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Maternal miR-202-5p is required for zebrafish primordial germ cell migration by protecting small GTPase Cdc42

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In many lower animals, germ cell formation, migration, and maintenance depend on maternally provided determinants in germ plasm. In zebrafish, these processes have been extensively studied in terms of RNA-binding proteins and other coding genes. The role of small non-coding RNAs in the regulation of primordial germ cell (PGC) development remains largely unknown and poorly investigated, even though growing interests for the importance of miRNAs involved in a wide variety of biological processes. Here, we reported the role and mechanism of the germ plasm-specific miRNA miR-202-5p in PGC migration: (i) both maternal loss and knockdown of miR-202-5p impaired PGC migration indicated by the mislocalization and reduced number of PGCs; (ii) *cdc42se1* was a direct target gene of miR-202-5p, and overexpression of *Cdc42se1* in PGCs caused PGC migration defects similar to those observed in loss of miR-202-5p mutants; (iii) *Cdc42se1* not only interacted with *Cdc42* but also inhibited *cdc42* transcription, and overexpression of *Cdc42* could rescue PGC migration defects in *Cdc42se1* overexpressed embryos. Thus, miR-202-5p regulates PGC migration by directly targeting and repressing *Cdc42se1* to protect the expression of *Cdc42*, which interacts with actin to direct PGC migration.

Keywords: primordial germ cell, miR-202-5p, *Cdc42se1*, *Cdc42*, migration

Introduction

In most vertebrates, primordial germ cells (PGCs), the precursors of germ cells, segregate from somatic cells and migrate across the embryo to reach the site where they carry out their function (Raz, 2003; Tang et al., 2016). The event of PGC migration is precisely regulated as the long-distance migratory path across various developing tissues before reaching to the genital ridge (Richardson and Lehmann, 2010). There is a close correspondence between proper migration and PGC survival. Usually, the mislocalized PGC cells cannot maintain their PGC characteristics and are eventually eliminated to prevent germ cell tumors in most animals (Paksa and Raz, 2015).

In lower animals such as fruit fly, frog, and zebrafish, maternally supplied materials in germ plasm are indispensable for germ cell development (Extavour and Akam, 2003). It has been demonstrated that loss of germ plasm components led to PGC developmental defects (Raz, 2003; Richardson and Lehmann, 2010; Strome and Updike, 2015). For example, the RNA-binding protein Dnd1 binds with messenger RNAs (mRNAs), such as *nos1*, *zeb1*, and *tdrd7*, and prohibits miR-430 from associating with these mRNAs. Knockdown of Dnd1 would impair the motility of PGCs, which resulted in abnormal migration of PGCs and eventually caused the reduction or complete loss of PGC at early developing stage (Weidinger et al., 2003; Goudarzi et al., 2012; Gross-Thebing et al., 2017). In zygotic embryos with loss of *vasa* and *tdrd12*, PGCs could normally specify and migrate; however, germ cells failed to maintain and the mutant fish developed into infertile males (Hartung et al., 2014; Dai et al., 2017). In addition, the germline component *nanos* family played essential roles in germ stem cells and ovary development in zebrafish (Draper et al., 2007; Beer and Draper, 2013; Cao et al., 2019).

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MicroRNAs (miRNAs) are a group of endogenous small non-coding RNAs, which usually bind to mRNAs of coding genes to direct their post-transcriptional repression in animals and plants (Bartel, 2009; Shenoy and Blelloch, 2014). Increasing evidences indicate that miRNAs are involved in the regulation of germ cell formation, migration, and maintenance (Hayashi et al., 2008; Shenoy and Blelloch, 2014; van den Driesche et al., 2014). In zebrafish, miR-430 played a robust role in PGC migration through the clearance of maternal stromal cell-derived factor 1 (SDF1) and its receptors (Staton et al., 2011). SDF1 is one of the master chemokine molecules, which guides PGC migration through germ-cell guidance receptors chemokine (C-X-C motif) receptor 4B (CXCR4B) and 7B (CXCR7B) (Doitsidou et al., 2002; Knaut et al., 2003; Boldajipour et al., 2008; Mizoguchi et al., 2008). PGC migration is regulated by transmembrane receptors that receive external chemoattractant signals, which are then translated to cytoskeletal change by effector molecules such as phospholipids and small GTPases (Richardson and Lehmann, 2010; Barton et al., 2016). A recent study indicated that expression of a dominant-negative form of small GTPase Cdc42 in the PGC led to round cell morphology, decreased formation of the membrane invaginations, and reduced blebbing (Goudarzi et al., 2017). However, the molecular mechanism underlying Cdc42 expression and regulation in PGCs was remained unclear in zebrafish.

Previously, we have identified a germ plasm-specific miRNA miR-202-5p, which was exclusively localized in PGCs during embryogenesis in zebrafish (Zhang et al., 2017). Here, we investigated the potential role and underlying mechanism of miR-202-5p in PGC migration. Our results demonstrated that miR-202-5p played a critical role in zebrafish PGC migration by maintaining the expression of Cdc42.

Results

miR-202 mutant zebrafish were fertile

To investigate the role of miR-202-5p in zebrafish germ cell development, we generated a miR-202-5p mutant line using Crispr/Cas9 gene-editing strategy. We observed five miR-202 mutants in F₀ embryos injected with Cas9 mRNA and gRNA. The F₀ embryos containing miR-202 mutant were raised to adulthood and crossed with wild-type (WT) zebrafish, and an 8-bp deletion (miR-202Δ8) mutant was chosen to establish the miR-202 mutant line for further analysis (Figure 1A). To confirm the deletion in miR-202 site, quantitative real-time PCR (qRT-PCR) was performed to check the expression of mature miR-202-5p. Indeed, no mature miR-202-5p was detected and the miR-202-3p level was also significantly decreased in the miR-202Δ8 mutant testis (Figure 1B). We observed that all miR-202 mutants developed normally, and the genotype of miR-202 mutants were inherited at the expected Mendelian ratio. There was no significant morphological difference among testes and ovaries of miR-202^{+/+}, miR-202^{+/-}, and miR-202^{-/-} adults, in which normal spermatozoa and oocytes were observed (Figure 1C and D). Mating between miR-202^{-/-} males and females produced offspring. Therefore, loss of miR-202 did not affect reproductive capacity in zebrafish.

