An miR-200 Cluster on Chromosome 23 Regulates Sperm Motility in Zebrafish

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Besides its well-documented roles in cell proliferation, apoptosis, and carcinogenesis, the function of the p53–microRNA axis has been recently revealed in the reproductive system. Recent studies indicated that miR-200 family members are dysregulated in nonobstructive azoospermia patients, whereas their functions remain poorly documented. The aim of this study was to investigate the function of the miR-200 family on zebrafish testis development and sperm activity. There was no substantial difference in testis morphology and histology between wild-type (WT) and knockout zebrafish with deletion of miR-200 cluster on chromosome 6 (chr6-miR-200-KO) or on chromosome 23 (chr23-miR-200-KO). Interestingly, compared with WT zebrafish, the chr6-miR-200-KO zebrafish had no difference on sperm motility, whereas chr23-miR-200-KO zebrafish showed significantly improved sperm motility. Consistently, ectopic expression of miR-429a, miR-200a, and miR-200b, which are located in the miR-200 cluster on chromosome 23, significantly reduced motility traits of sperm. Several sperm motility-related genes, such as *amh*, *wt1a*, and *srd5a2b* have been confirmed as direct targets of miR-200s on chr23. 17α-ethynylestradiol (EE2) exposure resulted in upregulated expression of p53 and miR-429a in testis and impairment of sperm motility. Strikingly, in p53 mutant zebrafish testis, the expression levels of miR-200s on chr23 were significantly reduced and accompanied by a stimulation of sperm motility. Moreover, the upregulation of miR-429a associated with EE2 treatment was abolished in testis with p53 mutation. And the impairment of sperm activity by EE2 treatment was also eliminated when p53 was mutated. Together, our results reveal that miR-200 cluster on chromosome 23 controls sperm motility in a p53-dependent manner. **(*Endocrinology* 159: 1982–1991, 2018)**

Spermatogenesis is an important reproductive event in which mature spermatozoa are developed from male spermatogonia in the testis. High-quality mature spermatozoa are essential for fertilization success and survival of the progeny. As a key determinant of sperm quality, sperm motility can be quantitatively evaluated by computer-assisted sperm analysis (CASA) in many vertebrates, including fish species (1), and displayed by some kinematic parameters such as the average path velocity (VAP), the straight line velocity (VSL), and the curvilinear velocity (VCL). Moreover, sperm motility has a positive correlativity with fertility (2–5) and hatching rates (3, 6–11).

Several master genes, such as *dmrt1*, *sf1*, *sox9*, *amh*, and *wt1*, have been identified as participating in the progress of male sex differentiation and spermatogenesis in vertebrates (12, 13). Specially, some secreted peptides, small organic compounds, iron concentrations, and signaling pathways are involved in sperm motility and essential for fertility (14). A number of genes, such as *wt1*, *galectin-1*, *crisp1*, *amh*, *srd5a*, *gsk3a*, *dmrt1*, and *sf1*, have been reported to be associated with spermatogenesis

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Abbreviations: 3′UTR, 3′ untranslated region; CASA, computer-assisted sperm analysis; EE2, 17α-ethynylestradiol; miRNA, microRNA; mRNA, messenger RNA; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; SEM, standard error of the mean; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WT, wild-type.

and sperm capacity. Aberrant expression of these factors results in impairment of sperm activity and male infertility (15–23).

MicroRNAs (miRNAs) play critical roles in controlling cellular self-renewal, differentiation, proliferation, and apoptosis by posttranscriptionally regulating the expression of multiple target genes (24, 25). Recently, miRNA were identified as important regulators in the reproductive processes including oogenesis, spermatogenesis, and fertilization. Several key factors for miRNA biogenesis, such as Argonaute 1, DROSHA, and Dicer, are required for oocyte formation, germ line cell division, or spermatogenesis in fly or mouse (26–28). In addition, a number of miRNAs, including miR-21, miR-34c, and let-7, regulate the self-renewal of spermatogonial stem cells and spermatogenesis in mouse or fly (29–31). However, the regulatory and functional roles of miRNA in the reproductive processes are still not clear in teleosts.

MiR-141, miR-429, and miR-200a/200b/200c belong to the conserved miR-200 family that has recently emerged as an important regulator of somatic growth (32) and neurogenesis in zebrafish (33). As transcriptional targets of p53, miR-200s directly regulate growth hormone/insulin-like growth factor axis genes and form a negative regulatory loop to control zebrafish embryo size (32). Extraordinarily, the miR-200 family plays an essential role in the reproductive processes. miR-200b and miR-429 are required for mouse ovulation and female fertility by targeting the zeb1 gene and inhibiting luteinizing hormone synthesis (34). In spermatozoa or testicular biopsies of nonobstructive azoospermia patients, miR-141 and miR-429 were significantly upregulated when compared with healthy controls (35, 36), which may be caused by the demethylation of these two miRNA (37). In the current study, zebrafish were used as a model system to dissect the physiological function of miR-200 family in mature testis. We provided evidence that miR-200 cluster in chromosome 23 regulates sperm motility by directly controlling the expression of multiple target genes. Further, we demonstrated that estrogen treatment leads to reduction of sperm motility and upregulation of miR-429a expression in testis in a p53-dependent manner.

