RESEARCH ARTICLE

FGF-4 Signaling Is Involved in mir-206 Expression in Developing Somites of Chicken Embryos

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The microRNAs (miRNAs) are recently discovered short, noncoding RNAs, that regulate gene expression in metazoans. We have cloned short RNAs from chicken embryos and identified five new chicken miRNA genes. Genome analysis identified 17 new chicken miRNA genes based on sequence homology to previously characterized mouse miRNAs. Developmental Northern blots of chick embryos showed increased accumulation of most miRNAs analyzed from 1.5 days to 5 days except, the stem cell–specific mir-302, which was expressed at high levels at early stages and then declined. In situ analysis of mature miRNAs revealed the restricted expression of mir-124 in the central nervous system and of mir-206 in developing somites, in particular the developing myotome. In addition, we investigated how miR-206 expression is controlled during somite development using bead implants. These experiments demonstrate that fibroblast growth factor (FGF) -mediated signaling negatively regulates the initiation of mir-206 gene expression. This may be mediated through the effects of FGF on somite differentiation. These data provide the first demonstration that developmental signaling pathways affect miRNA expression. Thus far, miRNAs have not been studied extensively in chicken embryos, and our results show that this system can complement other model organisms to investigate the regulation of many other miRNAs. ***Developmental Dynamics 235:2185–2191, 2006.***
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INTRODUCTION

One of the model organisms for studying vertebrate development is the chicken due to the experimental advantages of in ovo embryogenesis (Brown et al., 2003). Avian embryos also bridge the evolutionary gap between mammals and other vertebrates, and the chick is used as a model bird for approximately 9,600 avian species. The chicken genome consists of around a billion base pairs, which is about one third the size of the human genome and contains an estimated 20,000–23,000 genes, which is only slightly less than the 24,000 estimated human genes (Hillier et al., 2004; International Chicken Genome Sequencing Consortium, 2004). The difference in genome size is mainly due to a significant decrease in interspersed repeat content, pseudogenes, and segmental duplications within the chicken genome. Like the genome sequence of other organisms, the chicken genome encodes both noncoding RNAs (ncRNAs) and proteins. Recently, a new class of ncRNAs with a size of 20–22 nucleotides (nt) has been discovered in plants and animals and

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called microRNAs (miRNAs; for review, see Zamore and Haley, 2005). Biogenesis of miRNAs starts with the synthesis of a long precursor product (pri-miRNAs), which is cleaved by the RNaseIII type protein Drosha in the nucleus (Lee et al., 2003). The Drosha-generated pre-miRNA is a 70- to 80-nt RNA with a typical stem–loop structure. It is transported to the cytoplasm by Exportin-5 (Yi et al., 2003; Lund et al., 2004) where it is processed by another RNaseIII type protein, Dicer, to generate the mature (20- to 22-nt) miRNA (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). miRNAs have partially complementary sequences to certain mRNAs and guide a protein complex, RISC (RNA induced silencing complex), to the target mRNAs (Khvorova et al., 2003; Schwarz et al., 2003). If the sequence complementarity is very high, as in plants, RISC cleaves the target mRNA; however, if the miRNA is only partially complementary to the mRNA, RISC does not cleave it but suppresses the translation of the mRNA (Bartel, 2004). Recent work in zebrafish embryos has shown that most of the miRNAs are expressed during specific developmental stages and in particular cell types, although some are expressed ubiquitously (Wienholds et al., 2005).

A total of 122 miRNA genes have been identified in the chicken genome based on sequence homology to mammalian miRNA genes (Hillier et al., 2004; Hubbard et al., 2005; miRBAse Release 7.0 at http://microrna.sanger.ac.uk/sequences/), but none of them have been confirmed by Northern blot analysis. Hubbard et al. (2005) showed the expression of a few chicken miRNA genes by in situ hybridization using probes antisense to the precursor RNAs. This approach revealed restricted expression patterns in some cases; however, it did not allow the detection of several other miRNAs.

