MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling

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MicroRNAs (miRNAs) are genomically encoded small RNAs that hybridize with messenger RNAs, resulting in degradation or translational inhibition of targeted transcripts. The potential for miRNAs to regulate cell lineage determination or differentiation from pluripotent progenitor or stem cells is unknown. Here, we show that microRNA1 (miR-1) is an ancient muscle-specific gene conserved in sequence and expression in Drosophila. Drosophila miR-1 (dmiR-1) is regulated through a serum response factor-like binding site in cardiac progenitor cells. Loss- and gain-of-function studies demonstrated a role for dmiR-1 in modulating cardiogenesis and in maintenance of muscle-gene expression. We provide in vivo evidence that dmiR-1 targets transcripts encoding the Notch ligand Delta, providing a potential mechanism for the expansion of cardiac and muscle progenitor cells and failure of progenitor cell differentiation in some dmiR-1 mutants. These findings demonstrate that dmiR-1 may “fine-tune” critical steps involved in differentiation of cardiac and somatic muscle progenitors and targets a pathway required for progenitor cell specification and asymmetric cell division.

Deciphering the mechanisms by which specific cell lineages arise from pluripotent stem cells and subsequently differentiate is a fundamental challenge in stem cell and developmental biology. MicroRNAs (miRNAs) are 21- to 22-nt noncoding RNAs that are sometimes expressed in a lineage-specific fashion and thus have the potential to control cell fate decisions (1–3). There are >300 known miRNAs, and each is thought to target numerous messenger RNA (mRNA) transcripts for either degradation or, more often, translational inhibition. miRNAs typically bind to 3′ UTRs of mRNAs through inexact sequence matching. The lack of precise sequence homology between miRNA and targets has made target prediction difficult, although it does appear that sequence matching of the 5′ end of the miRNA and a permissive secondary structure of target mRNA are important features (4, 5). Despite recent advances in target prediction, only a handful of miRNA targets have been validated thus far, resulting in limited knowledge of biological roles for most miRNAs.

miRNAs may play a role in regulation of stem cell fates (6–8), but direct experimental evidence and a mechanistic understanding of miRNA regulation of cell lineages have been lacking. In Drosophila, the dorsal vessel, a primitive heart, is composed of distinct cell types, each arising from progenitor cells that follow stereotypic lineage decisions (9), providing a tractable system in which to study the possible involvement of miRNAs in cell fate decisions. We previously demonstrated that miR-1 and miR-1-2 (miR-1, microRNA1) are redundant muscle-specific mammalian miRNAs that play a role in cardiogenesis (5). Mouse miR-1-1 and miR-1-2 were regulated by serum response factor (SRF), a central transcriptional regulator of muscle differentiation, and excess miR-1 in vivo resulted in premature withdrawal of cardiomyocytes from the cell cycle. However, whether miR-1 is required for cardiac determination or differentiation is unknown.

In this study, we used the Drosophila system to investigate whether miR-1 is necessary for determination or differentiation of cardiac or somatic muscle progenitor cells. We found that the cardiac expression of the single orthologue of miR-1 in Drosophila (dmiR-1) is transcriptionally regulated through a conserved SRF-like binding site, and that overexpression of dmiR-1 in cardiac mesoderm results in fewer cardiac cells. Loss of dmiR-1 was uniformly lethal, with a spectrum of severity ranging from embryonic death to later demise in the larval stages after hatching. During the course of our work, Sokol and Ambros (10) reported similar loss-of-function effects of dmiR-1 and described milder defects present in hatchet larvae. Here, we focused on the dmiR-1 mutant flies that did not escape the early lethality and that died during embryogenesis and hatching. We demonstrate that in these embryos, dmiR-1 is involved in maintaining muscle gene expression and in some cases determination of specific cardiac cell types from pluripotent progenitors. In addition, we provide in vivo evidence that dmiR-1 targets the Notch ligand, Delta, for translational inhibition. Although dmiR-1 likely targets multiple miRNAs, regulation of the dosage of Notch signaling, which is involved in distinguishing cell types among equivalence groups, is consistent with the lineage defect observed in some dmiR-1 mutants.

Materials and Methods

Drosophila Strains. The dmiR-1 locus deletion was generated by using piggyBac insertion lines (f03931 and f03249 from the Exelixis collection at Harvard Medical School) and by following reported methods (11). The following fly lines were used: dmiR-1 4.6KB-GFP, 2.5KB-GFP, 0.72KB-SRFmut-GFP, UAS-miR-1, twi-Gal4, 5.1KB-rescue 24B-Gal4, Daughterless (Da)-Gal4, UAS-DSRF, UAS-DMRTF, dpp-Gal4. Overexpression of dmiR-1 was accomplished by using the UAS-Gal4 system (12). Oregon-R was used as the wild-type reference strain.

