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miR-196 regulates axial patterning and pectoral appendage initiation

Xinjun He [a], Yi-Lin Yan [a], Johann K. Eberhart [a,1], Amaury Herpin [b], Toni U. Wagner [b], Manfred Schartl [b], John H. Postlethwait [a,b,*]

[a] Institute of Neuroscience, University of Oregon, Eugene OR 97403 USA
[b] Department of Physiological Chemistry I, Biocenter, University of Wuerzburg, Am Hubland, 97074 Wuerzburg, Germany

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ABSTRACT

Vertebrate *Hox* clusters contain protein-coding genes that regulate body axis development and microRNA (miRNA) genes whose functions are not yet well understood. We overexpressed the *Hox* cluster microRNA miR-196 in zebrafish embryos and found four specific, viable phenotypes: failure of pectoral fin bud initiation, deletion of the 6th pharyngeal arch, homeotic aberration and loss of rostral vertebrae, and reduced number of ribs and somites. Reciprocally, miR-196 knockdown evoked an extra pharyngeal arch, extra ribs, and extra somites, confirming endogenous roles of miR-196. miR-196 injection altered expression of *hox* genes and the signaling of retinoic acid through the retinoic acid receptor gene *rarab*. Knocking down *rarab* mimicked the pectoral fin phenotype of miR-196 overexpression, and reporter constructs tested in tissue culture and in embryos showed that the *rarab* 3′UTR is a miR-196 target for pectoral fin bud initiation. These results show that a *Hox cluster* microRNA modulates development of axial patterning similar to nearby protein-coding *Hox* genes, and acts on appendicular patterning at least in part by modulating retinoic acid signaling.

Introduction

Hox cluster genes control animal body patterning in radiata and in bilateria, including both protostomes and deuterostomes (Finnerty et al., 2004; Postlethwait and Schneiderman, 1969; Wellik, 2009). In vertebrate deuterostomes, *Hox cluster* genes control the anterior–posterior body axis, including the identity of vertebrae and pharyngeal arches and the axes of body appendages (Krumlauf, 1994), and they are important for the development of mesodermal organ systems (Di-Poi et al., 2010). *Hox* clusters evolved by tandem gene duplication followed by whole genome duplication events in vertebrates that provided tetrapods with four *Hox* clusters and most teleost fish with seven or eight (Amores et al., 1998, 2004; Chambers et al., 2009; Gehring et al., 2009; Graham et al., 1989; Woltering and Durston, 2006).

Hox genes are expressed in a collinear fashion along the anterior–posterior body axis during early development, with genes located 3′ in the cluster controlling anterior development and those located 5′ regulating more posterior organ development (Duboule and Morata, 1994; Graham et al., 1989); as a result, *Hox* gene mutations can delete vertebrae or transform vertebral identity and remove or reduce limb skeletal elements (Chen and Capecchi, 1997; Davis et al., 1995). *Hox* genes act by controlling downstream transcription factors that regulate signaling events controlling body segmentation and organ initiation. Some *Hox* genes are themselves directly regulated by the extracellular signaler retinoic acid (RA), which controls axis and pectoral appendage development (Grandel et al., 2002; Hoffman et al., 2002; Nolte et al., 2003).

Bilaterian *Hox* clusters contain protein-coding genes and genes encoding microRNAs (miRNAs), small non-coding RNAs that generally bind to 3′ untranslated regions (UTRs) of messenger RNAs and regulate their stability or translation (Fjose and Zhao, 2010; Vella et al., 2004). The human genome has three *Hox cluster* miRNA genes, *MIR10*, *MIR196*, and *MIR615*. The *MIR10* gene is broadly distributed among bilaterians; *MIR196* is conserved among vertebrates; and *MIR615* is restricted to mammalian genomes (see miRBase collection at http://www.miRBase.org (Griffiths-Jones et al., 2008; Yekta et al., 2008)). In zebrafish, the *hoxdb* cluster lost all of its protein-coding genes (Amores et al., 1998), but surprisingly, retained *mir10* (Woltering and Durston, 2006). The similarity of *Hox* cluster miRNA expression patterns to those of nearby *hox* genes suggested that *Hox* cluster miRNAs and *Hox* cluster genes share regulatory mechanisms (Wienholds et al., 2005). Furthermore, the discovery that the 3′ UTRs of several *Hox* cluster genes contain predicted binding sites for either miR-196 or miR-10 suggested that some *Hox* genes might be regulated by *Mir10* and/or *Mir196* (He et al., 2009; Hornstein et al.,