Maternal loss of miR-202-5p impaired PGC migration

Subsequently, we investigated PGC development in miR-202 mutant embryos. The miR-202^{+/-} zebrafish were crossed to produce F₂ embryos that theoretically contained 25% miR-202^{-/-} genotypic embryos. The F₂ embryos developed normally, whose PGCs were visualized by whole-mount *in situ* hybridization (WISH) of *vasa* mRNA. WISH results indicated that there was no significant change in the number and localization of PGCs between F₂ mutant and WT embryos, suggesting that zygotic loss of miR-202-5p did not affect PGC development (Figure 2A–C). Since miR-202-5p was a maternal factor, we further explored whether PGC development was influenced in maternal miR-202-5p mutant embryos. No miR-202-5p and little miR-202-3p were detected in 1-cell stage embryos, indicating that miR-202 was maternally deleted (Figure 2D). We analyzed PGC development in maternal loss of miR-202 (*MmiR-202*) mutant embryos. At dome stage when PGCs specified and initiated to migrate, there was no significant difference in the number and localization of PGC between WT and *MmiR-202* embryos (Figure 2E and F). Whereas, at stage of 75% epiboly, PGCs were scattered widely and some mislocalized PGCs were found in *MmiR-202* embryos (Figure 2G and H), and the number of mislocalized PGCs increased at 3-somite and prim 5 stages (Figure 2I–L). In comparison with WT embryos, the expression levels of PGC markers such as *dnd*, *vasa*, and *nanos3* were significantly reduced during PGC migration in *MmiR-202* embryos (Figure 2M). The number of PGCs in *MmiR-202* embryos was significantly lower than that in WT embryos at corresponding stages during PGC migration (Figure 2N). Of the *MmiR-202* embryos at prim 5 stage, 11.2% had normally developed PGCs, 56.3% had reduction of PGC only, 12.9% had PGC mislocalization only, and 19.6% displayed both PGC reduction and mislocalization (Figure 2O and P). To verify whether the PGC developmental defects in *MmiR-202* embryos were caused by loss of miR-202-5p, miR-202-3p, or both, rescue experiments were performed in *MmiR-202* embryos by injection of miR-202-5p and miR-202-3p mimics, respectively. As shown in Figure 2O, Q, and R, injection of miR-202-5p mimics rather than miR-202-3p mimics significantly attenuated PGC developmental defects in *MmiR-202* embryos, suggesting that maternal loss of miR-202-5p was responsible for the PGC developmental defects in *MmiR-202* embryos. To further demonstrate this, miR-202-5p knockdown was carried out by injecting miR-202-5p inhibitor into WT embryos, and the number and localization of PGC were assessed. The expression of PGC markers was decreased in miR-202-5p inhibitor-injected embryos (Supplementary Figure S1A–D), and similar defects including PGC mislocalization and reduction were found in miR-202-5p inhibitor-injected PGC-labelled transgenic embryos at prim 5 stage (Supplementary Figure S1E–L). There was a close correspondence between the PGC number reduction and the injection dosage of miR-202-5p inhibitor (Supplementary Figure S1M–P). Moreover, PGC mislocalization and number reduction were found in miR-202-5p inhibitor-injected embryos during PGC migration (Supplementary Figure S2). Thus, maternal loss or knockdown of miR-202-5p impaired PGC migration in zebrafish.

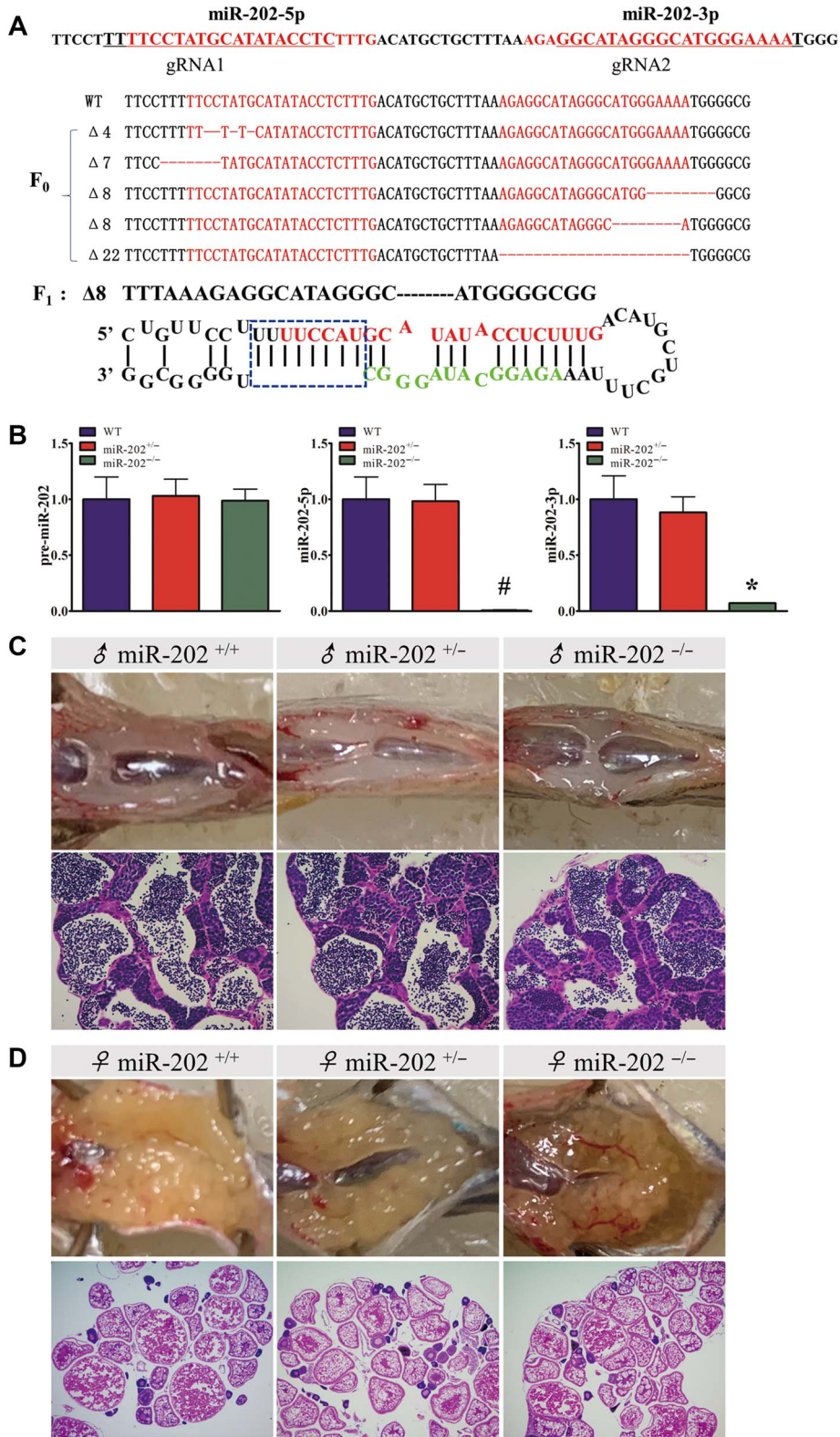


Figure 1 Analysis of gene expression and gonad structure in miR-202^{-/-} adults. **(A)** Generation of miR-202 mutant by CRISPR/CAS9 genome editing. **(B)** qRT-PCR analysis of pre-miR-202, miR-202-5p, and miR-202-3p in miR-202^{+/+}, miR-202^{+/-}, and miR-202^{-/-} testes. **(C and D)** Gonadal morphology and structure in miR-202^{+/+}, miR-202^{+/-}, and miR-202^{-/-} adults. **P* < 0.05; #, not detected.