Immunohistochemistry, in Situ Hybridization, and Microscopy. Embryos from different lines were collected and stained with various antibodies as described (13). The following primary antibodies were used: mouse anti-β-galactosidase 1:300 (Promega); rabbit anti-myosin heavy chain 1:100 (from D. Kiehart); rat anti-Eve 1:200, guinea pig anti-Odd 1:300 (from D. Kosman); rabbit anti-Tinman 1:500 (from R. Bodmer); rabbit anti-Dmef2 1:1,000 (from B. Paterson); rabbit anti-GFP 1:2,000 (Abcam, Inc., Cambridge, MA); mouse anti-GFP 1:1,000 (Invitrogen); rabbit anti-Twist 1:500 (R. Cripps); mouse anti-Delta 1:400 (DSHB); and Cy3, Cy5, biotin- or horseradish peroxidase-conjugated (with TSA plus Fluorescent Systems, PerkinElmer).

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Abbreviations: mRNA, messenger RNA; SRF, serum response factor; miRNA, microRNA; miR-1, microRNA1; dmiR-1, Drosophila miR-1; Eve, Even-skipped.

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secondary antibodies (The Jackson Laboratory). These antibodies were used to recognize the primary antibodies. In situ probe synthesis and hybridization were performed as described (14). Fluorescent images were obtained with a Zeiss LSM510-meta confocal microscope.

**Construct Generation, Transformation, and Transfection Assays.** GFP and 5.1-kb-rescue transgenes were generated by cloning the corresponding genomic DNA into the pH-Stinger vector (15). The SRF-like-binding site mutation was generated by substituting GCTATTATGTG by TGGATCCTATG (altered bases are shown in lowercase). For UAS-dmiR-1 generation, 552 bp around dmiR-1 (from 310 bp upstream to 220 bp downstream of dmiR-1) were cloned into pUAST. These constructs were introduced into flies by P-element-mediated germline transformation. The in situ probe was PCR-amplified by using the following primers: TGGCCATGTGGCAGCAGAATTGTCGCT and TCATCTAGACCTGTGGTTGGAATGGTATTTGTG. The dmiR1-luciferase, dmiR1-mut-luciferase, Delta 3′UTR, and Delta 3′UTRMut were generated by cloning the corresponding enhancers and three copies of a wild-type or mutant dmiR-1 target site present in the Delta 3′UTR into the pGL3 vector (Promega). Cell transfection and luciferase assays were performed as described (13). Luciferase activities are expressed as mean ± standard deviation from three experiments with constitutive activity of luciferase set at 100%.

**Results**

**Expression and Regulation of dmiR-1.** The single orthologue of mir-1 in Drosophila, dmiR-1, is nearly identical in sequence to mouse and human miR-1 (5). In situ hybridization revealed dmiR-1 transcripts in presumptive mesodermal cells as early as stage 5 (2.2–2.8 h) of Drosophila development (Fig. 1a). This pattern changed dynamically throughout gastrulation, but dmiR-1 consistently marked mesodermal cells (Fig. 1 b–d). Transcripts persisted in later stages of cardiac and somatic (body wall) muscle differentiation (Fig. 1e), as in mice, and were also found in visceral muscles of the gut (Fig. 1f). dmiR-1 expression overlapped, but preceded, that of dme2, a transcriptional regulator of muscle precursors (ref. 16, Fig. 1 a–d).

To determine whether transcriptional regulation of miR-1 was evolutionarily conserved, we aligned 10 kb of genomic DNA surrounding Drosophila melanogaster and Drosophila pseudoboscura miR-1 genes to find regions of sequence conservation (Fig. 2a). Transgenic flies containing conserved islands 4.6 kb upstream of dmiR-1 adjacent to the gene encoding nuclear GFP (nGFP) recapitulated the endogenous dmiR-1 expression in all muscle types (Fig. 2b and c). Cardiac nGFP expression coincided with dme2 expression in cardiac cells and was present in visceral muscles of the gut (Fig. 2e). Luciferase activities are expressed as mean ± standard deviation from three experiments with constitutive activity of luciferase set at 100%.

