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MiR-10 targets HoxB1a and HoxB3a
and is required for correct migration of the
Xth nerve and trunk motorneurons

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submitted

Abstract

Within the vertebrate Hox clusters the miR-10 microRNA is associated with the position of
the Hox-4 paralogue group genes. Using sensor RNAs, microRNA overexpression and
knockdown we show that miR-10 targets the more anterior HoxB1a and HoxB3a genes in
Zebrafish. The target genes have a dominant hindbrain domain of expression anterior to the
miR-10 expression domain. Their expression however overlaps in a weaker domain in the
spinal cord where miR-10 loss of function results in their up regulation. Overexpression of
miR-10 leads to a failure of the VIIth cranial nerve to migrate out of rhombomere 4 as a
direct result of repression of HoxB1a. Overexpression of miR-10 also phenocopies the
reported effects of HoxB3a, as shown by gcm-2 expression. Morpholino knockdown of
miR-10 results in abnormal morphology and branching of the Xth cranial nerve and a
failure to correctly innervate the branchial arches. We also observe consistent defects in
pectoral fin motor neuron innervation in morphant embryos. We identify a role for miR-10
in the restriction of functional domains of anterior Hox genes, the correct development of
the Xth cranial nerve and the correct migration of trunk motorneurons.
Introduction

The *Hox* genes are responsible for the regionalization of the anterior posterior axis of the metazoan trunk. In human and mouse this gene family comprises 39 closely related homeodomain transcription factors organized in 4 homologous clusters (A-D) (1-4). The genes are expressed along the body axis in a sequential fashion corresponding to their physical sequence within the clusters. The more 3' a gene is located in a cluster the more anterior its expression domain, something which is commonly referred to as ‘spatial colinearity’. The genes have sharply defined anterior boundaries of expression but their posterior boundaries are in general less clear and overlap with the expression of more posterior genes. Due to an additional genome duplication 7 or 8 *Hox* clusters (named *Aa*, *Ab*, etc.) are present in the fish lineages with in Zebrafish 7 and a total of 48 genes (5, 6). In addition to the *Hox* coding genes the miR-10, miR-196 and miR-615 microRNA gene families have been identified within the vertebrate *Hox* clusters (7-10). MicroRNAs are small (~22 nt) non-coding RNAs which are derived from stemloop forming precursor transcripts through processing by the RNAse III enzymes Dicer (11) and Drosha (12). MicroRNAs function in post-transcriptional gene silencing by binding to imperfect target sites in the messengerRNA and thereby induce translational inhibition and RNA destabilization (13, 14).

In the *Hox* clusters, miR-10 is closely associated with the position of the *Hox-4* paralogue members, miR-196 is located 5' of *Hox-9* paralogues genes and the more recently cloned miR-615 is located in the *HoxC5* intron in mammals but appears to be absent in Teleosts and *Xenopus tropicalis* and may therefore be restricted to either mammals or amniotes. In the Zebrafish genome miR-10 is present in 5 copies and 4 different isoforms (*a*, *b*, *c* and *d*) differing from each other at 1 to 3 positions and we have recently shown that the miR-10d microRNA corresponds to the degenerated *HoxDb* cluster (15, 16).

Mouse knockouts and a Zebrafish germline *Dicer* mutant have revealed important functions for microRNAs in the coordination of normal embryonic development (17, 18), but individual vertebrate microRNAs are in general still enigmatic genetic objects and only few
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have been characterized on a functional level. In Zebrafish miR-430 (19) has been shown to silence maternal RNAs, miR-214 has been shown to be involved in proper somite specification (20) and miR-375 is involved in the maintenance of embryonal pancreas integrity (21).

With respect to the Hox related microRNAs, HoxA7 and Hox-8 paralogous genes have been identified as targets of miR-196 (22, 23, 24) and in chicken the interaction with HoxB8 has been implicated in the mechanism that abolishes the competence of posterior lateral plate mesoderm to limb induction by retinoic acid (24). In Drosophila a conserved or possibly convergent interaction exists for the miR-196 homologue IAB-4 (4, 22) that appears to target the Ubx Hox gene (25). Till now the function of miR-10 has remained unclear but based on its evolutionary conservation within the Hox clusters a role in anterior posterior patterning similar to that of the Hox genes itself could be anticipated.

As key patterning genes in the posterior central nervous system, the Hox genes are at the basis of the segmental rhombomeric organization of the hindbrain (26, 27) and the regional differences in motoneuron characteristics in the spinalcord (28). The most anterior Hox genes (group 1-4) are involved in the patterning of the hindbrain and branchial arches and follow the rhombomeric boundaries in their expression. In the Zebrafish hindbrain HoxB1a and HoxB1b are expressed in rhombomere (r) 4, HoxB2a defies the rule of colinearity and is expressed more anterior in r 3 and r 4, HoxB3a and HoxA3a are expressed strongest in r 5 and 6 with a weaker domain extending more posterior in the spinal cord. Finally the Hox-4 paralogues are expressed from r 7 onwards throughout the spinal cord (reviewed 29). Their differential expression in the hindbrain contributes to the formation of localized neuronal structures like the rhombomere 4 specific mauthner neurons and the distinct pattern of cranial motor nerves in different regions of the hindbrain.

