The microRNA miR-8 is a conserved negative regulator of Wnt signaling

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Wnt signaling plays many important roles in animal development. This evolutionarily conserved signaling pathway is highly regulated at all levels. To identify regulators of the Wnt/Wingless (Wg) pathway, we performed a genetic screen in *Drosophila*. We identified the microRNA miR-8 as an inhibitor of Wg signaling. Expression of miR-8 potentially antagonizes Wg signaling *in vivo*, in part by directly targeting *wntless*, a gene required for Wg secretion. In addition, miR-8 inhibits the pathway downstream of the Wg signal by repressing TCF protein levels. Another positive regulator of the pathway, *CG32767*, is also targeted by miR-8. Our data suggest that miR-8 potentially antagonizes the Wg pathway at multiple levels, from secretion of the ligand to transcription of target genes. In addition, mammalian homologues of miR-8 promote adipogenesis of marrow stromal cells by inhibiting Wnt signaling. These findings indicate that miR-8 family members play an evolutionarily conserved role in regulating the Wnt signaling pathway.

adipogenesis | TCF | Wntless

Wnts are a family of highly conserved, secreted glycoproteins that act at a short range to regulate cell fate decisions during animal development (1, 2). The protein Armadillo (Arm; β -catenin in vertebrates) is central to the highly conserved signaling pathway by which cells respond to the *Drosophila* protein Wingless (Wg) and other Wnts. In the absence of the Wg/Wnt signal, a cytosolic pool of Arm/ β -catenin is phosphorylated and targeted for degradation. Wg/Wnt signaling inhibits this degradation, resulting in stabilization and nuclear translocation of Arm/ β -catenin. In the nucleus, Arm/ β -catenin forms a complex with members of the TCF/LEF family of transcription factors to regulate gene transcription. Wg/Wnt signaling is highly regulated, and inappropriate activation or inhibition of Wg/Wnt signaling results in developmental defects and diseases in humans, including colorectal cancer and inherited bone diseases (1, 2). Studies that add to our understanding of how the Wg/Wnt pathway is regulated are important, considering the key roles that this pathway plays in animal development and disease.

MicroRNAs are recently discovered regulators that influence cell physiology. These small (21–22 nt), noncoding RNAs post-transcriptionally silence gene expression in animals and plants by binding to specific mRNAs (3, 4). In animals, microRNAs generally bind to the 3'UTR of their mRNA targets and silence gene expression by causing degradation, decreased stability, or translational inhibition of target mRNAs. Hundreds of microRNAs have been identified, most of which are predicted to target multiple mRNAs, suggesting that microRNAs may function as part of an extensive gene regulatory network (3). Indeed, the regulation of conserved developmental signaling pathways (e.g., the Notch, Hedgehog, and TGF β pathways) by microRNAs has been reported (5–8). Activation of the Wg/Wnt pathway by the microRNA miR-315 was also been reported recently (9). Expression of miR-315 activates the pathway by targeting the negative regulators *Axin* and *Notum*.

Here, we report the identification of a microRNA, miR-8, in a genetic screen for antagonists of Wg signaling. We found that miR-8 inhibits Wg signaling *in vivo* and in cell culture by

targeting the Wg pathway at multiple levels. We demonstrate that miR-8 inhibits TCF protein expression and directly targets two positive regulators of the pathway, *wntless* (*wls*) and a gene, *CG32767*. These data illustrate how a single microRNA can modulate a signaling pathway at multiple levels. In addition, we demonstrate that mammalian homologues of miR-8 antagonize the ability of Wnt signaling to block adipogenesis in marrow stromal cells. Therefore, miR-8 and its homologues appear to play an evolutionarily conserved role in regulating Wnt signaling.

Results

miR-8 Is Identified in a Genetic Screen for Antagonists of Wg Signaling.

Ectopic activation of Wg signaling in the developing eye using the *GMR-Gal4* driver causes a dramatic reduction in eye size (Fig. 1B). To identify regulators of the Wg pathway, we performed a genetic screen to identify genes that, when misexpressed, suppress this small-eye phenotype (10, 11). Wg was coexpressed with random genes that were placed under the control of bidirectional Gal4-dependent (UAS) promoters by *GSV* transposable element insertions (12). Two *GSV* transposon insertions (*GSV*¹³⁰⁵⁻² and *GSV*²¹⁹⁶), known to suppress the *GMR/Wg* phenotype, were located upstream of the microRNA miR-8 (data not shown). Both *GSV* insertions also suppressed the phenotype resulting from ectopic expression of Arm*, a stable form of Arm, in the developing eye (Fig. 1C and data not shown). To verify that the phenotype of these insertions was because of expression of miR-8 and not to expression of surrounding genes, we generated transgenic flies expressing miR-8 under the control of a Gal4-dependent promoter (*UAS-miR-8*). Expression of miR-8 suppressed the small-eye phenotype caused by ectopic expression of Wg or Arm* (data not shown). These data suggest that miR-8 can inhibit ectopic Wg signaling in the developing fly eye.

