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Zebrafish *miR-214* modulates Hedgehog signaling to specify muscle cell fate

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Abstract

Numerous microRNAs (miRNAs) have been discovered in the genomes of higher eukaryotes, and functional studies indicate that they are important during development. However, little is known concerning the function of individual miRNAs. We approached this problem in zebrafish by combining identification of miRNA expression, functional analyses and experimental validation of potential targets. We show that *miR-214* is expressed during early segmentation stages in somites and that varying its expression alters the expression of genes regulated by Hedgehog signaling. Inhibition of *miR-214* results in a reduction or loss of slow-muscle cell types. We show that *su(fu)* mRNA, encoding a negative regulator of Hedgehog signaling, is targeted by *miR-214*. Through regulation of *su(fu)*, *miR-214* enables precise specification of muscle cell types by sharpening cellular responses to Hedgehog.

Multicellular organisms such as zebrafish use miRNAs to regulate gene expression in a tissue- or time-specific manner, guiding developmental decisions^{1,2}. To identify target genes regulated by miRNAs, we first developed a microarray to examine temporal miRNA expression patterns during the first 5 d post-fertilization (dpf) of zebrafish development (unpublished data). To understand the function of a subset of these miRNAs, we performed loss-of-function experiments using antisense morpholino oligonucleotides complementary to mature miRNAs. Morpholinos have been used extensively in zebrafish as antisense inhibitors of mRNA translation and splicing³ but are also capable of interfering with miRNA function (Supplementary Fig. 1 online). Injection of morpholinos designed to block the function of *miR-214* (214^{MO}) yielded embryos with U-shaped somites at 1dpf (1 dpf) (Fig. 1a–d). Expression of *miR-214* begins during early somitogenesis and continues throughout embryogenesis (Fig. 1e). *In situ* hybridization showed that *miR-214* is expressed in somites at 1 dpf (Fig. 1f,g; see also ref. 2).

Somites are transient embryonic structures derived from paraxial mesoderm that give rise to muscle and skeleton⁴. Presomitic mesodermal cells immediately adjacent to the notochord (adaxial cells) are highly influenced by Hedgehog and give rise to the slow-twitch muscle lineage^{5,6}. Lateral presomitic cells give rise to fast-twitch muscle fibers and experience little stimulation by Hedgehog initially, whereas later-developing fast-muscle fates are dependent on Hedgehog signaling⁷. There are two slow muscle cell types that require precise

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AUTHOR CONTRIBUTIONS

A.S.F., L.S.-K. and J.G.P. conceived and designed all experiments and wrote the paper. A.S.F. performed all experiments with help from N.L. on Figure 3 and help from E.J.T. with the developmental miRNA microarrays and supplementary statistics.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Hedgehog signals for proper development: superficial slow fibers (SSFs), which migrate from the midline to populate the surface of the myotome, and slow muscle pioneers that remain close to the midline^{6,8}. Muscle pioneers require higher levels of and longer exposure to Hedgehog for proper specification than SSFs and can be distinguished from slow muscle fibers by the expression of the transcription factor Engrailed (Eng)^{4,6}. *In situ* hybridization demonstrated that inhibition of *miR-214* function resulted in a loss of *eng2a*-positive cells during early segmentation (**Fig. 2a,b**), consistent with the U-shaped somite defects observed in 214^{MO}-injected embryos (**Fig. 1a–d**). Reduction of this marker suggested an overall decrease in Hedgehog signaling in the adaxial cells of 214^{MO}-injected embryos. To test this, we analyzed expression of *patched1* (*ptc1*), which encodes a Hedgehog ligand receptor whose transcription is activated by Hedgehog⁹. Embryos injected with the 214^{MO} showed decreased expression of *ptc1* in adaxial cells and upregulation in lateral cells. (**Fig. 2c–f** and **Supplementary Fig. 2** online). This suggested that *miR-214* might regulate the level of Hedgehog signaling during somite differentiation.