**Deletion of dmiR-1 Affects Cardiac Development.** To begin to define the functions of dmiR-1 in vivo, we used two Exelixis lines (11) of Drosophila containing FRT sites surrounding the dmiR-1 gene and generated a FRT-FLP-based deletion of the dmiR-1 locus (Fig. 3). Successful excision of dmiR-1, the only known or predicted gene in the 31-kb deleted interval, was confirmed by sequence analysis and RT-PCR (not shown). Homozygous dmiR-1 deletion was 100% lethal, but a spectrum of severity was observed, with approximately one-third dying at embryonic stages, one-third around hatching, and the remaining at larval stages. Homozygous mutant larvae were abnormally lethargic compared with their heterozygous siblings before death. The embryonic and larval lethality was fully rescued by overexpression of dmiR-1 by using a mesoderm-specific nvi-Gal4 driver (Fig. 3b) or by a 5.1-kb transgene encompassing the dmiR-1 genomic locus including the 4.6-kb enhancer and the sequence encoding dmiR-1 (not shown), consistent with dmiR-1 being the sole gene within the deleted region responsible for the lethal phenotype. The variability in phenotype may be related to previously described maternal dmiR-1 transcripts (19, 20), redundancy with other miRNAs or may simply reflect the role of dmiR-1 in “fine-tuning” whether cells achieve the thresholds of critical proteins to initiate critical developmental events.

Because one-third of all dmiR-1 mutants died around the time of hatching and another one-third at larval stages with poor mobility, we investigated whether there might be a discernable muscle defect. We found that nearly half of all dmiR-1 mutant embryos displayed severe defects in muscle gene expression with down-regulation of sarcomeric genes such as myosin heavy chain (MHC) (Fig. 3 c and d), indicating a late requirement for miR-1.
Fig. 2. Regulation of dmiR-1 in cardioblast and visceral muscle cells. (a) Map of the dmiR-1 locus showing the position of the 4.6-kb dmiR-1 enhancer (green) and subfragments, with expression domains summarized as follows: SM, somatic muscle; VM, visceral muscle; CB, cardioblast; PC, pericardial cell. An A/T-rich SRF-like binding site conserved in other Drosophila species is highlighted. (b–m) GFP expression in embryos carrying the 4.6-kb (b–g), 2.5-kb (h and i), 0.72-kb (j and l), or SRF-like site-mutated 0.72-kb (k and m) element. Embryos were costained with anti-Dmef2 (c and j) or anti-Twist (e and g). (n) Luciferase (luc) activity determined with luciferase reporters linked to the 0.72-kb element or the SRF-like site mutated (CArGm) 0.72-kb element in Drosophila S2 cells in the presence or absence of Drosophila SRF and myocardin-related transcription factor. Error bars indicate standard deviations. (b, c, h–k) Dorsolateral views of stage 16 embryos. (d–g) Lateral views of stage 11 embryos. F and g are ×40 images of d and e, respectively. I and m are inside views of j and k embryos, respectively, focusing on visceral muscles. Arrowheads indicate the presence (b, h, and j) or absence (k) of the heart tube, and arrows indicate somatic (b, h, k, and j) and visceral muscles (l).

An abnormal pattern of Eve+ cardiac progenitors (three to four cells per cluster) (Fig. 3 e). In contrast, 5–10% of all dmiR-1 mutants described above, the overabundance of poorly patterned Eve+ progenitors at this stage (Fig. 3 f). By stage 12 in wild-type embryos, eve+ progenitor cells normally differentiate in a defined pattern into two pericardial (Eve+ Dmef2+) cells and one DA1 muscle (Eve+ Dmef2+) per hemisegment (Fig. 3 g). A row of Dmef2+ cardioblasts also aligns at the dorsal edge of the mesoderm, separated from Dmef2+ somatic muscle progenitors by rows of pericardial cells including Eve+ pericardial cells (EPCs) (Fig. 3 g). At stage 12, Tinman expression is normally restricted to cardioblasts and the EPCs but is absent in the DA1 muscles (Fig. 3 g). However, in the subset of dmiR-1 mutants described above, the overabundant progenitor pool at stage 12 appeared arrested in development, similar to stage 11, and failed to differentiate into Eve+ Dmef2− pericardial cells (Fig. 3 h). Tinman was ectopically expressed in the expanded pool of Eve+ and/or Dmef2+ cells, indicating that these were cardioblasts and muscle progenitors that failed to terminally differentiate in the absence of dmiR-1 (Fig. 3 i and j).