miR-8 Inhibits Endogenous Wg Targets in Flies. To determine the effect of miR-8 expression on endogenous Wg signaling, we examined Wg readouts in the wing and leg imaginal discs. In the third larval instar wing imaginal disc, Wg was expressed in a stripe of cells along the dorsal-ventral boundary of the wing blade primordia (wing pouch; Fig. 2A) and activates target genes such as *Distal-less* (*Dll*, Fig. 2B) (10, 13). Expression of miR-8 along the anterior–posterior boundary of the wing pouch using *decapentaplegic* (*dpp*)-*Gal4* dramatically reduced *Dll* expression, as visualized by a loss of *Dll-lacZ* reporter expression in this domain (Fig. 2D). Expression of miR-8 inhibited all other Wg

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Fig. 1. miR-8 identified as a potential negative regulator of Wg signaling in a genetic screen. Micrographs of adult *Drosophila* eyes from flies containing the eye-specific driver *GMR-Gal4* alone (A) or in combination with *GMR-arm** (B) or *GMR-arm** and *GSV^{A2196}* (C). Each transgene is present as one copy/fly, and all flies were reared at 25°C.

targets tested, including *sens* and reporters for *fz3*, *nkd*, and *Notum*, caused notches in the adult wings [supporting information (SI) Fig. S1 and data not shown].

In the developing leg, Wg is expressed ventrally and *dpp* is expressed dorsally, as visualized with *dpp-lacZ* (Fig. 2E). If the Wg pathway is disrupted, then *dpp* expression becomes derepressed in the ventral portion of the leg disc (14–16). We expressed miR-8 using *ptc-Gal4*, a driver active in both *wg* and *dpp* expression domains. Expression of miR-8 in this domain

Fig. 2. miR-8 inhibits several Wg readouts *in vivo*. (A–D) Confocal images of third instar wing imaginal disc from a WT fly (A and B) or a fly containing *dpp-Gal4>UAS-miR-8* (C and D) to drive miR-8 expression along the anterior–posterior boundary. Expression of Dll, a positive target of Wg signaling, is monitored using the *Dll-lacZ* reporter. The discs were immunostained with antibodies against Wg (A and C) or lacZ (B and D). (E and F) Confocal images of third instar leg imaginal discs immunostained for lacZ. Expression of *dpp*, a negative target of Wg signaling, is monitored by using *dpp-lacZ*. (E) lacZ is repressed in WT discs by endogenous Wg signaling on the ventral side of the disc (arrow). (F) lacZ is derepressed on the ventral side of the *ptc-Gal4>UAS-miR-8* disc (arrow).

caused derepression of *dpp-lacZ*, as indicated by the extension of *dpp-lacZ* expression into the ventral portion of the leg disc (Fig. 2F). This derepression is consistent with miR-8 expression antagonizing endogenous Wg signaling.

miR-8 Inhibits Wg Secretion by Directly Targeting *wls*. One predicted target of miR-8 is *wls*, a transmembrane protein required for Wg secretion (17, 18). The 3'UTR of *wls* contains one putative miR-8-binding site, which is conserved in *Drosophila pseudobscura* (19) (Fig. 3A). To test whether miR-8 directly targets the 3'UTR of *wls*, we generated a sensor for *wls* by cloning the *wls* 3'UTR downstream of the coding region for lacZ. The *wls* 3'UTR sensor was suppressed by miR-8 in Kc167 cells, and mutation of the seed region (base pairs 2–8) of the putative miR-8-binding site partially blocked the ability of miR-8 to inhibit the *wls* sensor (Fig. 3A). Wls protein is expressed ubiquitously throughout the third larval instar wing imaginal disk, and Wls is up-regulated by the Wg pathway along the dorsal–ventral boundary (20) (Fig. 3B). Expression of miR-8 along the anterior–posterior boundary of the wing pouch using *dpp-Gal4* inhibited Wls protein expression (Fig. 3D). Secretion of Wg also was decreased by miR-8 expression, as indicated by decreased extracellular Wg (Fig. 3E). Together, these data suggest that miR-8 directly targets *wls* and inhibits Wg signaling in part by preventing Wg secretion.