The segmented nature of the vertebrate body plan is most obvious when looking at somites (Pourquie et al., 2003). Somites are generated sequentially from the unsegmented presomitic mesoderm (psm) at the posterior end of the embryo. During their maturation, somites undergo significant changes; cells in the ventral somite undergo an epithelial to mesenchymal transition and give rise to the sclerotome, which contains progenitor cells for cartilage and bone. The dorsal somite forms the dermomyotome containing precursors, which will give rise to migratory and nonmigratory skeletal muscle cells. The changes that are observed in the differentiation of somites over time are represented along the embryonic axis, with more mature somites present anteriorly and less mature somites present posteriorly. Somite morphogenesis is closely linked to the progressive commitment of cells to adopt a particular fate, for example skeletal muscle, and both processes are governed and coordinated by networks of developmental signaling molecules that regulate gene expression (Buckingham, 2001). We have demonstrated, using somite explant cultures and reverse transcriptase-polymerase chain reaction (RT-PCR), that Wnt and Sonic hedgehog (Shh) signaling molecules, derived from the dorsal neural tube and notochord/floor plate, respectively, promote skeletal muscle specific gene expression and, thus, the myogenic differentiation program (Münsterberg et al., 1995; Schmidt et al., 2000). Recent work demonstrated that myotome-derived fibroblast growth factor (FGF) signals, acting through extracellular regulated kinase–mitogen-activated protein (ERK-MAP) kinase, specify a population of progenitor cells in the ventral somite, which gives rise to ribs and tendons (Smith et al., 2005).

To investigate their role in the control of cell differentiation during embryogenesis, and in particular myogenesis, we set out to identify novel microRNAs expressed during development and to characterize their expression and regulation. We have cloned short RNAs from 2- and 5-day-old chicken embryos, which represent the early stages of organogenesis and found five new chicken miRNAs. A systematic basic local alignment search tool (BLAST) search identified a further 17 miRNA genes in the chicken genome based on homology to known mouse miRNAs. Northern blot analysis of different developmental stage chicken embryos showed that the expression of most miRNAs was very low at 1.5 days of development and steadily increased until day 5, except mir-302, which showed a reverse pattern. We carried out whole-mount in situ hybridization (WISH) of some mature miRNAs and found that mir-206 was expressed exclusively in somites of several vertebrate model organisms, the mouse, chick, and *Xenopus* embryo. In the chick, we investigated the regulation of mir-206 by implanting beads soaked in FGF-4 next to forming somites. The results show that FGF-4 suppressed mir-206 expression. Pharmacological inhibitor beads demonstrate that FGF receptor activity is required for inhibition of mir-206 expression and suggest that the signal is mediated by ERK-MAP kinase. In addition, loss of mir-206 in response to FGF-4 correlated with loss of MyoD in this scenario. These data illustrate that expression of miRNA genes is likely to be regulated through similar pathways to protein coding genes. Furthermore, it demonstrates that, due to its accessibility and the ease with which it can be manipulated, the chicken embryo is particularly suitable to investigate the spatiotemporal regulation of miRNA genes.

RESULTS AND DISCUSSION

Characterization of Chicken miRNAs

A short RNA library has been generated from total RNA extracted from 2-day-old chicken embryos, corresponding to Hamburger and Hamilton (HH) stages 8–12 (Hamburger and Hamilton, 1951). A total of 1,063 short RNA sequences were obtained representing 501 nonredundant sequences (Supplementary Table S1, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat). From these sequences, 289 were fragments of rRNA, tRNA, mRNA, and snRNA; 15 were known chicken miRNAs (Supplementary Table S2); 97 were mapped to the chicken genome but could not be folded into a stem–loop structure with their flanking sequences; and 100 could not be mapped to the current release of chicken genome (WASHUC1). Because little was known about the expression of chicken miRNAs during