Although the majority of embryos displaying early defects died early during embryogenesis, some survived to later embryogenesis and revealed varying degrees of gaps in the rows of cardiac cells that constitute the dorsal vessel, consistent with the requirement of dmiR-1 for determination and/or differentiation of cardiac cells (Fig. 3 k and l). These flies had morphologic...
late-stage 12 (Eve) and expression of both Dmef2 progenitor cells failed to differentiate into pericardial (arrows in red, white, black, dark, and light gray) heart morphology. (a–d) Lateral views of stage 13 WT (a and b) or dmiR-1 overexpressed embryos with 24B-Gal4 driver (c and d). a and d are ×40 images of white boxes in a and c, respectively. On average, four rather than six cardioblasts per hemisegment were observed in transgenic flies with occasional enlarged cardioblasts (arrowhead).

Fig. 3. Loss of dmiR-1 causes abnormal heart and muscle development. (a) Schematic of FRT-FLP-mediated dmiR-1 locus deletion and locations of neighboring genes. Embryonic and larval lethality was rescued by UAS-miR-1 with twi-gal4 driver (b) or by a transgene containing the 5.1-kb miR-1 locus (not shown). (c, e, g, i, k, and m) WT embryos. (d, f, h, j, l, and n) Homozygous miR-1 mutant (ΔmiR-1) embryos. Expression of MHC (myosin heavy chain) was dramatically reduced both in the heart and muscles (arrows in c and d) in half of all ΔmiR-1 embryos compared with WT, whereas expression of a pericardial cell marker, odd-skipped, was not affected (c and d). ΔmiR-1 embryos with the early defect had ectopic Eve progenitor cells at stage 11 (f). The ectopic progenitor cells failed to differentiate into pericardial (arrows in g) and dorsal muscles (asterisks) and maintained expression of both Dmef2 and eve at late-stage 12 (h). Ectopic Dmef2+ cardioblasts, identified based on their dorsal location relative to the Eve progenitors, were also observed (h). tinman was expressed in most of the ectopic cardiac progenitors that expressed eve (j). Another subset of embryos that fail to hatch showed reduced numbers of cardiac and muscle cells, indicated by gaps in the row of cardioblasts and missing dorsal muscles in ΔmiR-1 embryos (arrowsheads in l and n). (e–l) Lateral views; (c, d, m, and n) dorsal views.

Fig. 4. dmiR-1 overexpression in the mesoderm affects heart and muscle cell morphology. (a–d) Lateral views of stage 13 WT (a and b) or dmiR-1 overexpressed embryos with 24B-Gal4 driver (c and d). a and d are ×40 images of white boxes in a and c, respectively. On average, four rather than six cardioblasts per hemisegment were observed in transgenic flies with occasional enlarged cardioblasts (arrowhead).

defects in somatic muscle and heart formation, including frequent loss of cardioblasts and DA1 dorsal muscles (Fig. 3 m and n).

**dmiR-1 Overexpression Disrupts Dorsal Vessel Patterning.** Because dmiR-1 loss of function led to decreased muscle gene expression and an increased pool of undifferentiated muscle progenitors in a subset of embryos, we asked whether excess dmiR-1 might result in a decreased number and/or premature differentiation of precursor cells into muscle. Ubiquitous expression of dmiR-1 using the Daughterless (Da)-Gal4 or late mesoderm expression using the 24B-Gal4 driver resulted in 100% embryonic lethality at various stages of development. Twenty percent of dmiR-1 overexpressing flies showed disrupted patterning of cardiac and skeletal muscle with insufficient numbers of cardioblasts (Fig. 4). The dorsal vessel was reliably patterned with six cardioblasts per hemisegment in wild-type flies but had only three to four cardioblasts per hemisegment, with frequent enlargement of cardioblasts, in dmiR-1-overexpressing flies. Thus, excess dmiR-1, when sufficiently affecting the threshold of critical events, may result in early diversion of cardiac progenitors into an alternative cell fate or may cause premature differentiation of precursors, resulting in an insufficient pool of progenitors.

**dmiR-1 Can Target Delta for Translational Inhibition.** Because miR-NAs typically target numerous mRNAs, the phenotype of dmiR-1 mutants is likely due to down-regulation of multiple critical proteins. Despite the likely complexity of targets, we sought to identify mRNA targets of miR-1 in flies that might be involved in dmiR-1-dependent lineage determination and differentiation decisions. Although mouse miR-1 targets transcripts encoding the cardiac-enriched basic helix-loop–helix transcription factor Hand2 (5), we did not identify any miR-1-binding sites in the 3’-UTR of Drosophila hand, suggesting alternative targets in flies. Because the more severe dmiR-1 gain- and loss-of-function phenotypes were reminiscent of progenitor defects induced by altering Notch signaling, we searched the 3’-UTRs of genes involved in the Notch pathway for potential sequence matching and accessibility to dmiR-1.

