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# Protein Arginine Methyltransferase 5 Is Necessary for Embryonic Development in Medaka *Oryzias latipes*

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**Abstract:** Protein arginine methyltransferase 5 (*Prmt5*), conserved from yeast to humans, catalyzes arginine's dimethylation in proteins. *Prmt5* is necessary for embryonic development in mice because it maintains embryonic stem cells. However, the embryos of zebrafish (*Danio rerio*) remain viable with a deficiency in germ cells and sexual development after the knockout of *prmt5*. Therefore, it was considered whether *prmt5* is dispensable during embryogenesis in fish. Medaka (*Oryzias latipes*), another model fish organism, was used in this experiment. The medaka *prmt5* was mutated with Transcription Activator-Like Effector Nucleases (TALEN) causing the premature stopping of transcription. None of the homozygous *prmt5* mutant fish were viable, only the heterozygous offspring survived. Quantitative reverse transcription-polymerase chain reaction (qPCR) results showed a significant decrease in octamer-binding transcription factor 4 (*oct4*), homeobox transcription factor nanog (*nanog*), *vasa*, B-cell CLL/lymphoma 2 (*bcl2*), and the ratio of *bcl2* to *bax* (*bcl2* associated x), and a significant increase in *caspase3* and *caspase8* in the embryos of the heterozygous *prmt5* mutant compared with that of the wild type. The results showed that the mutation of *prmt5* caused down-regulation of the genes functioning in stemness and up-regulation of the genes in the cascade of cell death. These results suggested that *prmt5* is necessary for embryogenesis via maintaining stemness and repressing apoptosis in medaka.

**Keywords:** medaka; *prmt5*; gene editing; embryonic development; gene expression; stemness; apoptosis



**Citation:** Liang, X.; Duan, S.; Yang, Q.; Ma, X.; Li, Z.; Yao, Q.; Wu, K.; Chang, P.; Feng, G.; Hong, W.; et al. Protein Arginine Methyltransferase 5 Is Necessary for Embryonic Development in Medaka *Oryzias latipes*. *Fishes* **2023**, *8*, 19. https://doi.org/10.3390/fishes8010019

Academic Editor: Albert Kjartan Dagbjartarson Imsland

Received: 9 November 2022
Revised: 19 December 2022
Accepted: 24 December 2022
Published: 27 December 2022



## 1. Introduction

Protein arginine methyltransferases (Prmts) are a family of proteins that catalyze the methylation of arginine in histone or none histone proteins [1]. *Prmt5* is a major type 2 methyltransferase to catalyze ω-$N^G$-monomethylarginine (MMA) as an intermediate and ω-$N^G$, $N'^G$-symmetric dimethylarginine (sDMA). In addition, *Prmt5* is involved in many biological processes, such as DNA methylation, via the symmetric methylation of histone H4 arginine 3 (H4R3me2s) recruiting DNA methyltransferase (Dnmt) 3a [2] and small nuclear ribonucleoprotein (snRNP) biogenesis in the assembly pathway of Sm-class U snRNPs and the localization of the survival of motor neurons (SMN) in Cajal bodies [3,4]. It is also involved in cell survival through regulating the eukaryotic translation initiation factor 4E (eIF4E) expression and p53 translation [5]; apoptosis by regulating the p53 response [6,7]; tumorigenesis by regulating tumor suppressors such as p53 [5], polycomb repressor complex 2 (PRC2) [8], melanocyte inducing transcription factor (MITF), and p27 [9]; and cytokine interleukin 2 (IL-2) secretion by regulating the IL-2 gene expression in T lymphocytes [10].

*Prmt5* is necessary for embryogenesis in mice. Homozygous embryos lacking *Prmt5* die as zygotes [11]. Subsequent studies have shown that *Prmt5* plays a major role in

maintaining and inducing stem cells [12,13]. The loss of *Prmt5* resulted in the abrogation of pluripotent cells in blastocysts. *Prmt5* is upregulated with Stat3 in embryonic stem (ES) cells. *Prmt5* methylates cytosolic histone H2A to maintain stemness by repressing differentiation genes in ES cells [12]. *Prmt5* could reprogram mouse embryonic fibroblasts into pluripotent stem cells with Kruppel-like factor 4 (Klf4) and Oct3/4 [13]. In addition, *Prmt5* is needed for myogenic differentiation (MyoD) induced myogenesis via the dimethylation of histone 3 arginine 8 (H3R8) [14] and the expression of myogenic microRNAs [15].

*Prmt5* plays a major role in the functioning of germ cells. *Prmt5* is required for primordial germ cell (PGC) formation by methylating Piwi proteins in *Drosophila* [16]. *Prmt5* also plays a role in the maturation of spermatocytes in males and germ cell specification in females in *Drosophila* [17,18]. However, *Prmt5* is not required for PGC specification [19], but it is involved in their protection [20] and in the survival of germ cells during spermatogenesis [21] in mice. *Prmt5* functions in association with B lymphocyte induced maturation protein 1 (Blimp1) and Piwi proteins, MILI (miwi-like), MIWI (mouse piwi), and MIWI2 (mouse piwi 2), in mouse germ cells [22,23].

*Prmt5* is highly conserved, showing high degrees of similarity from yeast to humans [24–26]. Zebrafish (*Danio rerio*) *prmt5* functions in myogenesis with Prmt4 shown by knockdown with morpholinos. In addition, *Prmt5* regulates the *myod*, *myf5* (*myogenic factor 5*), and *myogenin* expression, thereby affecting slow and fast fiber formation [27]. Unlike mammals, zebrafish with a deficiency of *prmt5* normally develop during embryogenesis and survive to adult, but are infertile in males due to a loss of germ cells [28]. Whether it is common that *prmt5* is dispensable during embryonic development in fish is unknown to date.

Previously, *prmt5* was identified in medaka *Oryzais latipes*, another model fish used in developmental biology and toxicology [25]. Medaka *prmt5* is expressed ubiquitously in adult tissues and is a maternal factor in embryos [25,29]. Except for Mep50 (methylosome protein 50), other proteins such as Prdm (PR domain-containing protein) 1a, and Prdm1b, are identified binding with *prmt5* in medaka [29,30]. Therefore, we considered whether medaka *prmt5* functions similarly to its homologs in mammals or zebrafish. In this study, medaka *prmt5* was mutated by Transcription Activator-Like Effector Nucleases (TALENs), a gene-editing technology. The mutation of *prmt5* caused a developmentally severe problem in medaka embryos. The results showed that *prmt5* is essential for embryonic development in medaka.