To further characterize defects associated with inhibition of *miR-214* function, we monitored several markers of adaxial cell derivatives at later stages. All slow fibers express the homeodomain protein Prox1 as well as a myosin heavy-chain isoform specific to slow muscle (slow myosin HC) ¹⁰. Codetection of slow myosin HC and Prox1 by immunohistochemistry showed fewer slow muscles in *miR-214* morphants at 1 dpf (**Table 1** and **Supplementary Fig. 2**). Additionally, we monitored Eng proteins, which are expressed in both muscle pioneers and medial fast fibers, a fast-twitch muscle cell type that arises after SSF migration and is dependent on Hedgehog signaling but does not express Prox1 (ref. 6). Injection of 214^{MO} reduced the number of muscle pioneers during late somitogenesis (**Fig. 2g** and **Table 1**). To verify that the altered somite differentiation in *miR-214* morphant embryos is due to perturbation of Hedgehog signaling, we sought to rescue the 214^{MO} phenotype by coinjection of synthetic *sonic Hedgehog* (*shh*) RNA (**Fig. 2g–i**, **Table 1** and **Supplementary Fig. 2**). Notably, *miR-214* morphant defects were completely suppressed by Shh misexpression.

Next, we analyzed the effect of *miR-214* misexpression on Hedgehog-mediated cell fate specification (**Fig. 2j–m** and **Supplementary Fig. 2**). Unlike downregulation of *miR-214* function, injection of synthetic *miR-214* into one-cell embryos did not yield appreciable alterations of *ptc1* expression in somites (**Supplementary Fig. 2**). However, ectopic expression of excess *miR-214* did result in perturbation of Hedgehog-regulated markers in the ventral neural tube (**Fig. 2j–m** and **Supplementary Fig. 2**). *nkx2.2a* is expressed in the lateral floor plate of the neural tube, and its expression is stimulated by the highest levels of Hedgehog activity, whereas *olig2* expression is adjacent and dorsal to the *nkx2.2a* domain^{11–13}. Misexpression of *miR-214* resulted in expanded expression of *nkx2.2a* and a dorsal shift of *olig2* expression, further supporting a role for *miR-214* in the modulation of Hedgehog signaling.

Similar to most miRNAs, *miR-214* has many predicted targets, one of which is *suppressor of fused* (*su(fu)*)¹⁴ (**Fig. 3a**). *Su(fu)* is a well characterized negative regulator of Hedgehog signaling essential for proper specification of muscle cell types during somitogenesis^{6,15}. To test whether *miR-214* targets *su(fu)*, we monitored GFP fluorescence in embryos microinjected with mRNAs derived from the following reporter constructs: the 3' UTR of *su(fu)* cloned downstream of the GFP ORF (GFP_{*su(fu)*}), GFP fused to two perfect *miR-214* recognition elements (GFP 2XMRE) or GFP lacking heterologous 3' UTR sequences (GFP –UTR) (**Fig. 3b–h**). Consistent with the hypothesis that sequences in the *su(fu)* 3' UTR contain *bona fide* recognition elements, GFP fluorescence was substantially lower in embryos coinjected with *miR-214* RNA and GFP_{*su(fu)*} mRNA (**Fig. 3e**). We verified the

fluorescence experiments by protein blots performed with antibodies against GFP on whole-embryo lysates (**Fig. 3h**).

To confirm that the decrease in slow muscle cells caused by *miR-214* inhibition is directly due to *su(fu)* derepression, we sought to rescue the defect through simultaneous downregulation of both *su(fu)* and *miR-214* (**Fig. 4a–h**) (**Table 1**). Injection of two independent morpholinos targeted to *su(fu)* (*su(fu)*^{MO1,2}) caused an increase in the number of slow muscle cells but did not have a significant effect on the number of muscle pioneers (**Fig. 4g,h** and **Table 1**)⁶. In contrast, injection of a mismatched morpholino (*su(fu)* mm^{MO}) did not have any effect (**Fig. 4a,b** and **Table 1**). Notably, coinjection of 214^{MO} along with *su(fu)*^{MO1,2} resulted in a restoration of slow muscle cells (**Fig. 4e,f** and **Table 1**) compared with embryos coinjected with 214^{MO} and *su(fu)*-mm^{MO} (**Fig. 4c,d** and **Table 1**). Inhibition of *su(fu)* alone has been shown to increase the number of medial fast fibers while not affecting specification of muscle pioneers⁶. Colabeling of Eng and Prox1 demonstrated that the rescued Eng-expressing cells in 214^{MO} and *su(fu)*^{MO1,2} coinjected embryos are muscle pioneers and not medial fast fibers (**Fig. 4a,c,e,g**). Together, our results indicate that *su(fu)* inhibition suppresses the deficiency of muscle pioneers and SFFs associated with loss of *miR-214* function, supporting the hypothesis that *miR-214* modulates Hedgehog signaling largely by regulation of *su(fu)*.