embryonic development, we analyzed the distribution of 12 miRNAs between 1.5 and 5 days using a developmental Northern blot (Fig. 1). The amount detected for all but one analyzed miRNA increased significantly within this period and several miRNAs were not detectable at 1.5 and 2 days even after 4 days exposure. This general pattern is very similar to that observed in *Xenopus* (Watanabe et al., 2005) and recently in zebrafish embryos (Chen et al., 2005). The exception was mir-302, which was very abundant at 1.5 and 2 days but was not detectable after 3 days. This finding is consistent with the finding that mir-302 is stem cell specific (Houbaviy et al., 2003; Suh et al., 2004). The changing ratio of embryonic stem cells versus more restricted progenitor cells and differentiated cells would explain this expression profile. Because miRNAs were generally more abundant at 5 days and because we were interested in identifying miRNAs involved in cellular differentiation, we decided to carry out another round of cloning using total RNA isolated from 5-day-old embryos. From 1,017 short RNA sequences (613 unique), 436 were fragments of rRNA, tRNA, mRNA, and snRNA; 397 were known chicken miRNA (representing 39 different miRNAs; Supplementary Table S2, which is available online at http://www.interscience.wiley.com/jpages/1058-8388/suppmat); 94 could not be functionally annotated but mapped to the chicken genome; and 90 could not be mapped on the current version of the chicken genome (WASHUC1) (Supplementary Table S1). We further analyzed the 94 mapped sequences with unknown function and found that five of them were mature miRNAs from the complementary arm of the precursor stem–loops of known chicken miRNAs, three of them have been cloned in other species (mir-199*, mir-126-5p, and mir-140*) but two of them, mir-124a* and mir-128a*, have not been cloned from any other species (miRBase, Release 7.0). Of the 94 sequences, 3 were new miRNAs, subsequently published from other species during the preparation of this manuscript: mir-363, mir-454b, and mir-490 (Bentwich et al., 2005; Chen et al., 2005); we also cloned mir-455*, which is generated from the other arm of the recently published mir-455 gene (Berezikov et al., 2005; Chen et al., 2005) but has not been found in other species (Supplementary Table S3). Finally, one of the short RNAs was identified as mir-143, which was a known miRNA in mammals that had not been annotated in the chicken genome, although 19 of 22 nt were identical with mismatches at the 1st, 21st, and 22nd positions (Supplementary Table S3). This latter observation suggested that there were other not yet annotated chicken miRNA genes with similarity to known mammalian miRNAs. Therefore, we carried out a systematic BLAST search using mouse miRNA sequences. We accepted a maximum of 2 mismatches between mature miRNA sequences, and the secondary structures of the selected putative chicken miRNA genes were checked for stem–loop structures. Through this bioinformatics approach, we have identified an additional 17 chicken miRNA genes (Supplementary Table S3).

Somite-Specific Expression Pattern of miRNA-206

Expression profiling of miRNAs by different microarrays showed that many are expressed in a temporally controlled manner and in specific tissues (Babak et al., 2004; Sun et al., 2004). The most informative technique to investigate the spatiotemporal expression pattern of genes is in situ hybridization, but initially, it could not be used for miRNAs because of the small size of the mature product. Valoczi and colleagues used LNA (locked nucleic acid) containing oligonucleotides for the first time to detect miRNAs on Northern blots (Valoczi et al., 2004). This work demonstrated that these probes are at least 10 times more sensitive than traditional oligos. Subsequently, LNA probes were successfully used to analyze the expression pattern of miRNAs by in situ hybridization in zebrafish (Wienholds et al., 2005). We applied LNA WISH to chicken embryos, and to establish the technique, we investigated the expression of mir-124a, because it has been found to be specifically expressed in brain tissue at high levels (Babak et al., 2004; Sun et al., 2004). Figure 2 shows the central nervous system–specific expression of mir-124a in chicken embryos that is consistent with the recent finding by Wienholds et al. (2005) in zebrafish. Next, we analyzed several other miRNAs cloned from 2-day-old embryos (mir-18a, mir-19b, mir-20b, mir-26a, mir-130a, and mir-206), but most of them showed widespread expression in HH18 embryos (data not shown). However, mir-206 showed a very specific expression in developing somites of chick, mouse, and *Xenopus laevis* embryos (Fig. 3). In chick, mir-206 was first detected at HH14 where it was seen in somites in a medial location, consistent with the emergence of the epaxial myotome at this position. The timing of expression suggested that mir-206 was expressed after the first somite-specific myogenic marker, the basic helix–loop–helix (bHLH) transcription factor MyoD at HH10. Expression became stronger at later stages but remained restricted to developing somites. At HH17, the medial restriction of mir-206 was apparent in posterior somites and staining was first detected at somite stage VII. As the myotome expanded, mir-206 staining appeared in a triangular shape. In anterior somites, mir-206 was expressed throughout the myotomes, including in cells emerging from the ventrolateral lips (Fig. 3A). Sections at HH17 through a somite stage XI showed the first detectable expression in the myotome proper and demonstrated high levels of mir-206 localized in more mature myotomes (Fig. 3B–E). The mir-206 is similar to mir-1, with three mismatches between the mature miRNAs. In zebrafish, these two microRNAs appear to have an almost identical expression pattern (Wienholds et al., 2005), which is more extensive than in chicken and includes the heart, somites, and skeletal muscles in the head. Furthermore, using a lacZ knockin strategy, it has been shown that mouse mir-1 is expressed in the heart and somite (Zhao et al., 2005). We found, however, that mir-206 was not expressed in the heart of embryonic day (E) 10 mouse embryos (Fig. 3G). Similarly, the expression of mir-206 was somite specific in *Xenopus* tadpoles (Fig. 3I–K). In contrast, mir-1 expression was detected in somites and heart in both mouse and chick embryos (Fig. 3E, and data not