miR-8 Can Inhibit Wg Signaling Downstream of Arm Stabilization in Cell Culture. Expression of miR-8 suppressed the phenotype caused by expression of Arm*, a stable form of Arm, in the developing fly eye (Fig. 1). This suggests that miR-8 may target the Wg pathway downstream of Arm stability in addition to targeting *wls*. Expression of Arm* in Kc167 cells activates *TCF-luc*, a reporter gene with an enhancer that contains multimerized TCF-binding sites (21) (Fig. 4A). Activation of *TCF-luc* by Arm* is consistently decreased by 3- to 4-fold through coexpression of miR-8 (Fig. 4A). These data suggest that miR-8 can inhibit Wg signaling downstream of Arm stabilization both *in vivo* and in cell culture.

To explore the mechanism of miR-8 action, we took advantage of various chimeric constructs. miR-8 expression decreased TCF-reporter activation by Arm* but not by VP16-Lef1, a fusion protein between the activation domain of VP16 and Lef1 (Fig. 4A). This finding suggests that miR-8 is not a general inhibitor of transcription. miR-8 inhibited the activation of *TCF-luc* by Gal-Arm, a fusion protein between the Gal4-binding domain and full-length Arm; however, miR-8 did not suppress Gal-Arm activation of a Gal-dependent reporter gene (Fig. 4A). Together, these findings suggest that miR-8 can suppress Arm activation of gene expression only when Arm is recruited to its target gene by TCF, and that miR-8 has no effect on transcription if Arm is recruited independently of TCF via the Gal4 domain. These data suggest that inhibition by miR-8 of Wg target genes depends on TCF.

miR-8 Inhibits TCF Protein Without Affecting TCF mRNA Levels. To test for repression of TCF, we enriched for the population of cells transiently transfected with empty vector or miR-8. We found that TCF protein was decreased in cells expressing miR-8 (Fig. 4B), whereas *TCF* mRNA was unaffected (Fig. 4C). In contrast, we found no change in the levels of Arm protein or mRNA after miR-8 expression (Fig. 4B and C). The data are consistent with either miR-8 targeting translation of *TCF* mRNA or miR-8 targeting a gene required for TCF protein stability.

To examine the effect of miR-8 on TCF protein *in vivo*, we expressed miR-8 in developing imaginal discs using *U3-Gal4* (Fig. 4D). We found that TCF protein was decreased in cell lysates from wing and leg imaginal discs expressing miR-8, confirming that miR-8 inhibits TCF protein both *in vivo* and in cell culture.

The *TCF* locus, also called *pangolin* (*pan*), gives rise to

Fig. 4. miR-8 inhibits TCF protein without affecting mRNA in cell culture and *in vivo*. (A) Kc167 cells were transfected with TCF-luc or UAS-luc reporter gene, pAcLacZ, pAcArm*, pAcVP16-Lef, pAcGal-Arm, and pAc or pAc-miR-8, as indicated. Samples were normalized to β -galactosidase to control for transfection efficiency. Luciferase activity is reported as percent activation (mean $\pm$ standard deviation) relative to TCF-luc activated with pAcArm*, pAcVP16-Lef, or pAcGal-Arm, or to UAS-luc activated by pAcGal-Arm. (B and C) Kc167 cells transfected with pAc or pAc-miR-8 were purified and lysed for either protein or RNA. (B) Endogenous TCF and Arm protein were detected by immunoblot. Tubulin was detected as a loading control. (C) Endogenous TCF and arm mRNA were detected by quantitative RT-PCR and normalized to Tubulin mRNA. Values are reported as fold TCF mRNA (mean $\pm$ standard deviation) relative to cells transfected with control expression plasmid. (D) Endogenous TCF protein was detected by immunoblot in lysates from third instar larvae wing or leg imaginal discs from control *1J3-Gal4* or *1J3-Gal4>UAS-miR-8* flies, as indicated. Tubulin was detected as a loading control. The results shown here are representative of at least three independent experiments.

regulation of Wnt signaling by these microRNAs is consistent with a study reporting that all five miR-8 family members are highly expressed in the epidermis of the skin but are excluded from the hair follicle, a site of Wnt activity (28). Together, our data suggest that miR-8 may play an evolutionarily conserved role in negatively regulating Wnt signaling.