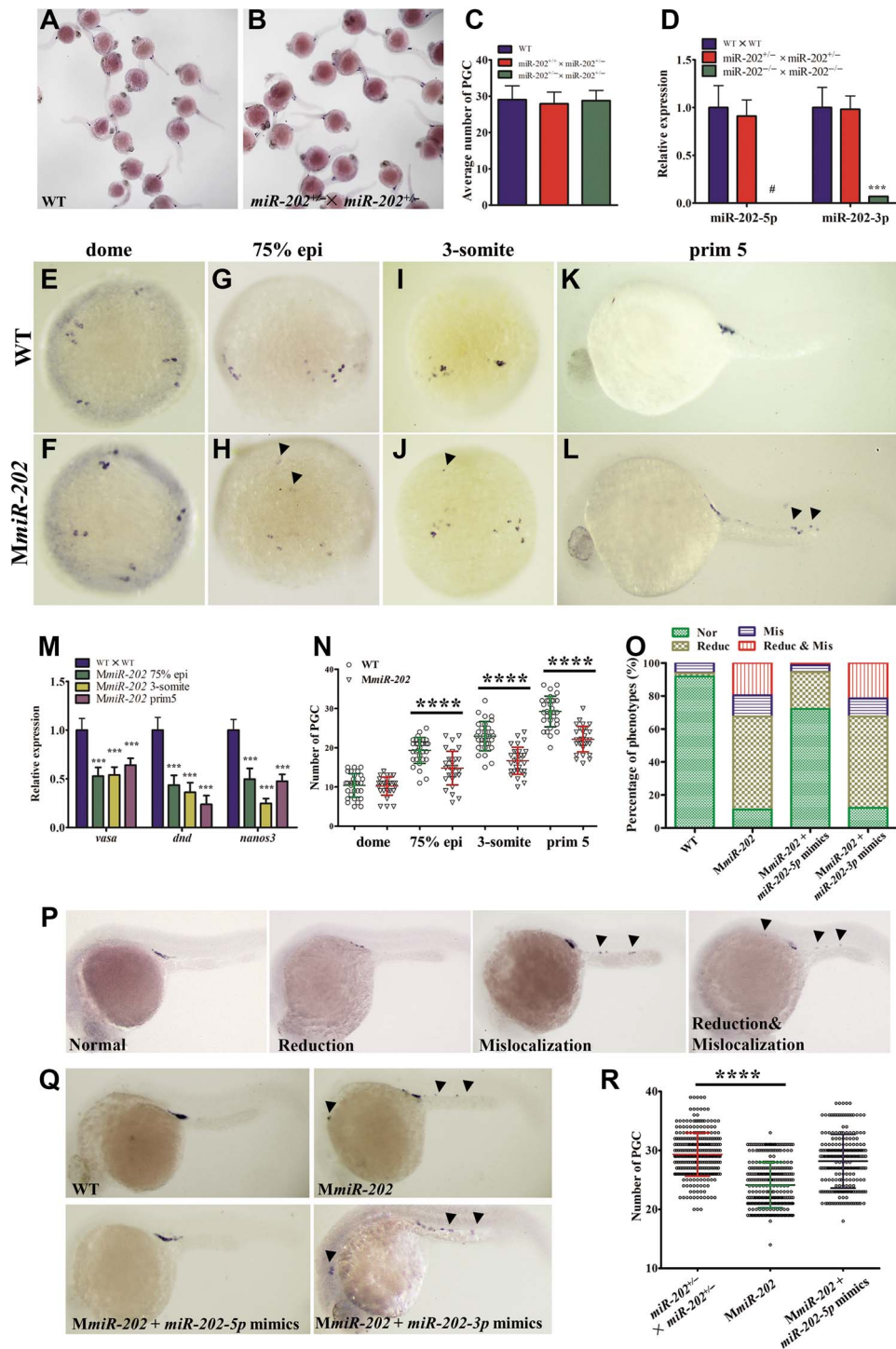


Figure 2 Maternal loss of miR-202-5p results in PGC reduction and mislocalization. (**A** and **B**) Whole-mount *in situ* hybridization of *vasa* in WT and F₂ embryos. (**C**) Average number of PGCs in embryos produced from WT, *MmiR-202*^{+/-} × *MmiR-202*^{+/-}, and *MmiR-202*^{-/-} × *MmiR-202*^{+/-} parents. (**D**) qRT-PCR analysis of miR-202-5p and miR-202-3p in 1-cell stage embryos produced from WT, *MmiR-202*^{+/-} × *MmiR-202*^{+/-}, and *MmiR-202*^{-/-} × *MmiR-202*^{+/-} parents. (**E–L**) Whole-mount *in situ* hybridization of *vasa* in WT and *MmiR-202* embryos at dome, 75% epiboly (epi), 3-somite, and prim 5 stages. (**M**) qRT-PCR analysis of PGC markers in WT and *MmiR-202* embryos from 75% epi, 3-somite, and prim5 stages. (**N**) PGC number in WT and *MmiR-202* embryos. (**O–Q**) Statistical analysis of PGC phenotypes in WT, *MmiR-202* embryos, and *MmiR-202* embryos injected with miR-202-5p or miR-202-3p mimics at prim 5 stage (**O**) and their representative images (**P** and **Q**). Nor, normal; Mis, mislocalization; Reduc, reduction; Mis & Reduc, mislocalization and reduction. (**R**) PGC number in WT, *MmiR-202* embryos, and *MmiR-202* embryos injected with miR-202-5p mimics. Arrowheads indicate the mislocalized PGCs. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; #, not detected.

miR-202-5p negatively regulated Cdc42se1 in PGC migration

miRNAs rarely produce functional protein products; they usually bind to mRNAs of target genes and initiate hierarchical biological events. To reveal the signaling pathway of miR-202-5p in PGC migration, potential target genes of miR-202-5p were bioinformatically predicted with PITA and miRanda (Supplementary Table S1). Among these candidates, *cdc42se1* (*cdc42* small effector 1) was one of the most promising candidates, whose 3'UTR contained a miR-202-5p binding site (Figure 3A). qRT-PCR results indicated that the embryonic endogenous *cdc42se1* mRNA was decreased in response to overexpression of miR-202-5p mimics (Figure 3B). Since the expression of endogenous Cdc42se1 protein in embryos was almost undetectable (data not shown), pcDNA3.1(+)-Cdc42se1 plasmid containing a *cdc42se1* open reading frame (ORF) that fused its own 3'UTR was co-transfected with control or miR-202-5p mimics in HEK293T cells. Western blotting showed that Cdc42se1 protein was significantly reduced in HEK293T cells in the presence of miR-202-5p mimics (Figure 3C). Furthermore, we analyzed the endogenous *cdc42se1* mRNA and protein in *MmiR-202* mutant individuals. In comparison with that in PGCs of WT embryos, the expression level of *cdc42se1* mRNA in PGCs of *MmiR-202* embryos was significantly upregulated (Figure 3D); the expression level of Cdc42se1 protein in *MmiR-202* testis was upregulated (Figure 3E). Next, the direct interaction between miR-202-5p and *cdc42se1* was examined by luciferase reporter assay by linking the 3'UTR of *cdc42se1* to the C-terminus of luciferase in psiCheck2 vector. As shown in Figure 3F, the relative activity of luciferase fusing WT 3'UTR of Cdc42se1 was strongly repressed by miR-202-5p mimics, whereas mutation of the miR-202-5p binding site abolished this repression. Furthermore, we employed RNA reporter by fusing the red fluorescent protein (RFP) ORF with WT or miR-202-5p binding site-mutated 3'UTR of *cdc42se1*. Quantitative pixel-intensity analysis revealed a dramatically elevated level of RFP in PGCs when the miR-202-5p binding site was mutant (Figure 3G and H). These results demonstrated that *cdc42se1* was a direct target gene of miR-202-5p.