Materials and Methods

Fish care and cell line

Experimental zebrafish used and relevant animal experimental protocol were reviewed and approved by the institution animal care and use committee of Huazhong Agricultural University. Experimental zebrafish were anesthetized using tricaine methanesulfonate (MS-222) before injection and sampling. Wild-type (WT) AB line and tp53 mutant zebrafish were raised at 28.5°C on a 14-hour light/10-hour dark cycle and maintained in accordance with the Guide for the Care and Use of Laboratory Animals. Tp53 mutant zebrafish was obtained from Wuhan Xiao (Institute of Hydrobiology, Chinese Academy of Sciences) and originally from A Thomas Look (Harvard Medical School) (38). HEK-293T cells were cultured in the recommended conditions and maintained in a constant temperature incubator at 37°C with 5% CO_2.

Generation of miR-200 mutant zebrafish

The zebrafish miR-200b, miR-200a, and miR-429a cluster locates on the chromosome 23, whereas the miR-200c, miR-429b, and miR-141 cluster locates on chromosome 6 (32). We generated the miR-200 knockout zebrafish in chromosome 23 (chr23-miR-200-KO) and chromosome 6 (chr6-miR-200-KO) using CRISPR and Cas9 technology, respectively. The guide RNA target sites for miR-200 clusters were designed by the online service of the Web site (http://zifit.partners.org/ZiFiT/CSquare9Nuclease.aspx); the knockout experiment was performed as described previously (39). For genotyping of mutant zebrafish, the genomic DNA was extracted from zebrafish tail fin and polymerase chain reactions (PCRs) were performed to amplify the genomic DNA containing target sites, followed by sequencing of the PCR products to identify the mutation type. The primers are listed in Supplemental Table 1.

Zebrafish treatment, sampling and determination of sperm motility, and fertilization rate

Three-month-old, sexually mature WT and p53 mutant male zebrafish were exposed to 17α-ethynylestradiol (EE2) at a gradient dose of 0, 1, 5, or 10 μg/L for 2 weeks. For overexpression experiments, each male zebrafish was intraperitoneally injected with miRNA agomiR and corresponding negative control (Genepharma, Shanghai, China) at a dose of 60 mg/kg of fish body weight, every 3 days 4 times. We pressed the male zebrafish abdomen and collected the milky sperms that flew from cloacal orifice into 1X Hanks balanced salt solution (Life Technologies, Grand Island, NY). After the sperm was activated when mixed with water, the sperm activity was measured by the CASA system. Spermatozoa motility and kinematic parameters were quantified by CASA II using Animal Motility Software Manual, version 1.4 (Hamilthon-Thorne Research, Beverly). Then the zebrafish were anesthetized using MS-222 before testes were sampled for following RNA isolation. The determination of fertilization rate of WT and chr23-miR-200-KO mutant male zebrafish was performed as described (40).

Quantitative real-time PCR analysis

Total RNA was isolated from each sample using miRNeasy Mini Kits (Qiagen, Hilden, Germany) and subjected to DNase I treatment (Invitrogen). Oligo(dT) primer and stem-loop RT primers were used for complementary DNA synthesis using Superscript II reverse transcription (Invitrogen, Carlsbad, CA). Then, quantitative real-time PCR (qRT-PCR) reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA), as described previously (32). Each experiment was performed in triplicate and data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The abundance of messenger RNA (mRNA) was normalized to that of elfa mRNA and miRNA was normalized to that of U6. Primers are shown in Supplemental Table 1.

