Medaka *piwi* is Essential for Primordial Germ Cell Migration

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Abstract: Piwi controls the number of primordial germ cells (PGCs) *via* protecting maternal mRNA from decay and adult germ stem cell division in *Drosophila*. In mouse and zebrafish, *piwi* controls maintenance and differentiation of adult germ stem cell during gametogenesis. Whether *piwi* plays a role in PGC development of vertebrates remains unsolved. We addressed this issue by using medaka (*Oryzias latipes*) as a vertebrate model. Molecular cloning, sequence comparison and analyses of genomic organization and chromosome synteny led to the identification in this fish of a single *piwi* gene, called *Opiwi*. By RT-PCR analyses and *in situ* hybridization, the *Opiwi* transcript is maternally supplied and becomes restricted to PGCs and the central nervous system (CNS). *Opiwi* knockdown did not prevent PGC formation even in the absence of any somatic structures but did significantly reduce the number of PGCs *in vivo* and *in vitro* and affect the distribution of PGCs in developing embryos. Surprisingly, depletion of zygotic *Opiwi* severely and specifically affected PGC migration. We conclude that *Opiwi* is required not only for determining the PGC number but also for controlling PGC migration. Our results demonstrate that *piwi* plays a generally conserved role in germ cell development from *Drosophila* to vertebrate and a specific role in PGC migration.

Keywords: Cell migration, embryo, germ cell, motility, *piwi.*

INTRODUCTION

Male infertility accounts for 40–50% of human infertility, which affects one-sixth of couples worldwide [1, 2]. A significant proportion of male infertility is associated with azoospermia or oligozoospermia, which is generally thought as being the consequence of genetic alterations [3]. To date, the DAZ gene family represents the best studied that encodes infertility factors in animal models [4] and human [5, 6]. Recently, an epidemiologic study provides evidence that genetic polymorphisms in Piwi genes are involved in spermatogenic failure [7]. The precise role of Piwi genes in human infertility has remained unresolved.

The *Drosophila piwi* was identified as “P-element induced wimpy testis” in a mutagenesis screen for maternal effect genes crucial for germline development [8]. Several studies have revealed that *piwi* plays an essential role in primordial germ cell (PGC) specification [9], germ stem (GS) cell (namely spermatogonia and oogonia) proliferation of both sexes and spermatogenesis [8, 10]. In *Caenorhabdits elegans* (*C. elegans*), both *piwi* homologs *prg*-1 and *prg*-2 are required for GS cell proliferation [10]. In mouse, there are three *piwi* genes encoding Miwi, Miwi2 and Mili, all of them show male-specific expression. Mutations in Mili and Miwi2 cause complete loss of male germ cells at three and six months, respectively, suggesting a role in maintaining GS cells [11, 12]. In addition, mutations in each of the three mouse *piwi* genes also cause meiotic defect in spermatogenesis [12-14]. In zebrafish, both Ziwi and Zili are required for maintaining germ cells, because loss of either Ziwi or Zili leads to germ cell loss by apoptosis [15, 16]. Thus, in these well-studied organisms, *piwi* plays a highly conserved role in the germline, ranging from PGC specification over GS cell self-renewal to germ cell survival and differentiation.

piwi is highly conserved across animal phyla and has been identified in diverse species including the sponge [17], planarian [18], *Xenopus* [19], zebrafish [20], mouse [21] and human [22]. Piwi is characterized by two major protein motifs: PAZ and PIWI domains. The PAZ domain has ~130 amino acid residues (aa) at the N-terminus and the ability to bind the 3’ end of short RNAs; the PIWI domain has ~300 aa at the C–terminus and is similar to the RNase H catalytic domain [23]. Piwi binds to *piwi*-interacting RNAs (piRNAs) [9, 24-27], a small class of small RNAs that are distinct from the small interfering RNAs (siRNAs) and microRNAs (miRNAs). piRNAs are 26-31 nt in length and processed in a Dicer-independent manner from single stranded precursors.

We and others make use of medaka (*Oryzias latipes*) to study stem cells and germ cells. This fish is excellent for studying vertebrate development [28], stem cell biology [29] and germ cell biology [30]. Specifically, this organism has diploid embryonic stem (ES) cells [31, 32], haploid ES cells [33, 34] and adult male GS cells [35]. Most recently, neural stem cells have also been identified in the medaka eye [36]. Medaka PGCs can easily be traced by transgenic GFP

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expression [37, 38] or by injection of germ cell-localized mRNA [39]. PGCs from early gastrula embryos can be maintained in culture [38]. Several medaka germ genes have been identified including *vasa* [40], *boule* and *dazl* [4], and *dnd* [41]. A transcription factor for germ genes has recently been identified in medaka [42]. Gene knockdown by morpholino has proven a powerful tool in medaka for analyzing PGCs development. For example, *vasa* knockdown in medaka leads to defects in PGC migration but not survival [38].

In this study, we cloned the medaka *piwi*, *Opiwi* and analyzed its temporospatial expression in developing embryos and adult tissues. We show that *Opiwi* depletion reduces the PGC number and affects PGC migration. Therefore, our work reveals that *piwi* exhibits a generally conserved function in germ cell development and also a previously unidentified function in PGC migration.

MATERIAL AND METHEDS

Fish and Embryos

Work with fish was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in Singapore and approved by this committee (Permit Number: 27/09). Medaka strains *af* and *orange* were kept at 26~28°C with a 14-h light/10-h dark daily cycle. Embryos were maintained at 28°C and staged as described [43]. Transgenic lines Ng and Vg were used to trace PGCs as described [38]. In medaka embryos, individual PGCs are easily identifiable by morphology (a round shape and a large size of ~20 μ in diameter), location (migration route at stages 19-23 and gonad after stage 27) and specific GFP expression. PGC counting was done at stages 21~23, when PGCs are bilaterally aligned to dorsal axis along the somites. Ectopic PGCs were scored at stage 32, when PGCs are normally within the gonad.

