

Regulation of multiple target genes by miR-1 and miR-206 is pivotal for C2C12 myoblast differentiation

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Accepted 31 March 2012

Journal of Cell Science 125, 3590–3600

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doi: 10.1242/jcs.101758

Summary

MicroRNAs are short non-coding RNAs involved in post-transcriptional regulation of multiple messenger RNA targets. The miR-1/miR-206 family is expressed during skeletal muscle differentiation and is an integral component of myogenesis. To better understand miR-1/miR-206 function during myoblast differentiation we identified novel target mRNAs by microarray and characterized their function in C2C12 myoblasts. Candidate targets from the screen were experimentally validated together with target genes that were predicted by three different algorithms. Some targets characterised have a known function in skeletal muscle development and/or differentiation and include Meox2, RARB, Fzd7, MAP4K3, CLCN3 and NFAT5, others are potentially novel regulators of myogenesis, such as the chromatin remodelling factors Smarcd2 and Smarcb1 or the anti-apoptotic protein SH3BGRL3. The expression profiles of confirmed target genes were examined during C2C12 cell myogenesis. We found that inhibition of endogenous miR-1 and miR-206 by anti-miRs blocked the downregulation of most targets in differentiating cells, thus indicating that microRNA activity and target interaction is required for muscle differentiation. Finally, we show that sustained expression of validated miR-1 and/or miR-206 targets resulted in increased proliferation and inhibition of C2C12 cell myogenesis. In many cases the expression of genes related to non-muscle cell fates, such as chondrogenesis, was activated. This indicates that the concerted downregulation of multiple microRNA targets is not only crucial to the skeletal muscle differentiation program but also serves to prevent alternative cell fate choices.

Key words: miR-1, miR-206, Skeletal muscle, C2C12 myoblast, MicroRNA, Cell fate

Introduction

MicroRNAs (miRNAs or miRs) are short non-coding RNAs, found in both plants and animals. In animals, miRNAs bind with imperfect complementarity to target sites that are typically found in the 3'UTR of messenger RNAs. This results in inhibition of translation and in many cases transcript degradation (Carthew, 2006; Guo et al., 2010). Post-transcriptional regulation of gene expression by miRNAs is an important feature of cell differentiation programs and it has been predicted that there are many as yet undiscovered miRNAs in the genomes of human and other higher vertebrates (Bartel, 2004). Although some miRNAs have been characterised in detail, in most cases there is only limited information about their function. This is in part due to the incomplete complementarity of miRNAs with their targets, which makes the identification of biologically relevant targets more challenging (Bartel, 2009). Different algorithms predict numerous potential targets, not all of which are genuinely affected by miRNAs (Krützfeldt et al., 2006).

C2C12 myoblast cells are widely used as a model for skeletal muscle cell differentiation. Undifferentiated C2C12 cells express Myf5 and MyoD; however, the ability of these MRFs to induce differentiation is blocked under growth conditions. The removal of growth factors or the downregulation of their receptors leads to myoblast differentiation, which correlates with expression of miR-1 and miR-206, two highly related but not identical

microRNAs, which are conserved across many species (Sweetman et al., 2008). The specific versus redundant functions of miR-1 and miR-206 are currently not well understood. In addition, miR-1 and miR-206 are co-expressed with a member of the miR-133 family, which is generated from the same primary transcript but has a completely different sequence and therefore a distinct set of targets (Chen et al., 2006; Kim et al., 2006; Rao et al., 2006; Sweetman et al., 2008). Experiments in C2C12 cells suggested that miR-1 and miR-206 promote differentiation of myoblasts through downregulation of HDAC4 and the p180 subunit of DNA polymerase alpha, whilst miR-133 promotes proliferation through downregulation of SRF (Chen et al., 2006; Kim et al., 2006). In addition, it has been shown that miR-1 and miR-133 influence the differentiation behaviour of rhabdomyosarcoma cells and it might be possible to exploit their pro-myogenic functions for therapeutic purposes (Rao et al., 2010).