## 2. Materials and Methods

### *2.1. Ethical Statement*

This study was carried out according to recommendations in the Regulation for the Management of Laboratory Animals of China's Ministry of Science and Technology of China. The animal protocol for this study was approved by the Animal Care and Use Committee of Hubei Province in China (no. SYXK(E)2015-0012, August 2015).

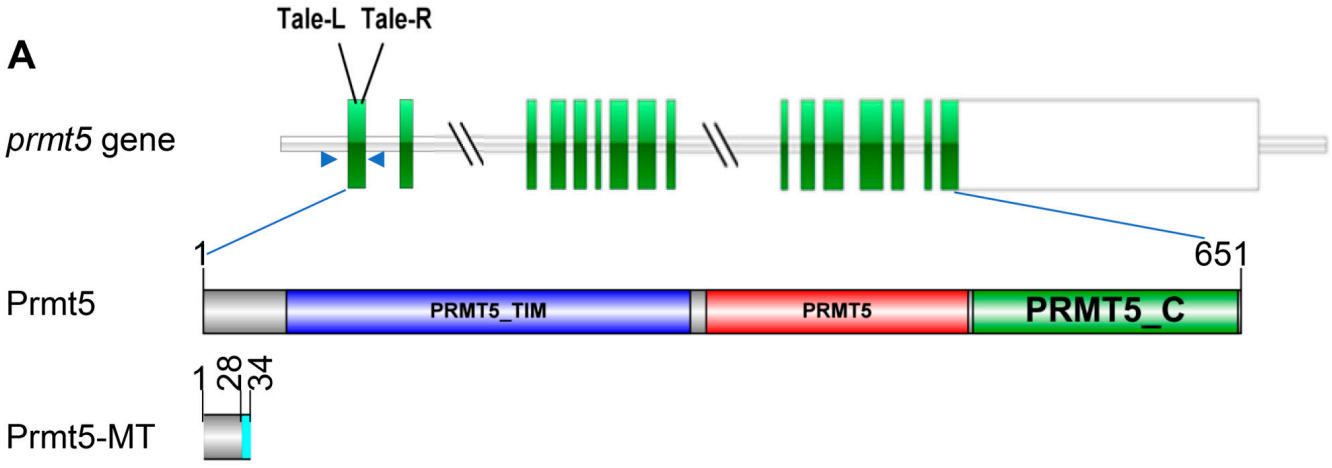
### *2.2. Experimental Animal*

A wild-type of medaka was used as the experimental fish, which was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China. The fish, including wild-type and mutants, were maintained in our aquarium in Central China Normal University, Wuhan, China, under an artificial photoperiod of 14 h light and 10 h dark and an ambient temperature of 26.0 °C. The mature male and female fish (2 to 3-month-old) were mated in the tank. Spontaneously spawned eggs were collected daily and incubated in the embryonic rearing medium (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2 \cdot 2H_2O$, 0.016% $MgSO_4 \cdot 7H_2O$ with or without 0.0001% methylene blue) at an ambient temperature of 26.0 °C. The embryos in development were observed and recorded under a stereomicroscope.

### 2.3. Gene Editing by TALENs

The specific TALEN target sites were identified online using TALEN Targeter (https://tale-nt.cac.cornell.edu/). The target sites were selected with the same criteria as described by Huang et al. [31,32]. The left and right TALEN targets, Tale-L and Tale-R, respectively, of medaka *prmt5* are GGCGTCCGCCAGTA and TCATATCTCTCCCG (Figure 1). The gene-specific TALEN constructs were assembled using the Unit Assembly method [31,32]. The two TALEN backbones, pCS2-C-PEAS and pCS2-C-PERR, and the four single-unit vectors, pA, pT, pC, and pG, were gifted from Dr. Bo Zhang (Peking University, Beijing, China). The final constructs were confirmed by sequencing.

The 5′-capped mRNA was produced by in vitro transcription from the constructed TALEN plasmids using SP6 mMESSAGE mMACHINE Kit as per the protocol provided by the manufacturer (ThermoFisher Scientific, Shanghai, China). The concentration of the transcribed mRNA was determined by NanoDrop 2000 (ThermoFisher Scientific). The left and right TALEN mRNA of 120 pg each were co-injected into the cytoplasm of one-cell stage embryos of medaka by microinjection with a MPPI-3 pressure injector (Applied Scientific Instrumentation, Eugene, OR, USA).




**B**

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                          TATCTCTCCC
 1 GTCATGGCGTCCGCCAGTACAGGCAGCA----GTCCTGCGGGAGAGATATGAACTGT    Δ4,+10
 2 GTCATGGCGTCC------------------------TGCGGGAGAGATATGAACTGT    Δ24
 3 GTCATGGCGTCC------------------------TGCGGGAGAGATATGAACTGT    Δ24
 4 GTCATGGCGTCCGCCAGTACAGGCA------------GCGGGAGAGATATGAACTGT    Δ12
 5 GTCATGGCGTCCGCCAGTACAGGCATT----TGCCCTGCGGGAGAGATATGAACTGT    Δ4
 6 GTCATGGCGTCCGCCAGTACAGGCAG-------TCCTGCGGGAGAGATATGAACTGT    Δ7
 7 GTCATGGCGTCCGCCAGTACAGGCAG-----AGTCCTGCGGGAGAGATATGAACTGT    Δ5
 8 GTCATGGCGTCCGCCAGTACAGGCAG----GTGTCCTGCGGGAGAGATATGAACTGT    Δ4
WT GTCATGGCGTCCGCCAGTACAGGCAGCAGGGTGTCCTGCGGGAGAGATATGAACTGT
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**Figure 1.** (**A**) Schematic structure of medaka *prmt5* gene and proteins. The blocks indicate the exons. The open reading frame (ORF) is shown in green. The arrowheads present the position of the primers used for screening. The target sites of the Tale-L and Tale-R are shown. The full protein of *Prmt5* in the length of 651 AA is shown with the functional domains. *Prmt5*-MT presents the mutant protein with 34 AA in length. The gray fragment is the same as it is in the full protein. The cyan fragment is deduced from the mutant sequence. (**B**) Alignment of the *prmt5* sequences in the founder embryos indicating the insertion and deletion (indel) after microinjection of the TALEN constructs. WT, wild type. The green letters indicate the left and right TALEN targets. The dash or Δ indicate the deletion, the red or + indicate the insertion. The start codon is in bold.