Here we address the function of miR-10. We show that it represses the more anterior HoxB1a and HoxB3a genes and that overexpression induces phenotypes associated with the loss of these genes. In miR-10 morphant embryos we observe posterior upregulation of these target genes. In addition we observe severe defects in the migration of the Xth cranial nerve and a disrupted pattern of trunk motoneuron migration.
Results

MiR-10 expression and upregulation by retinoic acid

MiR-10 is associated with the 5’ genomic region of Hox-4 genes and previous studies in Zebrafish and mouse with microRNA specific Locked Nucleic Acid (LNA) in situ hybridization (15, 30) and transgenic sensor lines (23) have shown that both genes are expressed in similar domains. RT-PCR with primers located 5’ of miR-10c and in the HoxB4a coding sequence reveals presence on the same primary transcript (fig.1A) and RT-PCR on the single genes reveals a similar temporal pattern of expression (fig.1B). The exact anterior boundary of expression for miR-10c was determined in double in situ hybridization with the anterior neighboring gene HoxB3a (fig.1C). Consistent with the transcriptionally implied co-regulation, miR-10c has the same anterior boundary of expression as described for HoxB4a and is expressed in a mutually exclusive domain with the anterior strong r 5/6 expression of HoxB3a (single in situ, supplementary fig.1A).

Under some circumstances LNA probes are known to exhibit single nucleotide resolution (15, 31). In situ hybridization with probes matching each of the miR-10 isoforms (supplementary fig.2) excludes that other isoforms not detected by the miR-10c LNA probe are expressed overlapping with or anterior to the main expression domain of HoxB3a. The expression patterns of the different miR-10 copies, as observed using LNA probes, differ in that the probes for miR-10b and miR-10d show a more posterior rostral boundary with highest intensity staining caudal to the hindbrain while miR-10a and miR-10c probes have an anterior boundary at r6/7.

Anterior Hox genes are regulated in the neural tissue by retinoic acid (26). Treatment of embryos with $10^{-6}$ retinoic acid shows upregulation of miR-10c in in situ hybridization (fig.1D). This is a response pattern as known for Hox-4 paralogue genes.
Figure 1) miR-10c expression and induction by retinoic acid. A) RT-PCR with primers located 5’ of miR-10c and within the coding region of exon 1 of hoxB4a, 35 cycles, -RT; no reverse transcriptase added. The miR-10c precursor is co-transcribed with the hoxB4a coding region. B) Temporal expression during development of hoxB4a (28 cycles) and mir-10c pre-miRNA (35 cycles) as determined by RT-PCR on different stages of Zebrafish development. C) Whole mount in situ hybridization on different stages Zebrafish embryos, red; hoxB3a coding region exon 1, purple; miR-10c LNA probe. Note the mutual exclusive expression with the strong r 5/6 domain of HoxB3a. D) miR-10c expression in wildtype embryos (WT) and embryos treated with 10⁻⁶M retinoic acid.
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MicroRNAs bind through a complementary fuzzy match to target sites in messengerRNA transcripts and the specificity of this interaction resides in nucleotide 2-7 (the seed) of the microRNA which requires a complete match usually flanked on position 1 by an adenosine or a perfect match (32). Accordingly, sequence information permits the prediction of target genes. In silico target analysis in Teleosts has predicted that miR-10 targets are present within the Hox clusters (33). Inspection of the Zebrafish Hox clusters for putative miR-10 target sites using the seed sequence (nucleotide 1-7, the sequence of miR-10 starts with an U so the perfect match for position 1 is already an A) indicates presence of them primarily in the parts 3’ to the microRNA itself (i.e. in parts of the cluster that are expressed more anteriorly). Detailed analysis of the HoxBa cluster results in the identification of seed target sequences associated with mature Hox transcripts: 2 located in the 3’ UTR of HoxB1a, 2 in the 3’ UTR of HoxB3a and 3 in the HoxB3a open reading frame (fig.2A). E-YFP sensor constructs containing the HoxB1a and HoxB3a 3’UTRs with wildtype or seed point mutant target sites predicted to abolish interaction with the microRNA, were tested for their sensitivity to silencing by miR-10 (fig.2B). 100pg sensor mRNA was injected with or without 1nl 20 μM miR-10 siRNA. Coinjection with 10pg E-CFP mRNA was used as a loading control. Both HoxB1a and HoxB3a wild type sensor constructs are strongly repressed by the microRNA (fig.2C) while the HoxB3a seed point mutant construct proves insensitive to repression by miR-10.

Figure 2) In the HoxBa cluster miR-10 target sites are associated with HoxB1a and hoxB3a.
A) MiR-10 seed bindingsites (nucleotide 1-7) (orange) within the sense strand of the HoxBa cluster; known and EST database inferred mature Hox transcripts are indicated in blue. The position of miR-10c is shown in green. B) Schematic presentation of the HoxB1a and HoxB3a E-YFP sensor constructs and the HoxB3a overexpression construct. The red sites represent target sites (seed 1-7). The light red site in the HoxB1a 3’ UTR is a site T 2-7 which is also mutated in the HoxB1a mutated sensor construct. C) Validation of the HoxB1a and HoxB3a E-YFP sensor constructs. Blastula stage embryos were injected with sensor construct and E-CFP with or without coinjection of a miR-10 siRNA. E-CFP is shown as loading control, wildtype (WT) are silenced by miR-10 while seed mutant (mut) constructs are insensitive. D) Validation of the HoxB3a ORF as miR-10 target. The phenotypic effects of HoxB3a RNA overexpression are rescued by coinjection of the miR-10 siRNA.
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The seed mutant construct for *HoxB1a* in which the two target sites are mutated is still partially silenced however after coinjection with *miR-10* (data not shown). Closer inspection reveals a 3rd possible target sequence corresponding to nucleotide 2-7 flanked by a T on position 1 (light red box fig.2B). After introduction of a point mutation in nucleotide 3 of this seed sequence the construct is no longer repressed by *miR-10* (fig.2C).

