Heritable and Lineage-Specific Gene Knockdown in Zebrafish Embryo

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Abstract

Background: Reduced expression of developmentally important genes and tumor suppressors due to haploinsufficiency or epigenetic suppression has been shown to contribute to the pathogenesis of various malignancies. However, methodology that allows spatio-temporally knockdown of gene expression in various model organisms such as zebrafish has not been well established, which largely limits the potential of zebrafish as a vertebrate model of human malignant disorders.

Principal Finding: Here, we report that multiple copies of small hairpin RNA (shRNA) are expressed from a single transcript that mimics the natural microRNA-30e precursor (mir-shRNA). The mir-shRNA, when microinjected into zebrafish embryos, induced an efficient knockdown of two developmentally essential genes chordin and -catenin in a dose-controllable fashion. Furthermore, we designed a novel cassette vector to simultaneously express an intronic mir-shRNA and a chimeric red fluorescent protein driven by lineage-specific promoter, which efficiently reduced the expression of a chromosomally integrated reporter gene and an endogenously expressed gata-1 gene in the developing erythroid progenitors and hemangioblasts, respectively.

Significance: This methodology provides an invaluable tool to knockdown developmental important genes in a tissue-specific manner or to establish animal models, in which the gene dosage is critically important in the pathogenesis of human disorders. The strategy should be also applicable to other model organisms.

Introduction

Understanding the development and disease-associated molecular and cellular processes in model organisms has largely relied on gene loss-of-function approaches. Homologous recombination-mediated gene knockout has not yet been achieved in zebrafish, due to the difficulty of generating embryonic stem cell line. The generation of zebrafish knockout has instead taken use of TILLING (targeting induced local lesions in genomes) strategy, in which a library of ENU-mutagenized F1 animals are generated and kept either as a cryopreserved sperm or as a living stock, and in which a library of ENU-mutagenized F1 animals are generated and kept either as a cryopreserved sperm or as a living stock, and the DNA of these animals is screened for genetic lesion in specific exons [1]. Recently, heritable targeted gene disruption with the DNA of these animals is screened for genetic lesion in specific exons [1].

Establishing zebrafish as a vertebrate model of human pathological conditions and diseases [1], because increasing evidence has demonstrated that haploinsufficiency and epigenetic suppression of tumor suppressor genes, other than complete mutational inactivation or permanent removal of genetic material from the host genome, might be a preferred mechanism in promoting cell transformation [5]. For instance, mice carrying hypomorphic Sftp1 enhancer allele that reduces Pu.1 expression to 20% of normal levels develop acute myeloid leukemia (AML), while a 50% or even a 100% loss of Pu.1 expression only induc accumulation of abnormal myeloid precursors [6]. Recent studies also indicate that haploinsufficiency of RPS14 and even lower levels of -catenin expression ranging from 10 to 30% of normal contribute to the pathogenesis of hematological malignant disorders [7,8]. These data suggest that a narrow window of reduced expression of a tumor suppressor is crucial for acute myeloid leukemia and solid tumor development.

RNA interference (RNAi) using either chemically synthesized small interfering RNAs (siRNA) or DNA-based vector systems expressing small hairpin RNAs (shRNA) driven by RNA
polymerase (pol) III promoter has been proved to be an efficient method to mediate sequence-specific, post-transcriptional silencing of virtually any gene in various model organisms [9]. The shRNA-mediated knockdown using either pol III or pol II promoter has been utilized to knockdown gene expression in mammalian cells and animals in a regulated fashion [10,11]. In combination with a natural backbone of the primary miR-30 microRNA (miRNA), higher amounts of synthetic shRNAs can be produced from the pol III promoter than from the simple hairpin design [12]. This miRNA-based shRNA (mir-shRNA), when embedded in an intron of β-actin genomic fragment that is in-frame linked to a fluorescent protein-coding reporter gene and placed under the ubiquitous or tissue-specific pol II promoter, is able to efficiently knockdown the expression of chromosomally integrated and endogenous genes in a heritable and tissue- or cell-specific fashion. Cells with reduced expression of targeted genes can be visualized and dynamically traced owing to the expression of the nontoxic actin-tagged fluorescent protein.

