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ORIGINAL ARTICLE

Germline-specific dgcr8 knockout in zebrafish using a BACK approach

Yun Liu[1,2] · Zeyao Zhu[2,4] · Idy H. T. Ho[2,3,4] · Yujian Shi[2,4] · Yuxin Xie[2,4] · Jianzhen Li[2,4] · Yong Zhang[1,2,5] · Matthew T. V. Chan[3] · Christopher H. K. Cheng[2,4]

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Abstract Zebrafish is an important model to study developmental biology and human diseases. However, an effective approach to achieve spatial and temporal gene knockout in zebrafish has not been well established. In this study, we have developed a new approach, namely bacterial artificial chromosome-rescue-based knockout (BACK), to achieve conditional gene knockout in zebrafish using the Cre/*loxP* system. We have successfully deleted the *DiGeorge syndrome critical region gene 8* (*dgcr8*) in zebrafish germ line and demonstrated that the maternal-zygotic *dgcr8* (MZ*dgcr8*) embryos exhibit MZ*dicer*-like phenotypes with morphological defects which could be rescued by miR-430, indicating that canonical microRNAs play critical role in early development. Our findings establish that Cre/*loxP*-mediated tissue-specific gene knockout could be achieved using this BACK strategy and that canonical microRNAs play important roles in early embryonic development in zebrafish.

Keywords Crispr-cas9 · Maternal-zygotic transition · Germ layer specification · Small regulatory RNAs

Introduction

Zebrafish is an important model to study developmental biology and human diseases. Engineered nucleases, including zinc-finger nucleases, transcription activator-like effector nucleases (TALENs) and the RNA-guided Cas9 system have been applied to achieve gene knockout in zebrafish [1–3]. However, these global gene knockout strategies were unable to investigate the functional roles of genes in a spatial and temporal manner. Conditional gene knockout can overcome these limitations [4–6]. Conditional gene knockout is usually achieved by employing the Cre/*loxP* system in which the *loxP* flanked sequence can be deleted in a Cre-dependent manner [7]. Using TALENs or the RNA-guided Cas9 system, single *loxP* sites have been inserted into the zebrafish genome as reported in some recent studies [8–10]. More recently, two *loxP* sites have been introduced into the zebrafish genome and Cre-mediated excision of the *loxP* flanked genomic fragment has been reported [11]. However, recombination of exogenous DNA into the zebrafish genome is still difficult, probably due to the low efficiency in the repair of the double DNA breakage by homologous recombination. Therefore, an alternative approach to achieve conditional knockout zebrafish is highly warranted.

Y. Liu and Z. Zhu contributed equally to this work.

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✉ Christopher H. K. Cheng
chkcheng@cuhk.edu.hk

1 State Key Laboratory of Biocontrol, Institute of Aquatic Economic Animals, and the Guangdong Province Key Laboratory for Aquatic Economic Animals, Sun Yat-Sen University, Guangzhou, China

2 School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China

3 Department of Anaesthesia and Intensive Care, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, China

4 School of Biomedical Sciences Core Laboratory, The Chinese University of Hong Kong Shenzhen Research Institute, Shenzhen, China

5 South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, Guangzhou, China

MicroRNAs are 22-nt noncoding small RNAs that negatively regulate the stability and translation of mRNA transcripts [12]. MicroRNA genes are transcribed by RNA polymerase II as primary miRNA transcripts and process to 70-nt precursors by Dgcr8 and RNase III enzyme Drosha [13]. Pre-miRNAs are exported from the nucleus and further processed into 21-nt mature microRNA by Dicer [13]. Non-canonical pathway has been identified that some microRNAs can bypass Dgcr8 cleavage while others can bypass Dicer processing [14]. Moreover, other small RNAs, such as endo-siRNA and endo-shRNAs, are required to be processed by Dicer [14]. The functional roles of microRNA during early development have been investigated. In mice, *dicer* mutant is embryonic lethal due to defects in gastrulation and definitive endoderm formation, whereas the maternal-zygotic *Dgcr8* mutant (MZ*dgcr8*) exhibits post-implantation embryonic development defects [15]. In zebrafish, Dicer is maternally provided and zygotic *dicer* mutant shows no phenotypes [16], whereas maternal-zygotic *dicer* (MZ*dicer*) mutant exhibits cell movement defects at the onset of gastrulation [17]. The developmental defects in MZ*dicer* mutant can be partially rescued by miR-430, suggesting that miR-430 play important roles in early development [17]. Given that the biogenesis of the other small endogenous RNAs was also disrupted in the MZ*dicer* mutant, the functional roles of microRNAs in early development remain to be established.