### *2.4. Genomic DNA Extraction and Polymerase Chain Reaction (PCR)*

The genomic DNA (gDNA) was obtained using the Universal Genomic DNA Kit (Cwbio, Beijing, China) according to the protocol provided by the manufacturer. The caudal fin was sampled after anesthesia of the fish with MS-222 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The sample DNA was used in PCR after extraction.

The PCR reaction was performed in a volume of 25 μL with 2 μL DNA, 1 μL each primer pair, dNTPs, buffer, and LaTaq (Takara, Beijing, China). The primers for medaka *prmt5* were *prmt5*F and *prmt5*R (Table 1). The program for PCR was denaturing at 94 °C 3 min, 30 cycles of 94 °C 30 s, 56 °C 30 s, and 72 °C 30 s, and a final incubation at 72 °C 10 min.

**Table 1.** The primers used in the experiments.

| Gene | Primer | Sequence (5′-3′) |
|---|---|---|
| *prmt5* | *prmt5*F | ACCTGTAGCTGTTAATTTGATCTGC |
| | *prmt5*R | GAATCCAAAAGCAGAGCATCCT |
| *β-actin* | *β-actin*F | CACACCTTCTACAATGAGCTG |
| | *β-actin*R | CCAGATCTGCTGGAAGGTGG |
| *oct4* | *oct4*-qF | TCTTTGGCGTAAACTCGTCTCA |
| | *oct4*-qR | CTTGCGTAAAACCCAAAGTGAT |
| *nanog* | *nanog*-qF | TACTCCAAACGCCCCGAAAG |
| | *nanog*-qR | GTGTCCTTCTGATGCCTCCTAA |
| *vasa* | *vasa*-qF | GCTCATCAACCAGATTTACCA |
| | *vasa*-qR | ATCTCCCTCATCTGGTAGCCG |
| *p53* | *p53*-qF | TCTACAAGAAGACGGAGCACG |
| | *p53*-qR | ACTGTAACACTCTGCCTTTTGGTAT |
| *bcl2* | *bcl2*-qF | TCGACAGTTTTCCCCTGCAA |
| | *bcl2*-qR | GAAACCCCCTGAACGGAACT |
| *bax* | *bax*-qF | GCGATCAAGGTAGCGAAAAAT |
| | *bax*-qR | CAGGTTTCTCCTGACCCGTT |
| *caspase3* | *caspase3*-qF | AACAAGACGCCGACCCTTAC |
| | *caspase3*-qR | TGTACCGTTACGAGGACCCA |
| *caspase8* | *caspase8*-qF | TGACCCTACCCTTTCCCAGT |
| | *caspase8*-qR | CACTTTGGGCTAGTGTGCCT |
| *caspase9* | *caspase9*-qF | AACTTCGTGGTGGAAGTCCG |
| | *caspase9*-qR | AGCTCCCAGCTGATCTGATCTT |
| *RPS18* | *s18*-qF | GTGTGGTGACCATCATGCAGAA |
| | *s18*-qR | TGGCAAGGACCTGGCTGTATT |

### *2.5. Detection of Mutation by T7E1 (T7 Endonuclease I) or Sequencing*

After the PCR reaction and purification by running on an agarose gel and gel extraction, the mutation of *prmt5* was detected by T7E1 digestion or sequencing.

For T7E1 detection, the purified PCR products were incubated with a T7E1 buffer in a tube at 95 °C in a water bath for 5 min. The T7E1 enzyme (New England Biolabs, Beijing, China) was added to the tube after cooling at room temperature. The tube was mixed well and incubated at 37 °C for 30 min. The mixture was electrophoresed on an agarose gel of 2%.

For sequencing, the purified PCR products were sequenced directly and separately for each sample. The purified PCR products were also subcloned into a pGEMT-easy vector (Promega, Beijing, China). Three colonies were selected randomly for sequencing after ligation, a transformation of *E. coli*, and incubation on an X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) LB (Luria Broth) agar plate with ampicillin.

### *2.6. Detection of the Gene Expression by Quantitative RT-PCR*

The total RNA from the embryos was extracted using an Ultrapure RNA kit (CoWin Biosciences, Beijing, China) following the protocol provided by the manufacturer. The cDNA (complementary DNA) was synthesized according to the protocol of the FastQuant RT kit (Tiangen Biotech, Beijing, China).

The quantitative RT-PCR (qPCR) of the triplicate samples was performed with a CFX96 real-time PCR detection system (BioRad Laboratories, Hercules, CA, USA) in a volume of 20 µL containing template cDNA, primers, and 2× SuperReal Pre Mix Plus kit (Tiangen). The cycling program was 95 °C 2 min followed by 39 cycles of 95 °C 10 s, 62 °C 30 s, and 65 °C 30 s. The relative expression of the genes in the samples was calibrated/normalized against *RPS18* (ribosomal protein S18) using the $2^{-\Delta\Delta Ct}$ method [33]. The primers are shown in Table 1. *RPS18* was used as the internal control [34].

### *2.7. Statistical Analysis*

Statistical analysis was performed with SPSS Statistics (IBM, Armonk, NY, USA). The gene expression values were calculated and described as mean ± standard errors with at least three independent experiments ($n \geq 3$). The differences among the treatments were calculated by one-way analysis of variance (ANOVA).

## 3. Results

### *3.1. Mutation of Medaka Prmt5*

After microinjection of the left and right TALEN mRNA into the zygotes, the embryos were incubated until four days post-fertilization (dpf). The live embryos at this stage were collected for detection of the editing efficiency. Twelve embryos were collected and detected by sequencing after DNA extraction, PCR, ligation into the pGEMT-easy vector, and transformation of *E. coli*. In totally, 20 colonies were selected and sequenced. Eight colonies were identified with insertion and deletion (indel) (Figure 1), which was also confirmed by the T7E1 method. The mutation efficiency was roughly 40%. The result showed that these TALEN constructs could efficiently induce indels in the target gene *prmt5* of the medaka embryos.