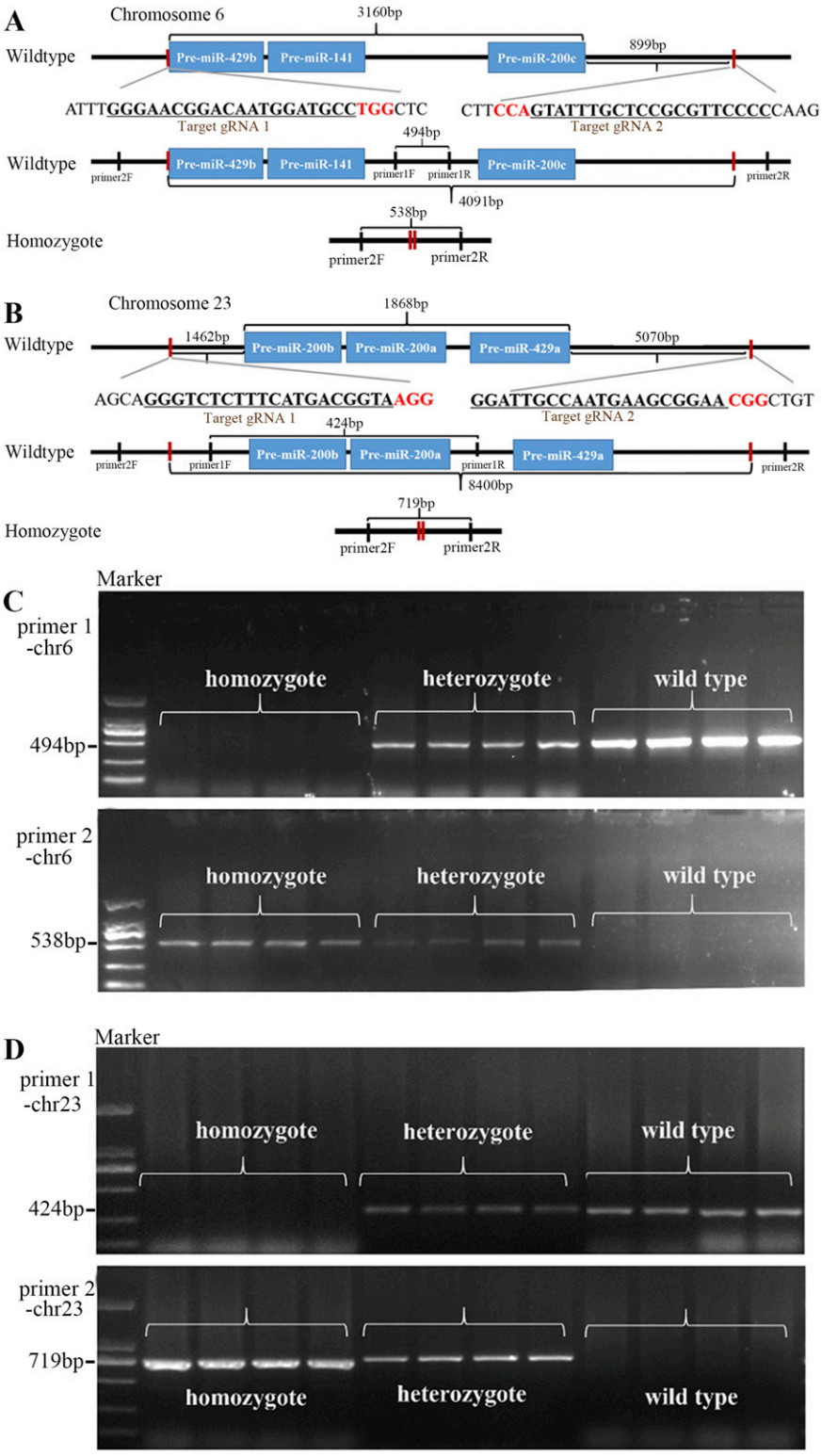

Figure 1. Knockout of miR-200b, miR-200a, and miR-429a in chromosome 23 and miR-141, miR-429b, and miR-200c in chromosome 6. (A) Generation of knockout of premiR-141, premiR-429b, and premiR-200c (chr6-miR-200-KO) zebrafish. (B) Generation of knockout of premiR-200b, premiR-200a, and premiR-429a (chr23-miR-200-KO) zebrafish. (C) Identification of homozygote, heterozygote of chr6-miR-200-KO and WT zebrafish. A 494-bp band was detected in the WT and heterozygote zebrafish and no band was observed in the homozygote zebrafish when using primers 1F and 1R. A 538-bp band was detected in the heterozygote and homozygote zebrafish and no band was observed in WT zebrafish when using primers 2F and 2R for genome of chromosome 6. (D) Identification of homozygote, heterozygote of chr-23-KO and WT zebrafish. A 424-bp band was detected in the WT and heterozygote zebrafish and no band was observed in the homozygote zebrafish when using primers 1F and 1R. A 719-bp band was detected in the heterozygote and homozygote zebrafish and no band was observed in WT zebrafish when using primers 2F and 2R for genome of chromosome 23. The DNA marker was DL-2000.

Plasmid construction and luciferase reporter assay

amh, *wt1a*, and *srd5a2b* were predicted to be the target genes of miR-200 using the Targetscan bioinformatics algorithm (Supplemental Fig. 1). The 3′ untranslated regions (3′UTRs) of *amh*, *wt1a*, and *srd5a2b* were cloned into the pmir-GLO plasmid (Promega) and corresponding mutant plasmids were construct to validate the targeted relationship. For luciferase reporter assay, HEK-293T cells were cultured in 24-well plates and cotransfected by 50 nM miR-200 mimic or negative control mimic with 25 ng constructed plasmid using DharmaFECT transfection reagent (Dharmacon). Twenty-four hours after transfection, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). Relative firefly luciferase activity was normalized with renilla luciferase activity. Experiments were conducted in triplicate.

Data analysis

Data of gene expression and sperm kinematic parameters are shown as mean ± standard deviation (SD) and mean ± standard error of the mean (SEM), respectively. Statistical analysis was performed with SPSS software (SPSS Inc.). Significant differences between two groups were analyzed by Student *t* test. $P < 0.05$ was considered statistically significant.