A phenotypic sensor assay was employed to validate the target sites located in the *HoxB3a* ORF; overexpression of 40pg *HoxB3a* RNA induces very strong phenotypes with both anterior and posterior truncations of the embryo (fig.2D). These effects can be completely rescued by coinjection of 1 nl 20μM *miR-10* siRNA which indicates absence of overexpressed *HoxB3a* protein. These experiments identify the predicted *HoxB1a* and *HoxB3a* 3’UTR and ORF target sites as genuine mediators of *miR-10* activity.

In the other *Hox-1* and *Hox-3* paralogue members in the *HoxAa*, *HoxBb*, *HoxCa* and *HoxDa* clusters one putative target site is present in the *HoxA3a* 3’ UTR (777 nt downstream of the ORF), one putative target site associated with the *HoxB1b* 3’ UTR (125 nt downstream of the ORF) and *HoxA1a* has a seed sequence located 5472 nt downstream from its ORF sequence. No seed sequences are associated with the *HoxC1a*, *HoxC3a* or *HoxD3a* coding region or 3’ UTRs.

**Response of endogenous target transcripts upon overexpression or knockdown of miR-10**

Besides interfering with translational processes, targeting by microRNAs leads to reduced transcript stability and decreases the amounts of transcript present (19, 34). RNA levels can therefore function as indicator of a transcript/microRNA interaction (19).

In situ hybridization on embryos injected at the one cell stage with 1nl 20μM *miR-10* siRNA, using probes derived from exon 1 coding sequences, shows a strong downregulation of the target genes *HoxB1a* and *HoxB3a* but not of *HoxB2a, B4a, B5a, B6a, B7a or B8a*, genes that are predicted not to be targets (fig.3C,h right panels marked *miR-10* siRNA and data not shown). Notably, *HoxB3a* is only downregulated in its anterior strong domain of expression and not in its weaker posterior domain, suggesting that within the latter domain the repressive effect of the endogenous *miR-10* is saturated and not elevated by any experimental surplus. In situ hybridization with a probe derived from the ‘sensor part’ of the 3’ UTR of *HoxB3a* shows that this region of the transcript is expressed.
both within the anterior and posterior domain of $HoxB3a$ and responds in the same way to overexpression of the microRNA (supplementary fig.1B). Relative quantitativeness of the method was assessed by control double in situ hybridization using $HoxB1a$ and $HoxB4a$.

These experiments show that it is well possible to visualize different responses of the genes (fig.3F). We also looked at the response of $HoxA3a$, $HoxC3a$, $HoxD3a$, $HoxB1b$ and $HoxA1a$ to overexpression of miR-10. Of these genes only $HoxA1a$ responds very strongly to overexpression of miR-10 and has a strongly reduced expression comparable to the effect of miR-10 on $HoxB1a$ (fig.3C).

Processing and production of a mature miRNA can be effectively blocked by a morpholino directed against a microRNA precursor (21). Since morpholinos allow mismatches with the target sequence it is possible to redundantly target several miRNA isoforms using fewer morpholino sequences. ClustalW alignment of the 5 microRNA precursors reveals a region of extended conservation in the stemloop 5’ to the mature miR-10 (fig.3A) allowing the design of two morpholinos with only minor overlap to control against off-target effects.

Morpholino 1 (MO 1) consist of a mix of two morpholinos directed against the miR-10a and miR-10b sequences, morpholino 2 (MO 2) is one morpholino directed against the upstream conserved sequence (fig.3A). Both morpholinos have maximally one nucleotide mismatch with any of the miR-10 isoforms. Injection of either 5ng of MO 1 or MO 2 leads to absence of the signal in in situ hybridization using LNA probes, showing that the processing is efficiently inhibited (fig.3E and data not shown).

Injected embryos were analyzed for the expression of Hox genes (fig.3C). In the morpholino injected embryos a second $HoxB1a$ expression domain appears more posteriorly in the hindbrain/spinal cord transition. When miR-10 knockdown embryos are stimulated with $10^{-6}$ retinoic acid which upregulates both $HoxB1a$ and miR-10c, a very strong upregulation of $HoxB1a$ but not $HoxB4a$ is observed (fig.4).

In morphant embryos $HoxB3a$ shows a similar upregulation in posterior domains, although to a lesser extent. More posterior $Hox$ genes and $HoxB2a$ which are also not affected by the overexpression of miR-10 siRNA do not respond to the morpholinos. None of the genes reacted to injection with up to 10ng of a Genetools standard control morpholino or a miR-10b antisense morpholino (data not shown). These data are consistent with the predicted targeting and silencing of $HoxB1a$, $HoxB3a$ by miR-10 within its domain of expression. Surprisingly both $HoxA1a$ and $HoxB1b$ are also upregulated strongly in
posterior domains in miR-10 morphant embryos (fig. 3C, E) suggesting direct de-repression by miR-10 or activation by the now de-repressed HoxB1a gene.
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Figure 3) Effect of miR-10 knockdown and overexpression on endogenous Hox target transcripts