The larvae from the microinjected embryos were cultivated into adults as $F_0$. In total, 7 of 20 $F_0$ fish were checked with different indels. The mutated fish with the reading shift mutation of *prmt5* were mated with the wild-type fish to produce $F_1$ fish. The $F_1$ fish were checked individually using the T7E1 method and/or sequencing (Figure 2). The $F_1$ fish with the deletion of four base pairs (bp) in the *prmt5* gene were selected. Deletion of the four bp caused a reading frame shift and premature stopping of *prmt5*'s translation (Figure 1). The mutated *prmt5* may produce a peptide of 34 amino acids (AA), and this peptide loses the main functional domains of *Prmt5*, PRMT5_TIM (TIM barrel domain), PRMT5 (PRMT5 arginine-N-methyltransferase), and PRMT5_C (PRMT5 oligomerization domain) (https://www.ncbi.nlm.nih.gov/cdd).

Male and female $F_1$ fish with the four bp deletion in the *prmt5* gene were mated to produce the homozygous $F_2$. Surprisingly, about 30% (245/819, dead/total numbers) of the $F_2$ embryos died during embryonic development (Table 2 and Figure 3). No homozygous mutated fish were detected in the adult $F_2$ fish. So, the heterozygous $F_2$ fish were mated with either wild type or heterozygous $F_2$ fish to produce the next generation. The fish were checked and mated generation after generation until $F_{12}$. Unfortunately, no homozygous mutant fish were obtained. Only the heterozygous fish were cultivated.

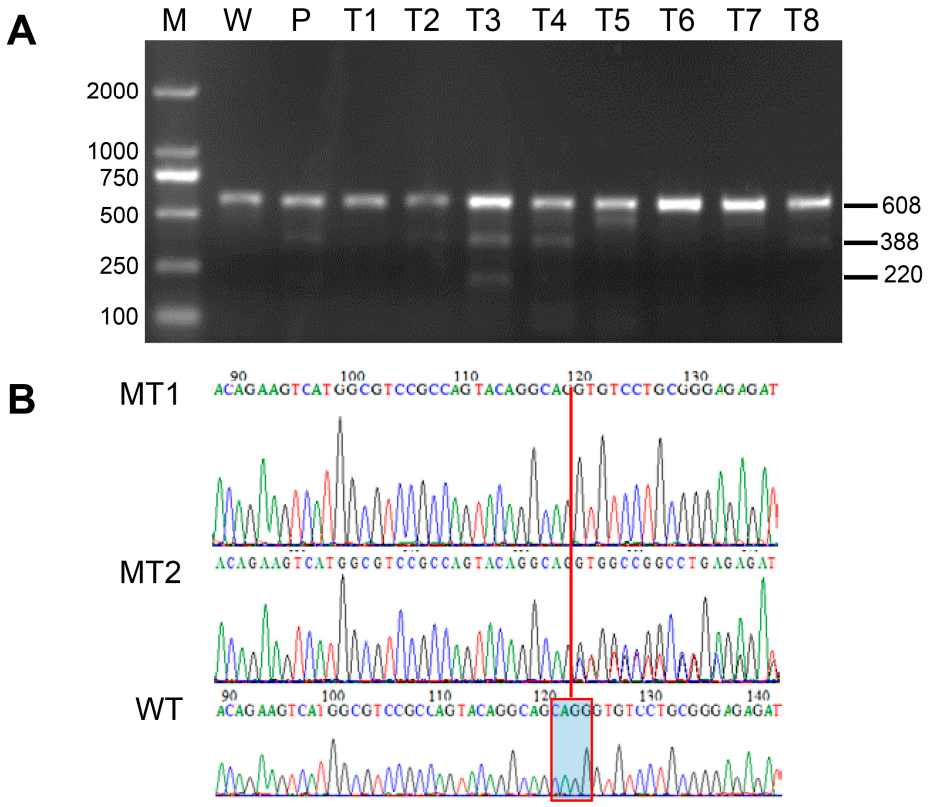


**Figure 2.** (**A**) A gel document presents the screening of the mutant by the T7E1 method. M, marker; WT, wild type; P, positive control; T1-T8 indicate the sample 1–8. The sizes of the marker and the fragments are indicated in bp beside the document. (**B**) The typical chromatogram of the partial sequence of *prmt5*. WT, wild type; MT, mutant. The deletion of the 4 bp in the wild type is highlighted in the red frame.

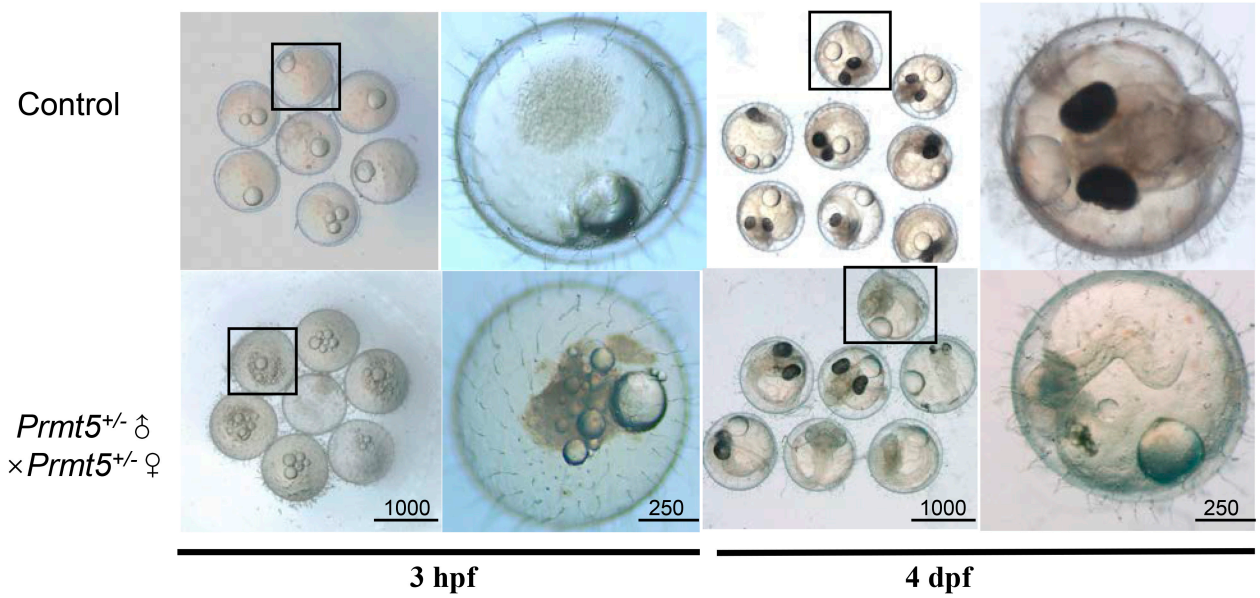


**Figure 3.** The embryos of the control and the offspring of the heterozygous fish at different stages (3 hpf and 4 dpf). The framed embryos are shown in enlarged pictures. The mutant offspring could have died in 3 hpf, developed slowly with minor or pigment-less eyes, abnormal body axis, and/or headless in 4 dpf. Scale bar, 1000 or 250 μm.