* Corresponding author at: Institute of Neuroscience, University of Oregon, Eugene OR 97403 USA. Fax: +1 541 346 4538.
E-mail addresses: xhe@uoneuro.uoregon.edu (X. He), yan@uoneuro.uoregon.edu (Y.-L. Yan), eberhart@mail.utexas.edu (J.K. Eberhart), amaury.herpin@biozentrum.uni-wuerzburg.de (A. Herpin), toni.wagner@biozentrum.uni-wuerzburg.de (T.U. Wagner), phch1@biozentrum.uni-wuerzburg.de (M. Schartl), jpostle@uoneuro.uoregon.edu (J.H. Postlethwait).
[1] Present address: Section of Molecular Cell and Developmental Biology, University of Texas at Austin, TX 78712, USA.

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2005; Kawasaki and Taira, 2004; Woltering and Durston, 2008; Yekta et al., 2004; Yekta et al., 2008). For example, *mir10* is involved in the regulation of metastasis by controlling *Hoxd10* in cell culture and *hoxb1a* and *hoxb3a in vivo* (Lund, 2009; Ma et al., 2007; Woltering and Durston, 2008). miR-196 binds to *Hoxb8* mRNA, thereby accelerating its cleavage, and this interaction has been hypothesized to be important for the outgrowth of hindlimb buds (Hornstein et al., 2005; Kawasaki and Taira, 2004; Yekta et al., 2004). In the CNS, miR-196 restricts motor neuron differentiation by regulating *Hoxb8* (Asli and Kessel, 2010). miR-196 is also involved in cancer progression by interaction with other *Hox8* paralogs (Li et al., 2010) (Chen et al., 2011). miRNA-196 can also repress *BACH1* expression in human liver cells (Hou et al., 2010) and is important for tail regeneration in the axolotl (Sehm et al., 2009). Knockdown of miR-196 in chick embryos leads to a homeotic transformation of a cervical vertebra to thoracic identity (McGlinn et al., 2009). Because no phenotype has yet been described for the overexpression of *mir196* in embryos and no phenotype has been described in other tissues where it is expressed, we do not yet fully understand its roles in development or the mechanisms by which it acts.

Here we show that precise levels of *mir196* are required to initiate development of the pectoral appendage, to develop the correct number of pharyngeal arches, and to specify the number and identity of rostral vertebrae and ribs. We show that miR-196 can alter *hox* gene expression patterns and that miR-196 acts on pectoral appendage development by altering retinoic acid signaling via fine-tuning the expression of the retinoic acid receptor Rarab.

Results

mir196 genomics

The human genome has three copies of *MIR196* located between paralogy groups 9 and 10 (Yekta et al., 2008), but due to the teleost genome duplication (Amores et al., 1998; Postlethwait et al., 1998; Taylor et al., 2003), zebrafish has five *mir196* genes (Supplementary Fig. S1A). The teleost whole genome duplication would have initially produced six *mir196* genes, but one of the two *hoxbb mir196* duplicates was lost and duplicates of only the *hoxa* and *hoxc* cluster genes were maintained. The five zebrafish *mir196* paralogs encode four different mature miR-196 sequences with a central nucleotide trio containing (C/G/T) A (A/T). The duplicate *hoxa* and *hoxc* clusters have *mir196* paralogs that differ by one nucleotide [(C/T) AT and (C/G) AT], respectively (Fig. 1A). Because miRNAs often bind their targets with some mismatch (He and Hannon, 2004; Yekta et al., 2004), all four miR-196 sequences probably regulate the same targets.