A) Clustal W alignment of the 5 Zebrafish miR-10 precursor sequences with indications of the positions of the mature microRNA, the hairloop and the miR-10* (antisense) position. The target sequences for both morpholino 1 and 2 are indicated yellow. B) Phenotypes of 72 hours post fertilization (hpf) embryos injected with miR-10 siRNA of morpholino. Note that the injected embryos look indistinguishable from the non injected controls (NIC). C) Whole mount in situ hybridizations with probes for HoxB1a, B2a, B3a, B4a and B5a on 24hpf embryos injected with morpholino 1 or 2 (MO 1 or MO 2), miR-10 siRNA or non injected controls (NIC). Embryos were stained equally long and staining was continuously monitored and stopped before reaching signal saturation. D) In situ hybridization with HoxB1b probe on 24 hpf embryos injected with MO1, MO2 or miR-10 siRNA. In the morphants there is upregulation, although no downregulation is observed in the miR-10 siRNA injected embryos. E) Effect of morpholino knockdown on the endogenous expression of miR-10b and miR-10c in 72 hpf embryos. F) Double whole mount in situ hybridizations on 24hr embryos using exon 1 coding sequence probes for HoxB1a and HoxB4a. Embryos were stained equally long till adequate staining was obtained for the HoxB4a probe.

Overexpression of miR-10 induces phenotypes associated with loss of HoxB1a and Hox-B3a but not HoxB1b

The phenotypes of both morphant and overexpression embryos during the first 5 days of development are remarkably normal without any apparent defects observed (72 hpf embryos shown fig.3B). However, as miR-10 appears to target HoxB1a and HoxB3a and possibly other 1 and 3 paralogue genes, the phenotype of miR-10 overexpression is expected to at least combine the loss of function phenotypes for these genes.

In Zebrafish, morpholino studies have shown that HoxB1a is required for the correct patterning of rhombomere 4 together with HoxB1b (35). Double knockdown of HoxB1a together with HoxB1b leads to absence of the primary mauthner neurons which run from rhombomere 4 down the spinal cord. Single knockdown of HoxB1a does not affect mauthner neurons but results in the failure of the branchiomotor neurons of the VIIth cranial nerve to migrate out of rhombomere 4. The knockdown of HoxB3a and HoxA3a has been shown to result in downregulation of the expression of the gcm-2 gene in the branchial arches (36). Loss of Hox-3 genes does not seem to lead to defects in the thyroid/parathyroid system as it does in mice (36, 37).

Analysis in 72 hr miR-10 overexpression and knockdown embryos by immunolabeling with primary neuron specific 3A10 antibody shows that the mauthner neurons which can be redundantly specified by both HoxB1a and HoxB1b still form normally (fig.5A).
Figure 4) Effects of miR-10 knockdown and treatment with retinoic acid. Shown are wildtype (WT), wildtype treated with $10^{-6}$M retinoic acid, control morpholino (CMO) treated with $10^{-6}$M retinoic acid, MO1 injected treated with $10^{-6}$M retinoic acid and miR-10 siRNA treated with $10^{-6}$M retinoic acid. In situ hybridization is done with probes for *HoxB1a* and *HoxB4a*. Note the strong upregulation of *HoxB1a* in retinoic acid treated morphants.

Figure 5) Overexpression of miR-10 phenocopies loss of *HoxB1a* and Hox-3 genes. A) 3A10 immunolabeling in 72 hpf embryo showing primary hindbrain motorneurons. The rhombomere 4 mauthner neurons are visible as horizontal stripes marked M. B) Confocal laserscanning microscope images of hindbrain regions of 5 days post fertilization retrograde labeled (RGL) embryos. All embryos show the same pattern of reticulospinal neurons, mauthner neurons are marked M. C) Flat mounted hindbrain region of 30 hpf embryos in situ hybridized with islet-1 and tag-1 showing the pattern of branchiomotor neurons. The VIIth nerve is non migratory in miR-10 siRNA injected embryos. D) *gcm-2* expression in 48 hpf embryos, note the downregulation in the miR-10 siRNA injected embryo. E) Rescue of the VIIth cranial nerve by coinjection of 5pg *HoxB1a* RNA together with the miR-10 siRNA. The normal pattern of VIIth nerve migration into r5/6 is restored.
Retrograde labeling in day 5 embryos also reveals a normal pattern of reticulospinal neurons projecting from the hindbrain into the spinal cord (fig.5B). Labeling by the secondary motoneuron specific marker *islet-1* or VIIth nerve specific *tag-1* in situ hybridizations shows that the VIIth nerve branchiomotor neurons do not migrate into rhombomere 5 and 6 anymore but stay in rhombomere 4 in miR-10 overexpression embryos (fig.5C). The pattern of the Vth, IXth and Xth branchiomotor neuron nerves as visualized by *islet-1* appears normal. To show that the VIIth nerve defect is directly due to targeting of *HoxB1a* by miR-10 and to exclude that it directly affects downstream processes, we rescued the miR-10 overexpression by coinjecting 5pg *HoxB1a* RNA from a construct that does not contain any of the target sites. 5 pg *HoxB1a* on its own does not induce any phenotype but is able to rescue the migration of the VIIth nerve in the miR-10 siRNA coinjected embryos (fig.5E). 72 hpf embryos were analyzed for the expression of *gcm-2*. In miR-10 overexpression embryos we observe downregulation of *gcm-2* (fig.5D) in the brachial arch region, as would be expected for embryos with impaired *HoxA3a* and/or *HoxB3a* expression. (36). These analyses show that miR-10 is able to induce specific phenotypes associated with the loss of function of *HoxB1a* and *HoxB3a* / *HoxA3a* genes but not *HoxB1b*. The same analyses in miR-10 morphant embryos show patterns similar to wildtype embryos (supplemental fig.3).