In this study, we describe a new approach, namely bacterial artificial chromosome-rescue-based knockout (BACK), to achieve conditional gene knockout in zebrafish using the Cre/*loxP* system. We have established the *dgcr8* germline-specific mutation line using this BACK approach and found that the MZ*dgcr8* mutant exhibits severe morphological defects in embryonic development. Our findings demonstrate that tissue-specific gene knockout could be achieved using this BACK approach and that canonical microRNAs play essential roles during early embryonic development.

Materials and methods

Zebrafish husbandry

AB zebrafish were maintained at 28 °C in the zebrafish facility of Sun Yat-Sen University and the Chinese University of Hong Kong. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Sun Yat-Sen University and the Chinese University of Hong Kong.

Generation of *dgcr8* mutant line

The TALENs for each target gene were assembled using the golden gate method as described previously [18–20]. The TALEN expression plasmids (pCS2-TALEN-ELD and pCS2-TALEN-KKR) were linearized by NotI restriction enzyme digestion. TALEN mRNAs were transcribed using the mMESSAGE mMACHINE SP6 kit (Ambion) and purified using the RNeasy Mini Kit (QIAGEN).

To generate zebrafish mutant lines, TALEN mRNAs (500 pg/embryo) were microinjected into one-cell stage zebrafish embryos. Two days after injection, genomic DNA was isolated from 8 to 10 pooled larvae. The target genomic regions were amplified by PCR and subcloned into the pTZ57R/T vector (Fermentas). Single colonies were genotyped by sequencing. To obtain germline mutations, the TALEN injected embryos were raised to adulthood and outcrossed with wild-type (WT) fish. The F1 progeny were genotyped by sequencing. To obtain homozygous mutants, heterozygous mutants of the same mutation were obtained and self-crossed. The primers used in this study are listed in Supplemental Table S1.

Whole mount in situ hybridization

Whole mount in situ hybridization was performed as described [21]. A cDNA fragment of zebrafish *dgcr8* was amplified by RT-PCR with specific primers, followed by in vitro transcription with either T7 or Sp6 RNA polymerase to generate the antisense probe using the DIG RNA Labeling Kit (Roche, USA).

BAC recombineering

The recombineering reagents including engineering bacterial strains (SW102, SW105, SW106) and plasmids (PL451, PL452) were obtained from the US National Cancer Institute (NCI) at Frederick. The piTol2 plasmids (*piTol2-amp, piTol2-kan* and *piTol2-galk*) were provided by Dr. Koichi Kawakami and Dr. Maximiliano L Suster. The BAC clone CH211-267E2 containing zebrafish *dgcr8* was purchased from the BACPAC Resources Center of Children's Hospital Oakland Research Institute.

BAC recombineering was carried out as previously reported [22]. The BAC DNA was electroporated into the engineering bacterial strain SW106. The first *loxP* cassette was amplified using primers with 45-bp homolog arms to the second intron of the *dgcr8* gene (Table S1). After induction of recombinase expression, the SW106 was transformed with the first *loxP* cassette PCR product and positive recombinants on kanamycin plates were screened by PCR. For excision of the *Neomycin* cassette, 10% L (+)-arabinose (Sigma) was added when the bacterial

culture OD_{600} reached about 0.5 and the targeting clones were screened by PCR and verified. Next, the first *loxP* positive BAC was transformed into SW105 strain by electroporation. The second *loxP* cassette was amplified using primers with 45-bp homolog arms to the third intron of the *dgcr8* gene. After recombineering and excision, the positive clones were screened by PCR and verified. The *iTol2* cassette was amplified using primers with 45-bp homolog arms to the BAC backbone to destroy the self-contained *loxP* site. After induction of recombinase, the *iTol2* PCR product was transformed into the BAC-containing strain SW105 and the *iTol2-amp* cassette positive colonies were identified by PCR and verified.

BAC transgenesis

The Tol2-mediated BAC transgenesis was performed as described [22]. The purified BAC (200 pg/embryo) (MN BAC purification kit) and pCS2FA transposase mRNA (100 pg/embryo) were co-injected into one-cell stage embryos. The injected embryos were raised to adulthood and outcrossed with WT fish. The founders were further crossed with $dgcr8^{+/-}$ fish to generate the $dgcr8^{+/-}$;*Tg:dgcr8* fish.

Q-PCR analysis of pri-miRNAs and mature miRNA

To quantify the pri-miRNA and mature miRNA level, RNA was isolated from shield stage embryos using the miRNeasy Mini Kit (QIAGEN). Mature miRNA expression was analyzed using the miRCURY LNA™ Universal RT microRNA PCR system (EXIQON). Real-time PCR was performed on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using the SYBR Green I Kit (Applied Biosystem). The pri-miRNA transcript levels were normalized against *ef1a* transcript level and mature miRNA levels were normalized against the U6 transcript level.