Results

Generation of miR-200 knockout zebrafish on chromosomes 6 and 23

To systematically investigate the functions of miR-200 family members *in vivo*, we deleted the miR-200 clusters on zebrafish chromosome 6 and 23 by CRISPR/Cas9, respectively. As shown in Fig. 1A and 1B, the targeting strategy deleted a 4-kb fragment containing the premiR-429b/-141/-200c on chromosome 6 (chr6-miR-200-KO) and an 8.4-kb fragment containing the premiR-200b/-200a/-429a on chromosome 23 (chr23-miR-200-KO). Two pairs of primers were designed to distinguish the WT, heterozygote, and homozygote zebrafish. When using the primers 1F and 1R to genotype zebrafish, 494- and 424-bp bands were detected in WT/heterozygote zebrafish of chr6-miR-200-KO and chr23-miR-200-KO, respectively, whereas no band was observed in the homozygote zebrafish (Fig. 1C and 1D). In addition, when using the primers 2F and 2R (the extending time for PCR was 1 minute), we could detect a specific band of 538 bp and 719 bp in the heterozygote/homozygote zebrafish of chr6-miR-200-KO and chr23-miR-200-KO, whereas no band was observed in WT zebrafish because the length of amplified fragments are too long (Fig. 1C and 1D). We developed an efficient method to identify the miR-200 knockout zebrafish.

Deletion of the miR-200 cluster on chromosome 23 promotes sperm motility in zebrafish

According to our morphology and histology observations, there was no important difference in testis tissues among WT, chr6-miR-200-KO, and chr23-miR-200-KO zebrafish (Supplemental Fig. 2). We then measured the kinematic parameters of sperm motility by CASA II. There is no statistical significance in the percentage of motile spermatozoa among WT, chr6-miR-200-KO, and chr23-miR-200-KO zebrafish (Fig. 2A). However, compared with WT, the values of several kinematic parameters including VAP, VSL, and VCL of motile sperm significantly increased in chr23-miR-200-KO zebrafish, whereas these values were not obviously changed in chr6-miR-200-KO zebrafish (Fig. 2B). The detailed videos of motile sperms are shown in Supplemental Video 1 (WT), Supplemental Video 2 (chr6-miR-200-KO), and Supplemental Video 3 (chr23-miR-200-KO). Meanwhile, the fertilization rate of male zebrafish was measured. When 3-month-old WT male zebrafish and chr23-miR-200-KO male zebrafish were crossed with WT female zebrafish in natural spawning, the chr23-miR-200-KO male zebrafish exhibited a higher fertilization rate than WT male zebrafish (Fig. 2C). These results suggest that miR-200b/-200a/-429a might play an important role in sperm motility.

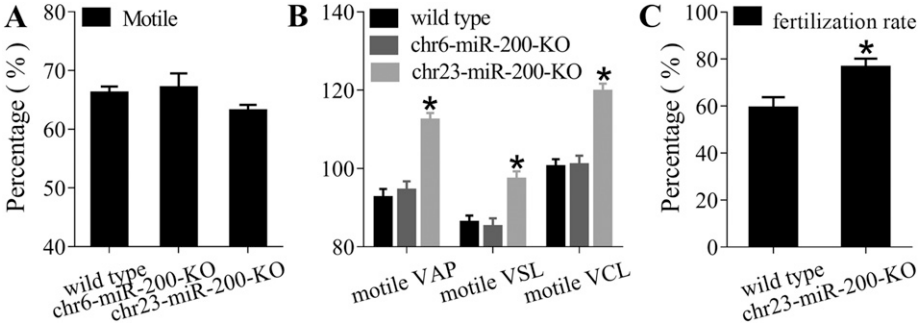

Figure 2. Knockout of miR-200 cluster in chromosome 23 promotes sperm motility. Representative kinematic parameters of sperm motility in chr6-miR-200-KO, chr23-miR-200-KO, and WT zebrafish. (A) Percentage of motile spermatozoa. (B) Values of VAP, VSL, and VCL. Error bars indicate mean ± SEM (n = 60). *$P < 0.05$. (C) Fertilization rates of WT and chr23-miR-200-KO male zebrafish that were crossed with WT females by natural spawning. Error bars indicate mean ± SD (n = 6). *$P < 0.05$.

Sperm motility-related genes are potential targets of miR-200 cluster on chromosome 23

Using the Targetscan bioinformatics algorithm (http://www.targetscan.org/fish_62/), we found several predicted target genes of miR-200b/-200a/-429a that are involved in sperm motility, such as anti-Müllerian hormone (*amh*), Wilms tumor 1a (*wt1a*), and steroid-5-alpha-reductase, alpha polypeptide 2b (*srd5a2b*) (Supplemental Fig. 1). Then, the expression of target genes in testis of chr23-miR-200-KO zebrafish was detected using qRT-PCR. The mRNA levels of *amh*, *wt1a*, and *srd5a2b* in testis were 6.1-, 2.3-, and 10.4-fold upregulated in chr23-miR-200-KO testis compared with that in WT zebrafish, respectively (Fig. 3A).