**Results**

**Efficient Knockdown of Reporter Gene In Vivo by Mir-shRNA**

It has been previously shown that the 5’ and 3’ flanking sequences of miRNA precursor are crucial for miRNA processing and maturation [16], and the hairpin shRNA can be expressed from a synthetic stem-loop precursor flanked by the 5’ and 3’ flanking sequences of either human miR-30-30 or mouse miR-155 gene [13]. We first identified zebrafish homologues of the hematopoietic intermediate cell mass (ICM) and forebrain in chordin expression during early embryogenesis. It has been shown that loss-of-function of chordin has been observed in the leukemia-initiating cells of del(5q)-associated acute myeloid leukemia/myelodysplastic syndrome and in the invasive solid tumors [8,20].

To test whether the mir-shRNA could knockdown endogenous genes in zebrafish, we selected chordin and alpha-catenin that were expressed during early embryogenesis. It has been shown that loss-of-function of chordin results in embryonic ventralization with the expansion of mesodermal hematopoietic tissue at the expanse of neuroectodermal development [19]. Significantly reduced expression of alpha-catenin has been observed in the leukemia-initiating cells of del(5q)-associated acute myeloid leukemia/myelodysplastic syndrome and in the invasive solid tumors [8,20].

Because the local secondary structure and the free energy (ΔG) of 3’UTR might affect the accessibility by mir-shRNA [16], we selected two sequences within the 3’UTR of chordin gene, which could be potentially targeted by mir-shRNAChordin-3’UTR-1 and mir-shRNAChordin-3’UTR-2, respectively (Figure 3A). These two sequences were selected with mFold software [21] based on the ΔG of these sites and their flanking sequence (60 bp 5’ and 3’), which the mir-shRNAChordin-3’UTR-1 appeared to have lower ΔG than miR-shRNAChordin-3’UTR-2 (Figure 3B). The capped mir-
shRNA\textsuperscript{chordin-3 UTR-1} and mir-shRNA\textsuperscript{chordin-3 UTR-2} was individually microinjected into one-cell stage embryos and whole-mount in situ hybridization (WISH) analysis with a dig-labeled antisense probe was performed to evaluate the level of \textit{chordin} transcripts. While the \textit{chordin} transcripts were appropriately detected in the dorsal shield of wild-type or mir-shRNA\textsuperscript{EGFP-ORF} control-injected embryos at 6 hpf as previously reported [22] (Figure 3C, left panel, white arrowhead), a dramatic reduction of \textit{chordin} transcripts was observed in the embryos injected with 200 pg of mir-shRNA\textsuperscript{chordin-3 UTR-1}, but not with the same amount of mir-shRNA\textsuperscript{chordin-3 UTR-2} likely due to its higher \textit{DG} (3.7 v.s. 0.2 kcal/mol) (Figure 3B and 3C, white arrowheads). As a result, an enlarged blood ICM (Figure 3C, black arrowhead, \(n = 61/99\)) with increased \textit{gata-1} expression (black arrow) and partial loss of neural tissues (white arrow) were observed only in mir-shRNA\textsuperscript{chordin-3 UTR-1}-injected embryos at 24 hpf, which were comparable to the embryos injected with 0.8 ng of \textit{chordin}-specific morpholino oligonucleotides [19] (Figure 3D).