Q-PCR analysis of mRNA expression

Total RNA was isolated from zebrafish embryos at various developmental stages (shield, 75%-epiboly, prim-6, prim-16) with RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript RT Reagent Kit (TAKARA). Real-time Q-PCR was performed on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using the SYBR Green I Kit (Applied Biosystem). The mRNA transcript levels were normalized against *ef1a* transcript level.

Reporter assays

The dsRED sequence was cloned into pCS2-3XIPT-miR-430 plasmid by BamHI and Xhol [17]. The zsYellow was amplified from ZsYellow1-N1 which was a gift from Michael Davidson (Addgene plasmid # 54701) and cloned into the pCS2 vector bone. Two nl of a mixture (100 ng/µl dsRED-3XIPT miR-430 mRNA and 150 ng/µl zsYellow mRNA) were injected into the MZ*dgcr8* and WT embryos at one-cell stage. The embryos were photographed and analyzed at 24 hpf on an Olympus FV1000 Confocal System.

Rescue experiments

To perform rescue experiments, we amplified the full-length *dgcr8* transcript from WT embryos using primer with T7 sequence. To obtain *dgcr8* mRNA, in vitro transcription was performed using the mMESSAGE mMACHINE T7 kit (Ambion) and polyadenylation was performed using the Poly (A) Tailing Kit (Invitrogen). The purified dgcr8 mRNAs (100 pg/embryo) were microinjected into one-cell stage MZ*dgcr8* embryos and GFP mRNA (100 pg/embryo) was injected as control.

The miR-430 mimics were synthesized by Shanghai GenePharma Co.,Ltd as described in a previous study [17]. For rescue, miR-430 mimics (10 pg/embryo) were injected into one-cell stage MZ*dgcr8* embryos and the phenotypic changes were recorded on a stereomicroscope.

Results

Global *dgcr8* KO by a dual TALEN approach in zebrafish

To disrupt *dgcr8* in zebrafish, we assembled two pairs of TALENs to delete exon 4 of *dgcr8* using our optimized TALEN platform (Fig. 1a) [19, 23]. The assembled TALENs were injected into 1-cell stage embryos and successful deletion of exon 4 was confirmed by sequencing (Fig. 1a). The injected embryos were raised to adulthood for founder screening. Of the eight fish screened, four have successfully transmitted the fragment deletions through the germline (Fig. 1b). We have further raised the F1 heterozygotes and obtained *dgcr8* homozygote mutant with a 256-bp deletion in F2 embryos (Fig. S1). These homozygotes developed normally in the first 5 days post fertilization (dpf) during which organogenesis is completed. At 10 dpf, the *dgcr8* homozygotes were smaller than the wild-type (Fig. 1c). No *dgcr8* homozygote survived to adulthood (0 of 48

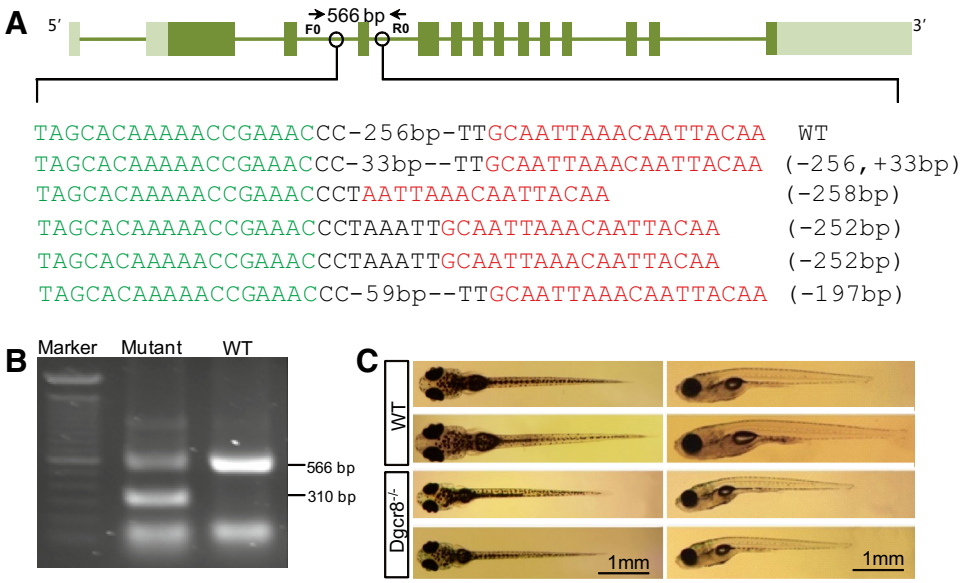

Fig. 1 Targeted deletion of *dgcr8*. **a** Schematic representation of the zebrafish *dgcr8* and the TALEN binding sites. Target sites for two TALEN pairs were chosen at intron 4 and intron 5 to delete exon 4 of *dgcr8* in zebrafish. Successful deletion of exon 4 in P0 generation was confirmed by sequencing. **b** Germline transmission of the deletion to F1. A PCR band of about 310-bp could be amplified after exon 4 deletion in F1 generation. The primer positions for genomic PCR were shown in **a. c** Representative pictures of wild-type and *dgcr8*$^{-/-}$ mutant zebrafish larvae at 10 dpf. The mutant larvae were smaller than the wild-type ones

genotyped in F2), indicating that canonical microRNAs function are essential for later life stages.