In vertebrate embryos, myogenic cells derived from embryonic somites give rise to skeletal muscles of the trunk and limbs, and skeletal myogenesis serves as a paradigm for the study of cell fate commitment in response to instructive cues. We, and others, showed that miR-1 and miR-206 expression is restricted to developing skeletal and cardiac muscle during vertebrate embryogenesis (Sweetman et al., 2006; Darnell et al., 2006; Wienholds et al., 2005). In addition, they are regulated by the

Myogenic Regulatory Factors (MRFs) in vivo (Hinits et al., 2011; Sweetman et al., 2008; Sweetman et al., 2006) consistent with the finding that MyoD and myogenin bind to regions upstream of *miR-1* and induce its expression in cell culture (Rao et al., 2006; van Rooij et al., 2008). Recently we demonstrated that miR-1 and miR-206 mediate appropriate timing of myogenesis in developing somites by contributing to the downregulation of Pax3 as committed myoblasts differentiate (Goljanek-Whysall et al., 2011). We have also identified additional microRNAs expressed in embryonic somites indicating that multiple microRNAs are involved in their differentiation (Rathjen et al., 2009). However, the precise roles of many of these miRs and how they act to regulate muscle development and differentiation remain to be identified.

Here we focus on miR-1 and miR-206 and identify novel targets using two different approaches: microarray screening of C2C12 cells transfected with miR-206 or anti-miR-206, as well as target predictions by three widely used target prediction algorithms, PicTar (Krek et al., 2005), TargetScanS (Friedman et al., 2009; Grimson et al., 2007; Lewis et al., 2005) and miRanda (Griffiths-Jones et al., 2008). Systematic analyses of predicted targets led to a list of candidates with potential biological relevance. A selection of these was experimentally validated using luciferase reporter assays with 3'UTR sensor constructs, leading to confirmation or elimination of predicted targets. Confirmed targets were then analysed in vivo, and we showed that endogenous expression levels of these genes are sensitive to miR-206/miR-1 overexpression, and that this miRNA-induced downregulation was inhibited by specific anti-miRs. This approach identified *CLCN3*, *Meox2*, *Smarcd2*, *Smarcb1*, *Fzd7*, *MAP4K3*, *NFAT5* and *RARB* as miR-1/miR-206 targets during differentiation of the C2C12 myoblast cell

line. The biological relevance of this regulation was confirmed by sustained overexpression of targets, which we show interferes with the myogenic differentiation program of C2C12 cells, leading to disruption of cell cycle exit, alteration of fibre morphology or activation of genes related to non-myogenic differentiation programs. Thus, this approach identified novel targets for miR-1/miR-206, whose negative regulation is crucial to orchestrate myogenic differentiation of C2C12 myoblasts.

Results

Transcript profiling identifies novel potential targets of miR-206

To identify effects of miR-206 on mRNA levels we generated transcript profiles of C2C12 cells on day one of myogenic differentiation, when the endogenous level of miR-206 is still low (D1-control). This was compared to C2C12 cells transfected with miR-206 (D1-miR-206), thus prematurely increasing the activity of miR-206. We also obtained samples on day two of differentiation (D2), when endogenous miR-206 expression is high. Cells were either untreated (D2-control) or transfected with an anti-miR that inhibits miR-206 (D2-anti-miR) (Fig. 1). Northern analysis showed that anti-miR-206 affected the expression of both miR-1 and miR-206 (Fig. 2D).