The mir-shRNA\textsuperscript{\textit{alpha-catenin}-3 UTR-1} and mir-shRNA\textsuperscript{\textit{alpha-catenin}-3 UTR-2} were also designed to target two regions within the 3' UTR of \textit{alpha-catenin} gene (Figure 4A). WISH analysis showed that the \textit{alpha-catenin} was maternally expressed (data not shown) and ubiquitously detected in wild type or control mir-shRNA\textsuperscript{\textit{alpha-catenin}-3 UTR-1} injected embryos at 8 hpf (Figure 4B, left panel). In contrast, a significant reduction in the \textit{alpha-catenin} transcripts was consistently observed in the 8 hpf embryos injected with 160 pg of either mir-shRNA\textsuperscript{\textit{alpha-catenin}-3 UTR-1} or mir-shRNA\textsuperscript{\textit{alpha-catenin}-3 UTR-2} (Figure 4B, right panels). Consistently, quantitative Western blot analysis showed that the \textit{alpha-catenin} proteins were dramatically decreased to 26% of normal level at 22 hpf (Figure 4C). To determine whether the mir-shRNA\textsuperscript{\textit{alpha-catenin}-3 UTR-1} can confer gene knockdown in a dosage-dependent fashion, embryos were injected with the same amount of duplex \(0^6\), duplex \(1^6\) and duplex \(2^6\), which harbored zero, one and two copies of shRNA\textsuperscript{\textit{alpha-catenin}-3 UTR-1}, respectively (Figure 4D, top). Northern and Western blot analyses showed that as expected, injection of duplex \(2^6\) generated about one-fold more shRNAs (Figure 4D, bottom) and one-fold less \textit{alpha-catenin} proteins than injection of duplexes \(1^6\) and control at 22 hpf embryos (Figure 4D, bottom). Consistently, injection of fourplex \(4^6\) also generated one-fold more shRNAs than injection of fourplex \(2^6\) (Figure 4E), suggesting that the \textit{alpha-catenin} protein could be further reduced (data not shown). Thus, the experimental design presented here provided not only an efficient means to screen and identify mir-
shRNA capable of reducing target gene level, but also a feasible tool to titer down the gene dosage in a controllable manner.

**Intronic Mir-shRNA Expression and Genetic Tractability under Pol II Promoter**

Natural miRNAs lying within the intron of protein-coding genes have been shown to be co-transcribed with message mRNAs under ubiquitous or tissue-specific pol II promoters [16]. We designed a cytomegalovirus (CMV) prompter-driven expression cassette in which the zebrafish $\beta$-actin genomic fragment containing an intact exon 2 (123 base pairs), an intact intron 2 (364 base pairs) and the first 21-base pairs of exon 3, was in-frame fused to the DsRed-Express (DsRed-EX) reporter followed by a bovine growth hormone (BGH) poly (A) site as 3' UTR (Figure 5A). After injection of the plasmid cassette into one-cell stage embryos, the injected embryos showed red fluorescence due to the expression of the chimeric $\beta$-actin-DsRed protein, and no any morphological and developmental abnormalities were observed during embryogenesis (Figure 5B). The precise splicing of intron 2 from the $\beta$-actin-DsRed fusion gene in vivo was confirmed by amplification of a predicted size of RT-PCR product and subsequent sequencing (Figure 5C, D). Furthermore, co-injection of the plasmid expression cassette carrying an introduced mir-shRNAEGFP-ORF or mir-shRNAEGFP-SV40-1 at the Bgl II restriction site within the intron 2, with the EGFP-SV40 reporter plasmid (Figure 5E), resulted in a dramatic decrease of EGFP fluorescence with correct splicing of the mir-shRNA-containing intron 2 in 22 hpf embryos (Figure 5F, G). The results provide a proof-of-concept that knockdown of chromosomally integrated or endogenous genes under a tissue- or lineage-specific pol II promoter might be feasible.