Su(fu) participates in Hedgehog signaling by altering the function of the Gli family of transcription factors. Su(fu) tethers both the activator and repressor forms of Gli in the cytoplasm, resulting in downregulation of both activities^{6,16}. Inhibition of Su(fu) by *miR-214* in adaxial cells, which experience high amounts of Hedgehog signaling, allows maximal activation of Gli-mediated transcription (**Fig. 5**). Regulation of *su(fu)* by *miR-214* in lateral muscle cells, which are exposed to lower levels of Hedgehog signaling, results in increased repressor activity, ensuring a commitment to fast-muscle cell fate (**Fig. 5**). These distinct effects on Hedgehog signaling are demonstrated by changes in the expression of *ptc1* in 214^{MO}-injected embryos. Inhibition of *miR-214* function permits increased expression of Su(fu), resulting in decreased *ptc1* expression in adaxial cells and increased expression in lateral presomitic mesoderm (**Fig. 2d**). The decreased Hedgehog response in adaxial cells causes loss (as in the case of muscle pioneers) or reduction (as in the case of SFFs) in cell types dependent on high levels of Hedgehog signaling, triggering the formation of U-shaped somites. In contrast, increased expression of *ptc1* in lateral somites can be explained by the decreased activity of Gli repressor forms, which are also negatively regulated by Su(fu). Likewise, aberrant expression of Hedgehog-regulated genes in the neural tube caused by ectopic expression of *miR-214* can be explained by disruption of Su(fu) expression. In the presence of ectopic *miR-214*, neural tube cells become more sensitive to Hedgehog signals, causing them to acquire more ventral fates, as demonstrated by the increase in *nkx2.2a* expression and a dorsal shift in *olig2* expression. Together, these data support a role for *miR-214*-mediated regulation of *su(fu)* that is essential for specification of muscle cell types during somitogenesis by sharpening the response to different levels of Hedgehog signals.

Compared with their function in invertebrates, fungi and plants, the function of miRNAs in vertebrates has been proposed to be more limited, subtly modulating cell types or acting as a redundant mechanism for ensuring appropriate gene expression patterns¹⁷. We have shown a requirement for *miR-214* in the specification of muscle cell fate during somitogenesis. This finding suggests that some vertebrate miRNAs have decisive roles during development, being required for the generation of specific cell types.

METHODS

Microinjections

Fertilized one-cell zebrafish embryos were injected with 1-nl volumes 2 ng of 214^{MO}, 2 ng of *miR-214*, 1 ng of *su(fu)*^{MO1} combined⁶ with 1 ng of *su(fu)*^{MO2}, 2 ng of *su(fu)* mm^{MO} (ref. 6), 100 pg of *in vitro*-transcribed, capped *shh* mRNA and/or 50 ng of *in vitro*-transcribed, capped GFP reporter. Zebrafish *su(fu)* 3' UTR sequences were amplified by RT-PCR and subcloned downstream of the GFP ORF that was inserted into pCS2+ (ref. 18). The sequences of oligonucleotides are listed in **Supplementary Table 1** online.

RNA blots and *in situ* hybridization

RNA blots were performed as previously described¹⁹. Detection of mRNAs and primary miRNAs was accomplished as previously described²⁰ using digoxigenin (DIG)-labeled antisense RNA probes and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) or fast red color development. Detection of mature *miR-214* RNAs was carried out using DIG-labeled Locked Nucleic Acid (LNA) probes (Exiqon) following previously reported methods² and was visualized using NBT/BCIP color development. Cryosectioning was performed as described previously²¹.