Isolation and Identification of Gene

Total RNA was isolated from the medaka ovary by using the Trizol Reagent (Invitrogen, Carlsbad, CA) and SMART cDNA libraries were synthesized by using the RACE cDNA Amplification Kit according to the manufacturer's manual (BD, BioSciences). When we started this project, the medaka genome sequence was not available. We cloned the *Opiwi* gene in four steps (Fig. **S1**). A sequence alignment of Piwi proteins from human, mouse and zebrafish revealed two conserved regions that were used for designing degenerate primers. By using primers CARATGAAYTGYAARATG GG (encoding QMNCKMG) and TTRTGNGCRAAYT GRCANGG (encoding PCQYAHK) for reverse transcriptase-polymerase chain reaction (RT-PCR), a ~700-bp cDNA fragment was first obtained, whose deduced amino acid sequence was most similar to Piwi protein. RT-PCR by using a degenerate primer (SCAGATHTC WGCTGGNTTTCAG encoding LQISAGFQQ) and a (specific primer CATCTTTAGCCCGTCCATGAG encoding LMDGLKM; located within the ~700-bp fragment) then led to a 1.7-kb fragment, whose deduced amino acid sequence was again most similar to the Piwi proteins. Third, 3'- and 5'-RACEs were run by using gene-specific primers TGCAAAATGGGAG GCGAGCT CT and CTGATACAAAACCCACTGAGGTC GGGA in combination with the universal primer mix (UPM). Finally, the full length cDNA was PCR-cloned by using a pair of terminal primers and validated by sequencing (Fig. **S2**).

Plasmids

The *Opiwi* coding sequence (CDS) was PCR-amplified with forward primer (aaaggatccgcggccgcATG TCcGGcCGaGCcCGcGCCAGATC; underlined are 5 mismatches introduced to change the target sequence for MOpw; see below) and reverse primer (aaagaattcgg gcccCAGGTAGAAGAGGTAGTTGTC), inserted to replace the *dnd* CDS in pCSdnd:chNS, resulting in pCS pw:chNS [41]. To construct pCVpwΔ:gfpNS (pw GFP, MOpw targeting sequence fused to GFP), pw GFP was PCR-cloned with forward primer (aaaggatcc AAAATGT CTGGTCGGGCTCGTGCCGTGAGCAAGGGCGAGG AGCTG; the underlined sequence is the target of MOpw) and reverse primer (aaactcgagTT ACTTGTAC AGCTCGTCCATG) and inserted to replace the pw:ch in pCSpw:chNS.

RT-PCR Analysis

cDNA synthesis from 1-2 μg of total RNA was primed by oligo dT_{18} by using the M-MLV (Promega, Madison, WI). RT-PCR was run for 35 cycles (10 sec at 94ºC, 10 sec at 60ºC and 50 sec at 72ºC) in a 25-μl volume containing 10 ng of cDNA by using primers maF (TTCAACAGCCCTGCCATGTA) plus maR (CCTCCAATCCAGACAGAGTATT) for β-actin, and pwF (ATGTCTGGTCGGGCTCGTGCCAGATC) plus pwR (ATACAAAACCCACTGAGGTCGGGA) for *Opiwi*. PCR products were separated and documented as described [44].

RNA Synthesis and *in situ* Hybridization

in situ hybridization on whole mount (WISH) and on sections (SISH) was done as described [44]. The 2.5-kb *Opiwi* ORF was inserted into pGEM-T vector (Promega) and sequenced. Plasmid was linearized for antisense or sense probe synthesis by using T7 or SP6 polymerase, respectively (Roche, Switzerland). Capped mRNA was synthesized from linearized pCSpw:chNS and pCVpwΔ:gfpNS by using the mMessage Machine kit, treated with the RNase-free TURBO DNase and purified by LiCl precipitation according to the manufacturer's instruction (Ambion, Austin).

Morpholinos and Microinjection

Three morpholino antisense oligos were synthesized (Gene tool, Oregon). One is MOpw (TGGCACGAGCCCGACCAGACATTTT), which targets the *Opiwi* mRNA sequence around the initiation codon

(underlined). The second is MOpwm (TGGCACGAGC TCGgCCgGACATaac), which was derived from MOpw by introducing 5 mismatches (lower case letter). The third is MOpws (atgtatggttttacTTTGTGAGAAC, lower case letters indicate intron and upper case exon sequences), a splicing morpholino targeting *Opiwi* PAZ domain. Medaka embryos were injected at the 1-cell stage [38]. For the GFP reporter assay, 50 pg of pCVpwΔ:gfpNS RNA and 2 ng of MOpw or MOpwm were co-injected at the 1-cell stage. For the rescue experiment, 100 pg of mRNA and 2 ng of MOpw were co-injected.

Cell Culture

Cell culture was done as described [31, 33]. Briefly, embryos were injected at the 1-cell stage and manually dissociated at the midblastula stage into single cells. Dissociated cells were seeded into 96-well plates in the medaka embryonic stem cell culture medium ESM2 [31, 33].

Microscopy

Observation and photography was carried out on Leica MZFIII stereomicroscope, Zeiss Axiovert invert and Axiovert upright microscopes with a Zeiss AxioCam M5Rc digital camera (Zeiss Corp., Germany) as described previously [44, 45].