Similar to the zygotic dicer mutant [16], the lack of phenotype during early embryonic development is probably due to the presence of the maternally provided *dgcr8* transcripts. Therefore, we examined whether *dgcr8* transcripts are present in the early embryos using transcriptome data [24]. *Dgcr8* is abundantly expressed in the 128-cell and oblong-sphere stage embryos but the expression is very low at the shield stage embryos (Fig. S2a). Whole mount in situ hybridization analysis further confirmed that the *dgcr8* transcripts were maternally provided (Fig. S2b).

Germline-specific *dgcr8* deletion using BACK approach

To eliminate the maternal *dgcr8* transcripts, we then generated the conditional allele of *dgcr8* using our BACK approach. The rationale of this BACK approach is that introduction of a *loxP*-modified gene *x* into an *x*-null background could rescue the *x*-null phenotype, and that conditional gene knockout can be achieved when the rescued line is crossed with a given *Cre* line (Fig. 2).

We first obtained the bacterial artificial chromosome (BAC) containing zebrafish *dgcr8* and electroporated the BAC into the engineering bacterial strain SW106. We next introduced two *loxP* cassettes flanking exon 3 of *dgcr8* via in vitro BAC recombineering (Fig. S3) [25–27]. We further engineered the BAC with the *iTol2* element to facilitate transposon-mediated transgenesis

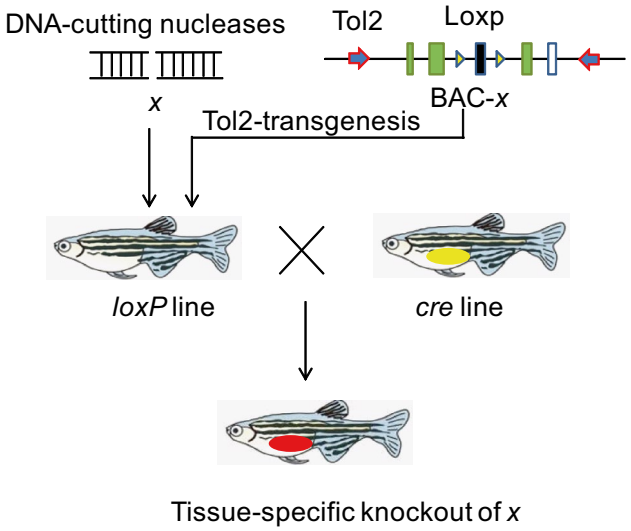

Fig. 2 Schematic diagram illustrating the BACK conditional gene knockout strategy. To achieve conditional knockout of gene *x*, the first step is to generate mutations of *x* using engineered nucleases. Then the BAC-containing gene *x* is modified to contain a Tol2 arm and two *loxP* sites. The modified BAC will be introduced to the *x* mutant background and crossed with a conditioned Cre line to delete the *loxP* flanking genomic sequence

[22]. Successful BAC engineering was confirmed by sequencing (Fig. S4). The *loxP*-modified *dgcr8* DNA and the transposase mRNA were co-injected into zebrafish embryos with a *dgcr8*$^{+/-}$ background. Two founders

of the ten fish screened were identified to contain the BAC (*dgcr8*$^{+/-}$; *Tg:dgcr8*). To achieve germline-specific expression of Cre, we used the *Tg*(*kop:cre*) fish line in which Cre expression is restricted to the primordial germ cells [28]. We crossed the *Tg*(*kop:cre*) fish with *dgcr8*$^{+/-}$ fish to obtain the *dgcr8*$^{+/-}$;*Tg*(*kop:cre*) fish. We then crossed the *dgcr8*$^{+/-}$; *Tg:dgcr8* male with the *dgcr8*$^{+/-}$;*Tg*(*kop:cre*) female fish (because the *kop* promoter is maternally active). Of the 16 offspring screened at adulthood, four fish were found to be of the *dgcr8*$^{-/-}$; *Tg:dgcr8* genotype (Fig. 3a, b). These fish possess no abnormal phenotype. Both male and female fish were fertile. The presence of *dgcr8*$^{-/-}$ genotype at adulthood indicates that the *loxP*-modified *dgcr8* transgene has successfully rescued the *dgcr8*$^{-/-}$ lethal phenotype.