Immunohistochemistry

Immunostaining was performed as described previously²². Antibodies included rabbit polyclonal antibodies against Prox1 (Abcam), mouse monoclonal antibodies against slow myosin HC (F59; see Acknowledgments), rabbit polyclonal antibodies against chick Engrailed (see Acknowledgments) and rabbit polyclonal antibodies against GFP (Torrey Pines Biolabs). The 4D9 monoclonal antibody against Engrailed was developed by C. Goodman, obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa Department of Biological Sciences. Secondary antibodies against mouse or rabbit IgG were Cy2 or Cy3 conjugated (Jackson ImmunoResearch).

Protein blotting

We manually dechorionated and deyolked 1-dpf embryos. Embryos were briefly sonicated in passive lysis buffer (25 mM HEPES (pH 7.5), 5 mM MgCl₂, 300 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10% glycerol, 1.0% Triton X-100 and 1 mM PMSF). We then separated 20 µg of total protein on 10% polyacrylamide gels and transferred it to nitrocellulose membranes. We blocked blots with 5% nonfat dry milk and probed them with antibodies against GFP (Santa Cruz) or α-tubulin (Abcam). For detection, we used HRP-conjugated secondary antibodies against mouse and rabbit, respectively, followed by visualization with ECL.

Imaging

Live embryos were mounted in 2% methylcellulose. We developed *in situ* hybridizations with NBT/BCIP and prepared whole mounts or flat mounts in 100% glycerol. Both live and NBT/BCIP-treated embryos were photographed using a Zeiss Axiophot compound microscope and an Axiocam digital camera. Images were acquired using Axiovision software and were imported into Adobe Photoshop for orientation. Embryos subjected to immunohistochemistry were mounted in 50% glycerol and imaged with a Zeiss LSM510 Meta Laser Scanning microscope. Stacks were acquired with LSM510 software, and Z-projections and contrast adjustments were made with NIH ImageJ. Images were imported into Adobe Photoshop for orientation. See the *Supplementary Note* for additional information concerning image acquisition.

Statistical analysis

We performed ANOVA to determine statistical significance between the number of cells counted from confocal images. Differences were established at a 95% confidence interval. Skewness and kurtosis tests showed that all data were normally distributed. Values used to determine statistical significance are summarized in **Supplementary Table 2** online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *miR-214* participates in somitogenesis. **(a,b)** Morphology of embryos injected with *214^{MO}* at the 14-somite stage. **(c,d)** Somite morphology in uninjected controls (UIC) **(c)** or in embryos injected with antisense morpholino oligonucleotides complementary to *miR-214* (*214^{MO}*) **(d)**. **(e)** Expression of *miR-214*, as determined by RNA blotting. Embryonic stages are listed above, and ethidium bromide-stained rRNA is shown as a loading control. **(f,g)** Expression of *miR-214* in somites at 1 dpf as determined by *in situ* hybridization in whole-mount embryos **(f)** and in a somite cross-section through the trunk region. Section in **g** was obtained from the region in **f** indicated by the black line.

Figure 2. Modulation of *miR-214* expression alters Hedgehog-mediated cell fate specification. (a,b) Loss of muscle pioneers shown by *eng2a* in situ hybridization in *214^{MO}* morphants (b) compared with uninjected controls (UIC) (a). (c,d) Aberrant *ptc1* expression in *214^{MO}*-injected embryos (d) compared with UIC (c). (e,f) Pixel intensities of *ptc1* stain in three embryos. From left to right, plot show the intensity from the bottom to the top of images in c and d (that is, from left to right in the embryo). e shows the UIC and f the *214^{MO}*-injected embryo. (g-i) Eng-positive nuclei in *214^{MO}*-injected embryos (g), in embryos injected with *214^{MO}* and *shh* (h) and in embryos injected with *shh* mRNA (i). (j-m) Expression of *nkx2.2a* and *olig2* in sections of spinal cord from UIC embryos (j,l) and in embryos injected with *miR-214* (k,m).