Statistics

Statistical analyses were calculated by using Graphad Prism v4.0. Data consolidated were presented as means ± s.d. and *p* values were calculated by using non-parametric student's t-test.

RESULTS

Isolation of *piwi* in Medaka Fish

We cloned the full length cDNA for *Opiwi*, the medaka *piwi*, in four steps (Fig. **S1**). *piwi* is an evolutionarily conserved gene which has PAZ domain at its N-terminal and PIWI domain at its C-terminal [23]. Alignment of Piwi proteins from different species led to the identification of several conserved sequences to design degenerate primers (Fig. **S1**). Two rounds of RT-PCR by using two pairs of degenerate primers in combination with 5' and 3' rapid amplification of cDNA ends (RACEs) led to a full-length cDNA for *Opiwi*, which was cloned by using a pair of terminal primers and sequenced (Fig. **S1**). The *Opiwi* cDNA is 2935 bp, including a 143-bp 5'-untranslated region (UTR), a 224-bp 3'-UTR with a polyA signal and an open reading frame (OPF) for 855 amino acids (aa; Fig. **S2**). The predicted protein OPiwi is most similar to Piwi proteins (Fig. **S3**). Phylogenetic analysis shows that OPiwi belongs to Piwi subfamily and is most similar to the zebrafish Piwi (Fig. **1A**). The OPiwi has a PAZ domain and a PIWI domain, which shows higher percentage of identity values between difference species than the full-length protein (Fig. **1B**). The *Opiwi* ORF spans 20 exons, as for its equivalent in zebrafish and human (Fig. **1C**). In addition, the *Opiwi* gene is on chromosome 9, which is syntenic to the *piwi*-containing chromosome in other organisms, which is chromosome 8 in zebrafish and chromosome 12 in human (Fig. **1D**). Taken together, *Opiwi* is the ortholog of the human *piwi* by sequence, gene structure and chromosome synteny.

piwi RNA Expression

By RT-PCR, the *Opiwi* RNA persists throughout embryogenesis. It is highly abundant in the adult gonads of both sexes and detectable in the brain and eye but absent in the kidney, liver and gut (Fig. **2A**). We performed SISH to study the temporospatial expression. On testicular sections, the *Opiwi* RNA was specific to germ cells. It was highly abundant in the peripheral region where spermatogonia and spermatocytes reside, undetectable in spermatids and sperm (Fig. **2B**). On ovarian sections, the *Opiwi* RNA was restricted to germ cells. The signal was weak in oogonia, strong in stage-I~II oocytes, declined in stage-III~IV oocytes and became hardly detectable in stage-V oocytes (Fig. **2B**). By WISH, the *Opiwi* RNA was present throughout cleavages stages and widely distributed to many cells until the blastula stage (Fig. **2C**). When embryogenesis proceeds, the *Opiwi* RNA was concentrated in PGCs, tail bud and organs of CNS, including the diencephalon, midbrain, hindbrain and spinal cord (Fig. **2C**). A sense probe as control did not produce any signal above background in the gonads (Fig. **2B**) and developing embryos (data not shown). Therefore, the *Opiwi* RNA is maternally inherited and expressed in embryonic and adult germ cells and CNS.

piwi Knockdown Affects Early Embryonic Development

We were interested in determining the role of *Opiwi* during embryonic development by using the morpholino (MO) mediated gene knockdown approach that has been widely used in zebrafish and medaka [38]. We designed MOpw, an antisense morpholino oligo that blocks *Opiwi* mRNA translation and its derivative MOpwm that contains five mismatches as a control (Fig. **S4A**). We introduced the MOpw target sequence in frame to the gfp ORF, leading to pCVpwΔ:gfpNS (Fig. **S4A**). Capped pwΔ:gfpNS RNA was synthesized from this plasmid, which contains the 3'-UTR of the medaka *nanos3* gene. Co-injection of this RNA and MOpw into 1-cell stage embryos resulted in the inhibition of GFP expression, while co-injection with MOpwm produced strong GFP expression (Fig. **S4B**), demonstrating the specificity and efficiency of MOpw, but not MOpwm, to suppress the *Opiwi* RNA translation.

We then analyzed the effect of MO-mediated *Opiwi* knockdown on embryonic development. Injection of MOpwm as control had no effect (Fig. **S5A**). However, MOpw injection caused dose-dependent developmental defects (Fig. **S5B**), which fall largely into three classes at a roughly equal ratio (Fig. **S5B**, **C**, **S6**). Class I displayed seemingly normal axis at stage