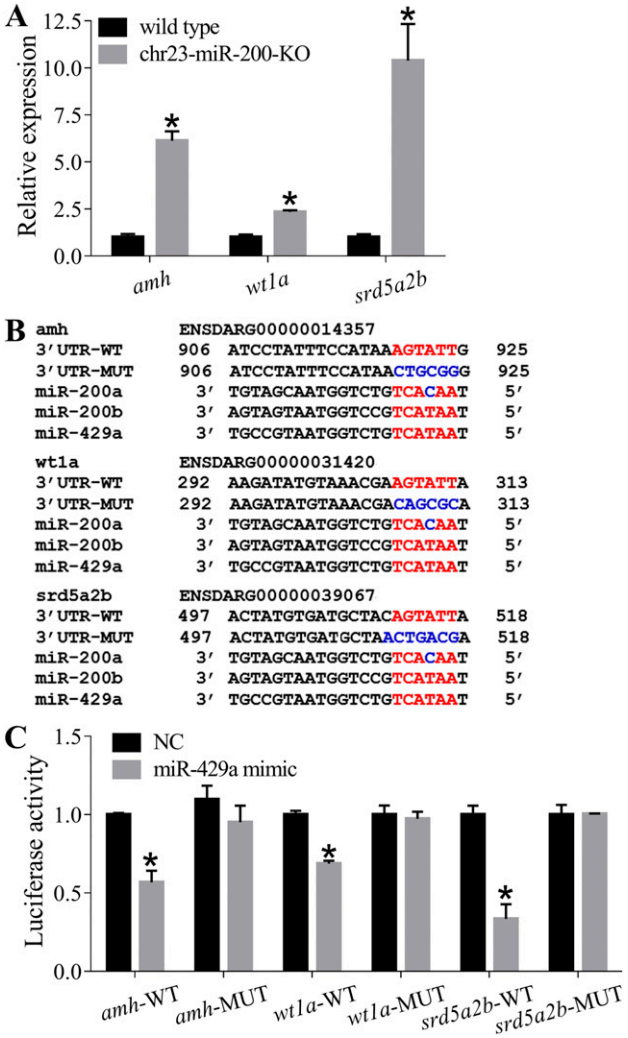

Figure 3. *wt1a*, *srd5a2b*, and *amh* are the target genes of miR-200s in chromosome 23. (A) Comparison of *amh*, *wt1a*, and *srd5a2b* expression in testis between chr23-miR-200-KO and WT zebrafish. (B) The construction of luciferase reporters. MiR-429a/200a/200b seed region and predicted target sites within 3′UTR of *wt1a*, *srd5a2b*, and *amh* are shown in red; the mutation sites are indicated in blue. (C) Validation of potential targets of miR-429a by a dual-luciferase reporter assay. Luciferase assays were performed in triplicate. Error bars indicate mean ± SD, n = 3. $^*P < 0.05$.

To confirm the regulation of zebrafish *amh*, *wt1a*, and *srd5a2b* by miR-429a/-200a/-200b, we performed luciferase reporter assays by linking 3′UTR of these putative target genes to the C-terminus of Firefly luciferase. The mutation sites in 3′UTR of *wt1a*, *srd5a2b*, and *amh* are shown in Fig. 3B. MiR-429a repressed the luciferase activity of *wt1a*, *srd5a2b*, and *amh* 3′UTR-pmirGLO to 68.7%, 33.4%, and 56.8%, whereas mutation of the predicted miR-429a binding sites abolished these repressions (Fig. 3C). Similarly, luciferase reporter assays indicated that miR-200a and miR-200b also especially repressed the luciferase activity of *wt1a*, *srd5a2b*, and *amh* 3′UTR-pmirGLO (Supplemental Fig. 3).

Ectopic expression of miR-200b/-200a/-429a inhibits sperm motility and downregulates expression of their target genes

To investigate the functions of miR-200b/-200a/-429a *in vivo*, synthetic miR-200b/-200a/-429a agomiR (200b-AGR/200a-AGR/429a-AGR) and scrambled controls were administered intraperitoneally into adult fish that were the same age and were cultured under the same conditions. qRT-PCR analysis demonstrated an 8.3-, 6.6-, and 5.7-fold increase of miR-200a, miR-200b, and miR-429a in the testes after injection of agomiR (Fig. 4A). The values of several kinematic parameters were evaluated, including percentage of motile spermatozoa, VAP, VSL, and VCL of motile sperm. Compared with the control, ectopic expression of miR-200a, miR-200b, and miR-429a agomiR resulted in a substantial decrease of the percentage of motile spermatozoa (Fig. 4B1 and 4B2) and the values of three kinematic parameters including VAP, VSL, and VCL (Fig. 4C1 and 4C2).

Generally, miRNA suppress mRNA expression of most target genes; therefore, qRT-PCR was used to detect the expression levels of these predicted target genes after ectopic expression of miR-200a, miR-200b, and miR-429a. Compared with the zebrafish injected with control agomiR, injection of miR-200 agomiR resulted in significant reductions of *amh*, *wt1a*, and *srd5a2b* expression in the testes to 21.8%, 62.8%, and 22.1% in 200a-AGR injected zebrafish; 68.3%, 76.4%, and 64.86% in 200b-AGR injected zebrafish; and 79.8%, 59.1%, and 73.53% in 429a-AGR injected zebrafish, respectively (Fig. 4D1 and 4D2).