Figure 3. *su(fu)* is a target of *miR-214*. **(a)** *su(fu)* 3' UTR sequence elements complementary to *miR-214*. **(b–g)** Fluorescence in embryos injected with synthetic mRNAs encoding GFP with or without coinjected *miR-214* RNA. **b, d** and **f** show embryos injected with GFP without the UTR sequence (–UTR; **b**), GFP fused to the *su(fu)* 3' UTR (*su(fu)*UTR; **d**) or GFP fused to two perfect *miR-214* sites (2XMRE; **f**) alone. **c, e** and **g** show embryos coinjected with the GFP reporters as in **b, d** and **f**, respectively, along with *miR-214* RNA. **(h)** Protein blot analysis of lysates prepared from embryos injected with GFP without the UTR (–UTR), *su(fu)*UTR or 2XMRE GFP with or without *miR-214* RNA coinjection. As a control, blots were performed on the same lysates with α -tubulin antibodies.

Figure 4. Rescue of the *miR-214*^{MO} phenotype by simultaneous inhibition of *su(fu)* expression. (a–h) Expression of Eng (green) and Prox1 (red) in 20-somite embryos (a,c,e,g) or expression of Prox1 (red) and slow-muscle myosin (green) in 1-dpf embryos (b,d,f,h). Embryos in a and b were injected with *su(fu)* mismatched morpholinos (*su(fu)* mm^{MO}), and embryos in g and h were injected with two morpholinos targeted to *su(fu)* (*su(fu)*^{MO1,2}). Embryos in c and d were coinjected with *su(fu)* mm^{MO} and 214^{MO}, and embryos in e and f were injected with *su(fu)*^{MO1,2} and 214^{MO}.

Figure 5.

Modulation of Hedgehog (Hh) signaling in somite cells by *miR-214*. Su(fu) acts on both activator (Gli^{act}) and repressor (Gli^{rep}) forms of Gli, inhibiting nuclear trafficking. *miR-214* downregulates Su(fu), allowing both maximal activation in the presence of Hedgehog and complete repression when Hedgehog signaling is minimal.

Table 1

Numbers of muscle cell types in morphants

Treatment	Slow muscle cells	Muscle pioneers
Uninjected control	23.7 ± 0.0.28 (47) ^a	3.4 ± 0.11(47) ^a
214 ^{MO}	16.0 ± 0.26 (73) ^b	1.7 ± 0.13 (68) ^b
214 ^{MO} + <i>shh</i>	43.0 ± 1.24 (53) ^c	6.4 ± 0.34 (25) ^c
<i>shh</i>	42.6 ± 1.68 (34) ^c	6.2 ± 0.31 (39) ^c
<i>su(fu)</i> mm ^{MO}	24.3 ± 0.33 (48) ^a	3.3 ± 0.11 (35) ^a
214 ^{MO} + <i>su(fu)</i> mm ^{MO}	16.5 ± 0.26 (71) ^b	1.4 ± 0.10 (103) ^b
214 ^{MO} + <i>su(fu)</i> ^{MO1,2}	22.5 ± 0.34 (62) ^a	3.6 ± 0.10 (108) ^a
<i>su(fu)</i> ^{MO1,2}	28.0 ± 0.44 (43) ^d	3.8 ± 0.09 (127) ^a

Slow muscle cells are positive for both Prox1 and slow myosin HC. Muscle pioneers are positive for Eng and Prox1. Markers were visualized by fluorescent immunohistochemistry and confocal microscopy as in **Figures 2 and 4** and **Supplementary Figure 2**. Values are the mean of the specified cell type per somite (± s.e.m.). The number of somites analyzed is indicated in parentheses. We performed analysis of variance (ANOVA) to determine statistical significance within a 95% confidence interval.

^a Superscript letters indicate significant differences within each column. Values in the same column that share the same superscript letter do not differ significantly from each other.

^b Superscript letters indicate significant differences within each column. Values in the same column that share the same superscript letter do not differ significantly from each other.

^c Superscript letters indicate significant differences within each column. Values in the same column that share the same superscript letter do not differ significantly from each other.

^d Superscript letters indicate significant differences within each column. Values in the same column that share the same superscript letter do not differ significantly from each other.