Microarray analyses were performed on a mouse Illumina array (Cambridge Genomic Services). The data set has been submitted to the GEO database at NCBI (accession number GSE34069). Bioinformatics analysis showed that anti-miR transfection affected the expression level of a greater number of genes than transfection with miR-206 (Fig. 1A,B). It is possible that at day one most targets are not yet expressed and therefore increasing miR-206 activity has a limited effect. Furthermore, the expression profile obtained with D2-anti-miR

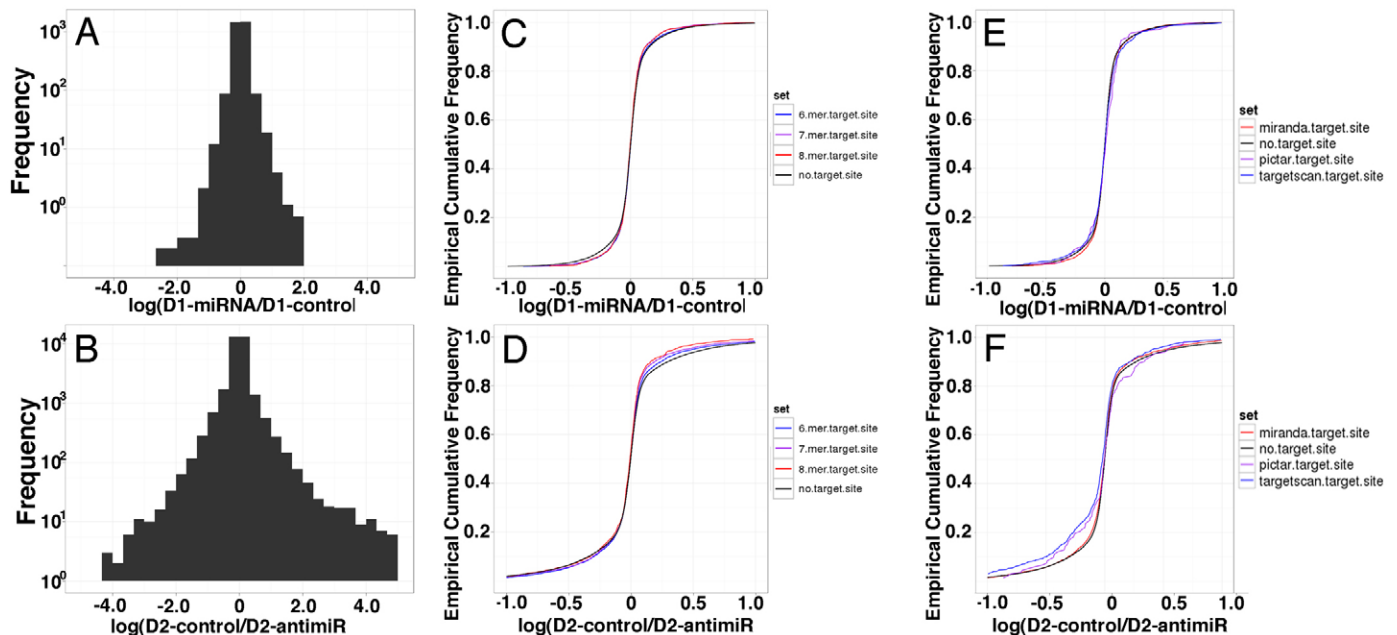


Fig. 1. Mouse Illumina array of C2C12 cells transfected with miR-206 or with anti-miR-206. (A,B) Differential expression histograms for pairwise comparisons: D1-control versus D1-miRNA (A) and D2-control versus D2-anti-miR (B). (C,D) Empirical cumulative distributions of differential expression for genes with and without miR-206 seed sites: D1-control versus D1-miRNA (C) and D2-control versus D2-anti-miR (D). (E,F) Empirical cumulative distributions of differential expression for genes predicted to be miR-206 targets by TargetScan, PicTar and Miranda: D1-control versus D1-miRNA (E) and D2-control versus D2-anti-miR (F).

