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Specific requirements of MRFs for the expression of muscle specific microRNAs, miR-1, miR-206 and miR-133

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ABSTRACT

The expression of three microRNAs, miR-1, miR-206 and miR-133 is restricted to skeletal myoblasts and cardiac tissue during embryo development and muscle cell differentiation, which suggests a regulation by muscle regulatory factors (MRFs). Here we show that inhibition of C2C12 muscle cell differentiation by FGFs, which interferes with the activity of MRFs, suppressed the expression of miR-1, miR-206 and miR-133. To further investigate the role of myogenic regulators (MRFs), Myf5, MyoD, Myogenin and MRF4 in the regulation of muscle specific microRNAs we performed gain and loss-of-function experiments in vivo, in chicken and mouse embryos. We found that directed expression of MRFs in the neural tube of chicken embryos induced ectopic expression of miR-1 and miR-206. Conversely, the lack of Myf5 but not of MyoD resulted in a loss of miR-1 and miR-206 expression. Taken together our results demonstrate differential requirements of distinct MRFs for the induction of microRNA gene expression during skeletal myogenesis.

Introduction

The development and differentiation of muscle has been instrumental in understanding both how cells acquire their fate during embryogenesis and how differentiation programmes are activated and maintained (Buckingham et al., 2003; Tapscott, 2005).

microRNAs, small non-coding RNAs of between 18 and 22 nucleotides, have recently been identified as novel regulators of gene expression. They negatively regulate genes by binding to specific sequences in the 3′ UTR of target genes and inhibiting their translation (He and Hannon, 2004; Lai, 2002). microRNAs have been implicated in a range of biological processes including cancer (Dalmay and Edwards, 2006; Zhang et al., 2007) development of the limb (Harfe et al., 2005; Hornstein et al., 2005), lung (Harris et al., 2006) and haematopoetic system (Chen et al., 2004). In addition, a number of microRNAs have been characterised as modulators of myogenic differentiation (Callis et al., 2007) and there is increasing evidence for microRNA involvement in myopathies such as muscular dystrophies (Eisenberg et al., 2007; McCarthy et al., 2007).

Some of the best-characterised microRNAs are miR-1, miR-206 and miR-133 (McCarthy, 2008; van Rooij et al., 2008). In situ hybridisation studies suggest that skeletal muscle specific expression of these microRNAs is conserved during *Xenopus*, zebrafish, chicken and mouse embryo development (Darnell et al., 2006; Sweetman et al., 2006; Wienholds et al., 2005). Genome analyses have shown that in human and mouse there are three loci encoding these microRNAs, each of which produces a transcript containing one of the miR-1/206 family and one of the miR-133 family. In the chicken genome four loci have been found, presumably resulting from a further duplication at one of these loci (http://www.ensembl.org/index.html). A schematic representation of these loci and alignments of the pre-microRNA and mature microRNA sequences is shown (Fig. 1).

Experiments in cell culture suggested that miR-1 and miR-206 promote differentiation of myoblasts, while miR-133 promotes proliferation through down-regulation of different target genes (Chen et al., 2006; Kim et al., 2006). ChIP on CHIP analysis indicated that the myogenic regulatory factors (MRF), MyoD and Myogenin bind to sequences upstream of miR-1 and miR-133 (Rao et al., 2006). Furthermore, MEF2, an essential regulator of skeletal muscle development, plays a critical role in the control of an intragenic enhancer located in the mir1–2 locus (Liu et al., 2007). We previously showed that ectopic FGF suppresses miR-206 expression during somite development. We provided evidence that miR-206 down-regulation

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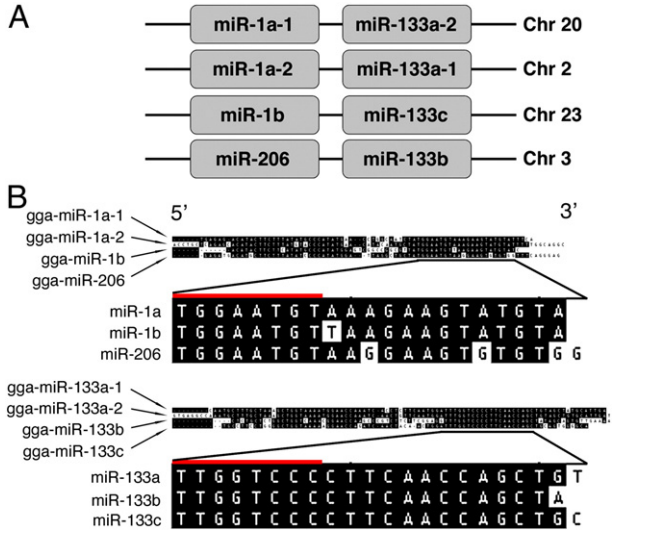

Fig. 1. Sequence alignments of chicken muscle specific microRNAs. (A) Four loci encode the muscle specific microRNAs in the chicken on chromosomes 2, 3, 20 and 23. Each has one member of the miR-1/206 family and one of the miR-133 family. (B) Alignments of the pre-microRNAs produced from these loci and the mature microRNAs. The four loci produce three distinct microRNAs as the mature microRNA sequences from miR-1a-1 and miR-1a-2 are identical as are those from miR-133a-1 and miR-133a-2. The red bar indicates the seed sequences of these microRNAs.

is mediated by inhibition of *MyoD* expression via ERK MAP kinase (Sweetman et al., 2006). As yet however a definitive role of MRFs in the regulation of microRNA expression during embryogenesis has not been demonstrated.

To explore further the regulation of microRNA expression during myogenesis we employed the skeletal muscle cell line C2C12, a mesenchymal cell line that is widely used as an in vitro model for muscle cell differentiation. Undifferentiated C2C12 cells express *Myf5* and *MyoD* but not *myogenin* and *Mrf4*. Although it is generally assumed that Myf5 and MyoD actively determine the committed state of C2C12 cells the ability of these MRFs to induce differentiation under growth conditions is blocked. Only the removal of growth factors and/or the down-regulation of growth factor receptors lead to myogenic differentiation as marked by a strong increase of the expression of *myogenin* and other muscle differentiation markers such as myosin heavy chain (*MyHC*) and several muscle specific microRNAs (Kim et al., 2006; Rao et al., 2006).

Here, we show that FGF signalling through ERK MAP kinase delayed expression of miR-1, miR-206, and miR-133 in C2C12 cells undergoing myogenic differentiation. Using gain-of-function and loss-of-function approaches in chicken and mouse embryos we demonstrate that ectopic expression of *Myf-5*, *MyoD*, *myogenin* and *MRF-4* in the developing chicken neural tube induced the expression of distinct muscle specific microRNAs while the lack of *Myf-5* resulted in a loss of miR-1 and miR-206 expression in developing somites. In contrast, the absence of MyoD had no discernable effect on expression of these microRNAs.

Materials and methods

C2C12 cell culture and transfection

Mouse C2C12 cells were maintained at sub-confluent densities in DMEM supplemented with 10% heat-inactivated FBS (growth medium; GM) and 1% penicillin/streptomycin. Myogenic differentiation was induced by changing the growth medium of sub-confluent cells to DMEM containing 2% heat-inactivated horse serum and 1% penicillin/streptomycin (differentiation medium; DM). Transfections were carried out using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. 300 ng plasmid DNA was used. Cells were incubated in transfection reagent–DNA complexes for 5 h. Afterwards, the cells were incubated in GM or DM depending upon the experiment, for 1–5 days and then harvested for RNA isolation. Transfection efficiency was quantified by counting GFP positive cells and found to be about 75%.