**Evolutionary conservation of the target sites in the HoxB cluster**

Evolutionary conservation of sequence information is considered a good indicator of functionality and is used in microRNA target prediction programs (33) to assign confidence levels. We searched the anterior part of the HoxB clusters in Medaka, Three spined stickleback, *Tetraodon, Takifugu, Xenopus*, Opossum, Mouse, Rat, Cow and Human for the presence of putative miR-10 bindingsites (seed nucleotide 1-7). In figure 6A the anterior parts of the HoxB and HoxBa cluster homologues are shown with indication of the identified seed sequences. Clear is the conservation of miR-10 target sites in the 3’UTR and coding regions of *HoxB3(a)* genes; all species investigated have at least 2 target sites associated with the ORF or 3’ UTR region. The sites in the HoxB1(a) show a weaker conservation profile and are most prominently present in Zebrafish. All the Teleosts HoxB1 genes for which sequence information could be found (note that the available HoxBa Medaka contig stops 300 nt downstream of HoxB1) posses a miR-10 target site in their 3’
UTR. This shows that repression of HoxB3 by miR-10 is probably conserved throughout the vertebrates and the interaction with HoxB1 is conserved in Teleosts.

**MiR-10 is required for correct branching of the Xth nerve and trunk motorneuron migration**

As shown above the loss of function of miR-10 leads to an upregulation of anterior Hox genes but information about the phenotypic consequences (i.e. what does miR-10 mean to the survival and live of the fishes) has to come from a loss of function approach. Most interesting therefore are the phenotypic consequences of the miR-10 knockdown in morphant embryos. As mentioned before the phenotypes of both MO 1 and MO 2 injected embryos are remarkably normal (fig.3B); embryos and larvae, although slightly delayed, seem to develop as controls till at least day 5. Also, analysis by transversal and sagittal sectioning of 5-8 day old embryos did not reveal any specific differences in morphology between miR-10 morphants, overexpression embryos, control morpholino injected embryos or wildtypes (data not shown).

Morphants were subjected to detailed marker analysis with respect to the developmental integrity of multiple anatomic structures positioned within the endogenous miR-10 expression domain. Neural and neuronal structures were investigated using amongst others zn-1, zn-5 and acetylated tubulin antibodies. Head and trunk muscles were studied using MyoD probe, MF-20 and 12/101 antibodies and the formation of the skeleton was assayed using alcian blue and alizarin red staining. No differences from controls were observed in neuronal patterns and musculature (data not shown). The formation of the endoskeletal disks as assayed by alizarin red and alcian blue at 5-8 day embryos also appeared essentially normal (data not shown). The thyroid, parathyroid and thymus were investigated using Nkx 2.1a, thyroglobulin, scl5a5 and rag-1 probes (Alt et al. 2006) but no defects were detected (data not shown).

Using in situ hybridization with islet-1 and immunostaining with anti acetylated tubulin antibody the formation and structure of the motorneurons were investigated. The motorneuron bodies are located in the hindbrain and spinal cord and have axonal projections innervating musculature in the rest of the body. Cranial nerves are the motorneurons located in the hindbrain that innervate the face and branchial arches; the Vth nerve originating from rhombomere 2 and 3 innervates the mandibular arch (1st arch), the
VIIth nerve originating from rhombomere 6 and 7 innervates the hyoid (2nd arch), the IXth nerve from rhombomere 7 the 3rd arch and the Xth nerve from rhombomere 8 arch 4 to 7 (38). In the spinal cord, motorneurons develop as spinal ganglia, one per somite, which project out of the neural tube and, amongst others, innervate the pectoral fins.

In 24 and 48 hpf miR-10 knockdown embryos there are no changes in the development of the motorneurons as visualized by islet-1 staining and the nerve bodies appear to form correctly and migrate to their proper positions in the hindbrain (supplemental fig.3). Also in the spinal cord no differences were observed using islet-1 or tag -1 as markers (data not shown). Islet-1 is a good marker gene for motorneurons in situ hybridizations but as the mRNA is only located in the nerve bodies (and the protein in the nucleus) it cannot be used as a marker to obtain a detailed image of the nerve morphology. Immunostaining with anti acetylated tubulin antibodies however is a good method to label outgrowing axonal structures. In wildtype 72 hr embryos the outgrowing axons of the VIIth, IXth and Xth nerves are clearly visible each with a distinct pattern. In miR-10 knockdown embryos there are severe disruptions of the morphology of the outgrowing Xth nerve; instead of branching and migrating into the 4 posterior pharyngeal arches, the Xth nerve more resembles the IXth nerve morphology and has only one or two branches or fails to split at all (fig.7B ). In the same larvae where we observe Xth nerve defects, the IXth and Vth nerve still have a wildtype morphology. Analysis of the branchial arch skeleton in 5 day embryos by alcian blue staining reveals no abnormalities, excluding that the failure of the Xth nerve to split and migrate correctly might be due to absence or mispatterning of one of the arches. All structures and patterns of cartilage formation appear normal data not shown.