The mutant offspring embryos died before or/and after the gastrula stage (Table 2 and Figure 3). Some mutant offspring developed beyond the gastrula stage with minor eyes, abnormal body axis, or retarded development during organogenesis (Figure 3). Compared with the control embryos, the death rate (43/177, 245/819) of the $F_2$-$F_3$ offspring was significantly higher. It was found that 18.1–19.9% (32/177, 163/819) of the embryos died from the zygote to the gastrula stage. We considered whether the homozygous offspring died early or died during organogenesis. However, 83.3% (335/401) of the $F_8$ embryos died in the gastrula stage due to some unknown reasons. The most inbred embryos of the heterozygous fish developed beyond the gastrula stage in $F_9$-$F_{11}$; the homozygous mutant could have died during organogenesis because no homozygote was obtained.

The genotypes of the mutant offspring at adulthood were checked. Because no homozygotes of the mutant were identified, the homozygotes were assumed to be dead. Theoretically, the heterozygotes should be 2/3, and the wild type should be 1/3. The heterozygotes and the wild-type numbers were not significantly different from the expected offspring values (Table 3). This confirmed the hypothesis that the homozygotes of the *prmt5* mutant died during development.

**Table 2.** The death rate of the embryos in different stages and different generations.

| Generation | *Prmt5* (+/−) × (+/−) | | Wild Type | | *p* Value |
|---|---|---|---|---|---|
| | All Stages | To Gastrula | All Stages | To Gastrula | |
| F2 | 29.9% | 19.9% | 4.2% | - | $2.3 \times 10^{-27}$ |
| F3 | 24.3% | 18.1% | 5.7% | - | $3.1 \times 10^{-5}$ |
| F8 | - | 83.3% | - | 6.9% | $1.9 \times 10^{-101}$ |
| F9 | - | 2.4% | - | 3.5% | 0.31 |
| F10 | - | 2.4% | - | 2.7% | 0.84 |
| F11 | - | 1.9% | - | 2.8% | 0.97 |

**Table 3.** The genotypes of the offspring with mutated *prmt5*.

| Mating Pattern | The Offspring Tested | (+/−) | (+/+) | (−/−) | *p* Value |
|---|---|---|---|---|---|
| F2 (+/−) × WT | 42 | 16 | 26 | - | 0.12 |
| F2 (+/−) × F2 (+/−) | 43 | 28 | 15 | 0 | 0.83 |
| F3 (+/−) × F3 (+/−) | 43 | 25 | 18 | 0 | 0.24 |
| WT × F8 (+/−) | 33 | 14 | 19 | - | 0.38 |
| F9 (+/−) × F9 (+/−) | 46 | 26 | 20 | 0 | 0.14 |
| F11 (+/−) × F11 (+/−) | 48 | 33 | 15 | 0 | 0.76 |
| Total | 255 | 142 | 113 | - | 0.81 |

Note: WT, wild type. The *p* value was obtained by the Chi-square test with Excel, and the offspring of *prmt5*(−/−) were expected not to survive.

### *3.2. Gene Expression of the Inbred Offspring of the Heterozygous Mutant during Embryogenesis*

Several important genes were selected and detected in the embryos by qPCR to understand the reasons for the homozygous decease. The selected genes were *oct4*, *nanog*, and *vasa* related to ES cells and PGCs [35–38], *p53* [39], *bcl2*, *bax* [40], *caspase3* [41], *caspase8* [42], and *caspase9* [43] related to apoptosis or cell death. In addition, because of lethal homozygotes, the inbred offspring of the heterozygotes were collected and checked.

Compared with the expression levels in the wild type embryos, *oct4* and *nanog* in the mutant offspring were higher at the blastula stage, but decreased significantly at the gastrula stage and 25 h post-fertilization (hpf) (Figure 4). In addition, *vasa* was significantly decreased at the gastrula and 25 hpf in the mutant offspring too. However, the *p53* level in the mutant offspring was not different from that in the control (Figure 4).