RNA isolation and Northern blotting

Total RNA was isolated from C2C12 cells as follows. Cells were washed twice with PBS, collected in guanidinium solution, transferred into an eppendorf tube and vortexed until lysis was complete. 2 M sodium acetate (pH 4) was added, followed by addition of 1 volume of acidic phenol (ph 4.5) and 0.2 volumes of chloroform/isoamyl alcohol. The samples were incubated on ice for 15 min and centrifuged at 13000 rpm for 15 min at 4 °C. The top phase was transferred into a new tube and RNA was precipitated with 2.5 volumes of 100% ethanol at −80 °C. After centrifugation at 13000 rpm for 15 min at 4 °C the pellet was washed with 70% ethanol, air-dried and resuspended in 20 µl DEPC-water. Northern blots were done as described in Sweetman et al. (2006). Briefly, 50 µg of total RNA was separated on 15% denaturating polyacrylamide gel electrophoresis (PAGE), run at 80 V for approximately 2 h, and visualized on a UV trans-illuminator after staining with 1 µg/ml ethidium bromide solution in 1×MOPS for 10 min. RNA was transferred to Hybond NX membrane (Amersham Biosciences) by semi-dry blotting using 20 V for 2 h. RNA was cross-linked using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 12.5 M 1-methylimidazole) at 60 °C for 2 h. The sequences of the probes were as follows, miR-1 probe: TACATACTTCTTTACATTCCA; miR-133c probe: GCAGCTGGTTGAAGGGGACCAA; miR-206 probe: CCACACACTTCCTTACATTCCA. Probes were end-labelled with γ-^{32}P-ATP using T4 polynucleotide kinase (Invitrogen) and purified on a Sephadex G-25 column (Amersham Biosciences). Blots were pre-hybridised in UltraHyb Oligo (Ambion) and hybridised overnight at 37 °C in a hybridisation oven. Membranes were washed twice with 0.2×SSC/0,5% SDS at 37 °C for 30 min and then exposed at room temperature to Fuji Bass cassette 2040 (Fuji). Next, the membranes were stripped and hybridised with probe detecting the U6 small nuclear RNA.

Generation of MRF expression constructs

Chicken MRF cDNAs were cloned by RT-PCR using template from 4 day old embryos and Phusion DNA polymerase (NEB) and standard molecular biology protocols. The following primers were used,

cMyf5F-NotI: GCGGCCGCATGGAGGTGATGGACAGC;
cMyf5R-EcoRI: GAATTCTCAAGCGTAATCTGGAACATCGTATGGGTATAGCGCCTGGTAGGTCG,
cMyoDF-NotI: GCGGCCGCATGGACTTACTGGGCCCC;
cMyoDR-EcoRI: GAATTCTCAAGCGTAATCTGGAACATCGTATGGGTATAGCACTTGGTAGATTGG;
cMgnF-BamHI: GGATCCATGGAGCTCTTTGAGACC;
cMgnR-EcoRI: GAATTCTCAAGCGTAATCTGGAACATCGTATGGGTAGTTTTGGACCCGCTCCTCTG;
cMRF4F-NotI: GCGGCCGCATGATGGACCTTTTCGAAAC;
cMRF4R-EcoRI: GAATTCTCAAGCGTAATCTGGAACATCGTATGGGTATTTCTCCACCGCTTCTTC

PCR products were A-tailed with Taq polymerase, cloned into pGEM-T-Easy (Promega), sequenced and subcloned into pCAβ-IRES-GFP using the appropriate restriction enzymes.

Electroporation

Expression plasmids were grown in *Escherichia coli* DH5α and isolated using a high-speed midi-kit (Qiagen). DNA for electroporation was further purified using a PCR purification kit (Qiagen) and eluted to give a concentration of 1 mg/ml. DNA was mixed with fast green to a final concentration of 0.05% and injected using glass capillary needles and a femtojet microinjector (Eppendorf) as described (Yue et al., 2008). Injections into the neural tube were performed in Hamburger–Hamilton (HH) stage 14 (Hamburger and Hamilton, 1951) embryos in ovo. Electroporation was done using a TSS20 Ovodyne Electroporator (Intracel) delivering ten 50 ms pulses of 30 V.

Whole mount in situ hybridisation

Detection of MRFs was as described (Smith et al., 2005). Dual-DIG-labelled LNA probes were obtained from Exiqon and hybridised as described in (Sweetman et al., 2006). LNA probes were hybridised and washed at the following temperatures, miR-1: 42 °C; miR-206: 50 °C and miR-133: 65 °C. Double in situs were first hybridised with Myf-5 probes at 65 °C, washed at 65 °C, then re-hybridised with miR-206 at 50 °C and washed at 50 °C.

Results

Muscle specific microRNAs are expressed during somite myogenesis

We examined the spatio-temporal expression of miR-1, miR-206 and miR-133 during chicken embryo development using whole mount LNA in situ hybridisation. Both, miR-1 and miR-133 were expressed in post mitotic cells in the somite myotome (Figs. 2A, C) similar to the expression pattern of miR-206 (Fig. 2B) (Sweetman et al., 2006), although the expression of miR-1 and miR-133 was restricted to the more anterior and therefore more differentiated somites compared to miR-206. At HH stage 20 the posterior expression boundary for miR-1 was at the level of the fore limb, for miR-133 at the level of the flank and for miR-206 at hind limb level (Figs. 2A–C, black arrowheads). Furthermore, miR-1 and miR-133 were expressed in the heart while miR-206 was not (Figs. 2A–C). Using LNA in situ hybridisation we cannot determine which miR-133 transcripts are expressed in the heart. It is likely that miR-133b, which is generated from the same primary transcript as miR-206, is only expressed in skeletal muscle. However we cannot exclude the possibility that differential processing of this primary transcript could produce miR-133b expression in cardiac cells in the absence of miR-206. There is precedence for this scenario, since it has been shown that miR-1-2 and miR-133a-1 can be generated independently from the same primary transcript, in at least some circumstances (Liu et al., 2007).

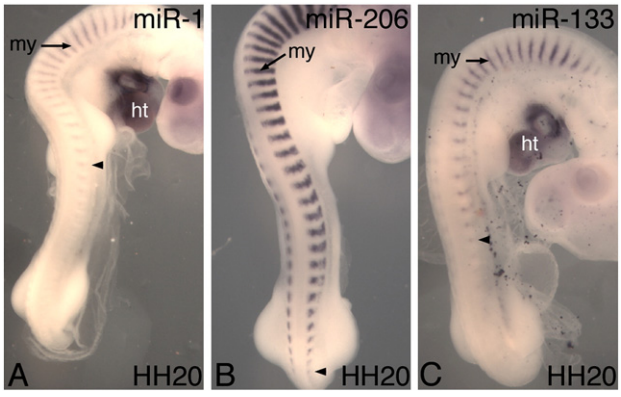

Fig. 2. Expression of muscle specific microRNAs during myogenesis. (A–C) Whole mount in situ hybridisation using LNA oligos in HH stage 20 chicken embryos showing expression of miR-1 (A), miR-206 (B) and miR-133 (C). Arrowheads show the most posterior somite with detectable staining. my — myotome, ht — heart.