Subsequently, we characterized the spatial and temporal expression patterns of *cdc42se1*. *cdc42se1* was a maternal factor universally distributing at gastrulation stage; then, its expression was gradually decreased and mainly concentrated at the central neural system and the yolk syncytial layer (Supplementary Figure S3). To investigate the possible role of Cdc42se1 in PGC migration, loss and gain of function were performed by injection of *cdc42se1* morpholino (MO) and mRNA, respectively. There was no observable defect of PGC development in *cdc42se1* MO-injected embryos (Figure 4A and B). Whereas, significant number reduction and mislocalization of PGC were observed in *cdc42se1* mRNA-injected embryos (Figure 4C and D), similar to the phenotype observed in maternal loss or knockdown of miR-202-5p embryos. Of the *cdc42se1* mRNA-injected embryos, 20.6% had normally developed PGCs, 17.8% displayed reduction only, and 16.0% showed mislocalization only, but 45.6% displayed both reduction

and mislocalization (Figure 4). In *cdc42se1* mRNA-injected embryos, co-injection of either *cdc42se1* MO or miR-202-5p mimics almost completely rescued the PGC abnormalities (Figure 4E, F, and J). In addition, there was a strong relevance between *cdc42se1* mRNA injection dosage and PGC number reduction (Figure 4G–I and K); there was no significant number reduction in embryos injected with 100 pg/embryo *cdc42se1* mRNA, whereas significant number reduction was found when the injection dosage of *cdc42se1* mRNA over 200 pg/embryo. Since overexpression of Cdc42se1 and knockdown of miR-202-5p caused similar phenotypes, we inferred that PGC developmental defects caused by knockdown of miR-202-5p might be due to an increase of Cdc42se1. To verify this, we co-injected *cdc42se1* MO to repress the expression of Cdc42se1 in miR-202-5p inhibitor-injected embryos. However, the results showed that co-injection of *cdc42se1* MO failed to rescue miR-202-5p inhibitor caused PGC reduction (Figure 4L). This might be because that *cdc42se1* was one of the key target genes in miR-202-5p inhibitor-injected embryos, and other regulating factors might function in PGC migration.

Furthermore, we performed specific overexpression of Cdc42se1 in a small portion of PGC population by injecting Cdc42se1-RFP-UTR-*nos3* mRNA into PGC-labelled transgenic embryos at 4-cell stage (Figure 5A). The results showed that overexpression of Cdc42se1 in PGCs resulted PGC mislocalization at prim 5 stage (Figure 5B–E), although some of Cdc42se1 overexpressed PGCs are still located at the genital ridge (Figure 5F). Of these mislocalized PGCs, some still possessed entire germ granule and maintained PGC identity (arrow in Figure 5E), whereas the remaining PGCs were degraded (asterisk in Figure 5D) or lost the PGC identity, germ granule (asterisk in Figure 5E). These data demonstrated that ectopic overexpression of Cdc42se1 in PGCs impaired PGC migration.

Cdc42se1 negatively regulated Cdc42 in zebrafish PGC

In mammal, Cdc42se1 was a negative regulator of Cdc42 and involved in actin polymerization, cytoskeleton change, and cell motility (Pirone et al., 2000; Ching et al., 2005; Block et al., 2012). However, the physic relationship among Cdc42se1, Cdc42, and actin in zebrafish was remained unclear. To illustrate it, HEK293T cells were co-transfected with Myc-tagged zebrafish actin (Myc-actin) and Flag-tagged zebrafish Cdc42 (Flag-Cdc42), followed by co-immunoprecipitation (co-IP) assay with antibodies specific to the Flag tag. As shown in Figure 6A, the protein complex immunoprecipitated by anti-Flag antibody was also recognized by anti-Myc antibody, indicating that actin was physically associated with Cdc42. Similarly, co-IP assay demonstrated that Cdc42se1 interacted with both WT Cdc42 (wtCdc42) and activated mutant of Cdc42 (Cdc42Q61L) (Figure 6B and C). However, in HEK293T cells co-transfected with Myc-actin and Flag-Cdc42se1, the protein complex immunoprecipitated by anti-Flag was not recognized by anti-Myc antibody, suggesting that actin did not interact with Cdc42se1 (Supplementary Figure S4). These data indicated that Cdc42

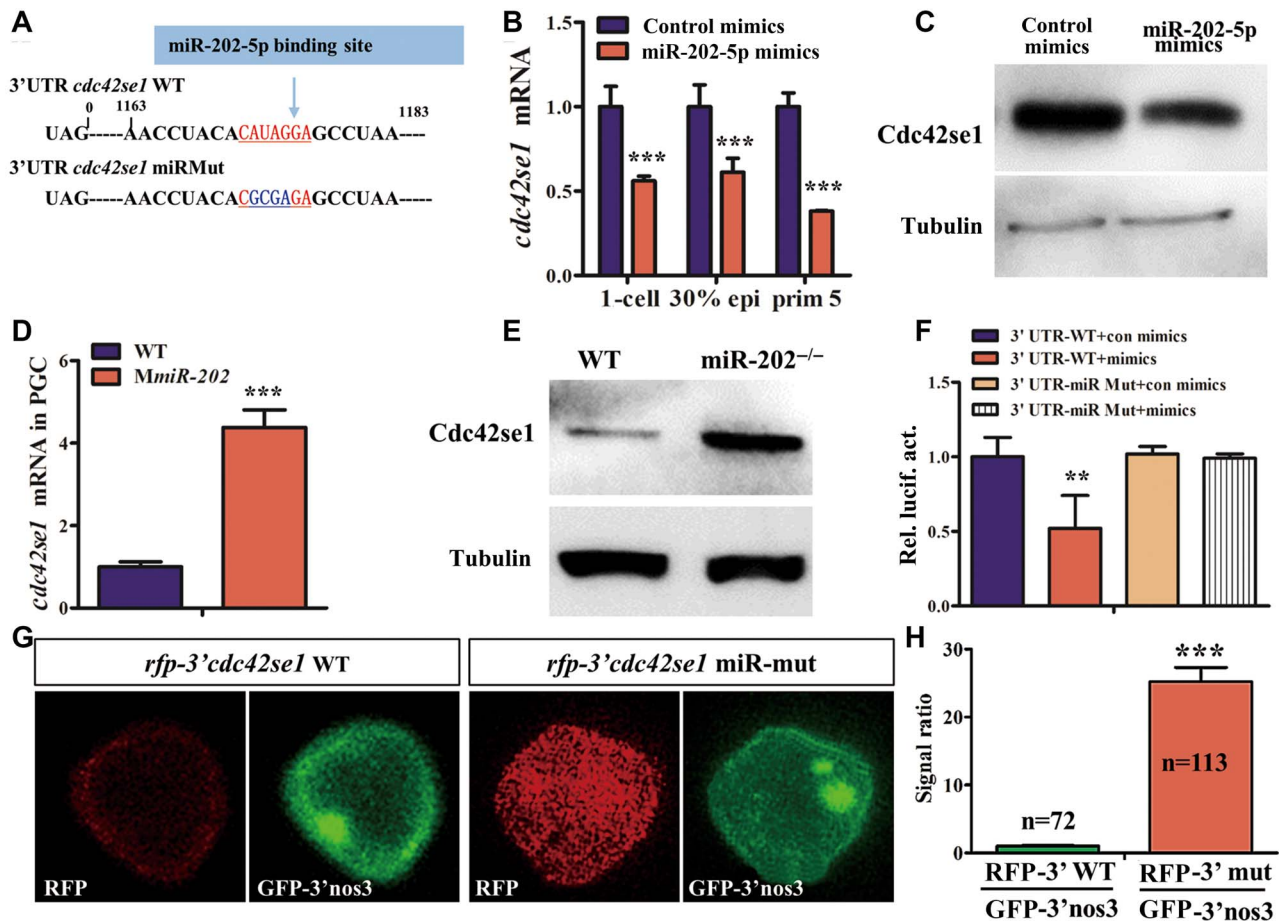


Figure 3 *Cdc42se1* is a target gene of miR-202-5p. (A) The miR-202-5p binding site (red) in 3'UTR of *cdc42se1* mRNA, and the mutant site is shown in blue. (B) qRT-PCR analysis of *cdc42se1* mRNA in control or miR-202-5p mimic-injected embryos. (C) Western blotting analysis of Cdc42se1 protein in HEK293T cells co-transfected with pcDNA3.1(+)-Cdc42se1 plasmid containing the ORF of *cdc42se1* fused with its own 3'UTR and control or miR-202-5p mimics. (D) qRT-PCR analysis of *cdc42se1* mRNA in PGCs of WT and *MmiR-202* embryos. (E) Western blotting analysis of Cdc42se1 protein level in WT and *MmiR-202* testes. (F) Relative expression of luciferase activity in HEK293T cells transfected with psiCheck2-WT 3'UTR-Cdc42se1 (3'UTR-WT) or 3'UTR-miRMut and control mimics (con mimics) or miR-202-5p mimics (mimics). (G) Mutation of miR-202-5p binding site in *cdc42se1* 3'UTR caused increased expression of RFP in PGCs as compared to the control WT 3'UTR. The co-injected RNA (GFP-3'UTR of *nos3*) served as a control. (H) Quantitative representation of the normalized signal intensity in the experiment presented in G. ** $P < 0.01$; *** $P < 0.001$.

separately interacted with actin and Cdc42se1 to form two complexes, Cdc42/actin and Cdc42/Cdc42se1.

