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Drosophila miR-960 negatively regulates Hedgehog signaling by suppressing Smoothened, Costal-2 and Fused

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ABSTRACT

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that post-transcriptionally regulate gene expression in eukaryotes. In *Drosophila melanogaster*, up to 240 miRNAs have been identified by computational methods or experimental approaches. However, most of their biological functions are still unknown. Here, we identified miR-960 as a suppressor of Hedgehog (Hh) signaling pathway. Ectopic miR-960 obviously represses the expression levels of target genes. This activity is mediated by direct inhibition of Smoothened (Smo), Costal-2 (Cos2) and Fused (Fu), which are essential signaling transduction components of Hh pathway. Through in vivo sensor assay and in vitro luciferase assay, we found that miR-960 directly binds to the 3′UTRs of *smo*, *cos2* and *fu* mRNAs to block their translation. Additionally, we demonstrated that miR-960 cannot suppress Wg and Dpp signaling pathways. Together, our results indicate that miR-960 can specifically suppress Hh pathway by directly targeting three important signaling transducers Smo, Cos2 and Fu.

1. Introduction

MicroRNAs (miRNAs) are small (18-24 nucleotides) endogenously expressed non-coding RNAs that mediate post-transcriptional gene silencing by annealing to complementary sequences in the 3′UTRs of target mRNAs [1]. This type of regulation modulates gene expression through the inhibition of protein synthesis and/or mRNA stability [2,3]. Since the genetic identification of the miRNAs *lin-4* and *let-7* in *Caenorhabditis elegans* [4,5], many miRNAs have now been identified by experimental and computational approaches in animals, plants, and viruses [6–8]. In addition, increasing evidence suggests that miRNAs play important roles in diverse physiological processes such as homeostasis, metabolism, development and cancer pathogenesis [5,9–11]. However, the biological functions of most miRNAs are still unknown.

The Hedgehog (Hh) signaling pathway, which is highly conserved from *Drosophila* to vertebrate, governs a wide variety of processes during embryonic development and adult tissue homeostasis [12]. Dysregulation of Hh signaling contributes to many human diseases including birth defects and cancers [12]. In *Drosophila* wing disc, Hh protein is produced by posterior compartment cells and forms gradient in the anterior compartment to specify distinct cell fates. The Hh signaling is transduced through a reception system that includes the 12 transmembrane protein Patched (Ptc) and seven transmembrane protein Smoothened (Smo). In the absence of Hh, Ptc inhibits Smo to transduce signals to an intracellular complex containing the Kinesin related protein Costal2 (Cos2), the protein kinase Fused (Fu) and the transcription factor Cubitus interruptus (Ci) [13]. This complex phosphorylates full-length Ci generating a truncated repressor form to block the expression of Hh responsive genes [14–16]. The presence of Hh relieves the inhibition of Ptc on Smo, inducing Smo cell-surface accumulation and Phosphorylation [17,18]. Then, phosphorylated Smo transduces signal to the intracellular Cos2–Fu–Ci complex to attenuate Ci cleavage [19–21], which further promotes nuclear translocation of Ci to induce the expression of target genes, such as *ptc*, *col* and *dpp* [22].

MicroRNAs are important regulators of the Hh pathway. Previous work in zebrafish shows that miR-214 enables precise specification of muscle cell types by sharpening cellular responses to Hh signaling through targeting the negative regulator Su(fu) [23]. In a miRNA high-throughput profile screening, miR-125b, miR-326 and miR-324-5p have been found to directly bind to *smo* 3′UTR and inhibit Hh signaling in mammalian cancer cells [24]. *Drosophila* miR-12/miR-283/miR-304 cluster can directly repress the expression of *smo*, *cos2* and *fu* [25]. miR-212 displays tumor-promoting properties in non-small cell lung cancer cells by targeting the receptor PTCH1 [26]. Recently, our work also identified that miR-5 can suppress Hh signaling by directly targeting Smo [27]. In this report, we investigated the role of