FGF signalling mediated by ERK MAP kinase suppresses the expression of microRNA miR-1, miR-206 and miR-133

To establish the expression profile of miR-1, miR-206 and miR-133 during differentiation of C2C12 cells Northern blot analysis was performed (Fig. 3A). miR-1, miR-133, and miR-206 were readily detected 2 days (D2) after transfer into differentiation medium (DM) (Fig. 3A). In contrast, cells grown in high serum (GM) did not reveal signs of myogenic differentiation as indicated by the lack of MyHC expression and failed to show expression of muscle specific microRNA until day 4 when cells became confluent (Fig. 3E).

Since FGF ERK MAP kinase signalling attenuates miR-206 expression in somites of developing chicken embryos (Sweetman et al., 2006), we wanted to examine the regulation of microRNAs during myogenic differentiation more closely in C2C12 muscle cells (Figs. 3B–D, F–H). Addition of recombinant FGF-4 or FGF-8 to DM significantly delayed the differentiation process resulting in the absence of any myotubes after 5 days of incubation. Concomitantly, the expression of muscle specific microRNAs was delayed and robust expression was not observed until day four (D4) (Figs. 3B, C). This result was consistent with the inhibitory effects of FGF-beads on miR-206 expression in somites (Sweetman et al., 2006).

Similarly, expression of a constitutively active form of MEK, MEK^{EE} (Smith et al., 2005) delayed myogenic differentiation in DM, as shown by MF20 immunostaining, and simultaneous expression of muscle specific microRNAs (Fig 3D). Next, C2C12 cells grown in GM were treated with a pharmacological inhibitor SU5402, known to block FGF receptor activation. Interestingly, expression of muscle specific microRNAs was enhanced under these conditions and was already detected at day two (D2) (Fig. 3F) indicating a direct regulation of the expression of muscle specific microRNA by FGF-signalling; myotube formation was evident by day three (D3) (not shown).

To determine which downstream effectors of FGF signalling mediated the repression of microRNA expression under GM conditions, C2C12 cells were treated with PD184352, a known MEK inhibitor (Sebolt-Leopold et al., 1999). This led to early expression of muscle specific microRNAs, with particularly strong effects on miR-1 and miR-133 (Fig. 3G), when compared to control cultures (Fig. 3E). Finally, we transfected C2C12 cells, cultured in GM, with the MKP3 phosphatase, a specific inhibitor of ERK MAP kinase (Groom et al., 1996). This resulted in premature expression of muscle specific microRNAs and myogenic differentiation (Fig. 3H). We provide a quantitative representation of the relative amounts of signal normalized to U6 loading control in Supplementary Fig. 1.

To confirm that the altered expression of miRs corresponded with changes in differentiation, cells were stained with the MF20 antibody to detect sarcomeric myosin heavy chain (MyHC) expression. In all cases delayed miR expression correlated with reduced MyHC expression, while early expression correlated with increased expression (see side panels in Fig 3).

Myogenic regulatory factors, MRFs, induce myogenic genes and ectopic microRNA expression in the neural tube

To determine if muscle specific microRNA expression can be induced in vivo by directed expression of MRFs we cloned chicken MRFs (*Myf5*, *MyoD*, *myogenin* and *Mrf-4*) into pCAβ-IRES-GFP for expression in embryos. This vector produces both the gene of interest and GFP from the same backbone and thus allows easy determination of electroporation efficiency. After electroporation into the chicken neural tube all embryos expressed GFP. To confirm that functional MRF proteins were produced by the plasmid, we first examined whether MRFs could activate each other's expression (Fig. 4). Electroporation of pCAβ-Myf-5-