More posteriorly in the trunk region the formation of the spinal ganglia shows the normal segmental pattern (fig.7A). The pattern of migration over the yolk is, however, often disturbed and there is more frequent branching and that the axonal projections follow non-parallel paths. The most frequent and distinct defect observed in the migration of the trunk motorneurons is a failure in ~ 80% of the embryos (MO1: 97/124, MO2: 81/107) of the spinal ganglion to correctly migrate into the posterior pectoral fin. Instead the axon bypasses the fin and migrates to the position of the posterior ganglion.
Figure 6) Conservation of miR-10 targetsites in the HoxB cluster.
A) The presence of putative miR-10 target sites (seed 1-7 sequence) in the anterior HoxB(a) cluster in all species for which relevant sequence information could be retrieved. The target sites are indicated in green and the Hox open reading frames in light blue. Conservation groups are indicated with dotted lines. B) Schematic representation of miR-10 and target gene expression in the Zebrafish hindbrain. MiR-10 is expressed posterior from the rhombomere 6/7 boundary. The target genes HoxB1a and HoxB3a are expressed in a strong domain (dark colour) anterior in the anterior hindbrain and in a weaker domain (light colour) in the area where they overlap with miR-10. HoxB1a shows a gap in expression in r5 and r6, possibly due to transcriptional repression by Hox-3 genes.

Figure 7) Neuronal defects in miR-10 morphant embryos.
A) Migration of motoneurons revealed by acetylated tubulin immunostaining in MO2 morphant embryos. The migration pattern over the yolk is chaotic compared to non injected controls and migrating neurons end up at different positions. B) Defects in 72 hpf embryos in the migration of the Xth cranial nerve revealed by acetylated tubulin immunostaining. The Xth nerve fails to split into the 4 branches innervating the posterior branchial arches in morphant embryos. In miR-10 overexpression embryos no defects are observed.

Discussion

MiR-10 is expressed in the hindbrain and spinal cord posterior from the rhombomere 6/7 boundary and occupies an axial domain similar to that of Hox-4 paralogue genes. We show an interaction between miR-10 and the anterior Hox-B1a and HoxB3a genes. These Hox genes have an anterior strong domain of expression in the hindbrain and are expressed at a low level in the spinal cord where they overlap with miR-10 expression (fig.6B).

In Zebrafish, Hox-1 paralogues are present in the HoxAa, HoxBa, HoxBb and HoxCa clusters and Hox-3 paralogues genes are present in the HoxAa, HoxBa, HoxCa and HoxDa clusters. Of these genes only the HoxA1a, HoxB1a, HoxA3a and HoxB3a genes are possible miR-10 targets. The HoxC1a, HoxC3a and HoxD3a genes all do not posses good candidate target sites in their vicinity and do not respond to either overexpression or knockdown of miR-10. It remains to be seen whether HoxA1a, HoxB1b, and HoxA3a are also true targets; HoxA1a, despite not being associated with a clear nearby target site, responds strongly to the loss and gain of miR-10. HoxB1b possesses a candidate target site and is upregulated in morphant embryos. The fact that the expression levels seem unresponsive to the overexpression of miR-10 may be caused by its expression exclusively within the miR-10
expression domain; just as we expect it to be the case for HoxB3a, it is possible be that no response is triggered because the addition of an experimental surplus of miR-10 does not elevate the level of silencing.

What, however, is pleading strongly against the targeting of HoxB1b is that the overexpression of miR-10 does not induce the phenotypic changes as observed in the double HoxB1a/HoxB1b knockdown (35). As there is extensive crossregulation between Hox paralogues, it is also possible that the effects observed are a direct result of altered levels of HoxB1a RNA. That HoxA3a, which also possesses one candidate target site, is not affected does not necessarily mean the gene is not targeted by miR-10. MicroRNAs affect target genes by both inhibition of translation and degradation of messengerRNA (19, 34). To which extend these processes are coupled and whether translational inhibition is always accompanied by an increase in messengerRNA decay is not yet known. It is thus theoretically possible that the effect of HoxA3a repression will only be noticeable at the protein level.

There could be several reasons why miR-10 targets particular Hox-1 and Hox-3 genes and not others. One explanation is that there is a high degree of subfunctionalization within the paralogues groups as was nicely illustrated for the Zebrafish Hox-1 genes (35). It appeared that the functions of HoxB1a, HoxA1a and HoxB1b are only partially interchangeable with only HoxB1a and HoxA1a having influence on the position of the VIIth nerve. These different abilities to interfere with particular developmental processes also suggest that different consequences will be associated with the expression outside their main domains. This could create needs for posttranscriptional silencing that differ from paralogue member to another.

The system of Hox regulation is characterized by the phenomenon of posterior prevalence. Already early on it was noticed that the knockout of a certain Hox gene in mouse would only lead to defects in the anterior most part of its expression domain. It was established that there is a certain hierarchy in the functioning of the Hox genes that makes posterior genes are always dominant in the determination of a regional phenotype over coexpressed anterior genes (2). In this model it is difficult to understand why anterior Hox-1 and 3 genes would need to be silenced in posterior domains, as there already should be phenotypic suppression by more posterior Hox genes in that region. However, a recent study has placed this model in a different light. It turned out that knockout of all paralogue 5 or 6
group genes leads to patterning defects throughout the thoracic region and much more posteriorly than expected (41). Based on these results it does not seem impossible that Hox-1 and Hox-3 genes could influence the phenotype despite coexpression of more posterior genes. The existence of the posttranscriptional repressive interactions between miR-10/HoxB1a and HoxB3a could in fact be contributing to the posterior dominance effect. The inhibition of miR-10 leads to posterior upregulation of the targeted genes. Since microRNAs cause downregulation of the target messengerRNA, it has been a frequently debated issue whether low or absent levels of target gene expression within the microRNA domain reflect an avoidance of expression or that they are a direct consequence of the downregulation by the microRNA (40). Inhibition of miR-10 leads to posterior target gene upregulation in the case of HoxB1a, HoxA1a and HoxB3a but certainly not to a level as found in the dominant anterior expression domain. It thus appears that the restriction to the dominant expression domain is occurring primarily at the transcriptional level and that it is in a posterior domain with an already low level of transcription where silencing by the microRNA occurs. This observation is consistent with the identification of rhombomere specific transcriptional Hox enhancers in mouse (41, 42). In the case of the microRNA target interactions described in this study it seems that both transcriptional avoidance and a direct repression by the microRNA are shaping the mRNA expression domains in the embryo. The effects at the transcript level likely reflect an on/off situation at the protein level, where all translation is silenced while there is still a significant amount of messengerRNA detectable.