**Heritable and Lineage-specific Knockdown of Chromosomally Integrated and Endogenous Genes in the Developing Erythroid Progenitors and Hemangioblasts during Embryogenesis**

We previously established a transgenic reporter line Tg(zgata-1:EGFP-SV40) with stable expression of EGFP under the erythroid-specific gata-1 promoter [23]. The transgenic line is unique in that the EGFP expression can be detected in multiple

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**Figure 2. Knockdown of EGFP sensors by mir-shRNA in vivo.** (A) Diagram of miRNA-based shRNAEGFP-ORF (mir-shRNAEGFP-ORF). The stem-loop region of miR-30e precursor was replaced with chemically synthesized shRNAEGFP-ORF oligonucleotides containing the same sequence as the miR-30e stem-loop, except that the miR-30e hairpin stem was changed to the sequence that was complementary to the open reading frame (ORF) of EGFP transcript. (B) Northern blot analysis of 12 and 24 hpf embryos injected with *in vitro* synthesized mir-shRNAEGFP-ORF mRNAs. The U6 promoter-driven expression of shRNAEGFP-ORF in 293T cells was used as a positive control (left lanes). (C) Diagram of various sensors containing one or two copies of binding sequence for mir-shRNAEGFP-ORF within the ORF or the 3' UTR. The 22-bp long binding sequence was inserted into the 3' UTR-SV40 of either EGFP or DsRed gene. (D) Individual capped sensor mRNAs was co-injected with either miR-30e precursor control or mir-shRNAEGFP-ORF. **(E–H)** Detection of EGFP and DsRed fluorescence in 24 hpf embryos injected with various sensor mRNAs. Red fluorescence was used as an injection control in **E**, **F** and **G**, and green fluorescence as injection control in **H**. (I) Western blot analysis of 24 hpf embryos shown in panels **E–G**. The $\beta$-actin was used as a loading control.

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tissues including the midbrain, forebrain, dorsal neurons other than in the erythropoietic ICM, which has also been observed in previous transgenic line with the same gata-1 promoter [24]. Thus, this line offers a unique advantage as a reporter line to detect miR-shRNA mediated knockdown effects in multiple lineages and tissues within an individual animal.

We screened and established 6 transgenic lines stably expressing the intrinsic mir-shRNA<sup>EGFP-SV40-1</sup> under the same gata-1 promoter. One of the lines designated as Tg(zgata-1:mir-shRNA<sup>EGFP-SV40-1</sup>-actin-DsRed-BGH)<sup>line 1</sup> was selected to determine its knockdown potency because the DsRed fluorescent proteins were also observed to be expressed in the same tissues as the reporter line Tg(zgata-1:EGFP-SV40). When the homozygous Tg(zgata-1:EGFP-SV40) reporter line was crossed to heterozygous Tg(zgata-1:mir-shRNA<sup>EGFP-SV40-1</sup>-actin-DsRed-BGH)<sup>line 1</sup> (Figure 6A), 421 (52.9%) and 375 (47.1%) of 796 F2 embryos collected from the reporter line Tg(zgata-1:mir-shRNA<sup>EGFP-SV40-1</sup>-actin-DsRed-BGH)<sup>line 1</sup> fathered by the homozygous and heterozygous parents, respectively, suggesting a dominant Mendelian ratio. The EGFP expression in both mRNA and protein levels was dynamically evaluated in the DsRed<sup>+</sup> and DsRed<sup>−</sup> embryos at 24, 48 and 72 hpf. The results demonstrated a significant reduction of EGFP fluorescence and transcripts in the midbrain (MB), hindbrain (HB), dorsal neurons (DN) and caudal hematopoietic tissue (CHT) only in the DsRed<sup>+</sup> siblings at 24, 48 and 72 hpf (Figure 6B, C, arrows; Figure S3). Western blot analysis further confirmed the results that a 45%, 58% and 62% of total EGFP protein was lost in the DsRed<sup>+</sup> embryos at 24, 48 and 72 hpf, respectively (Figure 6D). The results indicate that the mir-shRNA<sup>EGFP-SV40-1</sup> is able to mediate the cell subtype-specific knockdown of a chromosomally integrated gene in a genetically heritable manner.

