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Fgf-dependent depletion of microRNA-133 promotes appendage regeneration in zebrafish

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Appendage regeneration is defined by rapid changes in gene expression that achieve dramatic developmental effects, suggesting involvement of microRNAs (miRNAs). Here, we find dynamic regulation of many miRNAs during zebrafish fin regeneration. In particular, miR-133 levels are high in uninjured fins but low during regeneration. When regeneration was blocked by Fibroblast growth factor (Fgf) receptor inhibition, high miR-133 levels were quickly restored. Experimentally increasing amounts of miR-133 attenuated fin regeneration. Conversely, miR-133 antagonism during Fgf receptor inhibition accelerated regeneration through increased proliferation within the regeneration blastema. The Mps1 kinase, an established positive regulator of blastemal proliferation, is an in vivo target of miR-133. Our findings identify miRNA depletion as a new regulatory mechanism for complex tissue regeneration.

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Regeneration, the replacement of damaged or lost body parts, is a primary goal of stem cell research. Certain nonmammalian vertebrates like urodele amphibians and teleost fish restore complex tissues much more effectively than mammals, creating tantalizing examples of successful organ regeneration. For instance, the common laboratory model zebrafish regenerates heart muscle, retina, spinal cord, sensory hair cells, and appendages (Stoick-Cooper et al. 2007). Fin regeneration in zebrafish is a particularly efficient example of complex tissue regeneration, replacing bone, epidermis, blood vessels, nerves, connective tissue, and pigmentation within 2 wk of amputation of up to 95% of the organ. Following wound healing, spared mesenchymal cells disorganize, migrate distally, and accumulate to form the regeneration blastema, a proliferative mass of progenitor tissue. The blastema is maintained throughout the process of regenerative outgrowth, during which it creates new structures through a series of proliferation, patterning, and differentiation events. During fin regeneration and other examples of complex tissue regeneration, large-scale changes in gene expression programs occur to execute the rapid transformation of quiescent, differentiated tissue to proliferating, actively patterned tissue (Lien et al. 2006; Schebesta et al. 2006).

To implement these programmatic alterations in gene expression, it is almost certain that several modes of regulation are employed. These might include changes in chromatin accessibility and new production of transcriptional factors, as well as post-transcriptional mechanisms. Based on studies in other fields, microRNAs (miRNAs) present excellent candidates to mediate important post-transcriptional regulation during complex tissue regeneration. miRNAs are small, noncoding RNAs that base-pair with complementary sequences in the 3’ untranslated regions (UTRs) of target genes to promote mRNA degradation or inhibit protein translation (Kloosterman and Plasterk 2006). The first identified miRNAs, lin-4 and let-7, were discovered in genetic screens for mutations that disrupt developmental transitions in nematodes (Lee et al. 1993; Reinhart et al. 2000). miR-430 is the predominant miRNA species in early zebrafish embryos, where it refines the transition from maternal to zygotic miRNA utilization and helps balance levels of patterning factors (Giraldez et al. 2006; Choi et al. 2007). Investigators have also identified contributions by miRNAs to preventing or promoting the pathology of adult organs. In particular, miR-133 and miR-208 modulate pathologic cardiac muscle hypertrophy in mouse models of this common human cardiomyopathy, where they appear to have opposing functions (Care et al. 2007; van Rooij et al. 2007). Given the numerous roles for various miRNAs in different developmental processes like organogenesis, stem cell maintenance, and programmed cell death, as well as in adult pathogenesis, there is reason to suspect involvement in spectacular regenerative events of teleosts and urodele amphibians.

Here, we found that many miRNAs are differentially regulated during caudal fin regeneration in adult zebrafish. One of these miRNAs, miR-133, has relatively high levels in the uninjured fin, but these levels drop sharply during regeneration. By a combination of gain-of-function and loss-of-function experiments, our data indicate that miR-133 acts as a regenerative brake within a regulatory circuit for regeneration. Following amputation, Fibroblast growth factor (Fgf) signaling reduces miR-133 expression as part of the regeneration program, facilitating normal expression of targets like the mps1 kinase that direct blastemal proliferation and tissue renewal. Our findings identify miR-133 depletion as an important regulatory mechanism by which Fgfs promote appendage regeneration.