We analyzed the endogenous Cdc42 expression in embryos injected with *cdc42se1* mRNA or miR-202-5p inhibitor. The expression of both *cdc42* mRNA and protein were significantly decreased in *cdc42se1* mRNA or miR-202-5p inhibitor-injected embryos (Figure 6D and F). Furthermore, the endogenous *cdc42* mRNA level in PGCs of *MmiR-202* embryos was significantly decreased comparing with that in PGCs of WT embryos (Figure 6E). Since the expression level of *cdc42* mRNA decreased, we analyzed whether Cdc42se1 downregulated *cdc42* transcription by repressing *cdc42* promoter activity. Indeed, the relative activity of luciferase driven by *cdc42*

promoter was strongly decreased by Cdc42se1 overexpression (Figure 6G). In zebrafish, overexpression of domain negative Cdc42 affected bleb formation and resulted in PGC mislocalization (Goudarzi et al., 2017), similar to that observed in Cdc42se1 overexpressed embryos. We proposed that the PGC developmental defects in Cdc42se1 overexpressed embryos might be due to a decrease of Cdc42. To test it, we co-injected *cdc42* mRNA to rescue PGC migration in embryos injected with *cdc42se1* mRNA. The results indicated that overexpression of Cdc42 significantly attenuated the PGC migration defects in Cdc42se1 overexpressed embryos (Figure 6H–I). In comparison with Cdc42se1 overexpressed embryos, upon co-injection of Cdc42, the percentage of embryos with normal PGCs increased

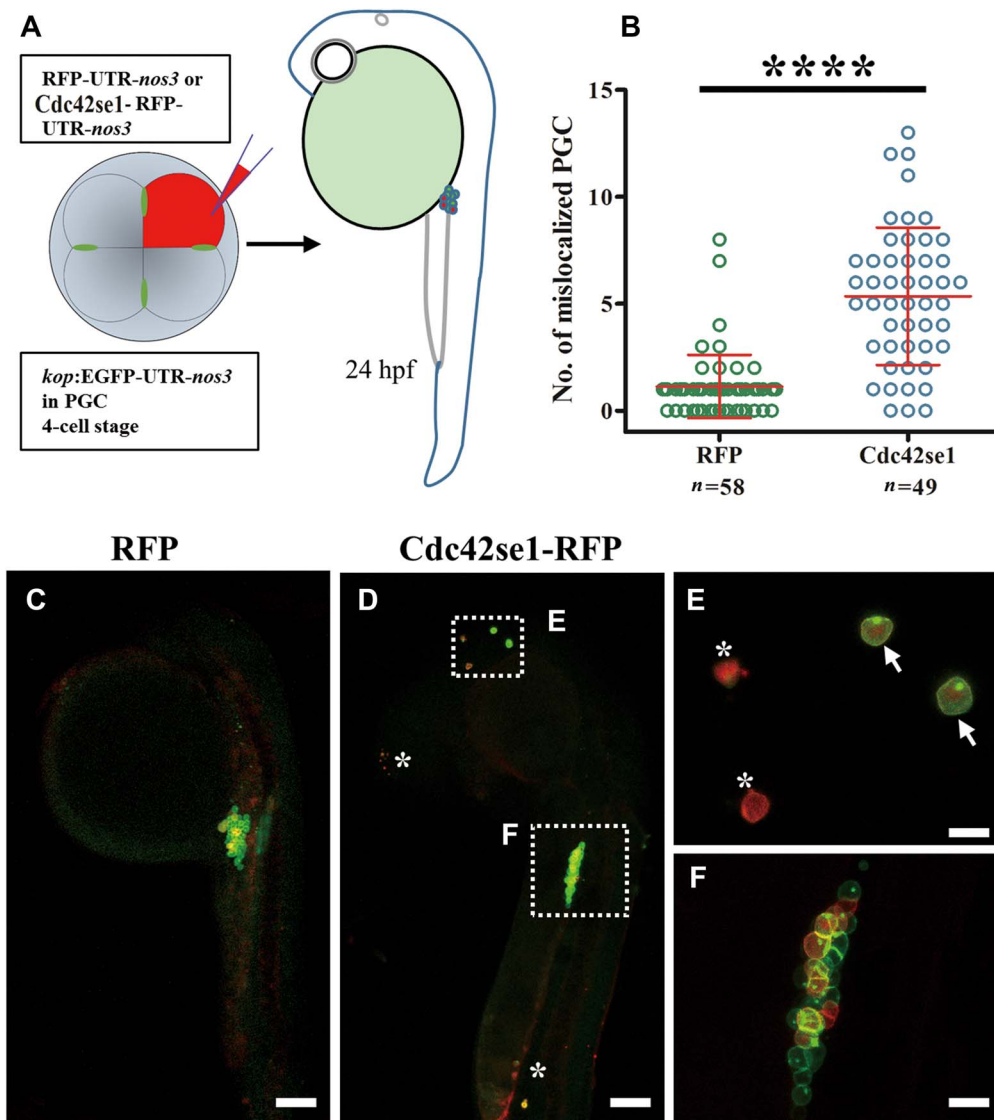


Figure 5 Overexpression of *Cdc42se1* in PGCs leads to mislocalization. (A) *Cdc42se1*-RFP-UTR-*nos3* mRNA was injected into 4-cell embryos of *Tg1 kop:EGFP-UTR-nos3* line. (B) The number of mislocalized PGCs in embryos injected with RFP or *Cdc42se1*-RFP-UTR-*nos3* mRNA. (C and D) Representative images of PGCs in embryos injected with RFP or *Cdc42se1*-RFP-UTR-*nos3* at prim 5 stage. (E and F) The magnified images as indicated in D. Arrows indicate the mislocalized PGCs, and asterisks indicate the dead or transforming PGCs. **** $P < 0.0001$. Scale bar, 100 μm (E and F) and 20 μm (D).

identification of the molecule module for bleb-based PGC motility revealed that PGC motility was controlled by a balance between miR-430 function and the action of germline-specific RNA-binding protein DND (Goudarzi et al., 2012).