In Zebrafish and Xenopus tropicalis (43) it has been shown that overexpression of let-7 induces similar phenotypes. We also overexpressed miR-10 in Xenopus laevis. Surprisingly, injection of already very small amounts of miR-10 siRNA blocks early cell divisions and is completely lethal (< 60 minutes after injection). The possibility therefore exists that miR-10 functions in a complete different way in Xenopus than in Zebrafish. As has been often noticed however (e.g. reference40), the accidental acquisition of a microRNA target site in a gene that is naturally never coexpressed with a microRNA in vivo would have no influence on the regulation of this gene in normal life or development. We believe that it is much more likely that in this case one or more early expressed Xenopus genes contain such biological irrelevant target sites. In the target prediction section of miRBase, where the output of the Miranda algorithm (http://microrna.sanger.ac.uk/) is listed, there are 1969
predicted target genes for Zebrafish miR-10. With respect to the number of predicted target genes, miR-10 scores second, only outnumbered by let-7 which has 2016 targets, while the average number of predicted targets for a microRNA in Zebrafish is 783. The high number of targets seems incompatible with the weak and very specific phenotypic defects observed in the miR-10 overexpression embryos, which are virtually indistinguishable from wildtypes, besides the VIIth nerve defect and altered gcm-2 expression. Absence of further severe developmental defects is further corroborated by a preliminary micro-array experiment where virtually no genes differ in expression levels upon overexpression of miR-10. These data strongly suggests, that, at least for miR-10, there is a very high component of false positives in the outcome of target prediction algorithms.

As a specific phenotype in morphant embryos we do consistently observe defects in neuronal migration of the Xth nerve and of the motorneurons innervating the pectoral fins (supplementary fig.4). In miR-10 knockdown embryos there are severe disruptions of the morphology of the outgrowing Xth nerve; instead of branching and migrating into the 4 posterior pharyngeal arches the Xth nerve more resembles the IXth nerve morphology and has only one or two branches or fails to split at all (fig.7B). In the same larvae where we observe Xth nerve defects, the IXth and Vth nerve still have a wildtype morphology.

Analysis of the branchial arch skeleton in 5 day embryos by alcian blue staining reveals no abnormalities excluding that the failure of the Xth nerve to split and migrate correctly might be due to absence or mispatterning of one of the arches. All structures and patterns of cartilage formation appear normal (data not shown).

More posteriorly in the trunk region the spinal ganglia are present in the normal segmental pattern. The pattern of migration over the yolk is however disturbed, as there is more frequent branching and that the axonal projections follow non-parallel paths (fig.7A). The relation of these phenotypes to the derepression of anterior Hox genes in the morphant embryos is however unclear; overexpression of HoxB1a, HoxA1a, HoxB3a or HoxA3a does not result in defects as observed in miR-10 morphants (data not shown). We have also performed double knockdown of miR-10 with either HoxB1a, HoxB3a or HoxA3a which did not rescue the neuronal migration defects. These experiments are however not very reliable, as the overexpression of Hox gene induces very severe early phenotypes (as for instance in fig.2D). To be able to analyze late stage developmental anatomy, like the morphology of the cranial nerves, lower RNA concentrations have to be used to avoid all
too heavy early effects. These doses, however, may simply not have sufficient impact to mimic the endogenous levels at these late stage developmental events. Unfortunately there is no good inducible system available in Zebrafish, enabling the timed overexpression of these genes. In case of a rescue by knockdown of target genes it may be necessary to knock all of them down at once; a complicated experiment due to nonspecific toxicity of high doses of morpholinos. Another explanation of course is that these phenotypic defects in the morphants are unrelated to the loss of repression on the anterior Hox genes and that they are induced by other target genes. Nevertheless, these phenotypes represent the first physical consequences reported for the loss of a Hox related microRNA.

The Hox genes are involved in specifying regional morphology in the axial skeleton where they define different types of vertebrae according to the so called ‘Hox code’ (44, 45). As the differences in vertebral development only become clear at the end of the larval stage in Zebrafish (about 1 month after fertilization) (46) we have not been able to investigate a possible involvement of miR-10 in the mesodermal Hox code which is also a candidate place of action.

Material & Methods

Zebrafish husbandry and embryo culturing
An AB x TL strain of Zebrafish was used; housing and embryo collection was according to standard procedures; embryos were cultured at 28°C.