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miR-959/miR-960/miR-961/miR962 cluster (miR-960C) in the Hh pathway. We found that miR-960 suppressed Hh signaling by targeting Smo, Cos2 and Fu. We confirmed the binding of miR-960 to the 3′UTRs of *smo*, *cos2* and *fu* by GFP sensor assay and dual luciferase assay. Furthermore, we found that miR-960 is not involved in the Wg and Dpp signaling pathways, which also control wing morphogenesis. Taken together, our data confirmed the importance of miRNAs in the Hh signaling regulation and introduced miR-960 as new suppresser of the Hh signaling pathway in *Drosophila*.

2. Materials and methods

2.1. Drosophila genetics

All stocks were maintained and crossed at 25 °C according to standard procedures. The *en-Gal4*, *ap-Gal4* and *MS1096-Gal4* lines were obtained from Bloomington stock center. The *UAS-GFP-GPI*, *UAS-GFP-GPI-miR-960C* and *UAS-DsRed-miR-960C* transgenic flies were generated using the PhiC31 integrase-mediated site-specific transgenesis system. The *miR-960-sensor*, *tub-EGFP*, *tub-EGFP-smo-3′UTR*, *tub-EGFP-cos2-3′UTR* and *tub-EGFP-fu-3′UTR* flies were generated by P-element transformation.

2.2. Plasmid construction

To generate the pWALIUM10-moe-GFP-GPI-miR-960C construct, GFP-GPI which contains GFP sequence followed by the GPI signal from Dlp (amino acids 695–765) was inserted in the BglII site of pWALIUM10-moe vector [28]. Approximately 706 bp of genomic DNA containing miR-959/miR-960/miR-961/miR-962 was amplified by PCR and cloned downstream of GFP-GPI in the XbaI site of pWALIUM10-moe vector. PCR primers are:

forward, 5′-GCTCTAGAGCTwvAATGCCCCAGTGGCGTG-3′;
reverse, 5′-GCTCTAGAATGTATCTCAATGAATTGCCG-3′.

A similar strategy was used to make the pWALIUM10-moe-DsRed-miR-960C construct; DsRed was inserted into the EcoRI site of pWALIUM10-moe vector, and the miR-960C fragment was cloned into the NdeI and NheI sites.

The tub-EGFP-smo-3′UTR was generated by cloning a 596 bp fragment of *smo* 3′UTR downstream of pCaSpeR-tub-EGFP [29] (a gift from T. Kai), through NotI and XhoI sites. The PCR primers are:

smo forward, 5′-TAGCGGCCGCAAGGTTCAAAAACTCTTACA-3′;
smo reverse, 5′-GACTCGAGTACACAAATTATTATGTATA-3′.

A similar strategy was used to make the tub-EGFP-cos2-3′UTR and tub-EGFP-fu-3′UTR constructs. The PCR primers are:

cos2 forward, 5′-GCGTTTAAACCCAGGACGATATTATAAGGTT-3′;
cos2 reverse, 5′-TAGCGGCCGCCCAACAATATCCACACAAAGC-3′.
fu forward, 5′-GCGTTTAAACCGTGCAGCGCATATGGAATGG-3′;
fu reverse, 5′-TTGCGGCCGCCGACGAACTGCTCGACTACTT-3′.

To generate the miR-960-sensor construct, the following primers are annealed (95 °C for 5 min, then slowly cold down to room temperature) in the annealing buffer (10 nM Tris-HCl, pH = 7.5, 100 mM NaCl, 1 mM EDTA), and then directly cloned into the pCaSpeR-tub-EGFP vector through NotI and XhoI sites.

miR-960-sensor forward, 5′-GGCCGCGCTATGCAATCTGGAATACTC AAATCACA CGCTATGCAATCTGGAATACTCAC-3′
miR-960-sensor reverse: 5′-TCGAGTGAGTATTCCAGATTGCATAGCG TGTGATT TGAGTATTCCAGATTGCATAGCGC-3′

2.3. Luciferase assay

For validation of miR-960 that target *smo* 3′UTR, *cos2* 3′UTR and *fu* 3′UTR, a 596 bp fragment of the *smo* 3′UTR was amplified by PCR from wild-type genomic DNA and cloned downstream of Renilla luciferase in the psiCheck-2 vector (Promega). PCR primers are:

smo forward, 5′-GGTTTAAACAAGGTTCAAAAACTCTTACA-3′;
smo reverse, 5′-TTGCGGCCGCTACACAAATTATTATGTATA-3′.