Maternally supplied germ plasm components played indispensable roles in germ cell formation, migration, and maintenance in zebrafish (Richardson and Lehmann, 2010). To date, studies of germ plasm components in PGC development were largely focused in the functional role and molecular mechanism of coding genes in PGCs. For example, *Dnd*, *Ca15b*, *Rgs14a*, and *Nanos3* were required for PGC survival and migration; *Vasa* and *Piwi* were essential for germ cell differentiation and maintenance (Kopranner et al., 2001; Houwing et al., 2007; Hartung et al.,

2014; Paksa and Raz, 2015). Germ plasm-specific long non-coding RNAs (lncRNA) such as *pgc* and *xlsirt* were involved in germ cell migration and transcriptional silencing in fruit fly and frog (Kloc and Etkin, 1994; Nakamura et al., 1996; Martinho et al., 2004). However, little was known about the role of maternal germ plasm associated lncRNAs and miRNAs and their functions in zebrafish PGC development. It has been found that miR-202-5p was gonad-specific miRNA in a broad of species. Mouse miR-202-5p was localized in Sertoli cells of primordial XY gonads and acted downstream of the testis-determining factor *Sox9a*, suggesting that miR-202-5p played an early role in testis development (Wainwright et al., 2013). Human miR-202-5p was expressed in Sertoli cells and possibly associated with

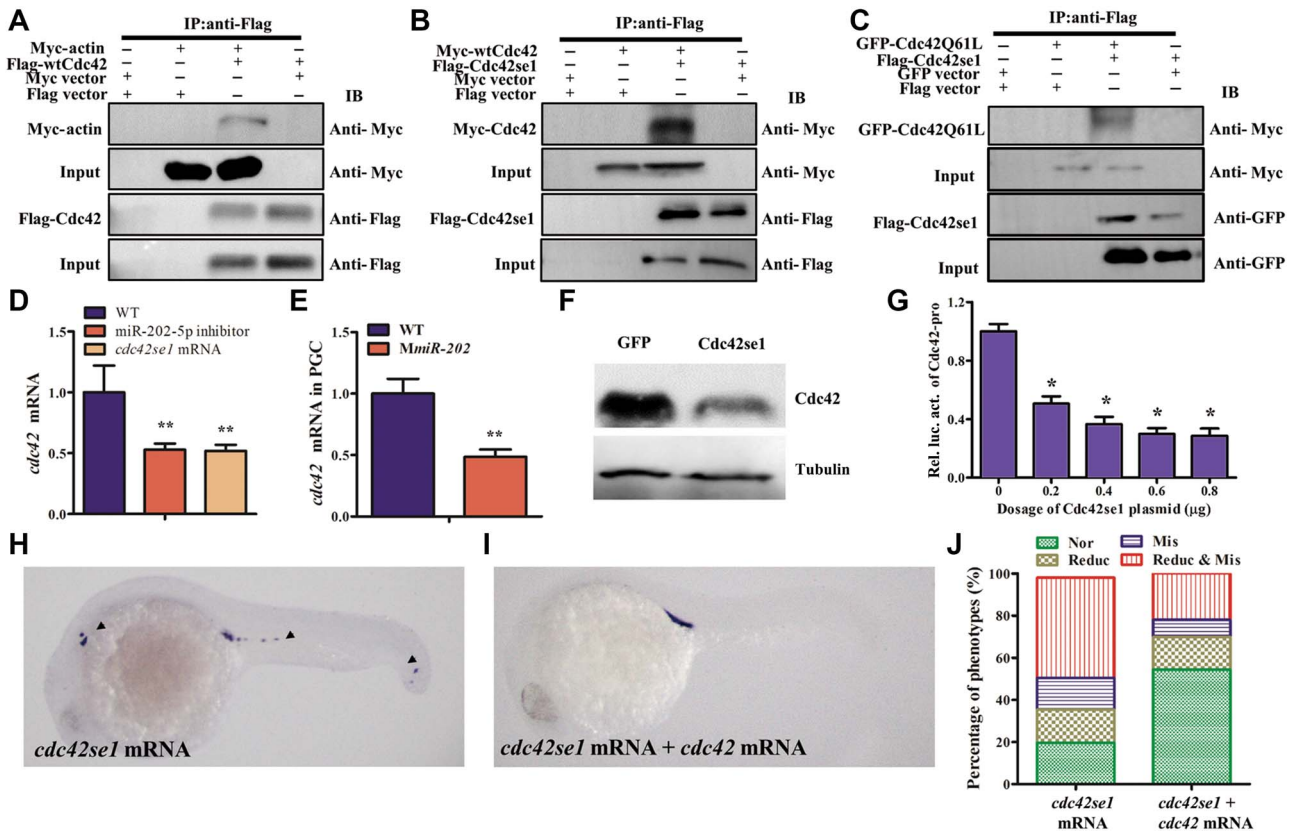


Figure 6 *Cdc42se1* negatively regulates *Cdc42* expression in PGCs. (A–C) The interactions among *Cdc42se1*, *Cdc42*, and actin were analyzed by co-IP. HEK293T cells were co-transfected with Myc-actin and Flag-*Cdc42* (A), Myc-*Cdc42* and Flag-*Cdc42se1* (B), EGFP-*Cdc42Q61L* and Flag-*Cdc42se1* (C) plasmids, and cells transfected pCMV-Myc, pCMV-Flag, or pEGFPN3 vector were used as controls. Twenty-four hours later, cells were immunoprecipitated with anti-Flag antibody, followed by analysis of the immunoprecipitates by western blotting. (D and E) qRT-PCR analysis of the endogenous *cdc42* mRNA in embryos injected with miR-202-5p inhibitor or *cdc42se1* mRNA (D) or in PGCs of WT and *MmiR-202* embryos (E). (F) The endogenous *Cdc42* protein in *cdc42se1* mRNA-injected embryos by western blotting. (G) Relative expression of luciferase activity driven by *cdc42* promoter in HEK293T cells, which were co-transfected with different dosages of pcDNA3.1(+)-*Cdc42se1* plasmid. (H and I) Representative images of embryos respectively injected with *cdc42se1* mRNA (H) and *cdc42se1* mRNA + *cdc42* mRNA (I) by WISH analysis of *vasa*. (J) Statistical analysis of PGC phenotypes in embryos injected with *gfp* mRNA, *cdc42se1* mRNA, and *cdc42se1* + *cdc42* mRNA. * $P < 0.05$; ** $P < 0.01$.

spermatogenesis (Dabaja et al., 2015). Frog miR-202-5p was highly expressed in stage I–II oocytes (Armisen et al., 2009). MiR-202-5p was a germ plasm-specific miRNA (Zhang et al., 2017), suggesting a potential role of miR-202-5p in PGC development.

Herein, we used loss of function strategy to explore the function of miR-202-5p in PGC development. The miR-202 mutant line were fertile and produced functional eggs and sperms, suggesting that miR-202 was not primary for reproductive capacity in zebrafish (Figure 1). However, maternal loss or knockdown of miR-202-5p significantly impaired PGC migration (Figure 2; Supplementary Figures S1 and S2). The PGC developmental defects in *MmiR-202* embryos, to a certain degree, was similar to that observed in *MZdicer* embryos (Mishima et al., 2006), demonstrating that miR-202-5p was a key miRNA in PGC development in zebrafish. Unlike other germ plasm-coding genes, such as *vasa*, *tdrd12*, and *dnd*, mutation of these did not affect PGC development during embryogenesis (Hartung et al., 2014;

Dai et al., 2017; Li et al., 2017); however, their mutant lines ultimately developed into infertile ‘males’. These suggested that different types of germ plasm components played diverse roles in zebrafish reproduction. Since reproduction was critical for species continuation, various species had adopted diverse regulatory mechanisms, such as genetic compensation response (GCR), to ensure reproduction works if some abnormalities happened. Recently, it was reported that there are two different GCR strategies in zebrafish, indicating that the occurrence of some loss-of-function mutations would activate expression of other genes in the same family, which resulted in recessive phenotypes (El-Brolosy et al., 2019; Ma et al., 2019). It was uncertain whether there was GCR that activated the expression of functionally similar miRNA to protect the gonad development in miR-202 mutant line. In medaka, Gay et al. (2018) reported that miR-202-5p was mainly localized in ovarian granulosa cells. Deletion of miR-202 impaired early oogenesis/folliculogenesis and decreased the number of large follicles, ultimately leading

to dramatically reduced female fecundity in medaka (Gay et al., 2018). The different cellular localization and function of miR-202-5p between zebrafish and medaka suggested that it might evolve diverse strategies for regulating reproduction in different fish species.