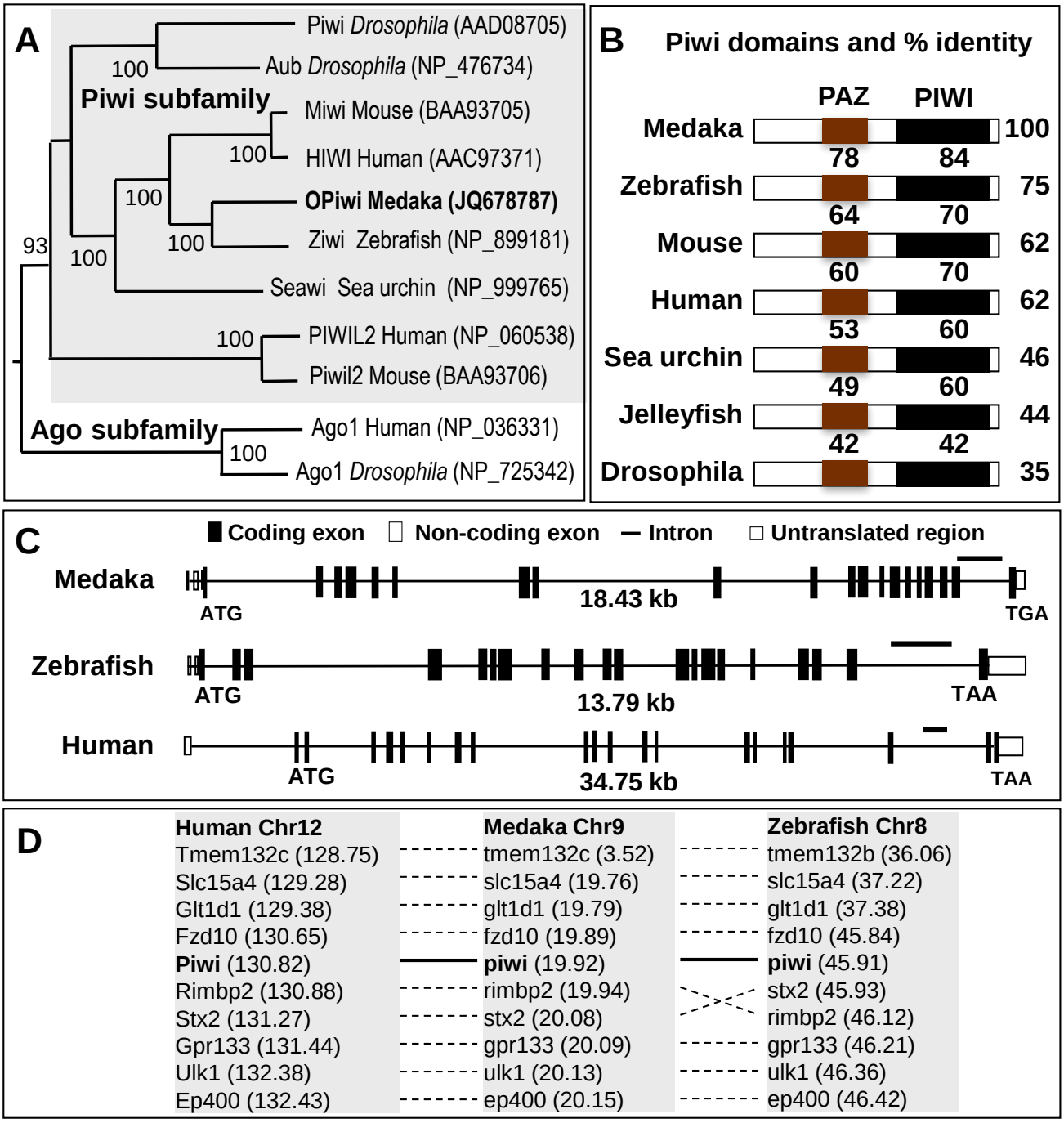

Fig. (1). *Opiwi* is the ortholog of human Piwi gene. (**A**) Phylogenetic tree of Piwi proteins. (**B**) Piwi domain structures and percentage of identity values between medaka and other organisms. (**C**) Genomic organization of *piwi* gene in medaka, zebrafish and human. (**D**) Chromosome synteny. Numerals in parenthesis denote chromosomal positions (http://www.ensembl.org).

17 and major structures and organs at stage 32; class II had visible but abnormal axis and eventually compromised CNS organs, the most evident defect was eye reduction or loss; class III failed to pass through gastrulation leading a disorganized cell mass until stage 32. Co-injection of *Opiwi* RNA was able to partially rescue MOpw-caused defects (Fig. **S6**). These results demonstrate that *Opiwi* plays a role in early embryonic development.

piwi Knockdown Affects PGC Development

In *Drosophila*, *piwi* functions primarily in PGC formation [8-10]. The role of *piwi* in early PGC development has not yet described in vertebrates.

