which can benefit further research on reproductive mechanisms.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fishes9060214/s1: Figure S1: Characteristics of *Opsariichthys bidens dazl* sequence. Figure S2: Characteristics of *Opsariichthys bidens dnd* sequence. Figure S3: Multiple alignment of Dazl proteins. Figure S4: Multiple alignment of Dnd proteins. Figure S5: FISH on adult ovary by using *Obdazl* sense probe and *Obdnd* sense probe.

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