mir196 expression patterns

mir196 genes and nearby *hox* genes share spatial expression patterns in the central nervous system (CNS) and pectoral fin bud (Wienholds et al., 2005; Woltering and Durston, 2006; Yekta et al., 2008) (compare Fig. 1C–J and Supplementary Fig. S1B–M). This result suggests that *hox* cluster miRNAs may share regulatory mechanisms with neighboring *hox* genes. To investigate temporal aspects of *mir196* expression, we used gene-specific primers for *mir196* primary transcripts and RT-PCR to discover that *mir196a1*(*hoxca*) transcript had begun to accumulate at 24 h post-fertilization (hpf), but *mir196a2* (*hoxaa*) transcript, which encodes the same mature miRNA sequence as *mir196a1*(*hoxca*), was maternally expressed (Fig. 1B). Transcripts from *mir196b*(*hoxba*) and *mir196c*(*hoxcb*) genes first appeared at bud stage, and transcript from *mir196d*(*hoxab*) first accumulated at 5 hpf (Fig. 1B) when gastrulation initiates. This gene-specific timing suggests that different *mir196* genes experience different regulations and may play different roles in development. In addition, whole mount *in situ* hybridization experiments showed that *mir196* genes are expressed in a pattern similar to but different from each other at 24 hpf (Fig. 1C–J).

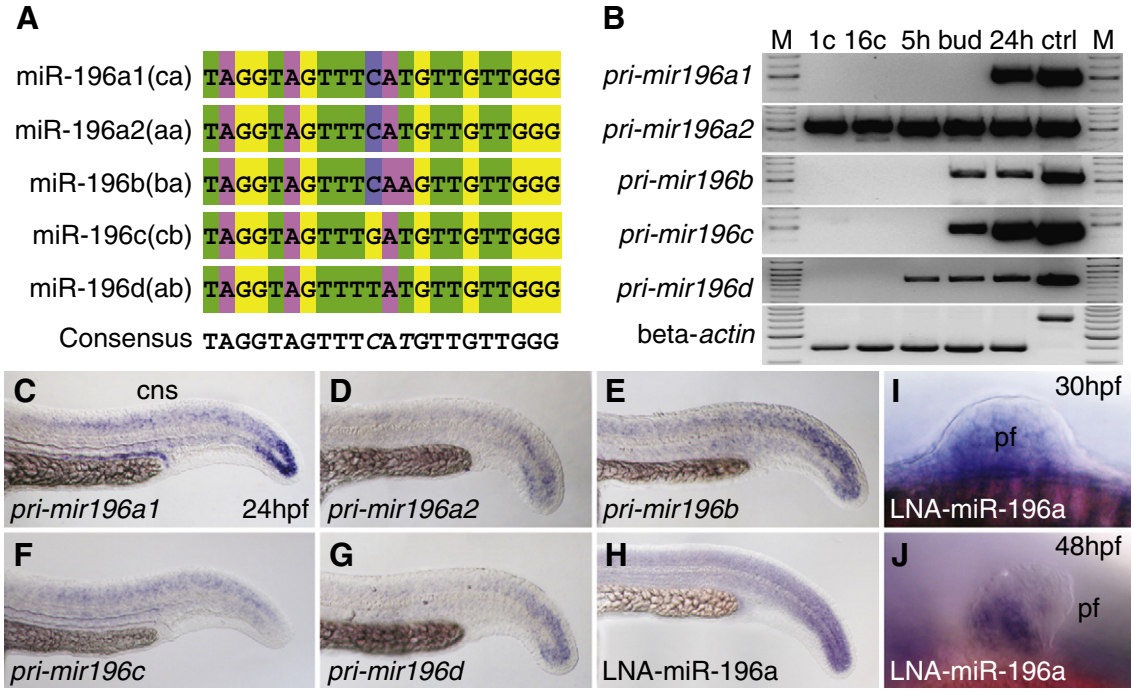

Fig. 1. Sequence and expression of *mir196* genes. (A) Alignment of mature miR-196 encoded by five *mir196* genes. (B) Expression of *mir196* paralogs studied by RT-PCR. Beta-*actin* (*bactin1*) was used as control for contaminating genomic DNA (ctrl lane). M, size marker; 1c, 1 cell stage; 16c, 16 cell stage; 5 h, 5 hpf (hours post-fertilization); bud, bud stage, about 10 hpf; 24 h, 24 hpf; ctrl, genomic DNA control. (C–H) Whole mount in situ hybridization for *mir196* primary transcripts showed expression in the tail bud and neural tube. (H) Linked nucleic acid (LNA) probe for miR-196a showed an expression pattern similar to the primary transcript. (I, J) LNA probes for miR-196a in the pectoral fin bud at 30 and 48 hpf. cns, central nerve system; pf, pectoral fin.