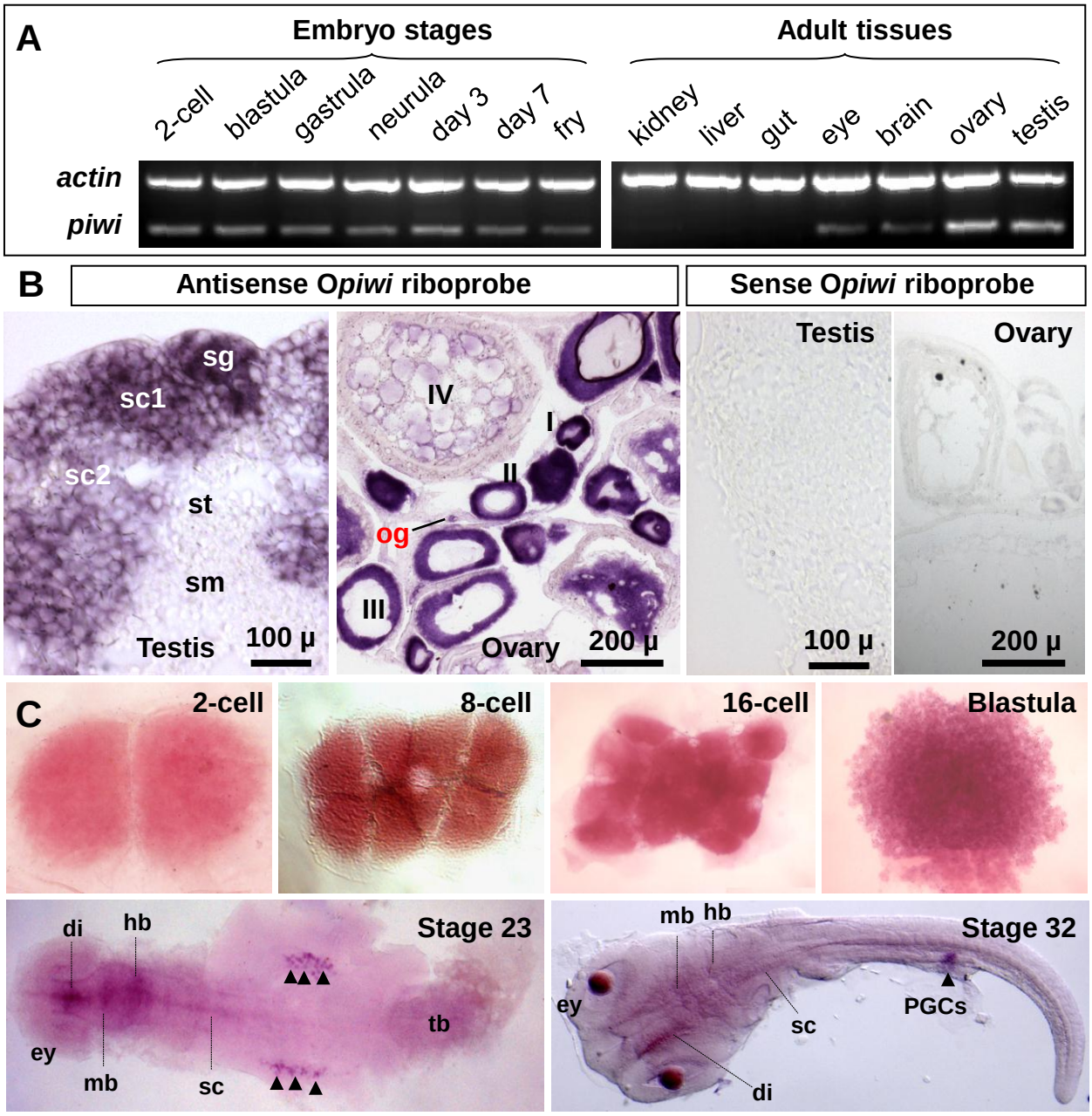

Fig. (2). *Opiwi* RNA expression. (**A**) RT-PCR analysis in embryos and adult tissues. (**B**) SISH on the adult testis and ovary by using antisense and sense *Opiwi* riboprobes. og, oogonia; I-IV, stages of oocytes; sg, spermatogonia; sc1, primary spermatocyte; sc2, secondary spermatocyte; st, spermatid; sm, sperm. (**C**) WISH by using an antisense *Opiwi* riboprobe in developing embryos, showing maternal supply and wide distribution until the blastula stage, preferential expression in PGCs (arrowheads) and faint but detectable expression in the central nervous system after gastrulation. di, diencephalon; ey, eye; mb, midbrain; hb, hindbrain; sc, spinal cord; tb, tail bud.

Maternal inheritance and PGC expression provoked us to examine the effect of *Opiwi* knockdown in PGC development. By stages 19~22, PGCs are clearly visible and easily countable in NgVg embryos (Fig. **3A**). NgVg is the hybrid between Ng female and Vg male and allows for PGC detection by transgenic GFP expression: Ng and Vg expresses GFP from the medaka *nanos3* and *vasa* promoters, respectively [38]. Zygotic injection with 5 ng of MOpw led to embryonic arrest at the blastula stage or to a disorganized cell mass. Strikingly, PGCs were clearly visible in all MOpw-injected embryos (n = 105), even in the absence of obvious embryonic structures and organs (Fig. **3B**, **B'**). On average, there were 32 PGCs in MOpwm-injected NgVg embryos (Fig. **3C**, **4A**), which is comparable to non-injected NgVg control embryos [38]. This number decreased to 19.9 PGCs upon MOpw injection (Fig. **3D**, **4A**). Thus, severe *Opiwi* knockdown does not affect PGC formation but reduces the PGC number.