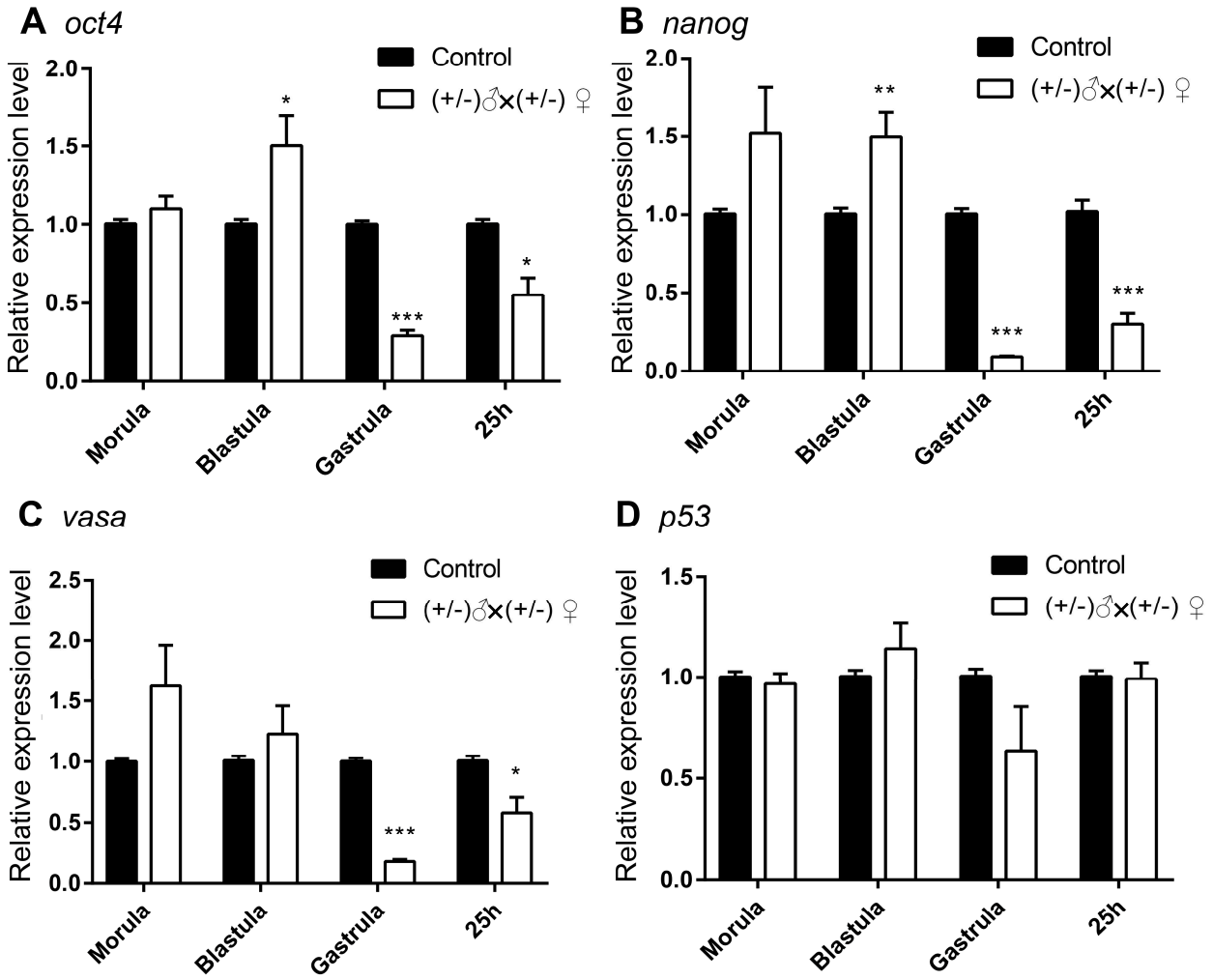


**Figure 4.** The relative expression of *oct4* (**A**), *nanog* (**B**), *vasa* (**C**), and *p53* (**D**) in the embryos from the morula stage to 25 hpf. The expression of each gene in the control at the same stage was set as 1. The asterisks show the significances. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Because the expression of *p53* was not different in the mutant and control offspring, the expression of *bcl2*, *bax*, *caspase3*, *caspase8*, and *caspase9* was checked (Figure 5). Comparing the expression in the control, the *bcl2* was decreased in the morula to blastula, but increased in 25 hpf; the *bax* was decreased only in the blastula. The pattern of the ratio of *bcl2*/*bax* was similar to that of the *bcl2* expression. The ratio was low from the morula to gastrula, but was high in 25 hpf in the mutant offspring. The *caspase3* level increased in the blastula stage and 25 hpf. The *caspase8* level increased in the gastrula stage to 25 hpf, but *caspase9* levels were decreased in the morula to gastrula stage.