Estrogen treatment leads to damage of sperm motility and upregulation of miR-429a expression in testis

High doses of EE2 have been reported to disrupt spermatogenesis and induce expression of miR-200 family members (41). To observe the effect of estrogen on sperm motility in testis, male adult zebrafish were

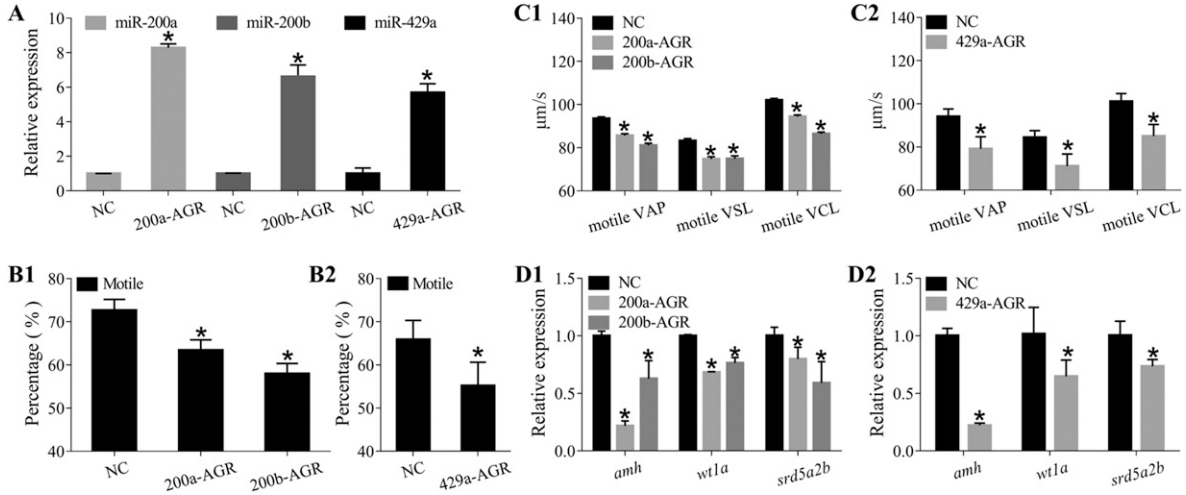

Figure 4. Injection of agomiRs of miR-200s on Chr23 impairs sperm motility. (A) Relative expression of miR-200s on Chr23 in testis after injection with control (NC) and the corresponding agomiRs. (B) Percentage of motile spermatozoa after injection with miR-200a, (B1) miR-200b agomiRs and (B2) miR-429a agomiR. (C) Values of VAP, VSL, and VCL after injection of miR-200a, (C1) miR-200b agomiRs and (C2) miR-429a agomiR. Error bars indicate mean ± SEM (n = 60). $^*P < 0.05$. (D) Relative expression of *amh*, *wt1a*, and *srd5a2b* in testis responding to injection with miR-200a, (D1) miR-200b agomiRs and (D2) miR-429a agomiR. Error bars indicate mean ± SD (n = 3). $^*P < 0.05$.

exposed to different doses of EE2 including 0, 1, 5, and 10 μg/g body weight for 2 weeks. The expression of miR-429a was significantly upregulated by EE2 treatment in a dose-dependent manner, with the 10 μg/g dose increasing approximately 15.7-fold compared with control (Fig. 5A). The values of several kinematic parameters including percentage of motile, VAP, VSL, and VCL of sperm were significantly reduced when treated by different doses of EE2 (Fig. 5B and 5C).

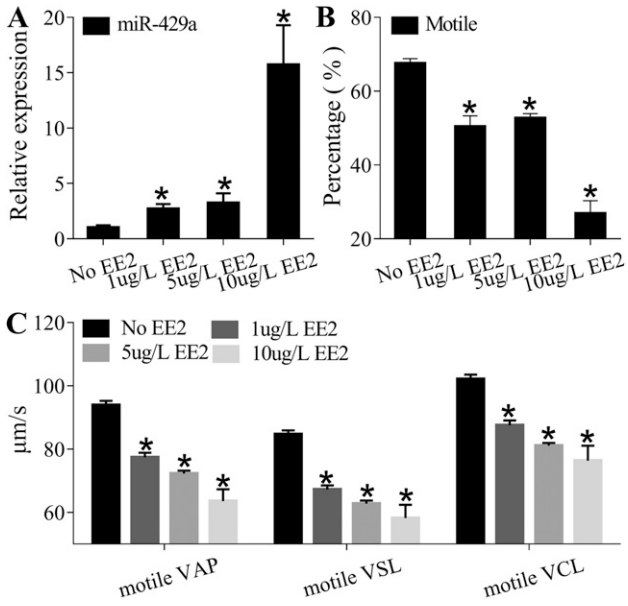

Figure 5. The effects of estrogen on expression of miR-429a and sperm motility. (A) Relative expression of miR-429a in testis after estrogen treatment. Error bars indicate mean ± SD, n = 3. $^*P < 0.05$. (B, C) Values of kinematic parameters of sperm after immersion in gradient concentration of EE2, compared with the control (0 μg/L). (B) Percentage of motile spermatozoa. (C) Values of VAP, VSL, and VCL. Error bars indicate mean ± SEM (n = 60). $^*P < 0.05$.