Results and Discussion

Caudal fin regeneration is accompanied by alterations in miRNA expression

To identify miRNAs present during caudal fin regeneration, we performed miRNA microarray experiments us-
miR-133 controls fin regeneration

Figure 1. miRNAs are dynamically regulated during zebrafish caudal fin regeneration. (A, left) Tissue distal to a region one ray segment proximal to the amputation plane (red arrow) was collected from wild-type (wt) fins at 0, 1, or 3 dpa and used for RNA isolation and miRNA microarray analysis. [Right] Northern analyses of various miRNAs present during fin regeneration. (B) A heat map comparing relative miRNA expression in wild-type and hsp70:dn-fgfr1 4-dpa regenerates collected 5 h after a single heat shock at 38°C. [Green] Lower expression; (red) higher expression; (asterisk) miR-133. (C) Ribonuclease protection assays (RPAs) were performed using RNA collected from wild-type regenerates at the indicated stages. (D, top) RPA using RNA collected from wild-type or hsp70:dn-fgfr1 samples. miR-133 levels are reduced during regenerative outgrowth (compare wild type, 0 dpa vs. wild type, 4 dpa), but are restored during a brief period of Fgfr inhibition at 4 dpa. [Bottom] Northern analysis with a 5s rRNA probe was used as a control.

miR-133 is depleted by Fgf signaling during fin regeneration

Because of its expression profile during regeneration, we focused our attention on miR-133, a highly conserved family of miRNAs that has recently implicated in regulating cardiac and skeletal muscle development, as well as differentiation and function of dopaminergic neurons (Chen et al. 2006; Care et al. 2007; Kim et al. 2007). The miR-133 family has four members encoded by different genes that vary only by 2 nucleotides (nt) in the mature species, outside of the predicted seed sequence. miR-133b was the only member displaying strong expression of the pre-
miR-133 functions as a regenerative brake

miR-133 levels were higher in uninjured fins than during the regenerative process, suggesting that miR-133 attenuates growth mechanisms. To determine the effects of experimentally increasing levels of miR-133 during regeneration, we injected and electroporated miR-133 RNA duplexes into wild-type fins (Fig. 2A,B). We found that dorsal lobe regenerates electroporated with miR-133 duplexes had an ~10% reduction in length compared with uninjected lobe regenerates when measured 24 h after introduction, and an ~13% reduction when measured 3 d after electroporation (Fig. 2B,D). These measurements conservatively estimate the effects on regeneration, since the first 3 d of regeneration proceed normally prior to electroporation. Also, it is possible that the percentage of cells affected or relative miR-133 increases in these studies are low. Electroporation with a mutated miR-133 RNA duplex, containing 3-nt changes in the predicted seed region, had no significant effects on regeneration (Fig. 2C,D). These results indicate that miR-133 slows regenerative outgrowth and support the idea that miR-133 is a significant regenerative brake.

miR-133 antagonism accelerates regeneration during Fgfr inhibition

To test the effects of antagonizing miR-133 function during regeneration, an antisense morpholino (MO) designed to disrupt miR-133 binding and activity was introduced into regenerating wild-type fins. No significant effects on regenerate length were observed during the first 2 d following miR-133 MO electroporation, however, an ~10% increase in length was evident by the third day (Supplemental Fig. 4). This minor effect may have been due to the fact that miR-133 levels are already quite low during regeneration. We hypothesized that we might achieve a greater effect during Fgfr inhibition, given that miR-133 levels increase during regeneration. We found that dorsal lobe regenerates electroporated with hsp70:dn-fgfr1 MO (top, red asterisk) were ~17% longer than uninjected ventral lobe regenerates (Fig. 2D). These results indicate that miR-133 slows regenerative outgrowth and support the idea that miR-133 is a significant regenerative brake.