To clarify the mechanism by which miR-202-5p regulated PGC migration, target genes of miR-202-5p were bioinformatically predicted, and a critical miR-202-5p target gene *cdc42se1* was identified (Figure 3). Previous studies showed that there was a weak interaction between *cdc42se1* and Dnd in zebrafish (Chen et al., 2010). In this study, we demonstrated that overexpression of *Cdc42se1* impaired PGC migration, similar to that observed in miR-202-5p knockdown or knockout embryos (Figures 4 and 5). However, knockdown of *Cdc42se1* could not significantly rescue miR-202-5p inhibitor-mediated PGC development defects, indicating that *cdc42se1* was just one of the critical dysregulated target genes of miR-202-5p. It was known that the PGC development was protected by transcriptional silence, which was regulated at the level of RNA polymerase II (pol II) activity and chromatin state (Strome and Updike, 2015). In *Drosophila*, the germ plasm-specific lncRNA *pgc* was important for PGC migration and maintenance by repression of RNA pol II-dependent transcription (Nakamura et al., 1996; Martinho et al., 2004). Prediction of target genes showed that numerous potential targets of miR-202-5p were associated with RNA pol II, translation, and chromatin structure (Supplementary Table S1), suggesting that other miR-202-5p targets might be involved in maintaining transcriptional silence in PGCs.

In zebrafish, small Rho-GTPase family, including Rac1, RhoA, and Cdc42, played essential roles in PGC migration. In migratory PGCs, Rac1 was responsible for formation of actin-rich structures and RhoA promoted retrograde actin flow, which were required for bleb formation (Ridley, 2015). Chemokine-guided germ cells within zebrafish embryos required the function of the small Rho GTPases Rac1 and RhoA (Kardash et al., 2010; Xu et al., 2012; Miranda-Rodriguez et al., 2017). Cdc42 was involved in cell cytoskeleton organization and cell migration (Heasman and Ridley, 2008) and controlled membrane invagination via the clathrin-independent pinocytotic pathway (Sabharanjak et al., 2002). Goudarzi et al. (2017) reported that overexpression of an inactivated form of Cdc42 in PGCs impaired PGC membrane invagination and bleb formation and ultimately resulted in PGC mislocalization. In mouse, *Cdc42se1* interacted with the activated form of Cdc42 through its N-terminal CRIB domain. *Cdc42se1* blocked Cdc42-induced c-JNK activity and altered Cdc42-induced morphology change in COS1 cells and NIH-3T3 fibroblasts in a manner depending on Cdc42 binding (Pirone et al., 2000). In this study, we found that zebrafish *Cdc42se1* interacted with both WT and activated mutant of Cdc42 (Figure 6B and C). Zebrafish and mouse *Cdc42* homologues shared 99.5% identities, and the N-terminus of zebrafish and mouse *Cdc42se1* homologues that contain the CRIB domain had 87.6% identities; therefore, it was possible that *Cdc42se1* also negatively regulated Cdc42 activity in

zebrafish. Moreover, *Cdc42se1* reduced the endogenous Cdc42 expression by downregulating the activity of the Cdc42 promoter, and overexpression of the WT form of Cdc42 could rescue the PGC developmental defects in *Cdc42se1* overexpressed embryos (Figure 6). Therefore, *Cdc42se1* overexpression negatively regulated the expression of Cdc42 and thus impaired PGC migration. However, it remains unclear how *Cdc42se1* affects Cdc42 transcription and whether *Cdc42se1* affects Cdc42 activity in zebrafish.

In conclusion, we revealed a novel miRNA involved in the Cdc42 signaling pathway in PGC migration; germ plasm-specific miR-202-5p repressed the expression of the Cdc42-negative effector *Cdc42se1* to ensure the sufficient expression level of Cdc42 in zebrafish PGC development.

Materials and methods

Ethics statement

All procedures with zebrafish were approved by the Ethics Committee of Sun Yat-sen University, and the methods were carried out in accordance with the approved guidelines.

Fish strains and cell lines

Zebrafish lines including AB line WT and PGC transgenic line Tg1 (Tg *kop:EGFP-UTR-nos3*) were purchased from the China Zebrafish Resource Center. Fish were raised at 28°C with 10 h darkness and 14 h light. All embryos were collected after natural spawning and staged as previously reported (Kimmel et al., 1995). HEK293T cells were maintained in DMEM supplemented with 10% FBS at 37°C in a 5% CO₂ incubator.

Targeted gene disruption by CRISPR/Cas9

MiR-202-5p knockout by CRISPR/Cas9 was performed as previously described (Guo et al., 2017). Two guide RNAs (gRNAs) were designed with an online tool (<http://crispr.mit.edu/>), gRNA1 (GGAAAAAGGATACGTATATGG) and gRNA2 (GGCATAGGGCATGGGAAAATGGG). The gRNAs were transcribed with T7 RNA Polymerase Kit (Ambion). *Cas9* mRNA (300 ng/μl) and gRNA (50 ng/μl) were injected into one-cell embryos, and the mutations were analyzed by sequencing.

Prediction of miR-202-5p target genes

Target genes of miR-202-5p were analyzed for sites complementary to the miR-202-5p seed sequence (UCCUAUG) using both PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html) and miRanda (<http://www.microna.org/microna/home>). Target genes predicted by both PITA and miRanda were considered as potential target genes of miR-202-5p (Supplementary Table S1).

Plasmid construction

The cDNA of *cdc42se1* (ENSDARG00000023724, ZV11), including its ORF and 3'UTR, and ORF of *cdc42* (ENSDARG000000044573, ZV11) were respectively inserted into the pCS2+ vector for mRNA synthesis by *Bam*HI and *Xba*I. The 2000-bp fragment

of the *cdc42* promoter was inserted to the pGL3B vector to generate the pGL3-Cdc42-pro plasmid. The ORF of *cdc42se1* and RFP and the 3'UTR of *nos3* were inserted into the pCS2+ vector to generate the pCS2-Cdc42se1-RFP-UTR-*nos3* plasmid. The ORFs of *cdc42*, *actin* (ENSDARG0000037746, ZV11), and *cdc42se1* were respectively inserted into both pCMV-*N*-Flag and pCMV-*N*-Myc vectors by *EcoRI* and *XhoI* to generate Myc-actin, Myc-Cdc42, Flag-Cdc42se1, and Flag-Cdc42 plasmids. The ORF of *cdc42se1* fused with its own 3'UTR was inserted into the pcDNA3.1(+) vector by *EcoRI* and *NotI*. The activated form of Cdc42 (Cdc42Q61L) was inserted into the pEGFPN3 vector by *EcoRI* and *BamHI* to generate the pEGFPN3-Cdc42Q61L (EGFP-Cdc42Q61L) plasmid. The 3'UTR of *cdc42se1* was inserted into the psiCHECK2 vector. Then, the miR-202-5p binding site (CATAGGA) in the constructed WT plasmids were replaced to (CGCGAGA) by Hieff MutTM Multi Site-Directed Mutagenesis Kit (Yeasen). Primers were list in [Supplementary Table S2](#).

mRNA synthesis

For mRNA synthesis, ORFs were inserted into the pCS2+ vector. The plasmid was linearized with *NotI* and transcribed using SP6 mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's instructions.