To examine whether exon 3 was specifically deleted in the germline, we collected genomic DNA of the tail fins and oocytes from the *dgcr8*$^{-/-}$; *Tg:dgcr8* line. Genomic PCR indicated that exon 3 of *dgcr8* was deleted in the oocytes but not in the tail fin (Fig. 3c, d). Sequencing of the genomic PCR product confirmed that the *loxP* flanked genomic sequence has been successfully deleted (Fig. 3e). These data indicate that we have successfully generated germline-specific deletion of *dgcr8* using our BACK approach.

Germline-specific *dgcr8* deletion disrupts microRNA processing

We subsequently crossed the *dgcr8*$^{-/-}$; *Tg:dgcr8* fish and obtained the MZ*dgcr8* embryos. Q-PCR analysis showed that the primary microRNA transcripts (pri-miR-21, pri-miR-25 and pri-miR-430) were increased but their mature microRNAs were depleted in the MZ*dgcr8* embryos (Fig. 4a), suggesting that microRNA biogenesis was disrupted in the MZ*dgcr8* mutant. Functional reporter assays showed that miR-430 failed to suppress reporter expression in the MZ*dgcr8* embryos but not in the WT embryos (Fig. 4b), indicating that miRNA mediated target suppression was abolished in the MZ*dgcr8* embryos. Moreover, the expression of the known miR-430 target mRNAs (gstm and cd82b) was significantly increased in the MZ*dgcr8* mutant and the increased mRNA level was suppressed by injection of miR-430 mimics [29] (Fig. S5). These results indicated that germline deletion of dgcr8 disrupts canonical microRNA processing and function.