We found that miR-8 inhibits TCF protein levels without affecting TCF mRNA, suggesting that TCF is a direct target of miR-8. However, the 3'UTRs of the reported TCF isoforms were not regulated by miR-8 (Fig. S2), suggesting that miR-8 may directly target TCF mRNA independently of its 3'UTR or through an indirect mechanism. An intriguing possibility is that miR-8 may directly target an unidentified gene that is required for TCF protein stability. Studies have shown that TCF/LEF transcription factor activity or subcellular localization is regulated by posttranslational modifications, such as sumoylation, phosphorylation, and acetylation; however, none of these studies have reported an effect of these modifications on overall TCF protein expression (29). Interestingly, Sox17 has been reported to negatively regulate both TCF-4 and β -catenin protein levels in human colorectal cell lines by a mechanism that appears to require the proteasome (30); however, a positive regulator of TCF protein stability has not yet been reported, and we found that knockdown of the miR-8 target *CG32767* did not affect TCF protein levels (data not shown).

Another microRNA, miR-315, was recently reported to be a positive regulator of the Wg pathway (9). miR-315 was identified in a cell culture-based screen for effects on Wg dependent reporter gene activity. In contrast to our findings, that study did

Fig. 5. miR-8 directly targets another positive regulator of the Wg pathway, *CG32767*. (A) Schematic of *CG32767* 3'UTR containing two putative miR-8 binding sites. The site depicted in black is conserved in *Drosophila pseudobscura*. (B) Kc167 cells were transfected with 3'UTR sensors containing the 3'UTR of *CG32767* along with pAc-luc and pAc or pAc-miR-8 as indicated. The seed regions of all putative miR-8 binding sites were mutated in the mutant 3'UTR sensors. Samples were normalized to luciferase to control for transfection efficiency. LacZ activity is reported as fold activation (mean $\pm$ standard deviation) relative to 3'UTR sensor activity with pAc5.1A.

not identify miR-8 as a pathway regulator in its screen. These discrepant findings may be because of our use of different cell lines (Kc167 vs clone8) and overall approaches (*in vivo* vs cell culture-based screen). We have not tested the effects of miR-8 expression in clone8 cells, although we have noted robust inhibition of the Wg pathway in the wing imaginal disk, the original source of the cell line.

miR-8 loss of function mutant flies were reported recently (31). The authors described a subtle mutant phenotype with defective leg and wing extension and behavioral defects, in part, because of an increased expression of *Atrophin*. Although the miR-8 mutants do not demonstrate an obvious Wg-related phenotype, Wg signaling does play an important role in *Drosophila* leg and wing development (14–16), and increased expression of other direct or indirect targets of miR-8 (e.g., TCF, *wls*, *CG32767*) also may contribute to the mutant phenotype in the leg and elsewhere.

The subtle phenotype of the miR-8 mutant is not surprising, given similar reports for other microRNA loss of function mutants (3). The differences in scale of the loss-of-function versus the gain-of-function phenotype that we have reported here may be, in part, because of redundancy, because all three targets that we have identified contain predicted binding sites for multiple microRNAs. Mutation of multiple microRNAs may be required to produce dramatic effects on some signaling and developmental pathways. In addition, negative and positive regulators of the Wg pathway, such as *nkd* and *fz3*, are dispensable in certain tissues, including the wing imaginal disk (32, 33). This may be indicative of redundant negative and positive regulator activities in these tissues. Redundancy may protect the Wg pathway in certain tissues from aberrations because of alterations in regulator gene expression. Overall, the studies of

Fig. 6. Murine miR-8 family members promote adipogenesis and antagonize inhibition of adipogenesis by Wnt3a. (A) Alignment of *Drosophila melanogaster* miR-8 mature sequence with *Mus musculus* miR-8 family members. Seed regions are shaded in gray. (B–D) ST2 marrow stromal cells expressing control cells (B), miR-200c/141 (C), or miR-200b,a/429 (D) were induced to differentiate into adipocytes. Cells were stained with Oil red-O to visualize lipid accumulation 6 d later. (E) ST2 marrow stromal cells expressing control vector, miR-200c/141, or miR-200b,a/429 were induced to differentiate into adipocytes in the presence of vehicle or indicated amounts of recombinant mouse Wnt3a (ng/ml). Cells were lysed 6 d later, and FABP4 was detected by immunoblot as an indicator of adipogenesis.

miR-8 suggest that the role of miR-8 is not as a developmental switch, but instead miR-8 may act as a modulator of multiple pathways *in vivo* by precisely tuning target gene expression in concert with other microRNAs and genes.

Materials and Methods

Drosophila Genetics. Insertions of a bidirectional EP element, Gene search vector (GSV) (12), were screened for the ability to suppress *GMR-Gal4>UAS-wg* and *GMR-Gal4>GMR-arm**, as described (10, 11) (see *SI Text*). Two GSV elements were mapped by inverse PCR to a region upstream of miR-8 (GSV^{A1305-2} and GSV^{A2196}). Both GSV lines gave similar phenotypes in all assays. *dpp-Gal4*, *ptc-Gal4*, *Dll-lacZ*, *dpp-lacZ* (B5 3.0), and *113-Gal4* were obtained from the Bloomington Stock Center.