Loss of p53 function abolishes the effect of estrogen on miR-429a expression and sperm motility

In a previous study, p53 could directly affect the expression of miR-200 in zebrafish (32), so we hypothesized that estrogen may affect sperm motility through p53. Interestingly, the expression of p53 was significantly upregulated by EE2 treatment in a dose-dependent manner, with the 10 μg/L dose increasing approximately 3.0-fold, compared with the control (Fig. 6A). Meanwhile, the expression levels of miR-200a, miR-200b, and miR-429a in p53 mutant zebrafish were all much lower than that in WT zebrafish (Fig. 6B). Consistently, the percentage of motile spermatozoa and the values of VAP, VSL, and VCL of motile sperm were more substantially increased in p53 mutants than in WT zebrafish (Fig. 6C and 6D).

In response to 1 μg/L EE2 exposure, there was a 2.1-fold increase of miR-429a expression in the testis of WT zebrafish, whereas no substantial change in miR-429a expression was detected in p53 mutant zebrafish (Fig. 6E). Moreover, the values of several kinematic parameters in sperm were evaluated in WT and p53 mutant zebrafish after EE2 treatment. As shown in Fig. 6F and 6G, after EE2 exposure, the percentage of motile spermatozoa and the values of VAP, VSL, and VCL of motile sperm significantly decreased in WT zebrafish, whereas there were no important changes in values of these kinematic parameters in p53 mutant zebrafish. These data suggest that EE2 activates miR-429a expression and impairs sperm motility in a p53-dependent manner.

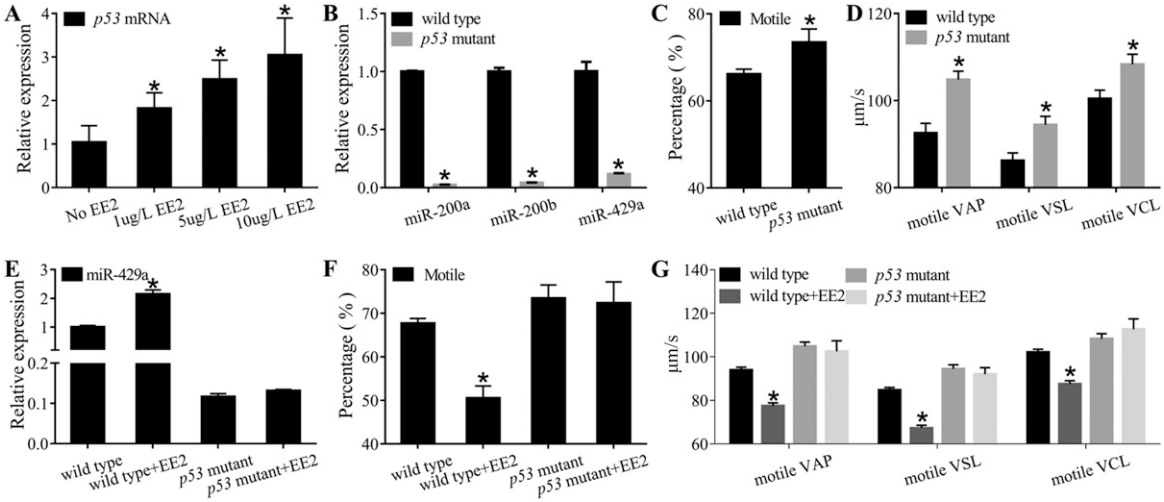

Figure 6. The effects of estrogen on WT and p53 mutant zebrafish sperm motility. (A) Relative expression of p53 mRNA responding to a gradient dose of estrogen. Error bars indicate mean ± SD, n = 3. $*P < 0.05$. (B) Relative expression of miR-429a/-200a/-200b in testis of WT and p53 mutant zebrafish. (C and D) Values of kinematic parameters of sperm motility in WT and p53 mutants. (C) Percentage of motile spermatozoa. (D) Values of VAP, VSL, and VCL. Error bars indicate mean ± SEM (n = 60). $*P < 0.05$. (E) Comparison of miR-429a expression in testis of WT and p53 mutant zebrafish responding to estrogen treatment. Comparison of kinematic parameters of sperm motility in WT and p53 mutant zebrafish responding to estrogen treatment. (F) Percentage of motile spermatozoa. (G) Values of VAP, VSL, and VCL. Error bars indicate mean ± SEM (n = 60). $*P < 0.05$.