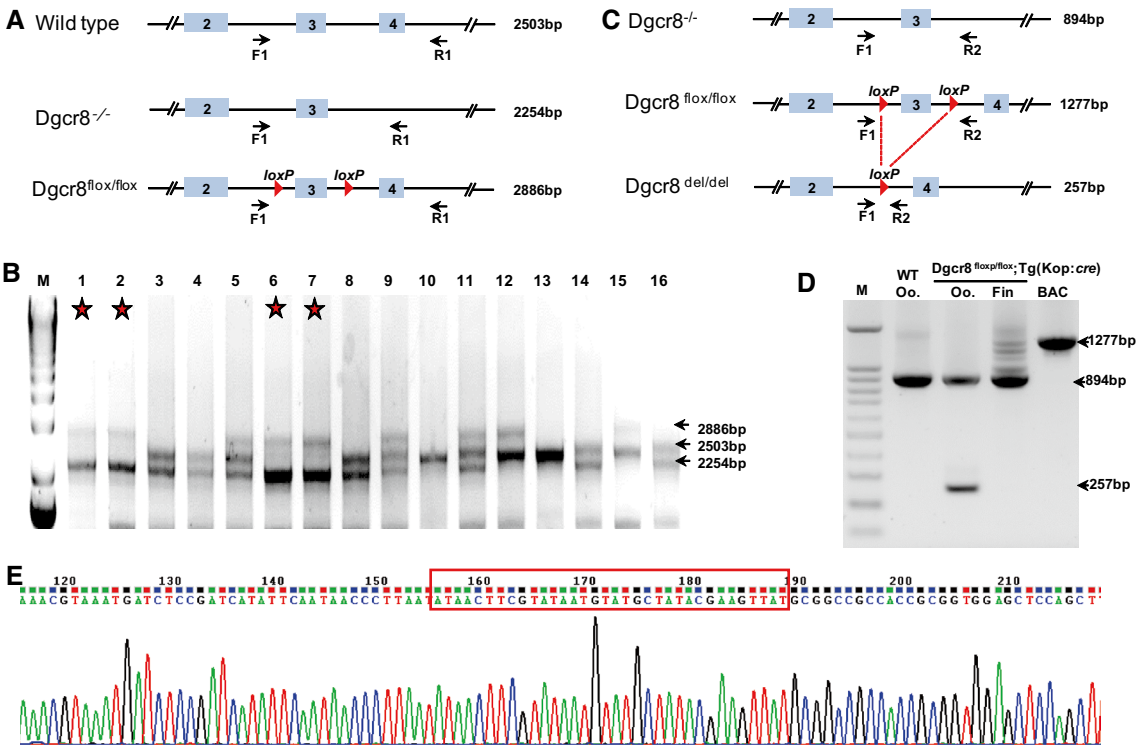

Fig. 3 Germline-specific *dgcr8* exon 3 deletion. **a** Primer positions and sizes of the target band for each genotype for genotyping of progenies from the *dgcr8*$^{+/-}$; *Tg:dgcr8* and *dgcr8*$^{+/-}$; *Tg*(*kop:cre*) cross. **b** Gel picture of genomic PCR of fish fin from the *dgcr8*$^{+/-}$; *Tg:dgcr8* and *dgcr8*$^{+/-}$; *Tg*(*kop:cre*) cross. The stars represent the *dgcr8*$^{-/-}$; *Tg:dgcr8* genotype with two bands of 2254 bp and 2886 bp. **c** Primer positions and size of the target bands after Cre-mediated *loxP* excision. **d** Deletion of exon 3 was detected in the oocytes (Oo) but not in the fin by genomic PCR. **e** Sequencing result confirmed successful deletion of the *loxP* flanked genomic region

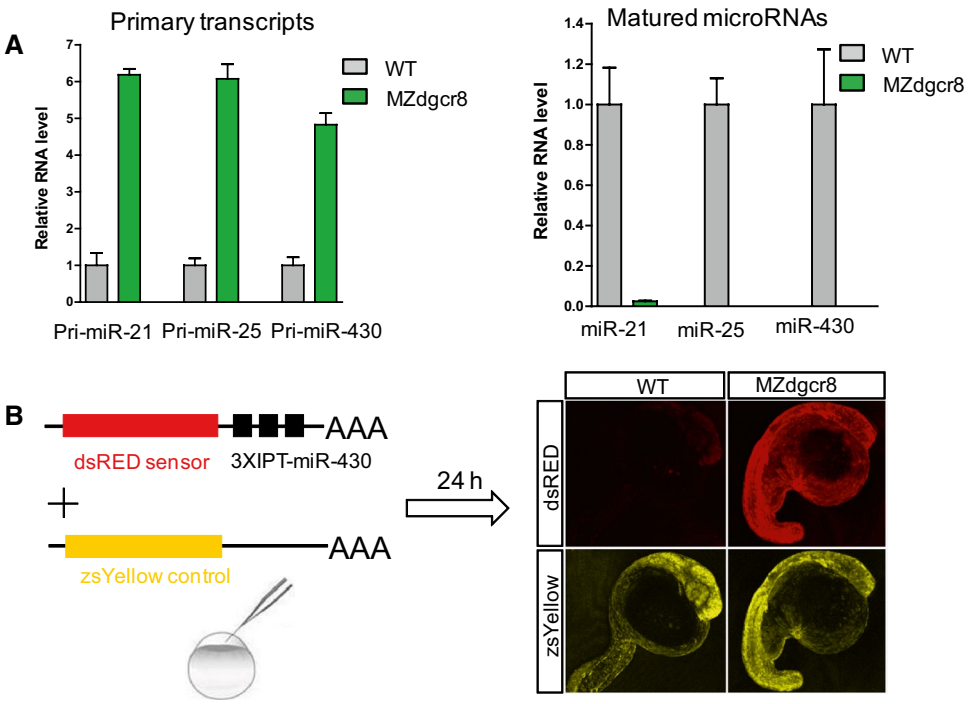

Fig. 4 Germline deletion of *dgcr8* disrupts canonical microRNA function. **a** Q-PCR analysis of pri-miRNA and mature miRNA expression in embryos from the wild-type and MZ*dgcr8* mutant line at 6 hpf. Data are expressed as the mean ± SEM ($n = 4$). **b** miR-430 efficiently suppresses a dsRED reporter containing three imperfect targets (IPT) in the wild-type embryos but not in the MZ*dgcr8* mutant at 24 hpf

Germline-specific *dgcr8* deletion disrupts early embryonic development

The MZ*dgcr8* embryos exhibited developmental delay from shield stage (6 h post fertilization, hpf) and developed slower than the WT for about 3–4 h at 24 hpf. The epiboly movements were disrupted with a longer animal–vegetal axis but a shorter dorsal–ventral axis (Fig. 5). The brain was smaller with no obvious brain boundaries (Fig. 5). No heart beat or circulation can be observed in the MZ*dgcr8* mutant at 30 hpf. To demonstrate that the observed phenotypes were due to the loss of function of *dgcr8*, we next performed rescue experiments. Injection of *dgcr8* mRNA into MZ*dgcr8* embryos efficiently rescued the mutant phenotypes (Fig. 5). In contrast to the MZ*dgcr8* mutant embryos which died within 5 dpf, the rescued embryos survived up to 12 dpf. Interestingly, the MZ*dgcr8* mutant phenotype was also well rescued by injection of miR-430 (Fig. 5), the most abundantly expressed microRNA in early embryonic development [17], suggesting that the early developmental defect observed in the MZ*dgcr8* was due to the loss of function of miR-430.