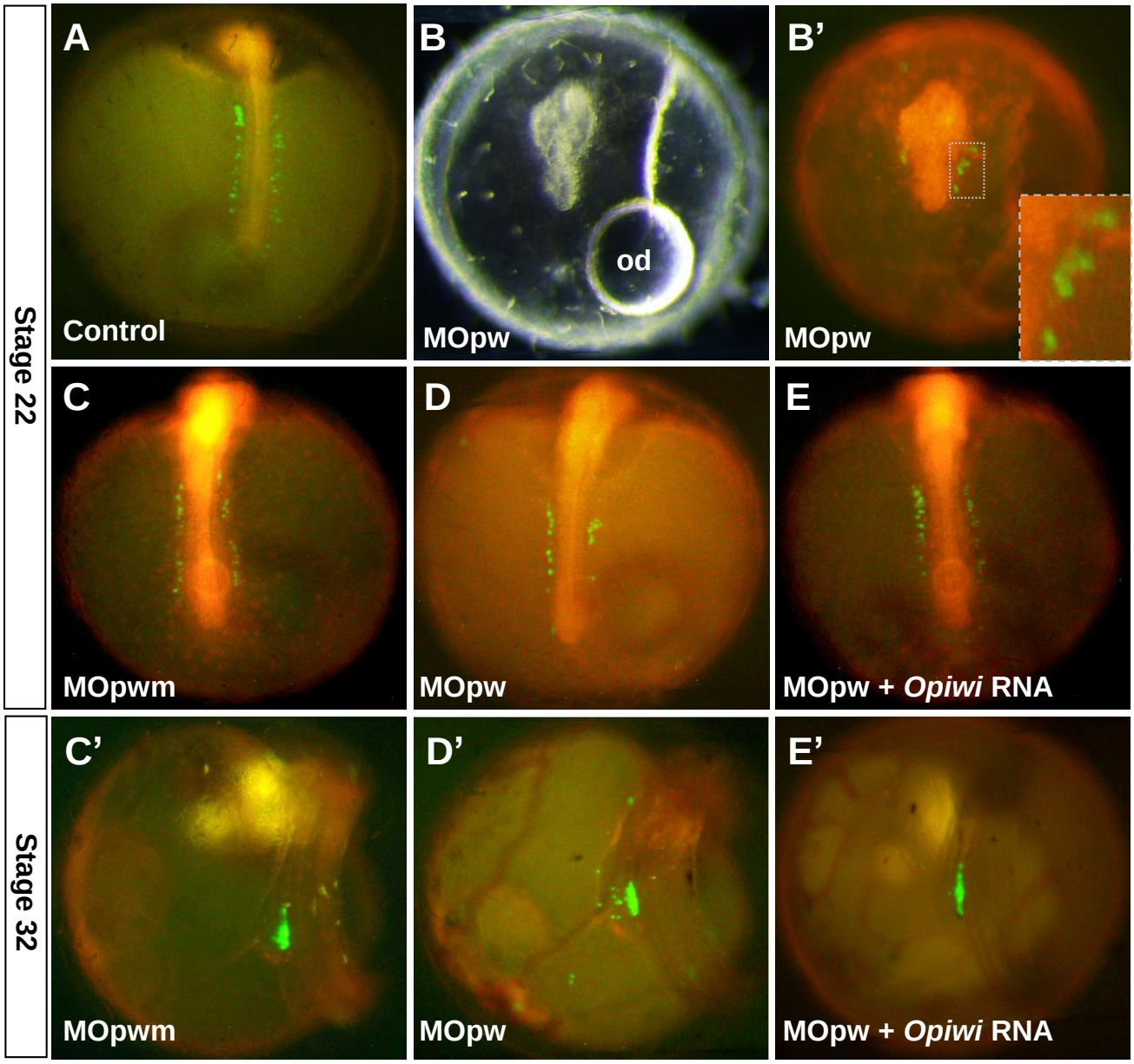

Fig. (3). *Opiwi* knockdown affects PGC development. (**A**) Control NgVg embryo. (**B** and **B'**) Embryo injected at the 1-cell stage with 2 ng of MOpw. (**C-E'**) Embryos injected with MOpwm, MOpw or MOpw plus *Opiwi* RNA, showing the number and distribution of PGCs (green) at stages 22 and 32. od, oil droplet.

piwi Knockdown Specifically Affects PGC Migration

Opiwi knockdown affected PGC distribution, producing PGCs at ectopic sites, which is most evident until stage 32, when 9 ectopic PGCs on average were seen in MOpw-injected embryos compared to only 1.2 ectopic PGCs in MOpwm-injected control embryos (Fig. **3D'**, **4B**). The normal number and distribution of PGCs were rescued in MOpw-injected embryos through co-injection of pw:ch RNA encoding a fusion between the full length *Opiwi* and cherry (Fig. **3E**, **E'**, **4**). These results suggest that *Opiwi* is involved in controlling the number and distribution of PGCs during early embryonic development.

Zygotic injection of MOpws, a splicing morpholino targeting PAZ domain of *O*Piwi (Fig. **5A**) that was able to effectively inhibit the splicing of zygotic *Opiwi* transcript (Fig. **6B**), had little effect on somatic development and PGC number, but led to severe defects in PGC migration (Fig. **5C-E**). Taken together, *Opiwi* knockdown affects PGC migration essentially independent of somatic development.

Effect of *piwi* Knockdown on the Number and Behaviors of PGCs

We have previously demonstrated in medaka that dissociated cells from gastrula embryos are capable of PGC formation in culture [38]. We made use of this cell

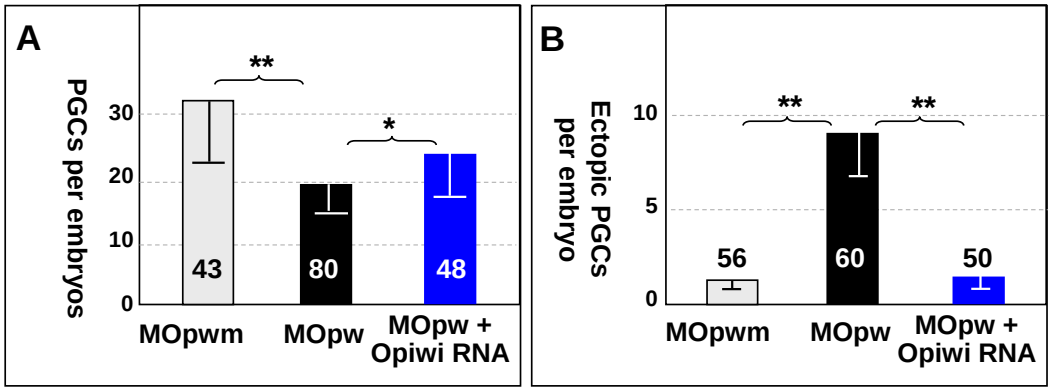

Fig. (4). *Opiwi* knockdown affects the number and distribution of PGCs. Embryos were injected at the 1-cell stage with 2 ng of MOpwm, MOpw or MOpw plus 100 pg of *Opiwi* RNA, and monitored for the number and position of PGCs. Total numbers of embryos observed are indicated within or above columns. Statistically significant (*; $p \leq 0.05$) and very significant (**; $p \leq 0.01$) differences were derived by comparison to the control value (MOpwm injection). (**A**) PGC number counted at stages 21 when PGCs are normally aligned along the axis. (**B**) Number of ectopic PGCs counted at stages 32 when PGCs are normally within the gonad.