shown). This finding suggested that the LNA-containing oligo probe can distinguish between mir-1 and mir-206 and indicates that they potentially can be used to dissect expression patterns of highly related miRNAs.

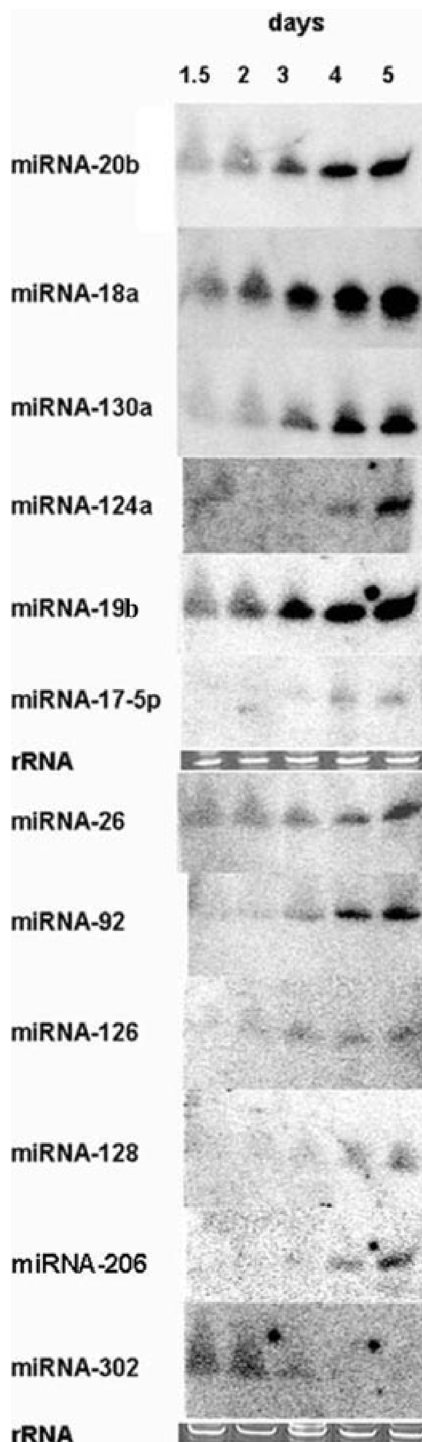

Fig. 1.

Fig. 1. Northern blot analysis of selected chicken microRNAs (miRNAs). Developmental stages are indicated at the top in days. Two membranes were probed at 37°C repeatedly with 5′ ^{32}P-radiolabeled oligonucleotides complementary to the cloned miRNA sequences using ULTRAhyb-Oligo Hybridization buffer (Ambion). Equal loading (30 μg of total RNA) is shown for both membranes by ethidium bromide staining of rRNA below each set. The membranes were exposed to phosporimager plates (Fuji BAS SR-type) overnight or 4 days. After exposure, the probes were removed from the membranes by 30 min incubation at 90°C in 0.1% sodium dodecyl sulfate, and the membranes were hybridized with other probes.