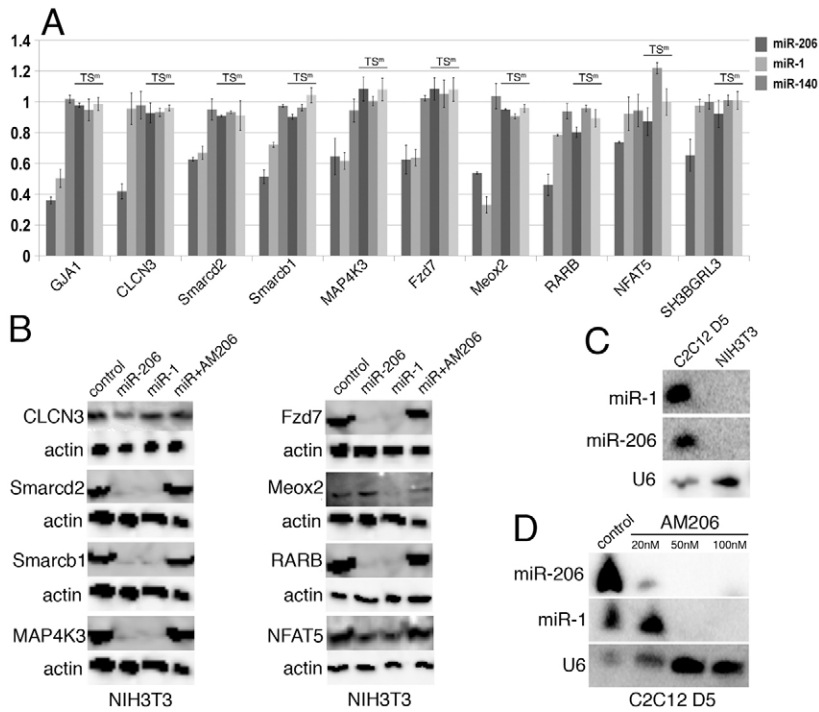


Fig. 2. miR-206 and miR-1 repress candidate target genes through elements in the 3'UTR. (A) Luciferase reporters were co-transfected with miRNAs identical to miR-206, miR-1, miR-140, as indicated. The 3'UTR-sensor constructs used are indicated below the columns. Relative luminescence units were plotted as a percentage of the control value obtained in absence of miRNA. A representative experiment of four independent experiments using two DNA plasmid preparations is shown. Error bars indicate s.d., $n=3$. Columns represent the wild-type or mutated (TS^m) 3'UTR sensor co-transfected with miR-1 or miR-206 ($P<0.05$, $n=3$), or with miR-140 (negative control). (B) Expression of endogenous proteins is regulated by miR-1 and/or miR-206 in NIH3T3 cells. Protein lysates were prepared from cells that were mock-transfected or transfected with miRNAs with or without anti-miR-206, as indicated above each lane. Western blots were probed with the antibodies indicated next to each panel. (C) Northern blot showing the expression of mature miR-1 and miR-206 in differentiated C2C12 cells. These miRNAs were not detected in NIH3T3 cells. U6 served as a loading control. (D) Northern blot showing inhibition of miR-1 and miR-206 expression in differentiated C2C12 cells. Cells cultured in GM were transfected with anti-miR-206 at concentrations indicated above each panel. Next, cells were cultured in DM for 5 days, RNA was isolated and probed against mature miR-1 and miR-206, as well as U6; 50 nM and 100 nM anti-miR-206 inhibited expression of both miR-1 and miR-206.

is more similar to either D1-control or D1-miR-206 than to D2-control (Table 1). One possible interpretation of these results is that the repression of miR-206 partially restores the profiles observed at day 1.