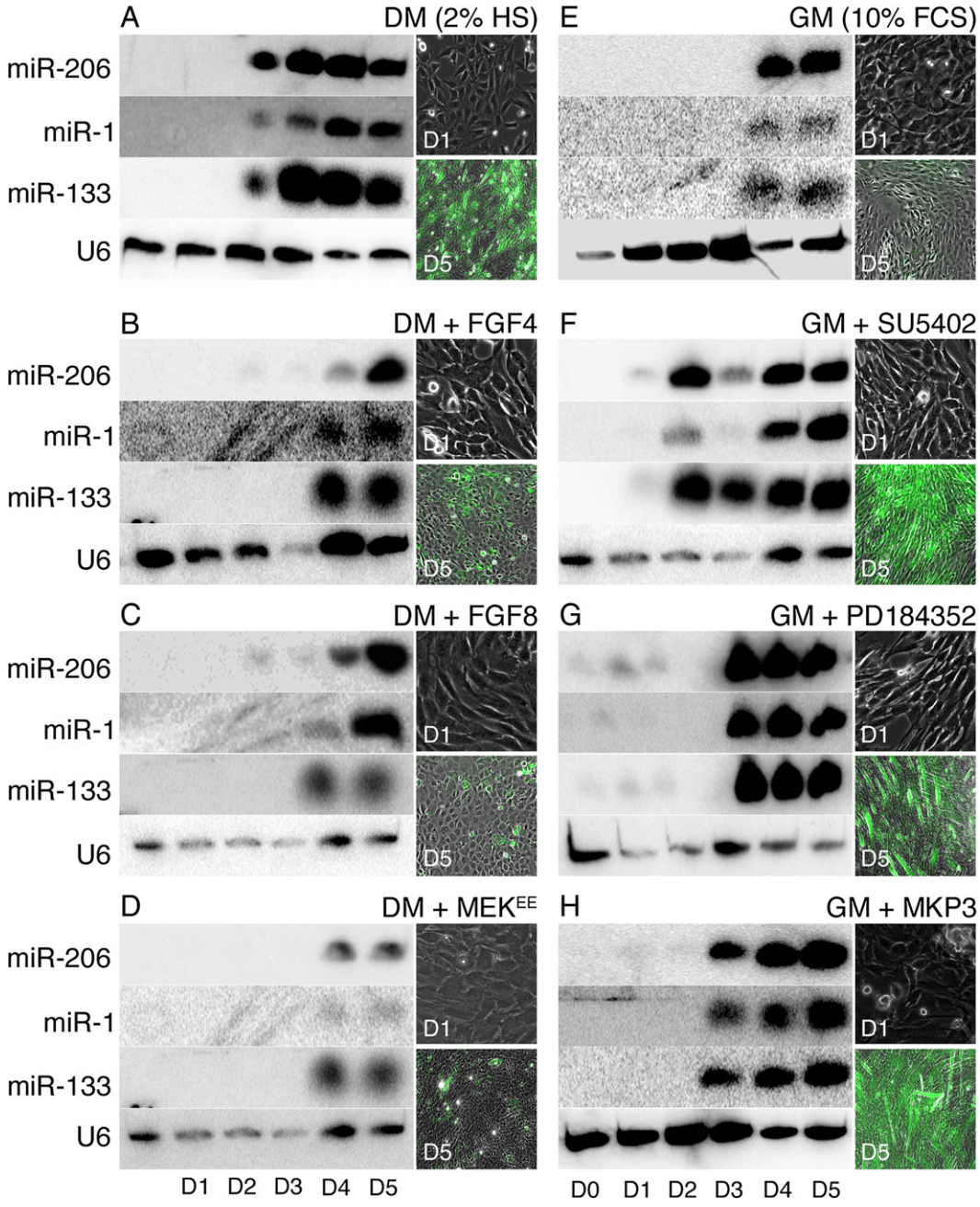

Fig. 3. Regulation of muscle specific microRNAs by FGF signalling in C2C12 cells. (A) MicroRNA expression beginning at day 2 following transfer to differentiation medium (DM). (B–D) Delayed microRNA expression in cells grown in DM following treatment with 10 ng/ml FGF4 (B), 20 ng/ml FGF8 (C) or transfection with ME^{KEE} (D). (E) Expression of microRNAs in growth medium (GM) first detected at day 4. (F–H) Induction of microRNA expression in GM following treatment with 20 μM SU5402 (F), 10 μM PD184352 (G) or transfection with MKP3 (H). Panels adjacent to blots show C2C12 cells at 1 and 5 days following treatment. Green staining shows MF20 positive cells expressing sarcomeric myosin which have undergone myogenic differentiation.

IRES-GFP led to ectopic expression of *MyoD*, *myogenin* and *Mrf-4* in neural tissue (Fig. 4A). Embryos electroporated with pCAβ-MyoD-IRES-GFP in the neural tube expressed ectopic *myogenin* (Fig. 4B), and expression of pCAβ-Mgn-IRES-GFP induced ectopic *MyoD* and *Mfr-4* (Fig. 4C). Finally, pCAβ-Mrf-4-IRES-GFP expression induced *myogenin* (Fig. 4D). These results confirmed that all MRF expression plasmids produced functional proteins.

Next, we investigated the ability of MRFs to activate expression of muscle specific microRNAs in neural tissue. We found that pCAβ-Myf-5-IRES-GFP electroporation led to expression of both miR-1 and miR-206 with high efficiency (Fig. 5A). Similarly, pCAβ-Mgn-IRES-GFP resulted in miR-1 and miR-206 expression in the neural tube (Fig. 5C). In contrast, pCAβ-MyoD-IRES-GFP and pCAβ-Mrf-4-IRES-GFP induced expression of miR-206 but not miR-1 (Figs. 5B, D). Activation of miR-206 expression by Mrf-4 was very inefficient, with 45% of embryos showing low levels of detectable expression (Fig. 5D).

To confirm that ectopic expression of microRNAs in the neural tube co-localised with ectopic MRF expression we performed double in situ hybridisation for *Myf-5* and miR-206 on embryos electroporated with pCAβ-Myf-5-IRES-GFP in the neural tube. Transverse sections of these embryos clearly revealed the presence of miR-206 in a subset of cells expressing *Myf-5* (Fig. 5E).

Muscle specific microRNA expression is downregulated in somites of Myf-5$^{(-/-)}$ but not MyoD$^{(-/-)}$ null mouse embryos

The directed expression of MRFs indicated that Myf-5 and myogenin are sufficient to promote expression of endogenous miR-1

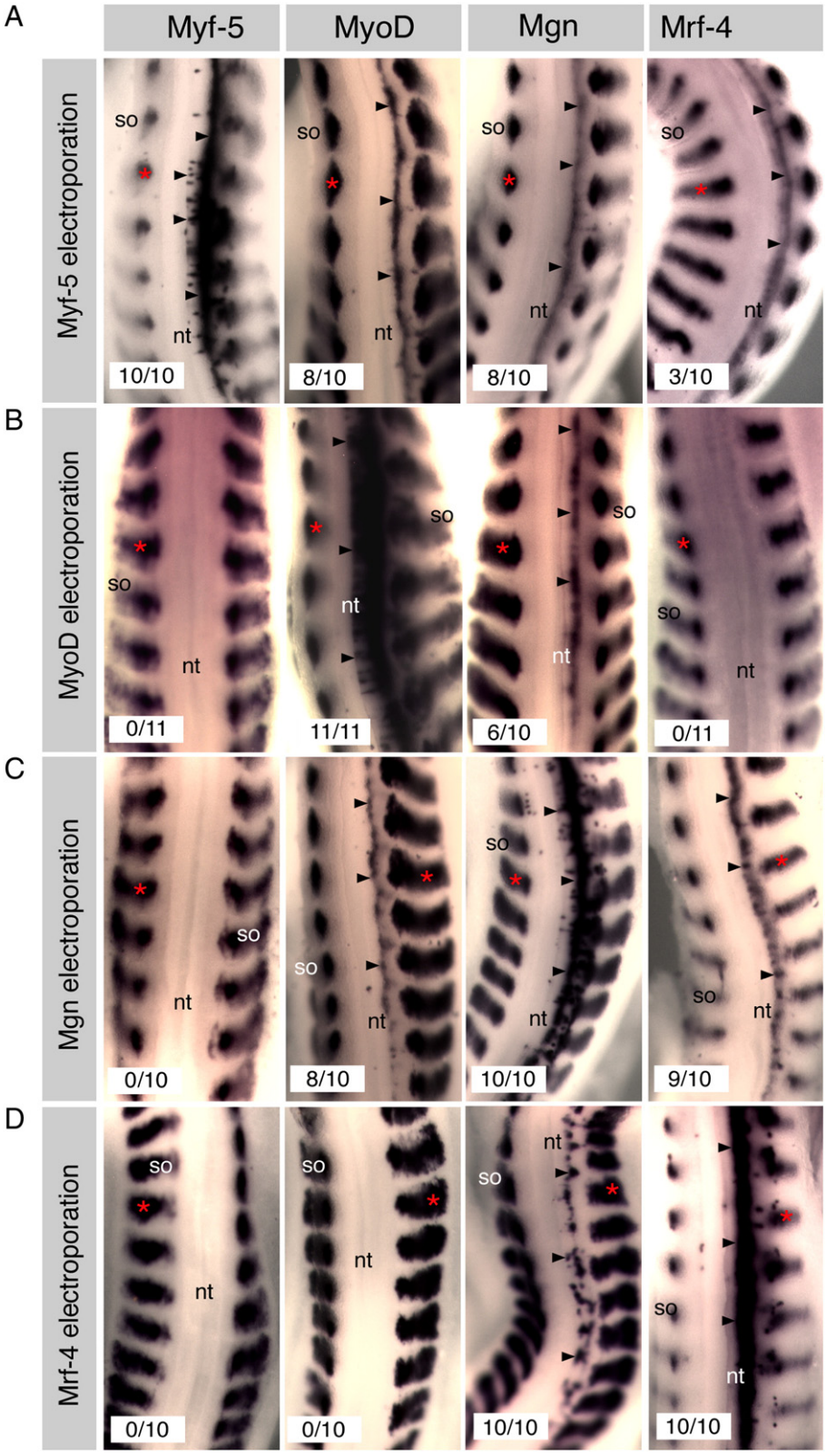

Fig. 4. Expression of MRFs in the neural tube activates muscle specific gene expression. (A) Electroporation of Myf5, (B) MyoD, (C) Myogenin and (D) MRF4. In situ hybridisation were performed for Myf5, MyoD, Myogenin and MRF4 as indicated above each column. Arrows show ectopic expression detected in the neural tube. The number of embryos with ectopic expression out of the total number of surviving embryos electroporated is shown in each panel. nt — neural tube, so — somite. Red asterisks show endogenous staining in somites.

and miR-206 loci in neural tissue, while MyoD and Mrf-4 were able to activate miR-206, but not miR-1 expression. To determine the effects of loss of function of MRFs on muscle specific microRNA expression, we examined the expression of miR-1 and miR-206 in embryos homozygous for null alleles of Myf-5 or MyoD compared to expression in wild type or heterozygous embryos (Fig. 6).