We then analyzed the MZ*dgcr8* phenotypes using whole mount in situ hybridization and qPCR analysis of marker gene expression (Fig. 6, Fig. S6). In the MZ*dgcr8* embryos, the expression of dorsal mesoderm markers (*chd* and *gsc*)

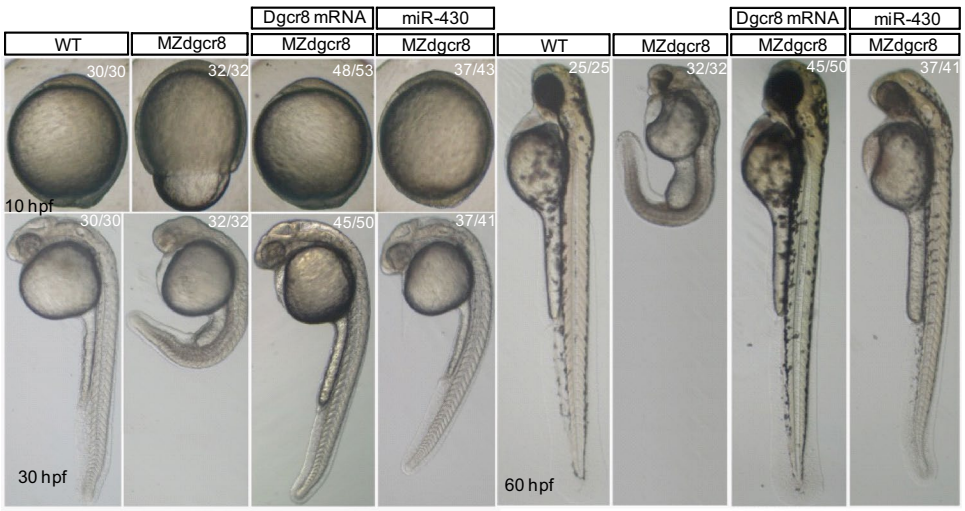

Fig. 5 Germline deletion of *dgcr8* causes severe developmental defects. Representative morphologies of embryos for each genotype exhibited at the indicated times during early development. The ratios of embryos with the presented phenotypes are indicated

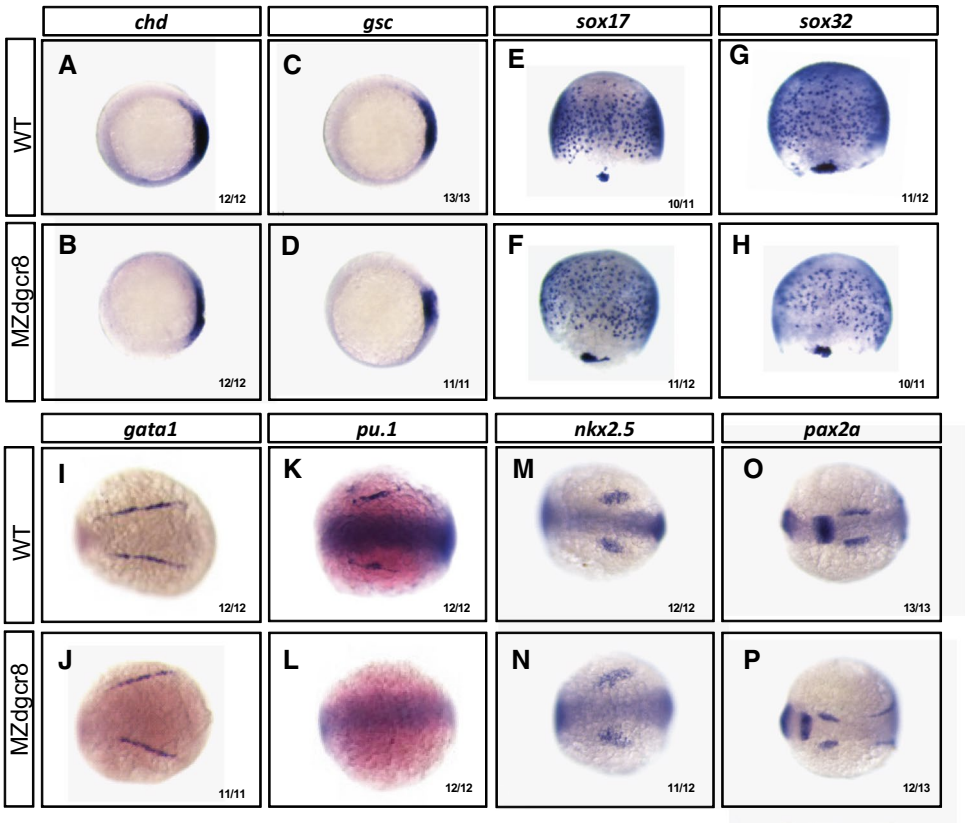

Fig. 6 Maker gene expression analysis of the MZ*dgcr8* mutant. The expression of the indicated marker genes in the control and MZ*dgcr8* mutant embryos at shield stage (**a**–**d**), 75% epiboly stage (**e**–**h**) and 6-somite stage (**i**–**p**). The ratios of the affected embryos are indicated