In the same way, a 979 bp fragment of *cos2* 3′UTR was cloned downstream of psiCheck-2 vector with the primers:

cos2 forward, 5′-GCGTTTAAACCCAGGACGATATTATAAGGTT-3′;
cos2 reverse, 5′-TAGCGGCCGCCCAACAATATCCACACAAAGC-3′.

A 910 bp fragment of *fu* 3′UTR including 136 bp Fu coding sequence was cloned downstream of psiCheck-2 vector with the primers:

fu forward, 5′-GCGTTTAAACCGTGCAGCGCATATGGAATGG-3′;
fu reverse, 5′-TTGCGGCCGCCGACGAACTGCTCGACTACTT-3′.

smo mutant 3′UTR, *cos2* mutant 3′UTR and *fu* mutant 3′UTR were generated by changing the miR-960 seed sequence (AATACTC) to (GGCGCGCC). Transfections were performed in 12 well plates by using effectene transfection reagent in S2 cells. In each well, 1 μg of total DNA was added. After 48 hours, the cells were lysed in passive lysis buffer, dual luciferase assays were carried out (Promega), and the results were analyzed on the luminometer.

2.4. Whole-mount staining and microscopy

Fixation and antibody staining in imaginal discs was performed as described [30]. Primary antibodies used for the immunostaining were: mouse anti-Ptc (1:40; DSHB, Apa-1), rat anti-Ci (1:10; DSHB, 2A1), rabbit anti-Col (1:1000; made in our laboratory), mouse anti-Smo (1:50; DSHB, 20C6), mouse anti-Cos2 (1:5; DSHB, 17E11), mouse anti-Fused (1:50; DSHB, 22F10), mouse anti-Wingless (1:5; DSHB, 4D4), guinea pig anti-Senseless (1:200; made in our laboratory), rat anti-Sal (1:100; made in our laboratory), mouse anti-Distal-less (1:50). The primary antibodies were detected by fluorescent-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories, Inc. Confocol images were collected using a Zeiss 780 confocal microscope with 40X/1.30 oil objectives. Adult wing images were obtained using a Zeiss Axio Imager Z2 microscope. Images were processed using Adobe Photoshop. For quantification of confocal images, the raw data were exported in tiff format. The plot values were measured from selected regions using Image J (NIH).

2.5. RNA extraction and quantitative RT-PCR

Total RNA was isolated from a pool of 80 wing imaginal discs with Trizol (Invitrogen) according to the manufacturer's protocol, and re-suspended in RNase-free water. First-strand cDNA was generated from 1 μg of these samples using random primers with the M-MLV Reverse Transcriptase System (Promega). Quantitative PCR was performed using the Brilliant SYBR Green QPCR Master Mix (Promega) on a CFX96 detection system (Bio-Rad). The primers were:

smo forward: 5′-AACGACTACTATGCCCTGAA-3′;
smo reverse: 5′-CGTATCTGTCGGAACCAAA-3′;
cos2 forward: 5′-TATCTATCCAAGCATCCGAAG-3′;
cos2 reverse: 5′-TCGCCCGAAGAACTTTCCAGA-3′;
fu forward: 5′-AACTGGTCTGCCACTTGGTAC-3′;
fu reverse: 5′-GACAATCCGTTCTACAAGCTC-3′;
Gapdh2 forward: 5′-GCCGAATACATCGTGGAG-3′;
Gapdh2 reverse: 5′-GGGTGTCGCTGAGGAAAT-3′.