As expected, expression of both miR-1 and miR-206 was detected in the myotomes of wild type (Figs. 6A, B) and heterozygous Myf-5$^{(+/-)}$

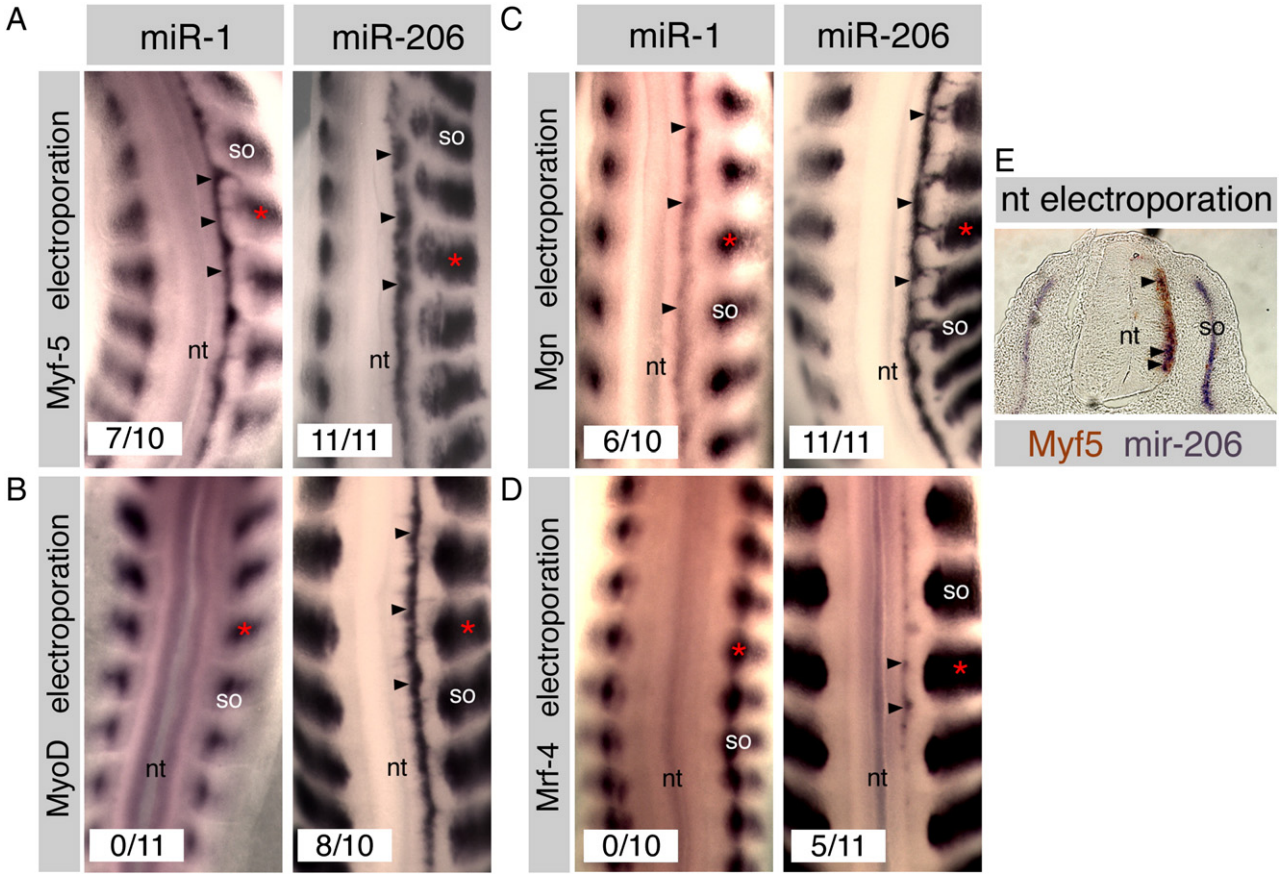

Fig. 5. MRFs can activate microRNA expression in the neural tube. (A) Electroporation of Myf5, (B) MyoD, (C) Myogenin and (D) MRF4. In situ hybridisation was performed for miR-1 and miR-206 as indicated above each column. Arrows show ectopic expression in the neural tube. The number of embryos with ectopic expression out of the total number of surviving embryos electroporated is shown in each panel. (E) Transverse section of embryo electroporated with Myf5 in neural tube followed by double in situ hybridisation for Myf5 (red) and miR-206 (blue). Arrow shows ectopic expression of both Myf5 and miR-206. nt — neural tube, so — somite. Red asterisks show endogenous staining in somites.

and MyoD$^{(+/-)}$ embryos at E10.5 (Figs. 6C, D, G, H). Similar to expression in chicken somites, miR-206 was more robustly expressed and was detected in more posterior, immature somites, when compared to miR-1. At E10.5, miR-206 was seen in somites at hind limb level (somites 26–29) and miR-1 was detected in flank level somites (somites 23–26). Importantly, we did not detect expression of either miR-1 or miR-206 in Myf-5$^{(-/-)}$ null embryos (Figs. 6E, F) whereas MyoD$^{(-/-)}$ null embryos retained robust expression of both miR-1 and miR-206 (Figs. 6I, J). These results demonstrated that Myf-5 is required for the activation of miR-1 and miR-206 expression in the myotome of developing somites, yet MyoD is dispensable.

Discussion

Our results illustrate that miRNAs are part of the differentiation programme of muscle cells. In particular, we show that the regulation of miR-1, miR-206 and miR-133 by FGF ERK MAP kinase signalling is conserved in myogenic C2C12 cells as well as in the embryo. We