UAS-miR-8 lines were generated by cloning a 500-bp genomic fragment containing the miR-8 hairpin into the pUAST vector (BestGene) (34). Ten independent lines were obtained, all of which gave phenotypes similar to those shown in Figs. 1–3. Two of the lines, 1F-20 (second chromosome insertion) and 1F-34 (third chromosome insertion), were used for experiments described in Figs. 2 and 3.

Plasmids. All expression plasmids used for transfection of Kc167 cells were expressed under control of the Actin promoter using pAc5.1/V5-His-A (Invitro-

gen). pAc-miR-8 was generated by cloning a 500-bp genomic fragment containing the miR-8 hairpin. pAcArm* expresses a stabilized form of Arm as described (35). VP16-Lef1 is a fusion protein of the activation domain of VP16 with Lef1 (36). A fusion protein of the DNA-binding domain of Gal4 and full-length Arm was reported (10). pMSCV-miR-200c/141 and pMSCV-miR-200b,a/429 were generated by cloning into pMSCV (Clontech) PCR-amplified genomic regions containing the clustered hairpins of miR-200c and miR-141 or miR-200b, miR-200a, and miR-429 from mouse genomic DNA.

The TCF-responsive reporter gene, *TCF-luc* (12XdtCF) (21), was obtained from N. Perrimon (Harvard Medical School, Boston). The Gal4-responsive reporter gene, *UAS-luc*, was reported (10). The CG32767 3'UTR reporter gene was generated by cloning a stop codon, followed by a 500-bp fragment of the CG32767 3'UTR containing the two predicted miR-8-binding sites downstream of the lacZ coding region in the pAcLacZ vector (Invitrogen). The *wls* 3'UTR reporter gene was generated by cloning the entire 3'UTR of *wls* downstream of lacZ in pAcLacZ. Mutant 3'UTR reporter genes contain CAG-TATT to ACTGCGG point mutations in the predicted seed matches. Cloning details are available on request.

Whole-Mount Staining and Microscopy. Immunostaining was performed as described (37). Anti-Wg and anti-lacZ were obtained from the Developmental Studies Hybridoma Bank and Abcam, respectively, and anti-Wls was obtained from K. Basler (University of Zurich, Zurich) (20). Immunostaining for extra-cellular Wg was performed as described (38). Fluorophore-conjugated secondary antibodies were obtained from Jackson ImmunoResearch and Molecular Probes. All confocal fluorescent pictures with Z-stack projection were obtained by using Leica apparatus. All adult fly head micrographs were taken with a Leica MZ APO light microscope.

Cell Culture. *Drosophila* Kc167 cells were routinely cultured as described (10). Mouse ST2 marrow-derived stromal cells were cultured and induced to differentiate into adipocytes as described (27). To visualize lipid accumulation, cells were stained with Oil Red-O 6 days after induction of differentiation (26). miR-200c/141 or miR-200b, a/429, or empty pMSCV vector were stably expressed in ST2 cells by retroviral infection, followed by antibiotic selection, as described (27).

Transient transfections of Kc167 cells and luciferase and β -galactosidase assays were carried out as described (39). Transfection details are available on request. All experiments using cell cultures were repeated at least three times, with two or more replicates in each independent experiment. All error bars represent a standard deviation of at least two replicates in a representative experiment.

Immunoblotting and Quantitative RT-PCR. Purification of transfected Kc167 cells was accomplished as described (39). Purified cells were either lysed for immunoblotting or isolation of total RNA. To determine TCF protein expression in wing and leg imaginal discs, control *113-Gal4* larvae or *113-Gal4>UAS-miR-8* larvae were raised at 18°C and switched to 29°C 24 h before dissection. Antisera raised against the N terminus of TCF (10), Arm antibody (Developmental Studies Hybridoma Bank), and Tubulin antibody (Sigma Aldrich) were used for immunoblotting. For quantitative RT-PCR, RNA was reversed-transcribed with Stratascript reverse transcriptase (Stratagene), followed by qualitative PCR analysis for *TCF*, *arm*, and *Tubulin56D* transcripts, as described (10).

Stable ST2 cell lines were treated with recombinant mouse Wnt3a (R&D Systems) for the first 4 d of adipocyte induction. Cells were lysed, and anti-FABP4 (R&D Systems) was used for immunoblotting 6 d after the start of induction.

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