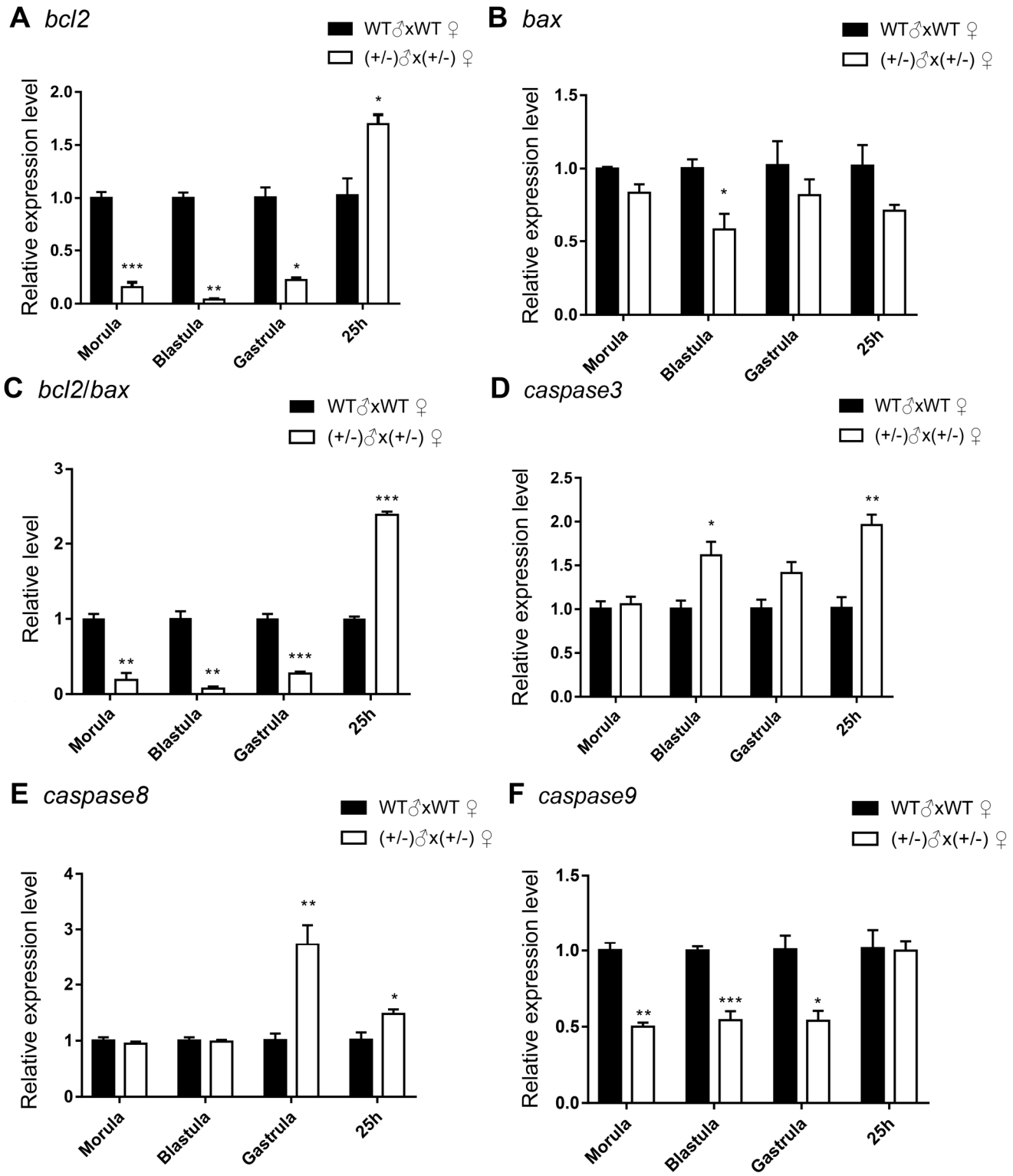


**Figure 5.** The relative expression of *bcl2* (**A**), *bax* (**B**), *caspase3* (**D**), *caspase8* (**E**), and *caspase9* (**F**), and the ratio of *bcl2*/*bax* (**C**) in the embryos from the morula stage to 25 hpf. The expression of each gene in control was set as 1 at the same stage. The asterisks show the significances. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

## 4. Discussion

In this study, the medaka *prmt5* gene was mutated by gene editing using TALENs. The mutation of *prmt5* resulted in a premature stopping mutation, which caused loss of

function in the *Prmt5* protein in homozygous offspring. As a result, the homozygotes of *prmt5* mutant died during embryogenesis. Moreover, the genes related to stemness and apoptosis were down- or upregulated. These results suggest that *prmt5* is necessary for the embryonic development of medaka by regulating cell stemness and apoptosis.

The mutation of *prmt5* seriously affects embryogenesis through its effects on stem cells. A defect in *Prmt5* is fatal for embryos in mice [11]. *Prmt5* is essential for maintaining the ES cells by repressing the differentiation genes [12,13]. Contrarily, *Prmt5* remarkably promotes the generation of induced pluripotent stem (iPS) cells from somatic cells in mice [13] and goats, *Capra hircus* [44]. *Prmt5* promotes the culture of human iPS cells [45] and regulates the proliferation of human ES cells by increasing the expression of the G1 cell cycle inhibitor P57 [46]. Our results are similar to previous findings. The mutation of *prmt5* caused homozygous death, and no homozygote was obtained in an extended period. The loss of *Prmt5* decreased the genes related to PGCs and ES cells, such as *nanog*, *oct4*, and *vasa*. *Oct4* and *nanog* are two factors expressed in the ES cells and PGCs of the medaka [37,47–50]. Although *oct4* and *nanog* were increased in the blastula stage, these two factors decreased significantly in the mutant offspring from gastrula to 25 hpf compared with that in the control. A decrease in *oct4* and *nanog* indicated that the stem cells were differentiated or dead in the embryos.

Along with *oct4* and *nanog*, the germ cell marker, *vasa*, was also decreased in the mutant offspring. This result indicates the loss of PGCs in the offspring with a deficiency of *prmt5*. A conditional loss of *Prmt5* in early PGCs caused complete male and female sterility in mice [20]. *Prmt5* is necessary for H2A/H4R3me2s chromatin modification to repress long interspersed repetitive element (Line) 1 and intracisternal A particles (Iap) transposons in mouse PGCs. *Prmt5* also participates in transposon silencing through the Piwi-interacting RNA (piRNA) pathway [20]. In zebrafish, the mutation of *prmt5* caused a loss of germ cells by apoptosis [28]. Mutating *prmt5* in zebrafish induces a decrease in *vasa* and *zili* (zebrafish piwi like) [28]. These results indicate a conserved function of *prmt5* in germ cells. Medeka's *oct4* and *nanog* are also expressed in germ cells [37,47–49]. Medaka Nanog mediates PGC migration by regulating the expression of cxcr4b [49]. The disappearance of PGCs may be as a result of deficits in migration and apoptosis.

*Prmt5* plays a significant role in organogenesis. The homozygous mutant of *prmt5* may die in the early stages before gastrulation or at the late stages during organogenesis in medaka. The mutation of *prmt5* decreases the expression of *oct4* in the gastrula stage. A decrease in *oct4* interferes with gastrulation, central nervous system development, and angiogenesis in medaka embryos [51]. In mice, *Prmt5* regulates glial cell differentiation [52]. *Prmt5* is essential for maintaining chondrogenic progenitor cells in the limb bud and for forming distinct cartilage identities in the knee and long bone in mice [53,54]. *Prmt5* is also needed for myogenesis [14,27] and regulates muscle stem cells [55]. The mutation of *prmt5* may also affect immune responses, such as the cholesterol biosynthesis-mediated Th17 responses [56]. *Prmt5* is necessary for B cell development by preventing p53-dependent and p53-independent blocks [57], and is required for T cell survival and proliferation [58]. A variety of effects may contribute to the homozygous death in the *prmt5* mutant of medaka.

*Prmt5* is a survival factor. *Prmt5* negatively regulates DNA damage-induced apoptosis in *Caenorhabditis elegans* [7]. Inactivation of *C. elegans Prmt5* induces excessive apoptosis in germline upon irradiation by a CEP-1 (*C. elegans* p53 homolog)-dependent up-regulation of the cell death initiator EGL-1 (egl eg, g-l aying defective) [7]. In mice, a deficiency in *Prmt5* causes cell-cycle arrest in G1. However, *Prmt5* promotes p53 expression, and the induction of p53 targets MDM2 (mouse double minute 2) and p21 upon DNA damage [5]. Growth suppression mediated upon *prmt5* knockdown is independent of p53, but is dependent on eIF4E [5]. Contrarily, *Prmt5* down-regulates *p53* and enhances iPS cell generation from dairy goat embryonic fibroblasts [44]. In this study, *p53* expression was not affected in the *prmt5* mutant offspring of medaka. This hints that the effect of *prmt5* on the expression of *p53* is different between species.

Bcl2 and Bax are a pair of factors for apoptosis and cell death [59,60]. Bcl2 functions as a repressor of cell death and Bax is a pro-apoptotic factor. The ratio of Bcl2/Bax determines the survival or death of cells following an apoptotic stimulus [59,60]. In the *prmt5* mutant offspring, *bcl2* was decreased from morula to blastula, and the ratio of *bcl2/bax* appeared in the same pattern. These results suggest a possible mechanism of *prmt5* on cell survival through the regulation of *bcl2* and the ratio of *bcl2/bax* in medaka embryos.