To further test the knockdown effects on endogenous genes, we selected an erythroid-specific gata-1 promoter to evaluate the knockdown the erythroid-specific gata-1 gene in the developing hemangioblasts. A transgenic line Tg[zm2:mir-shRNA<sup>gata-1</sup>-actin-DsRed-BGH]<sup>line 3</sup> was established under the hemangioblast lmo2 promoter [25,26] to be used in combination with cell- or tissue-specific pol II promoters using a dig-labeled full-length 3′ UTR of gata-1 as probe (upper panels). A significantly enlarged ICM (black arrowhead) with a partial loss of transcripts was only observed in the PBI of gata-1 expressing embryos injected with only shRNA<sup>chordin-3</sup>-UTR-1, but not with shRNA<sup>chordin-3</sup>-UTR-2 and control (middle panels). A higher level of gata-1 expression was also detected in the shRNA<sup>chordin-3</sup>-UTR-1-injected embryos, compared to control or shRNA<sup>chordin-3</sup>-UTR-2-injected embryos (bottom panels). (D) Morphology of embryos injected with gata-1 morpholino oligonucleotides and its 4-base pair mismatch control. Embryos at 6 hpf are dorsal view, and embryos at 24 hpf are lateral views with head to the left.

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**Figure 3. Knockdown of endogenous cellular gata-1 expression.** (A) Diagram of mir-shRNA<sup>chordin-3′UTR-1</sup> and mir-shRNA<sup>chordin-3′UTR-2</sup> against the 3′ UTR of gata-1 gene, whose predicted secondary structure was shown at the bottom. Red brackets denoted the targeted regions. (B) Energy of the targeted regions and corresponding flanking sequence predicted with mFold software. (C) Phenotypes of gata-1-deficient embryos, WISH analysis of gata-1 expression in the 6 hpf embryos injected with 200 pg of shRNA<sup>gata-1</sup>-ORF (control), shRNA<sup>gata-1</sup>-UTR-1 or shRNA<sup>gata-1</sup>-UTR-2 miRNAs using a dig-labeled full-length 3′ UTR of gata-1 as probe (upper panels). A significantly enlarged ICM (black arrowhead) with a partial loss of neural tissues (white arrow, 61/99) were observed in 24 hpf embryos injected with only shRNA<sup>chordin-3</sup>-UTR-1, but not with shRNA<sup>chordin-3</sup>-UTR-2 and control (middle panels). A higher level of gata-1 expression was also detected in the shRNA<sup>chordin-3′UTR-1</sup>-injected embryos, compared to control or shRNA<sup>chordin-3′UTR-2</sup>-injected embryos (bottom panels). (D) Morphology of embryos injected with gata-1 morpholino oligonucleotides and its 4-base pair mismatch control. Embryos at 6 hpf are dorsal view, and embryos at 24 hpf are lateral views with head to the left.

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### Discussion

In this study, we have developed a novel methodology that uses a microRNA-based shRNA (mir-shRNA) to reduce the dosage of a given gene in a controllable and tissue-specific manner. Although the miRNA-based shRNA knockdown strategy has been successfully used to mediate efficient and specific knockdown of genes *in vitro*, its use in combination with cell- or tissue-specific pol II
promoter in animals is still absent. The backbone of miR-30 is one of the most frequently used microRNA sequence to direct the processing and maturation of shRNA, because its stem sequence could be substituted with exogenous sequences that match different target genes and to produce 12 times more mature shRNAs than simple hairpin designs [12,14], and its ability to prevent interferon-stimulated gene expression and associated off-target effects and toxicity in cultured cells and mouse brain [17,28].