Several conserved putative miR-1-binding sites were found in the 3’-UTR of the gene encoding Delta, a membrane-bound ligand for Notch (Fig. 6, which is published as supporting information on the PNAS web site). Upon interaction with Delta, Notch is cleaved, allowing the Notch intracellular domain to translocate into the nucleus and regulate gene expression (23). Signaling between neighboring Delta- and Notch-expressing cells is necessary for lateral inhibition and asymmetric cell fates during lineage determination (24–26) and involves repression of Delta in Notch-expressing cells and similar repression of Notch in adjacent Delta-expressing cells (27–33). Notch signaling also later regulates differentiation of numerous cell types, including cardiac cells (22).

Introduction of one of the putative dmiR-1-binding sites from
Discussion

We have shown that dmiR-1 is an ancient gene that functions at multiple stages of Drosophila development, including regulation of specific cardiac and somatic muscle lineages from progenitor cells. Later in development, dmiR-1 is also involved in muscle differentiation and maintenance of muscle gene expression. Expression of dmiR-1 in cardiac and visceral cells depended on an A/T-rich DNA sequence that resembles a binding site for SRF and MEF2. Finally, we demonstrate that the Notch ligand, Delta, is a target of dmiR-1, providing in vivo evidence of miRNA-mediated regulation of Notch signaling.

The roles of some miRNAs have been revealed through genetic screens in Caenorhabditis elegans and Drosophila. The majority appears to be involved in fine-tuning biological processes by titrating precise dosages of regulatory proteins; however, targeted deletion of a specific miRNA in vertebrates has not yet been reported. The dmiR-1 mutant phenotype we characterized in this report is similar to that described by Sokol and Ambros (10), in that approximately 70–80% of homozygous mutants survive to around the period of hatching in both studies, with the remaining mutants dying during embryogenesis. However, we found that half of all mutants had a muscle differentiation defect marked by decreased muscle gene expression. In addition, our study revealed an interesting lineage defect in the subset that suffered embryonic lethality, suggesting that, in some cases, the dosage of proteins regulated by dmiR-1 could reach a critical threshold affecting cell determination events. It is worth noting that, in contrast to the mild muscle defect described by Sokol and Ambros (10), injection of dmiR-1 complementary 2′O-methyl oligoribonucleotides, which efficiently block miRNA function (35), resulted in significant embryonic lethality with substantial defects in embryonic morphology and few survivors (20). Although the discrepancy in phenotypes is difficult to resolve, the similar severity of defects in the dmiR-1 mutants we described, flies described by Leaman et al. (20), and the successful rescue of the mutant phenotype by dmiR-1 in our study suggest that the incompletely penetrant embryonic defects are due to loss of dmiR-1.

dmiR-1 likely controls numerous mRNA targets that are important for muscle development and maintenance but, interestingly, the Drosophila orthologue of Hand2, a mammalian miR-1 target (5), did not have any miR-1-binding sites. Previous reports that a reduction in Notch signaling results in excessive muscle progenitor cells and failure of pericardial cell formation (22, 26, 36), which is similar to severe dmiR-1 mutants, led us to examine members of the Notch signaling pathway as potential dmiR-1 targets. Our findings that dmiR-1 could regulate protein expression through a target site in the Delta 3′ UTR in vitro, could down-regulate Delta protein in vivo, and could mimic the Delta loss of function in the wing and leg upon misexpression...
together support Delta as a validated dmiR-1 target. It will be interesting to determine whether any of the Delta-like Notch ligands are also targets of mir-1 in mammals.

Whether targeting of Delta in cardiac progenitors by dmiR-1 is important during normal development remains to be determined, because interrogation of Delta protein in muscle progenitors has been limited by technical considerations. However, along with other dmiR-1 targets within the cardiac progenitors, it is worth considering that elevated levels of Delta protein in mesodermal cells upon loss of dmiR-1 might result in disruption of the normal pattern of cells sending (Delta+) or receiving (Notch+) the Notch signal during the process of lateral inhibition. In this scenario, inappropriate levels of Delta in Notch+ cells might result in feedback down-regulation of Notch expression in the same cells and consequent recruitment of excess progenitor cells that fail to undergo appropriate segregation into distinct lineages (Fig. 5f). Because dmiR-1 may only be titrating dosage of protein, increased Delta in Delta+ cells may not significantly affect the degree of Notch signaling in neighboring Notch+ cells, particularly if they have down-regulated expression of the Notch receptor. This is consistent with previous observations that high levels of Delta in active Notch domains inhibit Notch signaling (24, 27–33). Future studies may more directly test this hypothesis within cardiac precursors and determine whether miRNAs target Notch signaling in other cell types to regulate asymmetric cell division and lineage determinations.

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