The caspases are aspartate-specific cysteine proteases (reviewed by Galluzzi et al.) [61]. The activation and function of caspases can be regulated by various molecules, such as the Bcl2 family proteins (reviewed by Fan et al.) [62]. The caspase cascade plays vital roles in apoptotic signaling. In the caspase family, caspase3 is an apoptosis executioner, and caspase8 and caspase9 are two apoptosis activators or initiators [61,62]. *Caspase8* and *caspase3* were upregulated in the blastula, gastrula, and 25 hpf in the *prmt5* mutant offspring. However, the expression of *caspase9* was decreased in the *prmt5* mutant offspring from the morula to gastrula. These results suggest that *Prmt5* can regulate the expression of the caspases in the embryos and that embryonic death is induced by apoptosis through caspase8 and caspase3 cascade after the mutation of *prmt5*.

In this study, the expression of the genes related to stemness, PGCs, and apoptosis was studied in the offspring embryos of the heterozygous *prmt5* mutant fish due to homozygous death. The results are not the same as those in the homozygotes. To further study the role of *prmt5* in medaka fish, conditional editing must be applied. However, the results from the offspring of the heterozygotes can provide some rough information to understand the functions of *prmt5* in medaka embryos.

Taken together, *prmt5* is necessary for embryonic development in medaka. The mutation of *prmt5* can induce apoptosis in the embryos by decreasing *bcl2/bax* and increasing *caspase 8* and *caspase3*. In addition, the mutation of prmt5 causes a decrease in the stemness of ES cells and PGCs through down-regulation of *oct4*, *nanog*, and *vasa*. A decrease in the stem cells and increase in cell apoptosis results in death of the embryos in the homozygous *prmt5* mutant (Figure 6).

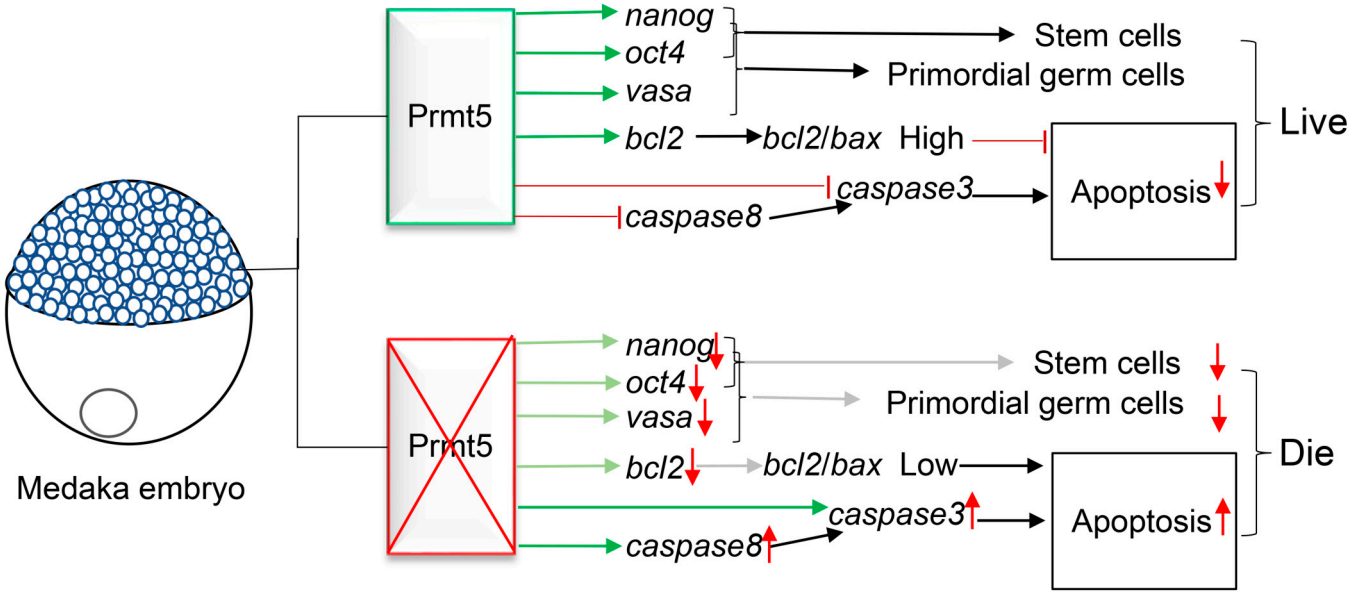


**Figure 6.** A diagram indicating the functions of *Prmt5* in medaka embryos. In the wild type, *Prmt5* is functional (in green frame) to maintain the *nanog*, *oct4*, and *vasa* expression in the stem and primordial germ cells. *Prmt5* promotes the expression of *bcl2*, which results in a high *bcl2/bax* ratio and represses the expression of *caspase8* and *caspase3* to prevent apoptosis. So, the embryos of the wild type are live. The contrary events occurred in the homozygotes of the *prmt5* mutant (in red frame) and the homozygous mutant embryos died.

## 5. Conclusions

*Prmt5* is necessary for embryonic development in medaka. A decrease in the stem cells and increase in cell apoptosis results in death of the embryos in the homozygous *prmt5* mutant.

**Author Contributions:** Q.Z., X.Z. and H.Z.: conceptualization and designing the experiment; X.L., S.D., Q.Y. (Qing Yang), X.M., Z.L., Q.Y. (Qiting Yao), W.H., K.W., P.C. and G.F.: experimental set up and execution; X.L., Q.Y. (Qiting Yao) and M.C.: data analysis; X.L. and H.Z.: manuscript writing. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the Natural Science Foundation of China (grant no. 31672284, 31272645 to H.Z.) and the Open Fund of Hubei Key Laboratory of Environmental and Health Effects of Persistent Toxic Substances (no. PTS2021-01 to QZ).

**Institutional Review Board Statement:** This study was carried out according to recommendations in the Regulation for the Management of Laboratory Animals of China's Ministry of Science and Technology of China. The animal protocol for this study was approved by the Animal Care and Use Committee of Hubei Province in China (No. SYXK(E)2015-0012).

**Data Availability Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Conflicts of Interest:** The authors declare no conflict of interest.

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