Transcriptional Regulation of mir-206

Next, we wanted to investigate the regulation of mir-206 gene expression during somite development by candidate signaling pathways that are known to control somite patterning and differentiation. We used heparin beads soaked in recombinant FGF-4 and implanted these beads adjacent to forming somites at different stages of embryo development. This strategy led to a dramatic loss of detectable mir-206 expression after overnight incubation (Fig. 4A). Between three and six somites were affected adjacent to the bead, depending on the size of the bead and its proximity to the paraxial mesoderm. Control somites on the opposite side expressed high levels of mir-206. Implanting an FGF-4 bead together with a bead soaked in SU5402, which blocks the FGF receptor tyrosine kinase, antagonized the inhibitory effect on mir-206 expression (Fig. 4B), demonstrating that receptor activity was required. Implantation of an SU5402 bead alone did not lead to ectopic or premature up-regulation of mir-206, suggesting that blocking endogenous FGF signals was not sufficient. The MEK-1 inhibitor PD184352 (Davies et al., 2000) also prevented effective FGF-4–mediated inhibition of mir-206 expression, suggesting that active ERK-MAP kinase may be involved (Fig. 4D). It is well documented that both FGF-4 and FGF-8 are expressed in the somite myotome concomitant with MyoD (Fig. 4E,F,H; Kahane et al., 2001; Stolte et al., 2002). We showed previously that FGF-8 beads and increased levels of ERK-MAP kinase inhibit MyoD expression in developing somites (Smith et al., 2005); similarly, FGF-4 beads blocked MyoD expression (Fig. 4G). Therefore, loss of detectable mir-206 correlated with loss of MyoD transcripts following FGF-4 bead implantation. Together with its expression in the myotome, this finding suggests a role for mir-206 in the myogenic differentiation program, which is sensitive to the levels of FGF-mediated signaling. Taken together, this study identified several new chicken miRNAs, established the expression profile of some of these miRNAs during embryogenesis and determined the specific expression pattern of mir-124 and mir-206. We also showed that FGF signaling affects mir-206 expression, possibly by disrupting the expression of myogenic bHLH transcription factors.

EXPERIMENTAL PROCEDURES

RNA Isolation

Total RNA was harvested from chick embryos of different ages using the method described by Chomczynski and Sacchi (1987). Briefly, embryos were lysed in 300 μl of lysis buffer (25 g of guanidinium thiocyanate, 1.76 ml of 0.75 M sodium citrate, pH7, 2.64 ml of 10% Sakosyl, 38 μl of β-mercaptoethanol, and 29.3 ml of H_2O). After adding 30 μl of sodium acetate, pH 4, 300 μl of acidic phenol (Sigma), and 60 μl of chloroform/isoamylalcohol (49:1) and vortexing, the mixture was incubated on ice for 10 min. After centrifugation, the supernatant was transferred to a fresh tube and RNA was precipitated with 750 μl of ethanol at −80°C for at least 1 hr. After centrifugation, the pellet was washed with 80% ethanol, air-dried, and resuspended in water. For older embryos, the volumes were adjusted. RNA quality and concentration was assessed on agarose gels.

Short RNA Cloning

Short RNAs were cloned using 300 μg of total RNA extracted from either 2- or 5-day-old embryos following the