Discussion

As a noticeable subject in recent years, male infertility is caused by both environmental and genetic factors (42). Sperm motility, the main index to evaluate sperm quality, is a primary determinant of male fertility in vertebrates, including fish species (2–5). A number of small molecules have been revealed to regulate sperm motility, such as adenosine triphosphate, glycine, acetylcholine, g-aminobutyric acid, and prostaglandin E (14). However, the roles of miRNAs in sperm motility are still not clear. High levels of miR-141 and miR-429 have been reported to be associated with impaired spermatogenesis in human patients (35, 36). Nevertheless, the role of the miR-200 family during spermatogenesis is obscure *in vivo*. The aim of this study was to explore the function of miR-200 family *in vivo* through knockout of premiR-200s in chromosome 6 (chr6-miR-200-KO) and chromosome 23 (chr23-miR-200-KO) in the zebrafish genome. In this study, we knocked out miR-200 family members in zebrafish and developed an efficient method to identify the miR-200 knockout zebrafish. Compared with the WT control, we found that the chr23-miR-200-KO zebrafish displayed a remarkably increase of sperm motility, whereas the chr6-miR-200-KO had no change in sperm motility. As a result, miR-200b, miR-200a, and miR-429a on chromosome 23 play an important role in the regulation of sperm motility.

miRNAs are involved in diverse biological processes through regulating multiple target genes in post-transcriptional process (43, 44). Many important genes have been reported to be associated with spermatogenesis and sperm motility in vertebrates. Loss of *Wt1* protein mutation resulted in spermatogenic failure (17). The level of Anti-Müllerian hormone (AMH) in seminal plasma and serum is closely associated with sperm count and sperm motility, whereas low AMH levels were regarded as a potential marker of subfertile male patients (45, 46). The polymorphisms of *SRD5A2* were correlated with human sperm concentration and motility (19). Fortunately, we found that *amh*, *wt1a*, and *srd5a2b* are the targets of miR-200. Following injection of miR-429a agomiR, we detected a reduction of *amh*, *wt1a*, and *srd5a2b* expression and sperm motility (Fig. 4), contrary to the results in chr23-miR-200-KO zebrafish.

A number of miRNAs have been identified by high-throughput sequencing in infertile male patients (46–48). Also, many miRNAs are involved in spermatogenesis (49). Mir-18 functions in spermatogenesis through targeting HSF2 activity (50) and miR-34c plays a role in spermatogenesis and promotes mouse spermatogonial stem cells differentiation by targeting *nanos2* (51, 52). In our previous study, a high dose of EE2 impaired spermatogenesis and upregulated the expression of miR-141 and miR-429a, whereas lower expression of these two miRNA was associated with higher degree of testis maturity in YY supermales compared with XY males (41). However, the regulatory and functional roles of miRNA in sperm motility are still not clear. Present study indicated that miR-200 cluster on chromosome 23 could directly regulate *amh*, *wt1a*, and *srd5a2b* to affect sperm motility in zebrafish.

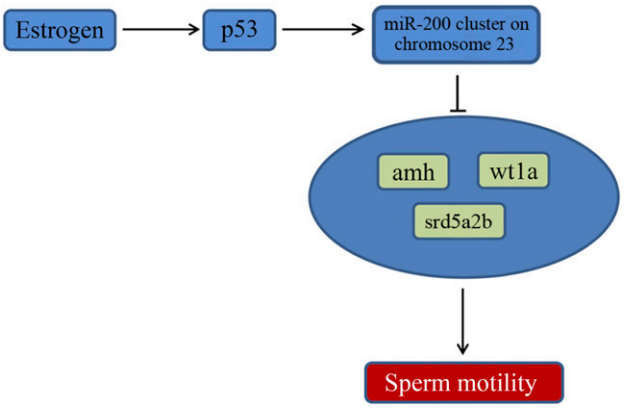

Figure 7. Schematic diagram of the roles of p53 and miR-200 cluster on chromosome 23 in sperm motility. In zebrafish testis, p53 activity is induced by EE2, thereby resulting in the upregulation of miR-200s that are transcriptional targets of p53. Subsequently, sperm motility was impaired by an miR-200 cluster on chromosome 23 that targets a number of genes related to sperm motility, including *amh*, *wt1a*, and *srd5a2b*.

P53 protein has been revealed to regulate spermatogenesis and semen quality parameters. Loss of p53 function resulted in an increase of testes weight, sperm concentration, and percentage of abnormal sperm (53–55). Moreover, sperm motility was elevated in p53 mutant mouse (56). Interestingly, deletion of p53 could rescue the male fertility defects of $Kit^{W\text{-}v}$ mice (57). In zebrafish, we also observed an increase of sperm motility when p53 was mutated (Fig. 6C and 6D). In response to a series of DNA damage, p53 was induced to reduce sperm activity and reproductive ability (58–60). In fish species, EE2 treatment could induce the expression of p53 and resulted in the damage of sperm motility and fertilization (61, 62). In zebrafish, sperm motility was also reduced when exposed to EE2 (Fig. 5B and 5C). However, the effect of EE2 on sperm motility is abolished when p53 function is lost, suggesting that EE2 impairs sperm motility by inducing expression of p53 and its transcriptional targets. However, no putative estrogen-responsive elements were detected on the promoter of *p53* by bioinformatics analysis, suggesting that EE2 induces p53 expression by an indirect manner. Together, we put forward a molecular cascade model revealing the roles of estrogen, p53, and miR-200 cluster on chromosome 23 and their downstream target genes involved in sperm motility (Fig. 7). The function of the miR-200 target genes in zebrafish sperm motility could be further studies by gene editing technologies, such as TALEN and CRISPR/Cas9.

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