culture system to analyze the number and behavior of PGCs in more detail at the single cell level. Individual cells were dissociated from midblastula embryos injected with 2 ng of MOpwm or MOpw and seeded in 96 well plates, one embryo one well, and PGCs were observed and counted daily. On average, a MOpwm-injected control embryo in culture (n = 9) gave rise to 25 PGCs, whereas a MOpw-injected embryo (n = 9) produced only 15.4 PGCs (Fig. **6A**). An observation on the time-course variation in PGC number revealed a similar curve during 8 days of culture between control and MOpw-injected embryos (Fig. **6A**), suggesting that once specified, PGCs from MOpw- and MOpwm-injected embryos are not different in proliferation and/or survival. Therefore, the reduced number of PGC in *Opiwi*-depleted embryo might be due to compromised divisions before PGC formation.

A defect in PGC migration may result from aberrant motility and/or the loss of an ability to sense migration

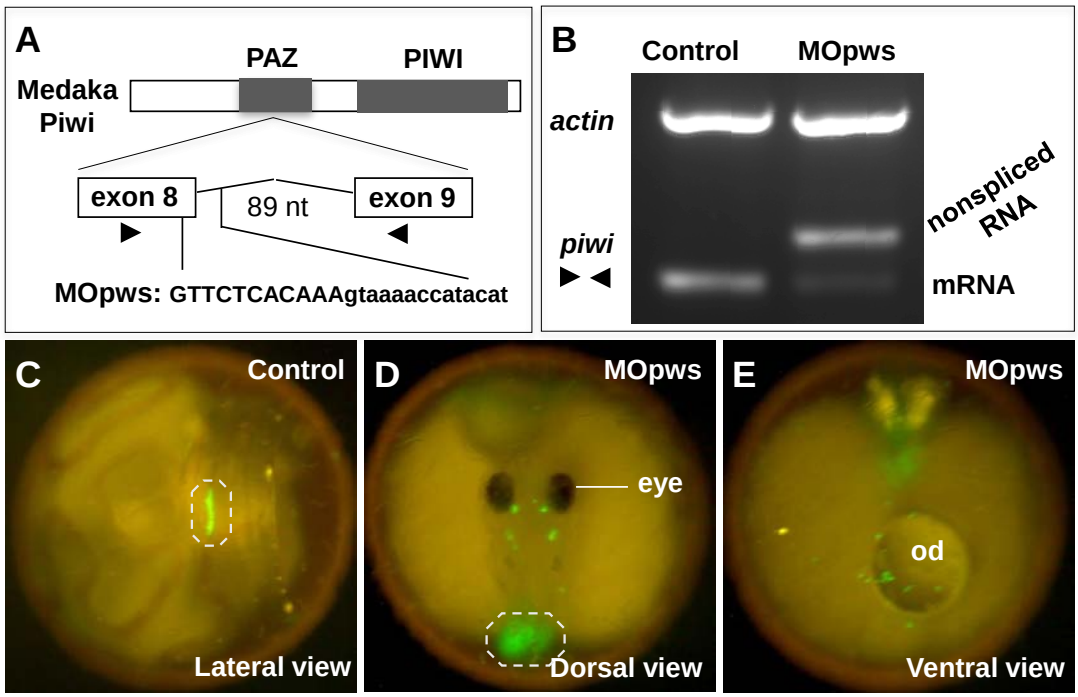

Fig. (5). Zygotically expressed *piwi* is required for PGC migration. (**A**) Position and target sequence of the splicing MOpws. Horizontal arrowheads define the positions and directions of PCR primers. (**B**) MOpws prevents splicing, showing that the appearance of a non-spliced RNA variant accompanies the reduction in the mRNA. (**C**) Control Vg embryo, showing PGCs within the gonad (circle). (**D** and **E**) MOpws-injected embryo at dorsal (**D**) and ventral view (**E**), highlighting the presence of many PGCs at ectopic sites.