To assess the effect of the treatments on the expression level of putative miR-206 targets, we compared the differential expression empirical distributions of the set of genes containing putative target sites for miR-206 with that of all other genes (Fig. 1C,D). A putative target site was defined as a sequence complementary to the seed region of the miRNA (Rajewsky, 2006). We generated lists of target sites for perfect matches of six, seven and eight base pairs, required to mediate miRNA targeting (Grimson et al., 2007). Using the Mann–Whitney–Wilcoxon test we assessed the significance of the difference between the sets of genes containing each of these types of target sites and the set of genes without target site. The only significant results were obtained for the sets of genes containing 7-mer and 8-mer matches, in the D2-anti-miR and D2-control pairwise comparison (P -values of 0.009 and 0.006, respectively). The absence of significant differences between D1-control and D1-miR-206 confirms the small effect of miR-206 overexpression on day one. The significant differences between D2-control and D2-anti-miR indicate that there is a large number of genes containing a putative target site that are de-repressed due to the inhibition of miR-206 by anti-miR-206 transfection.

We also examined the set of targets predicted by three major target prediction algorithms TargetScanS (TargetScan4.1)

Table 1. Spearman correlation coefficient between pairs of samples

	D1-control	D1-siRNA	D2-control	D2-anti-miR
D1-control	1.00	0.96	0.91	0.95
D1-siRNA		1.00	0.92	0.96
D2-control			1.00	0.92
D2-anti-miR				1.00

(Friedman et al., 2009; Grimson et al., 2007; Lewis et al., 2005), PicTar (Krek et al., 2005) and miRanda (Griffiths-Jones et al., 2008) (supplementary material Fig. S1A). We repeated the comparison between the empirical distributions between the set of targets of each program and the set of genes not predicted to be targets (Fig. 1E,F). Once again, no significant difference was observed for the D1-miRNA and D1-control pairwise comparison. For the D2-anti-miR and D2-control, the most significant difference was observed for the set of targets predicted by TargetScan ($P<10^{-8}$, $P<0.74$ and $P<0.02$ for TargetScan, PicTar and miRanda, respectively).

To assess the impact of miR-206 on the expression profile not only through direct targeting but also through indirect effects we functionally annotated genes differentially expressed between D2-control and D2-anti-miR. For genes upregulated in D2-anti-miR, that is, the set of genes that negatively correlates with the activity level of miR-206, and which therefore should include most direct targets, the list of significantly enriched annotation terms contained only generic terms, such as 'cell cycle' and 'acetylation'. For the set of genes downregulated in D2-anti-miR (and that should constitute almost exclusively indirect targets) most of the enriched terms are related to muscle (supplementary material Table S1). This would suggest that miR-206 directly targets a relatively small number of genes that are repressors of a larger number of muscle-related genes (supplementary material Table S2 for the list containing these genes). This list includes for example myogenin, Laminin $\alpha 5$, Myh1, Myh3, Myh8, integrin $\alpha 7$, integrin $\beta 6$, fibrillin 1, desmin, troponin 1, troponins T1 and T3, dystroglycan 1, titin, actinin $\alpha 2$ amongst other interesting genes that are all associated with muscle structure and function.

Experimental validation of targets for miR-206 and miR-1

We generated a list of candidate miR-206 target genes that contained a seed site for miR-206 and were upregulated in the D2-anti-miR sample or that were predicted to be miR-206

targets by at least two of three target prediction databases (supplementary material Fig. S1). From these we selected putative targets associated with skeletal muscle development or differentiation. The transcripts of interest included *Meox2*, *Fzd7* and *RARB*, which are known to be expressed in embryonic muscles, including developing limb muscles (Blentic et al., 2003; Cauthen et al., 2001; Mankoo et al., 2003; Mollard et al., 2000; Reijntjes et al., 2007), *CLCN3*, a chloride channel expressed in adult muscle and heart (Yamamoto-Mizuma et al., 2004), *MAP4K3*, a mitogen activated kinase primarily activating the JNK signalling pathway (Ramjaun et al., 2001) *Smarcd2*, *Smarca1*, chromatin remodelling factors (Klochender-Yeivin et al., 2006; Lickert et al., 2004; Wang et al., 1996), *SH3BGL3* (*TIP-B1*), a novel TNF inhibitory protein (Berleth et al., 2001) and *NFAT5*, a transcription factor expressed in myoblasts and regenerating muscles (Lopez-Rodríguez et al., 1999; O'Connor et al., 2007). The putative miR-206 and miR-1 target sites are conserved in human, mouse, rat and chicken, except for *Smarcd2* where alignments showed that the seed sequence was poorly conserved (supplementary material Fig. S1). Sensor constructs were generated containing the 3'UTR with the potential microRNA binding site(s) (see Materials and Methods) and tested in luciferase assays. Chicken DF1 cells were transfected with 3'UTR-sensor constructs, Renilla luciferase vector and miR-1 or miR-206, or with miR-140, which is not predicted to target these transcripts (Goljanek-Whysall et al., 2011) (Fig. 2A).