RT-PCR
Whole embryo RNA was isolated using Tri-pure (Roche) and reverse transcribed using MuMLv Reverse transcriptase (Roche) using oligo-dT N=18. Primer sequences;
miR-10c up; (AGCTGGCTTTCTCAATACC),
miR-10c down; (TACATACTCCCCTAGATACGAA),
HoxB4a exon1 up; (ATGGCCATGAGTTCCTATTTG ),
HoxB4a exon1 down; (TTGGTTCACCCCTGAATTAG),
HoxB4a exon1 5’down; (TTGTGGTGTTGAGTGCCTTCTGAATAG). DNA oligos were obtained from Biolegio, Malden, the Netherlands.
Micro-injection

Embryos were injected with 1 or 2 nl at the one cell stage; RNAse free phenol red was added as tracer to injection mixes prior to injections.

Morpholinos were obtained from genetools, OR, USA; miR-10 morpholino 1 corresponds to a mix of miR-10a (CACAAATTCGGATCTACAGGGTA) and miR-10b (CACAAATTCGGTTCTACAGGGTA) antisense morpholino, the sequence of miR-10 morpholino 2 is (TCTACAGGGTATATAGACGAC).

RNA oligos were obtained from Biolegio, Malden, The Netherlands. The miR-10 siRNA sense strand corresponds to miR-10a (UACCCUGUAUCCGAUUUGUGUG) and miR-10b (UACCCUGUAAGAACCUGGGTA) and the sequence of the antisense strand is (CACAAAUUCGGAUCUACAGGGCAU). Note that the antisense sequence has mismatches with the miR-10 sense strand at its 3’ end resulting in the specific incorporation of the sense miR-10 strand in the microRNA silencing complex. Oligos were annealed to siRNAs by gradually cooling from 98°C to 20°C in buffer in 500ml H2O beaker glass; 30ul 50μM of each oligo, 15ul annealing buffer (50mM Tris, pH 7.8, 100mM NaCl RNAse free) in 75 ul, final concentration of siRNA is 20μM.

RNA for injection was transcribed using Ambion Sp6 message machine kit and purified using an RNA easy column (Qiagen), from the CS2+ plasmids; CS2+HoxB1a sensor wt, CS2+HoxB1a sensor mut, CS2+HoxB3a sensor wt, CS2+HoxB3a sensor mut, CS2+Dre-HoxB3a ORF, CS2+Xl-HoxB4-Myc, CS2+E-YFP, CS2+E-CFP.

In situ Hybridization

In situ hybridization was performed according to standard procedures. Hybridization temperatures were 65°C for normal probes and 56°C for LNA probes. In double in situ hybridizations with a LNA probe 56°C was used. In double in situ hybridization DIG and fluorescein labeled probes were used. Embryos were stained using BM-Purple (Roche) and Fast Red (Roche). Probes were synthesized using T7 and Sp6 polymerase (Roche) in the presence of labeled nucleotides (RNA DIG or fluorescein labeling mix, Roche) from pGEM-TE plasmids containing: HoxB1a and HoxB3a-HoxB8a exon 1 coding sequence, HoxB2a exon 2/3’UTR, HoxB1b exon1/2 coding sequence; HoxB3a splv2 was synthesized from PCR product from a partial cDNA cloned in pGEM-TE.
LNA probes were obtained from Exiqon, Denmark and sequences are:

\( miR-10a \) ;(CACAAATTCCGGATCTACAGGGTA),
\( miR-10b \) ;(ACAAATTCGGTTCTACAGGGTA),
\( miR-10c \) ;(CACAAATCCGGATCTACAGGGTA),
\( miR-10d \) ;(ACACATTCCGGTTCTACAGGGTA).

Our step by step in situ protocol is available on request.

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**Supplementary figure 1) Expression of HoxB3a exon1 and HoxB3a sensor.**
A) HoxB3a coding region and 3’ UTR. Probe regions exon1 and sensor are indicated and expression of HoxB3a exon1 and HoxB3a sensor in 24 hpf embryos. B) Response of HoxB3a sensor to miR-10 siRNA injection in 24 hpf embryos. A similar down regulation is observed as for HoxB3a exon 1.

**Supplementary figure 2) Expression of HoxB3a with miR-10a, miR-10b, miR-10c and miR-10d.**
Expression of HoxB3a exon 1 (red) and LNA probes for all 4 Zebrafish miR-10 isoforms. All miR-10 isoforms are expressed more posterior than the r5/6 domain of HoxB3a. MiR-10b and miR-10d seem to be expressed slightly more posterior than miR-10a and miR-10c.
Supplementary figure 3) Patterns of primary and secondary hindbrain motorneurons in miR-10 overexpression and morphant embryos.

Column 1; wildtype, column 2; morpholino 1 injected, column 3; morpholino 2 injected, column 4; miR-10 siRNA injected (also shown in main figure 5)

A) 3A10 immunolabeling. Mauthner neurons are present in morphant and overexpression embryos and are indistinguishable from wildtype embryos. B) Confocal images of hindbrains of 5 day old retrograde labeled embryos. No differences are observed between non injected, morphant or overexpression embryos. C) Flatmounts of 30 hpf embryos in situ hybridized with islet-1. Patterns of brachiomotorneurons are indicated. Note the failure of the VIIth cranial nerve to migrate into r 5/6 as shown before. The motorneuron patterns in the morphant embryos are like wildtypes. D) Sideview of 48 hpf embryos stained with islet-1. In miR-10 siRNA injected embryos the VIIth nerve is located near the Vth nerve and there is a large gap between the VIIth and the IXth nerve. The patterns in the morphant embryos are like wildtypes.