Figure 2. Experimental manipulation of miR-133 controls fin regeneration. [A] Design for RNA duplex (blue) and MO (red) electroporation studies. [B] Electroporation of miR-133 RNA duplex into the dorsal lobe (top, blue asterisk) slows regeneration in wild-type fins, as compared with a mutated miR-133 RNA duplex (bottom, black asterisk). [C] Electroporation of miR-133 MO into the dorsal lobe (top, red asterisk) enhances regeneration during Fgfr inhibition, as compared with a standard MO (bottom, black asterisk). [D,E] Quantification of average intralobal length ratios of injected versus uninjected fin lobes with miR-133 RNA duplex [D] or miR-133 MO [E]. The injected:uninjected length ratio is significantly reduced by miR-133 MO (E). The injection:uninjected length ratio is ~10% higher by miR-133 duplex introduction, but significantly increased by miR-133 MO introduction (mean ± SEM; [*] P < 0.05, t-test; n = 10 per group).
miR-133 controls fin regeneration

miRNAs typically have from several dozen to hundreds of different mRNA target genes (Lewis et al. 2005; Giraldez et al. 2006). We searched the miRanda and miRBase databases and identified many potential targets, as predicted by complementarity of miR-133 seed sequences with mRNA 3’ UTR sequences. These potential targets include factors that support cellular and molecular functions previously implicated in regeneration, including cell division and growth, metalloproteinase activity (Vinarsky et al. 2005), and ion flux (Supplemental Table 1; Adams et al. 2007). One gene containing a single predicted miR-133-binding site is mps1, which encodes a kinase that regulates multiple aspects of cell proliferation during morphogenesis, including centrosome duplication, spindle checkpoint activation, and normal mitotic progression (Fisk and Winery 2004). Most importantly, mps1 is one of only four genes to date that have been shown by forward genetic approaches to be essential for fin regeneration. mps1 expression is specifically induced in blastemal tissue during regeneration, and a temperature-sensitive mutation in mps1 arrests regeneration during outgrowth, the phase at which our data implicated miR-133 function (Poss et al. 2002).

To determine if miR-133 regulates mps1 mRNA in vivo, we used zebrafish embryo sensor assays (Giraldez et al. 2005). We injected a sensor mRNA construct consisting of EGFP fused to the 3’ UTR of mps1 into one-cell zebrafish embryos, in the presence or absence of miR-133 RNA duplex (Fig. 4A). One day later, we quantified EGFP fluorescence as an indicator of Mps1 expression. Injections of the mps1 sensor mRNA alone resulted in high EGFP expression; however, this fluorescence was dampened almost 50% by coinjection of miR-133 RNA duplex (Fig. 4A). Three additional experiments provided evidence of specific interaction between miR-133 and the EGFP-mps1-3’ UTR sensor mRNA. First, no interaction was observed between miR-133 and a sensor mRNA containing a mutated miR-133-binding site. Second, coinjection of miR-101 RNA duplex, not predicted to regulate the mps1 3’ UTR, did not reduce EGFP sensor fluorescence. Finally, coinjection of miR-133 MO or an LNA-133 antisense oligonucleotide effectively prevented miR-133-induced dampening of fluorescence (Fig. 4A,B). Northern blot analysis indicated little or no effect of miR-133 duplex on EGFP-mps1-3’ UTR sensor mRNA levels [data not shown]. Therefore, we suspect that miR-133 regulates mps1 in these assays through translational repression. This finding suggests that the increases in mps1 mRNA levels we observed after antagonism of miR-133 during fin regeneration were likely contributed by other direct and indirect regulatory inputs [Fig. 3C]. In summary, the known blastemal regulator mps1 is a target of miR-133.

Conclusions

In conclusion, our experiments implicate post-transcriptional regulation by miRNAs in the process of complex...
miR-133 targets the blastemal regulator mps1. [A,B] EGFP embryo sensor assays demonstrating specific regulation of the mps1 3' UTR in vivo by miR-133. For each experimental group in B, EGFP-mps1 3' UTR fluorescence is expressed as a percentage of fluorescence observed from the sensor injected alone. (A, bottom) DsRed mRNA is injected as a control. (Mean ± SEM, *P < 0.05; n = 10 embryos per group.) (C) Model for miR-133 function during fin regeneration. Fin amputation triggers Fgf signaling, which attenuates miR-133 levels through unknown mechanisms. The reduction in miR-133 levels boosts expression of target genes important for blastemal proliferation like Mps1, optimizing regeneration.