A known target, *Connexin-43* (*GJA1*) (Anderson et al., 2006; Kim et al., 2006), served as a positive control and the *GJA1*-3'UTR sensor was regulated as predicted by miR-1 and miR-206. We defined validated targets as those that were efficiently downregulated by miR-1/miR-206 but where the unrelated miR-140 had no effect on the expression of the sensor construct. This approach confirmed *Smarcd2*, *Smarca1*, *MAP4K3*, *Fzd7*, *Meox2* and *RARB* as targets for both, miR-1 or miR-206. Interestingly, the 3'UTRs of *CLCN3* and *SH3BGLR3* were regulated by miR-206 but not miR-1. The *NFAT5* sensor was only weakly regulated by miR-206 and miR-1. For all confirmed targets mutation of the putative target site in the 3'UTR rendered the sensor construct insensitive to miR-1 or miR-206 indicating that interaction with the target site is required (Fig. 2A).

Next, we investigated the ability of miR-1 and miR-206 to regulate the expression of target genes in a cellular context (Fig. 2B). We found that NIH3T3 cells expressed robust levels of most candidate targets (data not shown) but miR-1 and miR-206 were not expressed (Fig. 2C). Therefore these cells were ideal to investigate whether transfected miR-1 and miR-206 could regulate expression of the endogenous proteins. To determine if the effects of miR-1/miR-206 expression were specific we conducted rescue experiments with co-transfected anti-miR-206, which suppressed the expression of both miR-1 and miR-206 in C2C12 myoblasts at 50 nM and 100 nM concentration (Fig. 2D).

Consistent with luciferase reporter assays *CLCN3* protein level decreased after transfection with miR-206 but not after transfection with miR-1 compared to untransfected cells (Fig. 2B). The reduced levels of *CLCN3* protein were restored by co-transfection of anti-miR-206. Western blots of *Smarca1*, *Smarcd2*, *MAP4K3*, *Fzd7* and *RARB* showed decreased protein levels after transfection with miR-1 or miR-206 compared to untransfected cells (Fig. 2B). In all cases protein levels were restored to that of controls by co-transfection of anti-miR-206. *Meox2* protein level was reduced by miR-1 but was not affected

by miR-206 transfection, although in luciferase assays both miRNAs were effective in reducing expression levels. The decreased levels of *Meox2* protein could be restored by co-transfection of anti-miR (Fig. 2B). Consistent with our results from luciferase assays *NFAT5* protein levels were only slightly affected by transfection of miR-206 or miR-1 (Fig. 2B) and co-transfection of anti-miR restored protein levels to that of controls.

miRNA dependant downregulation of candidate genes during C2C12 differentiation

To determine whether interactions between miR-1/miR-206 with these novel targets are biologically relevant we examined their expression profiles during C2C12 myoblast differentiation. When grown in differentiation medium (DM) containing low serum, C2C12 cells differentiate into myotubes within 5 days and miR-1 and miR-206 are expressed by day 2 (Chen et al., 2006; Kim et al., 2006; Rao et al., 2006; Sweetman et al., 2008). Consistent with previous reports transfection of C2C12 cells with miR-206 or miR-1 enhanced myogenic differentiation (Chen et al., 2006; Kim et al., 2006; Nakajima et al., 2006), whereas transfection of anti-miRNAs inhibited both, miRNA expression (Fig. 2D) and myogenesis (data not shown).