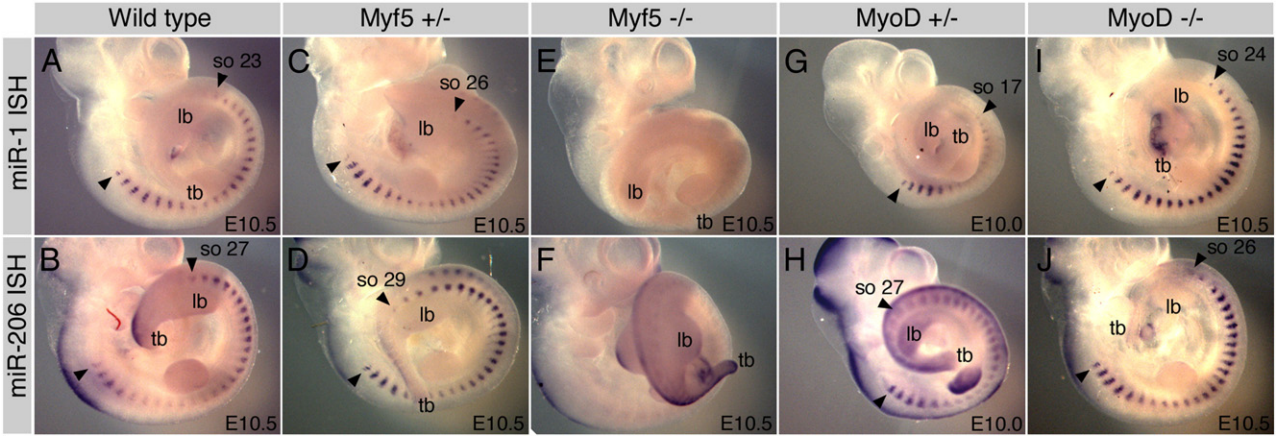

Fig. 6. miR-1 and miR-206 expression is lost in Myf-5$^{(-/-)}$ null mice. LNA in situ hybridisation was performed on Myf-5$^{(-/-)}$ and MyoD$^{(-/-)}$ mouse mutants, heterozygous animals and wild type, genotypes are indicated on each panel. Expression of miR-1 (A, C, E, G, I) and miR-206 (B, D, F, H, J) in either wild type (A, B), Myf5$^{(+/-)}$ (C, D), Myf5$^{(-/-)}$ (E, F), MyoD$^{(+/-)}$ (G, H) or MyoD$^{(-/-)}$ (I, J) embryos. lb — limb bud, tb — tail bud, so — somite. Arrowheads mark the most anterior and posterior somites expressing microRNAs.

propose that FGF ERK MAP kinase inhibition of miR expression acts by abrogating MRF activity and, consistent with this, we demonstrate that MRF transcription factors are sufficient to activate microRNA expression in vivo. In addition, our analyses reveal distinct roles of individual MRFs to induce microRNA expression in the chicken neural tube. Finally, investigation of mouse mutants suggests a previously unrecognized requirement of Myf-5 for the expression of miR-1 and miR-206 in developing somites.

FGF ERK MAP kinase regulates muscle specific microRNAs during C2C12 cell myogenesis

Implantation of FGF4 or FGF8 beads adjacent to developing somites led to down-regulation of both miR-206 and MyoD (Smith et al., 2005; Sweetman et al., 2006), which raised the question whether FGF-mediated inhibition of muscle specific microRNAs is a general regulatory phenomenon. To explore this possibility we utilized C2C12 muscle cells, a well characterised myogenic cell line derived from adult mouse satellite cells, that undergoes myogenic differentiation under defined conditions (Portier et al., 1999). We found that in low serum conditions (DM) addition of FGF-4 or FGF-8 was sufficient to delay the onset of muscle specific microRNA expression (Figs. 3B, C), while in high serum (GM) FGF receptor inhibition, using the pharmacological inhibitor SU5402, led to early differentiation (Fig. 3F). A number of pathways providing both positive and negative signalling cues for myogenic differentiation could be affected by these manipulations (de Alvaro et al., 2005; Kuwahara et al., 2005; Lluis et al., 2006). However, with increased density the cells differentiated eventually and formed myotubes, which is probably due to the well-documented down-regulation of FGF-receptors in confluent muscle cells in culture (Olwin and Hauschka, 1988). Consistent with this observation, expression of muscle specific microRNA was detected in the presence of FGF, albeit delayed when compared to the normal expression profile (Figs. 3A–C). Specific activators of ERK MAP kinase also led to delayed microRNA expression (Fig. 3D), while ERK inhibitors had the opposite effect and led to earlier expression of muscle specific microRNAs even under high serum (GM) conditions (Figs. 3G, H). These findings suggest that FGF ERK MAP kinase signalling promotes proliferation and inhibits differentiation of myogenic cells most likely through ERK-mediated inhibition of MRF activities described by several groups (Bennett and Tonks, 1997; Kontaridis et al., 2002; Pena et al., 2000; Perry et al., 2001). Here we characterise muscle specific microRNAs as new targets of this regulatory network.

Differential regulation of microRNAs by MRFs in the developing chicken neural tube

The loss of muscle microRNA expression following FGF treatment of somites or C2C12 cells was likely to be indirect, since FGF treatment leads to loss of MyoD as well as miR-206 in embryos (Smith et al., 2005; Sweetman et al., 2006). Furthermore, MyoD and myogenin are known to bind regions upstream of miR-1 (Rao et al., 2006). Thus, we examined whether the effects of FGF were mediated via the MRF family of transcription factors. Ectopic expression in the developing chicken neural tube has previously revealed the myogenic potential of Pax3 (Maroto et al., 1997) and Myf5 and MyoD (Delfini and Duprez, 2004). Interestingly, ectopic expression of MRFs revealed unexpected differences in the ability of MRFs to activate expression of different microRNAs (Figs. 4, 5). We found that Myf-5 induced both miR-1 and miR-206 together with *MyoD*, *myogenin* and *Mrf4* indicating that Myf-5 is able to activate the whole repertoire of MRFs and microRNAs (Figs. 5A, 4A). We were not able to distinguish between the electroporated and the endogenous Myf-5 transcripts, since both were from avian origin. Therefore, we could not determine whether Myf-5 is able to induce its own expression, although extensive characterisation of the *Myf-5* locus in transgenic mice suggests that it cannot (Carvajal et al., 2001; Carvajal et al., 2008). In the neural tube, myogenin had a similar activity as Myf-5 and induced *MyoD*, *Mrf-4*, miR-1 and miR-206 but clearly did not induce *Myf-5* expression (Figs. 4C, 5C). Interestingly, ectopic expression of either MyoD or Mrf-4 led to ectopic expression of only *myogenin* and miR-206 (Figs. 4B, D, 5B, D). These results demonstrate that even when expressed at high levels there seem to be differences in the ability of the MRFs to activate microRNA genes in the non-myogenic cells of the developing chicken neural tube. Although our data implies a role for the MRFs in the activation of miR gene expression, a single MRF is unlikely to be sufficient. Closer analysis of Myf5 electroporation into the neural tubes shows that only a subset of the cells expressing Myf5 undergoes myogenic differentiation and activates microRNA expression. This implies additional requirements found in only some of the electroporated neural cells, for example, the levels of Myf5 may be sufficient in only a subset of cells and/or additional MRFs have to be expressed.

The fact that MyoD and Myogenin are known to bind to putative regulatory regions of miR-1 suggests that the induction of ectopic microRNA expression by MRFs may indeed be direct (Rao et al., 2006). ChIP on Chip experiments revealed binding of both MyoD and myogenin to miR-1 upstream regions in the mouse genome. However, it is difficult to determine whether the effects we observed can be directly attributed to the MRF that was electroporated since other MRFs were induced, although their expression tended to be weaker (Fig. 4). Nevertheless, it is noteworthy that the differences observed between Myf-5/myogenin and MyoD/Mrf-4 with respect to their ability to induce microRNA gene expression (Fig. 5A–C) strongly correlated with their ability to induce the network of myogenic regulators.