Although the sequences targeted by mir-shRNA in this study are derived from 3'UTR, the mir-shRNA should be able to target sequences within other part of a given transcript such as the open reading frame as described previously [12,17]. The observations that the targeted site in the 3'UTR, rather than in the ORF of EGFP, confer robust knockdown effects by mir-shRNAEGFP-ORF (Figure 2), suggest that the mir-shRNA might preferably target sequence in the 3'UTR in vivo. Interestingly, the similar phenomenon has also been observed in cultured Schneider S2 cells, although the underlying mechanism remains elusive [18]. On the other hand, to optimize the site that mediate maximal knockdown effects, two to three potential target sequences for a given gene should be designed with mFold software and selected based on the predicted secondary structure and DG. Furthermore, as shown in Figure 4D and 4E, taking use of mir-shRNA duplex or fourplex also provides a potential means to maximally knockdown the target genes whose dosage can be regulated in a controllable fashion.

The use of pol II promoter-driven mir-shRNA expression cassette provides a unique advantage in that the cells or tissues with reduced expression of target gene can be genetically traced and visualized in the transparent zebrafish embryos, because of the simultaneous expression of the chimeric red fluorescent protein, β-actin-DsRed. Transgenic embryos and adults stably expressing this chimeric fluorescent protein appear morphologically and developmentally normal and have been fertile for three generations, suggesting a lack of detectable toxicity. Given the facts that...
reduced expression of many disease-associated and developmentally important genes due to either epigenetic inactivation or haploinsufficiency, contribute to the pathogenesis of myeloid malignancies and tumorogenesis [6,8], the methodology described in this study highly complements the recently reported zinc finger-mediated gene knockout strategy in zebrafish, and provide an

Figure 5. Design of pol II promoter-driven knockdown construct. (A) Diagram of pol II-type promoter CMV driven-knockdown vector (CMV promoter-actin-DsRed-BGH). DS: donor site; AS: acceptor site. The first 21-base pairs of exon 3 of zebrafish actin gene have been in-frame fused to the DsRed fluorescent protein gene followed by a bovine growth hormone (BGH) sequence as 3′ UTR. (B) Red fluorescence was observed in 22 hpf embryos injected with the plasmid shown in panel A. (C) RT-PCR analysis with total RNAs derived from 22 hpf embryos shown in panel B. The primers used are indicated by horizontal arrows in panel A. (D) The sequence of RT-PCR product shown in panel C. Note that the entire intron 2 of the actin gene has been spliced out (arrow). (E) The mir-shRNAEGFP-SV40-1 was inserted into the intron 2 at the Bgl II site and co-injection with CMV-EGFP-SV40 reporter plasmid. (F) Knockdown of EGFP fluorescence in the 24 hpf embryos co-injected with EGFP-SV40 reporter plasmid plus CMV promoter-actin-DsRed-BGH plasmid carrying either mir-shRNAEGFP-ORF or mir-shRNAEGFP-SV40-1. (G) RT-PCR analysis of total RNAs derived from 22 hpf embryos shown in panel F.
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Figure 6. Tissue-specific knockdown of chromosomally integrated EGFP expression. (A) Diagram of transgenic lines Tg(zgata-1:EGFP-SV40) and Tg(zgata-1:mir-shRNAEGFP-SV40-1-actin-DsRed-BGH) line 1 under control of zebrafish gata-1 promoter. (B) Knockdown of EGFP fluorescence was observed in the mid- and hindbrain of the DsRed−, but not DsRed− F2 sibling at 48 hpf. Embryos are dorsal view with head to the left. (C) Knockdown of EGFP fluorescence was observed in the dorsal neurons and caudal hematopoietic tissue of the DsRed−, but not DsRed− F2 sibling at 72 hpf. MB: midbrain; HB: hindbrain; ys: yolk sac; DN: dorsal neurons; CHT: caudal hematopoietic tissue. Embryos are lateral view with head to the left. (D) Western blot analysis of EGFP expression in the DsRed− and DsRed− F2 embryos at 24, 48 and 72 hpf. The α-tubulin protein was used as a loading control.
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invaluable tool to knockdown disease-associated gene in specific tissues or cells in the model organisms. In combination with Cre-loxP recombination and drug-inducible strategy [25,29], the pol II promoter-driven mir-shRNA knockdown system could be further optimized to prevent embryonic lethality from reduced expression of the developmentally crucial genes and tumor suppressors.