Target gene expression was examined by QPCR and Western blots of differentiating C2C12 cells in the presence or absence of anti-miR-206 (Fig. 3A–C). Expression of *Connexin-43* (*GJA1*) served as a positive control and, as previously reported by others, transcripts were downregulated significantly from day 2 of C2C12 cell myogenic differentiation (Kim et al., 2006); this was inhibited by blocking miRNA function through transfection of anti-miR-206 (data not shown). Similarly, *CLCN3*, *Smarcd2* and *Smarca1* transcripts were reduced during C2C12 myogenic differentiation (Fig. 3A) and inhibition of miR-1/miR-206 with anti-miR-206 prevented the downregulation of these transcripts and levels remained at 80–100% of that seen in undifferentiated cells at day 0 (Fig. 3B).

Transcripts for *Meox2*, *RARB* and *NFAT5* were reduced during myogenesis from day 1 or day 2 onwards (Fig. 3A). However, downregulation of *Meox2*, *RARB* and *NFAT5* transcript levels was only partially inhibited in the presence of anti-miR-206 (Fig. 3B). This indicates that additional mechanisms, independent of miR-1/miR-206 function, are involved in the negative regulation of *Meox2*, *NFAT5* and *RARB* expression during C2C12 cell differentiation.

There was no detectable change in *Fzd7* and *MAP4K3* transcript levels during C2C12 cell myogenesis (Fig. 3A), indicating that miR-1/miR-206 may downregulate *Fzd7* and *MAP4K3* proteins mainly through inhibition of translation.

Next, we determined the effects of miR-1/miR-206 on protein expression in differentiating C2C12 cells. Western blot analyses demonstrated that endogenous *CLCN3*, *Smarcd2*, *MAP4K3*, *NFAT5* and *RARB* proteins were downregulated significantly during myogenic differentiation from day two onwards. *Smarca1*, *Fzd7* and *Meox2* protein levels were downregulated from day 3 or later (Fig. 3C). Transfection of anti-miR-206, which blocks expression of miR-1/miR-206 in differentiating C2C12 cells (Fig. 2D; Fig. 3D), inhibited the downregulation of these target genes.

Sustained expression of miR-1/miR-206 targets inhibits C2C12 cell myogenesis

To investigate whether miRNA-mediated target repression is essential for myogenesis, we examined the consequences of