Isolation of PGC by FACS

GFP-3'UTR-*nos3* mRNA (400 pg/embryo) was injected into 1-cell stage WT and *MmiR-202* embryos. At 10 hpf, PGCs from 400 WT or *MmiR-202* embryos were isolated by FACS. A total of 1200 PGCs were sorted and reverse-transcribed by Single Cell Sequence Specific Amplification Kit (Vazyme, P621-01). The *cdc42se1* and *cdc42* mRNA levels in PGCs of WT and *MmiR-202* embryos were analyzed by qRT-PCR.

RNA isolation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The first-strand cDNA was synthesized using PrimeScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa). qRT-PCR analyses of *cdc42se1*, *cdc42*, and *vasa* were performed as previously described ([Xiang et al., 2017](#)). The relative gene expression was normalized with β -*actin* using $2^{-\Delta\Delta Ct}$ methods. Expression analysis of miR-202-5p/miR-202-3p was performed as previously described ([Zhang et al., 2017](#)). Data were shown as mean \pm SD from three independent experiments in triplicate. The primer sequences were listed in [Supplementary Table S2](#).

WISH

The digoxin-labelled antisense probes of *vasa* and *cdc42se1* were synthesized using the DIG RNA Labeling Mix with T7 RNA polymerase (Roche). WISH was performed as previously described ([Thisse and Thisse, 2008](#); [Zhang et al., 2017](#)).

Dual luciferase reporter assay

HEK293T cells in 24 well plate were transiently co-transfected with 10 ng plasmid (WT or mutant psiCHECK2-3'UTR-Cdc42se1) and 25 nM miR-202-5p mimics or negative control with Lipofectamine 3000 (Invitrogen). The cells were lysed at 24 h post-transfection, and the luciferase activities were measured by a dual-luciferase reporter assay system (Promega). HEK293T cells in the 24-well plate were transfected with 250 ng pGL3B-Cdc42-pro plasmid and 25 ng pRL-TK, and cells transfected with the pGL3B vector and 25 ng pRL-TK were used as control. Furthermore, the cells were co-transfected with a total of 1 μ g plasmids containing different amounts of pcDNA3.1(+)-Cdc42se1 plasmid/pcDNA3.1(+) vector (0.2/0.8, 0.4/0.6, 0.6/0.4, and 0.8/0.2 μ g). The cells were lysed for luciferase assay after 24 h post-transfection. At least three independent experiments were performed.

Micro-injection

The miR-202-3p mimics, miR-202-5p inhibitor (a special modified antisense single RNA that could pair with miR-202-5p), mimics and corresponding negative controls were designed and purchased from RiboBio. *cdc42se1* MO (CCGGAGAUGAGUGCGU-UCUGGCAUA) targeting the start codon and its mismatch MO were designed from Gene Tools.

Three dosages of 10, 40, and 160 pg/embryo miR-202-5p inhibitor were respectively injected into 1-cell stage embryos, and embryos injected with equal amount of control inhibitors were used as control. To analyze the effect of miR-202-5p knockdown on PGC migration, embryos were respectively injected with 40 pg/egg miR-202-5p or control inhibitor and collected from dome to prim 5 stages, followed by whole-mount *in situ* hybridization of *vasa*.

The Cdc42se1-RFP-UTR-*nos3* or *rfp* mRNA (300 pg/embryo) were injected in 4-cell embryos of the Tg1 line. The PGC migration was analyzed by a Zeiss LSM 880 confocal microscope.

Rescue experiments

To rescue miR-202-5p inhibitor (40 pg/embryo) knockdown embryos, embryos were co-injected with miR-202-5p mimics (40 pg/embryo) or *cdc42se1* MO (4000 pg/embryo).

To rescue the PGC defects in *MmiR-202* embryos, miR-202-5p, or miR-202-3p mimics (40 pg/embryo) were injected, and the PGC number and localization were analyzed. The experiment was independently performed in three repeats, and each group had >100 embryos.

Three dosages of *cdc42se1* mRNA with its own 3'UTR (100, 200, 300 pg/embryo) were respectively injected into 1-cell stage embryos, which were collected for whole-mount *in situ* hybridization of *vasa* at prim 5 stage. To rescue Cdc42se1 overexpressed embryos, miR-202-5p mimics (40 pg/embryo), *cdc42se1* MO (4000 pg/embryo), or *cdc42* mRNA (150 pg/embryo) was injected into 1-cell stage embryos, respectively. Each experiment was independently performed in three repeats, and each group had >40 embryos.

PGC phenotype observation

To score PGC phenotypes, embryos were fixed at 24 hpf in 4% paraformaldehyde, and PGCs were labelled by *in situ* hybridization using the *vasa* anti-sense probe. Phenotypes of PGCs in each embryo were recorded as normal, number reduction only, mislocalization only or both reduction and mislocalization. To quantify the different phenotypes, embryos with over 25 PGCs at the genital ridge and with <3 ectopic localized PGCs were counted as normal; embryos with at least three mislocalized PGCs were counted as mislocalization; embryos with <10 PGCs were counted as number reduced. To determine the phenotype, sets of 30–50 embryos were scored. Standard deviations were calculated for multiple repetitions of each experiment.

Western blotting and co-IP assay

For analyzing miR-202-5p repressed *Cdc42se1* expression *in vitro*, HEK293T cells were co-transfected with 5 µg pcDNA3.1(+)-*Cdc42se1* plasmid and 100 nmol control mimics/miR-202-5p mimics. Forty-eight hours later, cells were lysed and analyzed by western blotting using anti-*Cdc42se1* antibody.

For analyzing *Cdc42* expression *in vivo*, 200 pg *cdc42se1* mRNA was injected into 1-cell stage embryos, which were collected at 10 hpf for western blotting with anti-*Cdc42* antibody. The testes from WT and *MmiR-202* adults were collected and lysed for western blotting with anti-*Cdc42* antibody.

For co-IP assay, HEK293T cells were co-transfected with 10 µg of different plasmid combinations as follows: Myc-*Cdc42* and Flag-*Cdc42se1*, Myc-actin and Flag-*Cdc42*, Flag-*Cdc42se1* and Myc-actin, Myc vector and Flag-*Cdc42se1*, Myc-*Cdc42* and Flag vector, or Flag vector and Myc vector. Cells were lysed with lysis buffer (10 mM Tris-HCl [pH 7.5], 0.4 M NaCl, 1% NP-40, 0.4% Triton X-100, 0.2% sodium deoxycholate, 1 mM EDTA, protease inhibitors [Calbiochem]) at 48 h post-transfection. Western blotting and co-IP were performed as previously described (Jia et al., 2013). Anti-Flag antibody (M20008) and anti-Myc antibody (M20002) were purchased from Abmart; anti- α -tubulin antibody (ab15246) and anti-*Cdc42* antibody (ab187643) were purchased from Abcam; anti-mouse IgG (7076S) was purchased from Cell Signaling Technology; anti-rabbit IgG (A0208) was purchased from Beyotime Technology.

Statistics analysis

All statistics were calculated using SPSS version 20. Differences between control and treatment groups were assessed by one-way ANOVA. *P*-values <0.05, <0.01, <0.001, and <0.0001 are identified with 1, 2, 3, and 4 asterisks, respectively. ns, *P* ≥ 0.05.

Supplementary material

Supplementary materials are available at *Journal of Molecular Cell Biology* online.

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