Mutant mice indicate a requirement of Myf-5 for muscle specific microRNA expression in somites

Overexpression of MRFs in the neural tube suggested that Myf-5 and myogenin were sufficient to elicit ectopic expression of both miR-1 and miR-206, whereas MyoD was able to induce only miR-1. This observation was consistent with the considerable redundancy of function that is known for MRFs. Analysis of *Myf-5* and *MyoD* mutant mice indicated a surprising specific requirement of Myf-5 for miR-1 and miR-206 expression (Fig. 6). At E11.5 both miR-206 and miR-1 expression was almost entirely absent in somites of Myf-5$^{(-/-)}$ null embryos, suggesting that other MRFs, including myogenin, which are expressed in these mice (Rudnicki et al., 1993) cannot compensate for the loss of Myf-5. In contrast, embryos lacking MyoD$^{(-/-)}$ showed an apparently normal expression of both microRNAs. This is particularly notable given that both MyoD and Myf-5 null animals will produce morphologically normal skeletal muscle, as these transcription factors are able to compensate for each other during development (Braun et al., 1992; Rudnicki et al., 1992). Interestingly, this functional redundancy seems not to apply to the expression of miR-1 and miR-206 and this suggests a number of possibilities. It is possible that miR-1 and miR-206 do not have an essential function during myogenesis. Alternatively, Myf5 mutants, which lack miR-1 and miR-206 expression, might suffer from a yet undetected embryonic phenotype. Finally, other microRNAs may compensate for the lack of miR-1 and miR-206 in Myf5 mutants. Further identification of microRNAs and characterisation of their expression patterns may well reveal other miRs in muscle, which could fulfil this role.

Owing to the functional compensation between the MRFs in knockout mouse models (Braun et al., 1992; Kassar-Duchossoy et al., 2004; Rawls et al., 1995; Rudnicki et al., 1992) their distinct biological activities have only recently been recognized. Different MRFs have been shown to be important in different myogenic lineages (Tajbakhsh, 2005) and there is a specific requirement for Myf-5 in adult homeostasis and regeneration (Gayraud-Morel et al., 2007;

Ustanina et al., 2007). Thus, it is tempting to speculate that miR-1 and miR-206 are potential mediators of Myf5 in these processes.

Myogenesis has been reported to be delayed in Myf-5$^{(-/-)}$ mice, therefore, it is possible that expression of miR-1 and miR-206 is eventually restored (Braun et al., 1992). However, we have examined Myf5$^{(-/-)}$ embryos as late as E14 and there was no expression of miR-206 detected in somites at that stage (data not shown). This suggests that loss of miRs in E11.5 Myf5$^{(-/-)}$ embryos is not just the result of delayed differentiation.

It was shown recently that in mouse embryos where all Myf5 expressing cells have been ablated a population of MyoD positive cells is able to compensate and generate apparently normal muscle (Gensch et al., 2008; Haldar et al., 2008). This raises the possibility that miR-1 and miR-206 are only expressed in Myf5-dependant lineages and that Myf5 independent muscle lineages may have entirely separate regulatory mechanisms either involving different microRNAs or microRNA independent regulation. The conditional deletion of *Dicer* resulted in skeletal muscle hypoplasia, clearly demonstrating a role for microRNAs in both embryonic and postnatal development (O'Rourke et al., 2007).

A number of microRNA targets are predicted through different algorithms and a few of these have been experimentally confirmed. These include Connexin-43 (Anderson et al., 2006), HDAC4, Id1-3, DNA polα (Kim et al., 2006), follistatin and Utrophin (Rosenberg et al., 2006), which are targeted by miR-1/206, and the HERG K+ channel (Xiao et al., 2007) and SRF (Chen et al., 2006), which are targets of miR-133. In addition, the muscle specific microRNAs have been implicated in muscle hypertrophy and a mouse model of muscular dystrophy (McCarthy and Esser, 2007; McCarthy et al., 2007).

How the interactions between upstream regulators of microRNAs and the targets of miR activity lead to muscle differentiation during development and the maintenance of healthy muscle physiology and function will require further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.06.019.

References

Anderson, C., Catoe, H., Werner, R., 2006. MIR-206 regulates connexin43 expression during skeletal muscle development. Nucleic Acids Res.

Bennett, A.M., Tonks, N.K., 1997. Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. Science 278, 1288–1291.

Braun, T., Rudnicki, M.A., Arnold, H.H., Jaenisch, R., 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell 71, 369–382.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., Relaix, F., 2003. The formation of skeletal muscle: from somite to limb. J. Anat. 202, 59–68.

Callis, T.E., Chen, J.F., Wang, D.Z., 2007. MicroRNAs in skeletal and cardiac muscle development. DNA Cell Biol. 26, 219–225.

Carvajal, J.J., Cox, D., Summerbell, D., Rigby, P.W., 2001. A BAC transgenic analysis of the Mrf4/Myf5 locus reveals interdigitated elements that control activation and maintenance of gene expression during muscle development. Development 128, 1857–1868.

Carvajal, J.J., Keith, A., Rigby, P.W., 2008. Global transcriptional regulation of the locus encoding the skeletal muscle determination genes Mrf4 and Myf5. Genes Dev. 22, 265–276.

Chen, C.Z., Li, L., Lodish, H.F., Bartel, D.P., 2004. MicroRNAs modulate hematopoietic lineage differentiation. Science 303, 83–86.

Chen, J.F., Mandel, E.M., Thomson, J.M., Wu, Q., Callis, T.E., Hammond, S.M., Conlon, F.L., Wang, D.Z., 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat. Genet. 38, 228–233.

Dalmay, T., Edwards, D.R., 2006. MicroRNAs and the hallmarks of cancer. Oncogene 25, 6170–6175.

Darnell, D.K., Kaur, S., Stanislaw, S., Konieczka, J.K., Yatskievych, T.A., Antin, P.B., 2006. MicroRNA expression during chick embryo development. Dev. Dyn. 235, 3156–3165.

de Alvaro, C., Martinez, N., Rojas, J.M., Lorenzo, M., 2005. Sprouty-2 overexpression in C2C12 cells confers myogenic differentiation properties in the presence of FGF2. Mol. Biol. Cell 16, 4454–4461.

Delfini, M.C., Duprez, D., 2004. Ectopic Myf5 or MyoD prevents the neuronal differentiation program in addition to inducing skeletal muscle differentiation, in the chick neural tube. Development 131, 713–723.

Eisenberg, I., Eran, A., Nishino, I., Moggio, M., Lamperti, C., Amato, A.A., Lidov, H.G., Kang, P.B., North, K.N., Mitrani-Rosenbaum, S., Flanigan, K.M., Neely, L.A., Whitney, D., Beggs, A.H., Kohane, I.S., Kunkel, L.M., 2007. Distinctive patterns of microRNA expression in primary muscular disorders. Proc. Natl. Acad. Sci. U S A 104, 17016–17021.

Gayraud-Morel, B., Chretien, F., Flamant, P., Gomes, D., Zammit, P.S., Tajbakhsh, S., 2007. A role for the myogenic determination gene Myf5 in adult regenerative myogenesis. Dev Biol 312, 13–28.

Gensch, N., Borchardt, T., Schneider, A., Riethmacher, D., Braun, T., 2008. Different autonomous myogenic cell populations revealed by ablation of Myf5-expressing cells during mouse embryogenesis. Development 135, 1597–1604.

Groom, L.A., Sneddon, A.A., Alessi, D.R., Dowd, S., Keyse, S.M., 1996. Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. Embo. J. 15, 3621–3632.

Haldar, M., Karan, G., Tvrdik, P., Capecchi, M.R., 2008. Two cell lineages, myf5 and myf5-independent, participate in mouse skeletal myogenesis. Dev. Cell 14, 437–445.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.