Figure 4. miR-133 targets the blastemal regulator mps1. [A,B] EGFP embryo sensor assays demonstrating specific regulation of the mps1 3' UTR in vivo by miR-133. For each experimental group in B, EGFP-mps1 3' UTR fluorescence is expressed as a percentage of fluorescence observed from the sensor injected alone. (A, bottom) DsRed mRNA is injected as a control. (Mean ± SEM, *P < 0.05; n = 10 embryos per group.) (C) Model for miR-133 function during fin regeneration. Fin amputation triggers Fgf signaling, which attenuates miR-133 levels through unknown mechanisms. The reduction in miR-133 levels boosts expression of target genes important for blastemal proliferation like Mps1, optimizing regeneration.

Materials and methods

Zebrafish caudal fin amputations
Zebrafish of the Ekkwill (EK) strain or EK/AB mixed strain 4–6 mo old were used for all experiments. Amputations were performed with a razor blade and removed one-half of the caudal fin. In Figure 1A experiments, samples were collected from wild-type animals and allowed to regenerate at 33°C, a temperature that accelerates the process. For all other experiments, regeneration occurred at 26°C. Heat shocks were given at 38°C to wild-type and hsp70:dn-fgfr1 animals as described (Lee et al. 2005). Gene expression analysis
Total RNA was isolated (Tri-Reagent, Sigma) from tissue distal to a region one segment proximal to the amputation plane and used for miRNA microarray hybridizations, Northern analysis, quantitative PCR, and ribonuclease protection assays (see Supplemental Material).

MO and RNA duplex injections
Wild-type and hsp70:dn-fgfr1 caudal fins were amputated and allowed to regenerate for 3 d at 26°C. MOs (Gene-Tools) or RNA duplexes (IDT Technology, design based on miR-133b sequence) (see Supplemental Table 2; Supplemental Fig. 2) were injected into each ray of the dorsal lobe, followed by electroporation of the entire caudal fin using a 5-mm electrode (Thummel et al. 2006; Visvanathan et al. 2007). Fluorescein-tagged MOs were used to monitor electroporation efficiency. When hsp70:dn-fgfr1 regenerated were treated with MOs, electroporation occurred 5 h after a single heat shock at 38°C. Regenerates were imaged daily for 3 d following electroporation and measured from the amputation plane to the distal tip of regenerating rays 2 and 3 (with respect to the most lateral ray) for dorsal and ventral lobes, using Openlab software. The lengths of these rays were averaged to give one length value each for the dorsal and ventral regenerates per animal. Intrafin ratios represent injected dorsal regenerate length/uninjected ventral regenerate length, calculated for each animal at each time point. Student’s t-tests (two-tailed, unequal variance) were performed to determine P-values.

BrdU analysis
A 2.5 mg/mL BrdU solution was injected intraperitoneally into hsp70:dn-fgfr1 animals 5 h after MO injection. After 30 min, caudal fins were collected and processed for immunofluorescence (Lee et al. 2005). We quantified the number of BrdU-positive cells by counting within a 0.036-mm² box, based on the average size of 4-dpa, BrdU-dense blastemas of wild-type animals that had been heat-shocked and electroporated with a standard control MO. Each square was aligned at the distal edge of the regenerate in hsp70:dn-fgfr1 MO-treated fin rays. For each fin, BrdU-positive cells in dorsal rays 2 and 3 were counted for MO effects, while ventral rays 2 and 3 were counted for the uninjected control.
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EGFP sensor assays
One-cell zebrafish embryos were injected with 1–2 nL of a solution consisting of 150 ng/µL sensor mRNA and 100 ng/µL Dsred mRNA. When applicable, 10 µM miRNA duplex was added with or without 0.5 ng/µL miR-133 MO or 0.8 ng/µL LNA-133. EGFP fluorescence was quantified at 24–28 h post-fertilization as described (Giraldez et al. 2006).

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