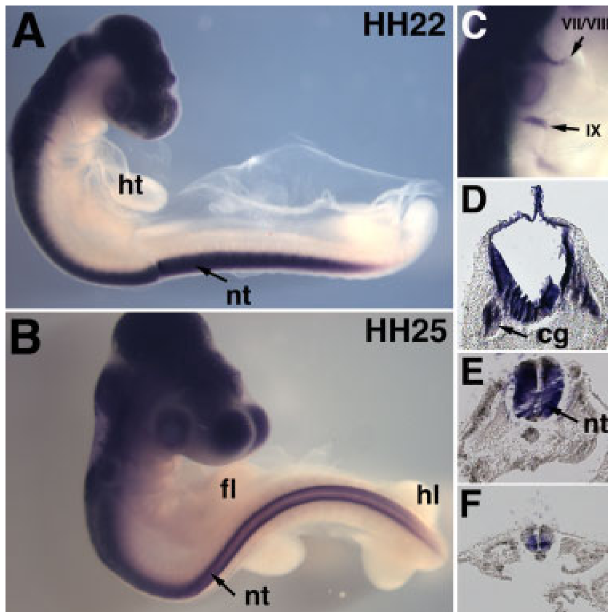

Fig. 2. The mir-124a expression in chicken embryos. Whole-mount in situ hybridization using Locked nucleic acid (LNA) probes in chick embryos reveals highly restricted expression of mir-124a in the central nervous system. **A,B:** Hamburger and Hamilton (HH) stage 22 and 25 embryos show expression in the brain and neural tube. **C:** Expression in cranial ganglia VII–IX. **D:** Transverse section through the hindbrain region shows expression in cranial ganglia and hindbrain. **E,F:** Transverse sections show expression in the neural tube. (F) In posterior regions, expression levels are higher in the ventral neural tube. cg, cranial ganglia; ht, heart; nt, neural tube; VII, VIII, IX are cranial ganglia (facial, acoustic, glossopharyngeal).

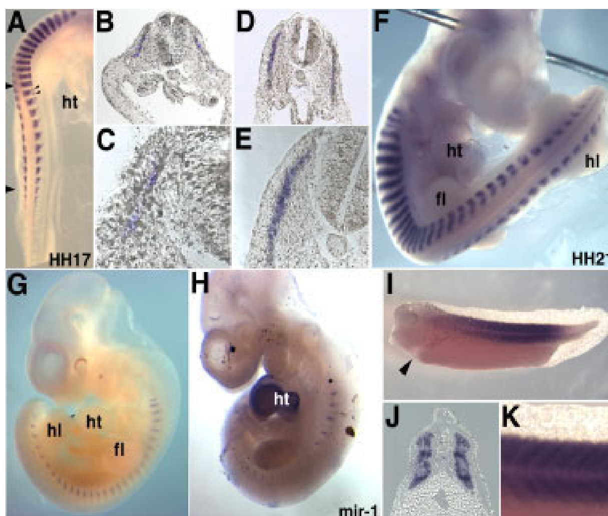

Fig. 3. The mir-206 is expressed in somites of chick, mouse, and *Xenopus* embryos. **A–F:** Locked nucleic acid (LNA) probe was used to show mir-206 expression in chick Hamburger and Hamilton (HH) stage 17 (A–E) and HH21 (F) embryos; small arrowheads in A indicate mir-206 expression in the lateral myotome. B–E: Transverse sections through the trunk of an HH17 embryo, arrowheads in A indicate the axial levels; in C,E, higher magnification shows staining in the myotome. **G,I–K:** Somite specific expression of mir-206 in an embryonic day (E) 10 mouse embryo (G) and a stage 35 *Xenopus laevis* tadpole (I–K); the arrow in I indicates the lack of mir-206 expression in the heart. Section through the trunk shows somite staining (J); K is a higher magnification of I. **H:** Somite- and heart-specific expression of mir-1 in an E10 mouse embryo the arrow indicates the heart. fl; forelimb, ht, heart; hl, hind limb.

protocol of Lau et al. (2001). The approximately 60mer monomers were digested with *Ban*I and ligated to each other using 2,000 U of T4 DNA ligase (M0202T, New England Biolabs) overnight at room temperature. The concatemerized PCR products were cloned into pGEM-Teasy vector (Promega) and sequenced using the BigDye 3.1 sequencing kit (Applied BioSystems).

Sequence Analysis

Obtained short RNA sequences were compared with all sequences in Genbank using BLAST (Altschul et al., 1990). Sequences matching rRNA, tRNA, sn/snoRNA, and mRNA were removed, and the remaining sequences were compared with known miRNAs listed in the miRBAse Release7.0 (previously: The miRNA Registry; http://microrna.sanger.ac.uk/sequences/). Potential new miRNA sequences were localized in the chicken genome sequence using ENSEMBL (www.ensembl.org) and the current release of chicken genome (WASHUC1). Secondary structures of potential miRNA precursors were predicted by MFOLD 3.2 (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/; Zuker, M. 2003).