and the endoderm markers (*sox17* and *sox32*) was reduced (Fig. 6a–g). The erythroid progenitor (marked by *gata1*) was specified but the myeloid progenitor (marked by *pu.1*) was absent at the 6-somite stage (Fig. 6h–k). The cardiac progenitor (marked by *nkx2.5*) was specified at the 6-somite stage but failed to migrate to the middle line at 32 hpf in the MZ*dgcr8* mutant (Fig. 6m, n, Fig. S7). In the brain, the mid-hind boundary domain was reduced and the optic placode was expanded (Fig. 6o, p). The embryonic body was expanded mediolaterally and the anterior-posterior axis was reduced (Fig. 6i, j, m–p), suggesting that the convergence and extension movements was disrupted in the MZ*dgcr8* embryos. Collectively, this data indicating that canonical microRNA function are required for germ layer specification, organ progenitor formation and cell movements.

Discussions

The development of engineered artificial nucleases enables targeted genome editing across species [30]. To achieve genome editing, the engineered nucleases were applied to introduce targeted DNA double strand breakages in the genome [31, 32]. The DNA repair pathway will be activated and targeted mutagenesis could be achieved by error-prone repair pathway while DNA replacement could be achieved by homologous recombination pathway [33]. In human cell lines and mouse, gene replacement is relatively well established [34, 35]. However, a high frequency of targeted mutagenesis could be easily achieved but targeted knock-in of an exogenous DNA is difficult to achieve in zebrafish. Indeed, efforts have been made to knock-in a *loxP* site into targeted locus in several studies [8, 9], but thus far only one study reported successful Cre-*loxP*-mediated conditional knockout in zebrafish [11]. Therefore, other approaches for conditional knockout are in high demand in zebrafish as well as in other species in which knock-in is difficult to perform.

In this study, we have described a BACK approach. Compared to the established conditional knockout approach, our BACK approach has several advantages. First, this BACK approach is not dependent on the efficiency of the precise repair pathway. Second, the replacement of DNA to a specific genomic site is limited by whether the specific locus is targetable by engineered nucleases, but there is no such limitation in our BACK approach. Moreover, this BACK approach also provides an opportunity to rescue the knockout phenotype to confirm the target specificity of the engineered nucleases.

Using the BACK approach, we have successfully generated germline-specific knockout of *dgcr8*. We found that the processing of microRNAs and early embryonic

development were disrupted in the MZ*dgcr8* mutant. The MZ*dgcr8* embryos resemble MZ*dicer* phenotypes in several aspects [17]: the exhibition of marked developmental delay, the disruption of brain development, and the shorter body axis and the lack of circulation. These data suggested that the canonical microRNAs but not other small RNAs processed by Dicer play important roles in early development. Furthermore, the MZ*dgcr8* phenotype could be well rescued by miR-430, indicating that miR-430 is the key microRNA in the early embryonic stage. Deletion of the miR-430 cluster could produce MZ*dgcr8*-like phenotypes (our unpublished data), providing further support that miR-430 is the major functional microRNA in early development. Therefore, most of the observed MZ*dgcr8* phenotypes in the early embryonic stages could be due to disruption of miR-430 function. An important function of miR-430 is to remove the maternal transcripts after zygotic genome activation [28, 36, 37]. Several hundreds of maternal transcripts were not efficiently removed in the MZ*dicer* mutant [29], and this may lead to development delay. The observed development delay in the MZ*dgcr8* mutant may also due to the delayed clearance of maternal transcripts by miR-430. Moreover, miR-430 plays important roles in promoting nodal signaling [38–40]. Both agonist and antagonist of the nodal pathway were directly regulated by miR-430 [38]. In the MZ*dicer* mutant, nodal signaling activity was decreased and mesoendoderm development was disrupted [38]. Similar mesoendoderm development was observed in the MZ*dgcr8* mutant, probably due to decreased nodal signaling upon loss of function of miR-430. Cell movement defects were observed in both MZ*dicer* and MZ*dgcr8* mutant. Further investigations are highly warranted to understand how this process is regulated.

In summary, we have demonstrated that the Cre/*loxP*-mediated tissue-specific gene knockout strategy could be achieved in zebrafish using our BACK approach. This approach could conceivably be applied to other genes in zebrafish and possibly in other species as well. Moreover, the *dgcr8* line produced in this study could be crossed with other *Cre* line to investigate the functional roles of canonical microRNAs in other biological processes.

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