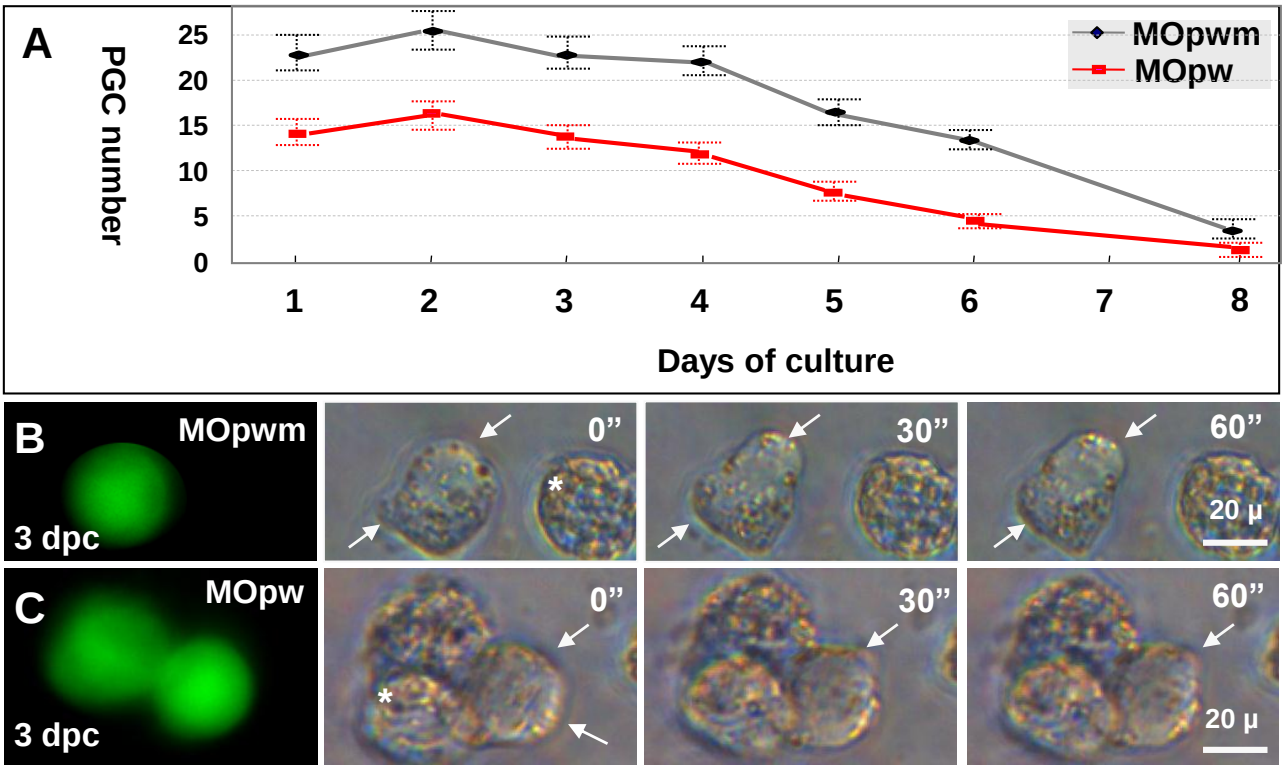

Fig. (6). Number and behaviors of PGCs *in vitro*. (**A**) Time course of changes in the number of PGCs in culture. Data are shown as means ± s.d. (bars) from six wells each containing cells equivalent to an embryo injected with MOpwm or MOpw. (**B** and **C**) Serial micrographs showing PGC motility at 3 days of culture from MOpwm-injected control (**B**) and MOpw-injected embryos (**C**), as evidenced by pseudopod formation (arrows).

cues. In cell culture, we observed that PGCs from *Opiwi*-depleted embryos exhibited motility as their counterparts from control embryos, as they steadily formed filopodia and changed their positions by ameba-like movement throughout the entire period of culture (Fig. **6B**, **C**). Thus, *Opiwi* knockdown does not affect the PGC motility *in vitro*.

DISCUSSION

In this study, we have identified *Opiwi* as the human *piwi* ortholog by sequence, gene structure and chromosome synteny. By RT-PCR and ISH we find that *Opiwi* expression occurs not only in embryonic and adult germ cells of both sexes but also in the embryonic and adult CNS. Expression in the CNS and tail bud has also been reported for the zebrafish *piwi* [20]. Medaka and zebrafish are distantly related and separated ~320 million years ago [46]. Therefore, the *piwi* expression pattern may be conserved in fish. Importantly, we show for the first time that *Opiwi* is essential for PGC migration.

In all organisms examined so far, *piwi* functions in the germline, although there are a wide variety of variations in sex- and stage-specific processes and aspects [9-12, 14, 15]. Except for *Drosophila* where *piwi* is essential for PGC formation [8, 9], *piwi* has mostly been implicated in adult GS self-renewal and meiotic differentiation in diverse organisms ranging from *C. elegans* to all vertebrates examined so far. In this study, we provide first evidence in medaka that *piwi* functions also in PGC development, namely regulating the PGC number and migration. This is consistent with the maternal inheritance of *Opiwi* in this organism. Maternal inheritance of *piwi* has also been documented in zebrafish [20] and *Xenopus* [27].

In *Drosophila*, *piwi* overexpression can increase the PGC number in a dose-dependent manner, whereas its mutations lead to a reduction in, but not complete loss of PGCs [9]. However, unlike *oskar* capable of inducing ectopic PGCs [47], *piwi* is not able to induce PGCs at ectopic sites [9]. In this study, we reveal that *Opiwi* depletion allows for timely PGC formation even in the absence of any visible somatic tissues/structures, corroborating with our previous finding for PGC preformation in medaka. Although the knockdown experiments described in this study does not establish whether *Opiwi* is absolutely required for PGC formation, the role of *piwi* in determining the number of PGCs appears to be conserved from *Drosophila* to medaka.

A striking finding in this study is that *Opiwi* is required fro PGC migration, which is most evident in embryos in which the zygotic *Opiwi* expression is suppressed by using a splicing morpholino antisense oligo. In these embryos, somatic development is

apparently normal, and many PGCs are present at ectopic sites, including the dorsal trunk and the extraembryonic yolk sac. Therefore, *Opiwi* appears to control PGC migration through a cell-autonomous mechanism, a situation that has previously been reported also for the medaka *vasa* knockdown [38]. *vasa* is a highly conserved germ gene that is essential for normal PGC formation in the posterior pole and *oskar*-induced PGC formation at ectopic sites in *Drosophila* [47].

In human, Piwi genes are implicated in male fertility as revealed by an epidemiologic study that suggests the involvement of genetic polymorphisms in Piwi genes in spermatogenic failure [3]. Our study in medaka as a model provides the first evidence that *piwi* genes may play an essential role in PGC development of vertebrate animals.

In summary, we reveal that *piwi* performs a generally conserved role in germ cell development from *Drosophila* to medaka and a specific role in PGC migration in medaka as a lower vertebrate model.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary materials include 6 figures and are available on the Publishers website along with the published article.

ABBREVIATIONS

hpf or dpf = Hour(s) or day(s) post fertilization

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