In Situ Hybridization

WISH of chicken and *Xenopus* embryos were performed essentially as described in Smith et al. (2005), but hybridizations were performed at 42°C. The LNA oligonucleotides (Exiqon) were labeled with digoxigenin using the 3′-end labeling kit (Roche), and one labeling reaction was added to 5 ml of hybridization solution. Results from hybridizations improved after repeated use of labeled probe probably due to the removal of shorter oligonucleotide products from the solution, which caused background hybridization usually in the head but also neural tube and notochord. WISH of *Xenopus* and mouse embryos were carried out as described by Wheeler et al. (2006) and Harrison et al. (2004).

Bead Implants

Heparin beads soaked in FGF-4 (40 μg/ml; R&D Systems) were prepared as described in Smith et al. (2005) and implanted adjacent to presegmented mesoderm or adjacent to somite 1 of HH14–HH16 embryos. After overnight incubation (16–18 hr), embryos were fixed and processed for in situ hybridization.

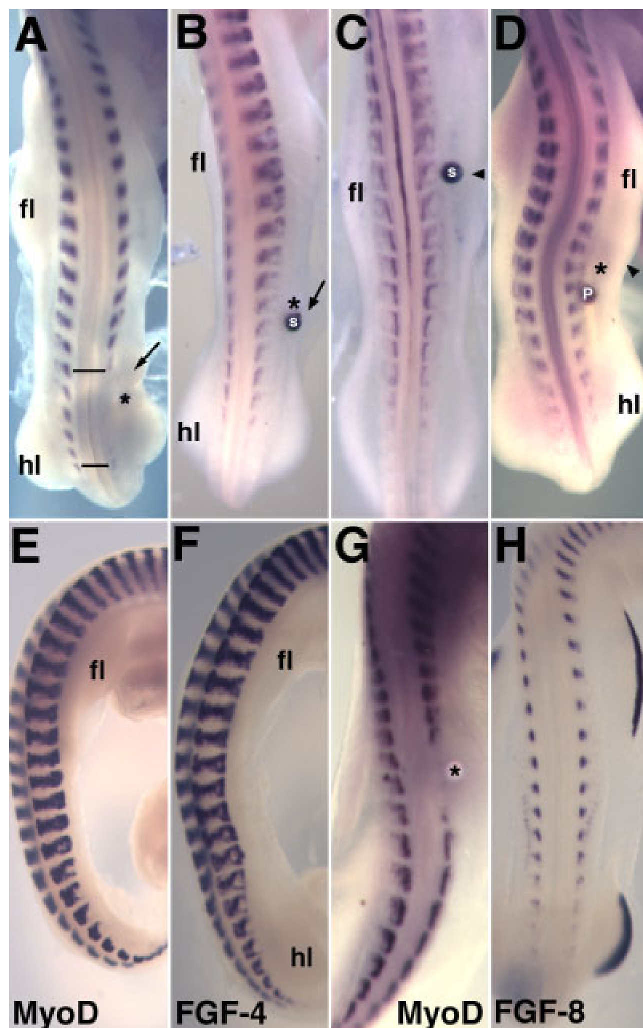

Fig. 4. Bead implants demonstrate a role for fibroblast growth factor-4 (FGF-4) in the regulation of mir-206 expression. **A:** FGF-4 beads (asterisks) were implanted adjacent to a newly formed somite at hindlimb level. The arrow indicates outgrowth of an ectopic limb bud. **B:** Implantation of an FGF-4 bead adjacent to an SU5402 bead; the arrow indicates ectopic thickening. **C:** Implantation of an SU5402 bead; the arrowhead indicates indentation in the ridge of the forelimb bud. **D:** Implantation of an FGF-4 and a PD184352 bead; the arrowhead indicates ectopic thickening in the flank. C,D: The signal in the neural tube is due to background hybridization. Embryos in A–D were incubated overnight followed by in situ hybridization with the mir-206 probe. D: Expression of MyoD in a Hamburger and Hamilton (HH) stage 20 embryo; the hindlimb bud was removed. **F,H:** Expression of FGF-4 (F) and FGF-8 (H) at HH20. **G:** Implantation of an FGF-4 bead (asterisk) resulted in loss of MyoD expression. fl, forelimb; hl, hindlimb; P, PD184352; S, SU5402.

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