Harfe, B.D., McManus, M.T., Mansfield, J.H., Hornstein, E., Tabin, C.J., 2005. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. Proc. Natl. Acad. Sci. U S A 102, 10898–10903.

Harris, K.S., Zhang, Z., McManus, M.T., Harfe, B.D., Sun, X., 2006. Dicer function is essential for lung epithelium morphogenesis. Proc. Natl. Acad. Sci. U S A 103, 2208–2213.

He, L., Hannon, G.J., 2004. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. 5, 522–531.

Hornstein, E., Mansfield, J.H., Yekta, S., Hu, J.K., Harfe, B.D., McManus, M.T., Baskerville, S., Bartel, D.P., Tabin, C.J., 2005. The microRNA miR-196 acts upstream of Hoxb8 and Shh in limb development. Nature 438, 671–674.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., Tajbakhsh, S., 2004. Mrf4 determines skeletal muscle identity in Myf5: Myod double-mutant mice. Nature 431, 466–471.

Kim, H.K., Lee, Y.S., Sivaprasad, U., Malhotra, A., Dutta, A., 2006. Muscle-specific microRNA miR-206 promotes muscle differentiation. J. Cell. Biol. 174, 677–687.

Kontaridis, M.I., Liu, X., Zhang, L., Bennett, A.M., 2002. Role of SHP-2 in fibroblast growth factor receptor-mediated suppression of myogenesis in C2C12 myoblasts. Mol. Cell. Biol. 22, 3875–3891.

Kuwahara, K., Barrientos, T., Pipes, G.C., Li, S., Olson, E.N., 2005. Muscle-specific signaling mechanism that links actin dynamics to serum response factor. Mol. Cell. Biol. 25, 3173–3181.

Lai, E.C., 2002. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. Nat. Genet. 30, 363–364.

Liu, N., Williams, A.H., Kim, Y., McAnally, J., Bezprozvannaya, S., Sutherland, L.B., Richardson, J.A., Bassel-Duby, R., Olson, E.N., 2007. An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. Proc. Natl. Acad. Sci. U S A 104, 20844–20849.

Lluis, F., Perdiguero, E., Nebreda, A.R., Munoz-Canoves, P., 2006. Regulation of skeletal muscle gene expression by p38 MAP kinases. Trends Cell. Biol. 16, 36–44.

Maroto, M., Reshef, R., Munsterberg, A.E., Koester, S., Goulding, M., Lassar, A.B., 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell 89, 139–148.

McCarthy, J.J., 2008. MicroRNA-206: the skeletal muscle-specific myomiR. Biochim. Biophys. Acta.

McCarthy, J.J., Esser, K.A., 2007. MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. J. Appl. Physiol. 102, 306–313.

McCarthy, J.J., Esser, K.A., Andrade, F.H., 2007. MicroRNA-206 is over-expressed in the diaphragm but not the hindlimb muscle of mdx mouse. Am. J. Physiol. Cell Physiol.

O'Rourke, J.R., Georges, S.A., Seay, H.R., Tapscott, S.J., McManus, M.T., Goldhamer, D.J., Swanson, M.S., Harfe, B.D., 2007. Essential role for Dicer during skeletal muscle development. Dev. Biol.

Olwin, B.B., Hauschka, S.D., 1988. Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. J. Cell. Biol. 107, 761–769.

Pena, T.L., Chen, S.H., Konieczny, S.F., Rane, S.G., 2000. Ras/MEK/ERK up-regulation of the fibroblast KCa channel FIK is a common mechanism for basic fibroblast growth factor and transforming growth factor-beta suppression of myogenesis. J. Biol. Chem. 275, 13677–13682.

Perry, R.L., Parker, M.H., Rudnicki, M.A., 2001. Activated MEK1 binds the nuclear MyoD transcriptional complex to repress transactivation. Mol. Cell. 8, 291–301.

Portier, G.L., Benders, A.G., Oosterhof, A., Veerkamp, J.H., van Kuppevelt, T.H., 1999. Differentiation markers of mouse C2C12 and rat L6 myogenic cell lines and the effect of the differentiation medium. In Vitro Cell Dev. Biol. Anim. 35, 219–227.

Rao, P.K., Kumar, R.M., Farkhondeh, M., Baskerville, S., Lodish, H.F., 2006. Myogenic factors that regulate expression of muscle-specific microRNAs. Proc. Natl. Acad. Sci. U S A 103, 8721–8726.

Rawls, A., Morris, J.H., Rudnicki, M., Braun, T., Arnold, H.H., Klein, W.H., Olson, E.N., 1995. Myogenin's functions do not overlap with those of MyoD or Myf-5 during mouse embryogenesis. Dev. Biol. 172, 37–50.

Rosenberg, M.I., Georges, S.A., Asawachaicharn, A., Analau, E., Tapscott, S.J., 2006. MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. J. Cell Biol. 175, 77–85.

Rudnicki, M.A., Braun, T., Hinuma, S., Jaenisch, R., 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71, 383–390.

Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H., Jaenisch, R., 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75, 1351–1359.

Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S., Leopold, W.R., Saltiel, A.R., 1999. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat. Med. 5, 810–816.

Smith, T.G., Sweetman, D., Patterson, M., Keyse, S.M., Munsterberg, A., 2005. Feedback interactions between MKP3 and ERK MAP kinase control scleraxis expression and the specification of rib progenitors in the developing chick somite. Development 132, 1305–1314.

Sweetman, D., Rathjen, T., Jefferson, M., Wheeler, G., Smith, T.G., Wheeler, G.N., Munsterberg, A., Dalmay, T., 2006. FGF-4 signaling is involved in mir-206 expression in developing somites of chicken embryos. Dev. Dyn. 235, 2185–2191.

Tajbakhsh, S., 2005. Skeletal muscle stem and progenitor cells: reconciling genetics and lineage. Exp. Cell. Res. 306, 364–372.

Tapscott, S.J., 2005. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development 132, 2685–2695.

Ustanina, S., Carvajal, J., Rigby, P., Braun, T., 2007. The myogenic factor Myf5 supports efficient skeletal muscle regeneration by enabling transient myoblast amplification. Stem Cells 25, 2006–2016.

van Rooij, E., Liu, N., Olson, E.N., 2008. MicroRNAs flex their muscles. Trends Genet. 24, 159–166.

Wienholds, E., Kloosterman, W.P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H.R., Kauppinen, S., Plasterk, R.H., 2005. MicroRNA expression in zebrafish embryonic development. Science 309, 310–311.

Xiao, J., Luo, X., Lin, H., Zhang, Y., Lu, Y., Wang, N., Yang, B., Wang, Z., 2007. MicroRNA miR-133 represses HERG K+ channel expression contributing to QT prolongation in diabetic hearts. J. Biol. Chem. 282, 12363–12367.

Yue, Q., Wagstaff, L., Yang, X., Weijer, C., Munsterberg, A., 2008. Wnt3a-mediated chemorepulsion controls movement patterns of cardiac progenitors and requires RhoA function. Development.

Zhang, B., Pan, X., Cobb, G.P., Anderson, T.A., 2007. microRNAs as oncogenes and tumor suppressors. Dev. Biol. 302, 1–12.