Materials and Methods

Fish care

The maintenance, breeding and staging of zebrafish lines (Tubingen and Shanghai) were performed as described previously [30].

Cloning and plasmid construction

The precursor sequences of zebrafish mir30e (409 bp) and mir155 (447 bp) were cloned from the genomic DNA of Tubingen adult fish into the pCS2+ vector. The 68-bp mir30e stem-loop region was replaced with a linker sequence containing two Bbs I sites. The shRNA sequences were synthesized as DNA oligonucleotides (Invitrogen) and inserted at the Bbs I sites. The sensor sequences were synthesized as DNA oligonucleotides (Invitrogen) and placed into the 3′UTR of the pCS2+EGFP plasmid. The genomic sequence of zebrafish β-actin containing an intact exon 2 and intron 2, and the first 21-bp of exon 3 was in-frame infused to the open reading frame of DsRed-3′UTR BGH, and cloned into the pCS2+ plasmid through BamHI and EcoRI sites. The mir-shRNA was inserted into an endogenous Bgl II site within the intron 2. The resultant intronic mir-shRNA was then inserted downstream of the gata-1 promoter or lmo2 promoter and cloned into the I-SceI-containing plasmid. All primer sequences were available in Table S1.

Microinjection and establishment of transgenic zebrafish line

All capped mRNAs were synthesized with SP6 mMessage mMachine (Ambion) and microinjected into one-cell stage embryos. The transgenic plasmids flanked by the I-SceI sites were prepared with endotoxin-free miniprep kit (Axogen). Microinjec-
tion was performed at one-cell stage embryos with 2 ml of injection solution containing 40 pg/ml of DNA, 0.5 x 10^6 I-SceI buffers and 0.5 units/ml I-SceI meganuclease (New England Biolabs). Injected embryos were raised to sexual maturity (F0 founders) and crossed to wildtype zebrafish to generate F1 progeny, which were screened for red fluorescent DsRed expression in the ICM at 24 hpf. The DsRed^+ F1 embryos were raised to adults to establish the stable transgenic lines. Embryos were imaged using a Zeiss SteREO Discovery V12 fluorescent stereomicroscope.

shRNA Northern blot analysis and RT-PCR

Total RNAs of embryos injected with capped mRNAs were extracted with Trizol (Invitrogen), and separated on 12% of UREA-PAGE gel. Northern blot was probed with dig-labeled antisense probe, and visualized using DIG luminescent detection kit for nucleic acids (Roche). RT-PCR was performed with One-step RT-PCR kit (Qiagen) as previously described [30].

Whole-mount mRNA in situ hybridization

Whole-mount mRNA in situ hybridization was performed as described previously [30]. Dig-labeled antisense probes of τ-catenin and chordin were generated from a 975-nt cDNA fragment encoding N-terminal 325 aa of τ-catenin, and entire 570 bp 3’UTR, respectively.

Western blot analysis

Embyros were deyolked as described previously [31], and dissolved directly by 2xSDS-PAGE loading buffer (2 μl per embryo). The samples were separated on 8% or 12% SDS-PAGE gel (for detection of τ-catenin and EGFP protein, respectively). The antibodies against EGFP (Santa Cruz), τ-catenin (BD), β-actin (Sigma) and α-tubulin (Sigma) were diluted in 2% BSA as a ratio of 1:1000, 1:500, 1:2000 and 1:10000, respectively.

Cell culture and transfection

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% calf serum in an atmosphere containing 5% CO2. H 1 pol III promoter-driven shRNA and EGFP reporter (pEGFP-C1, Clontech) and DsRed plasmid were cotransfected with a ratio (20:1:2) into the plasmid were cotransfected with a ratio (20:1:2) into the HEK293T cells using the calcium phosphate method.

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