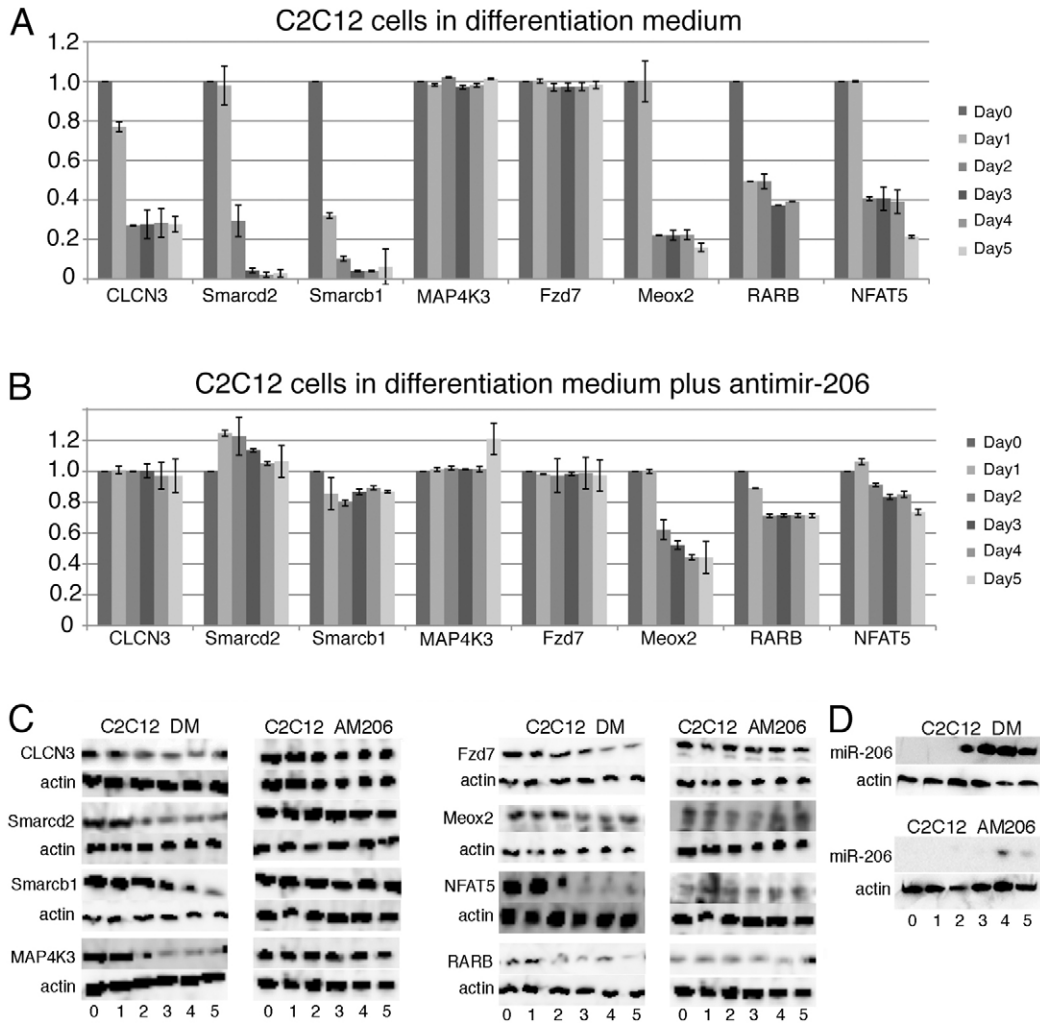


Fig. 3. Transcript and protein levels of candidate targets are regulated by miRs during C2C12 myogenesis. (A,B) qPCR for the transcripts indicated was performed on cDNA from C2C12 myogenic cells on the days indicated by different shaded columns. Cells were grown in differentiation medium (DM) in the absence (A) or in the presence of antimir (B), as indicated. Normalised values are plotted. (C) Protein lysate was harvested from C2C12 cells or from C2C12 cells treated with antimir on the days indicated below each lane. Western blots were probed with the antibodies indicated next to each panel. Inhibition of miR-1/miR-206 results in loss of downregulation of target genes. (D) Northern blot shows expression of miR-206 during C2C12 cell differentiation in the presence or absence of antimir-206 as indicated. U6 served as loading control. Antimir-206 was efficient in blocking miR-206 expression during differentiation.

forced expression of target genes. We transfected C2C12 cells with eukaryotic expression constructs lacking the 3'UTR. Based on GFP controls the transfection efficiency was around 30%. Western blots showed that this led to significantly increased levels of target proteins. In all cases, elevated protein levels were still evident at day 5 of differentiation (supplementary material Fig. S2) and there was no evidence of increased apoptosis (supplementary material Fig. S4). After transfection C2C12 were cultured in growth (GM) or differentiation medium (DM) and myogenic differentiation was assessed by immunostaining of myosin heavy chain (MyHC) with MF20 antibody (Fig. 4A,B). Mock-transfected C2C12 cells cultured in GM did not differentiate until day 5, when a few myotubes were detected due to depletion of growth factors. In contrast, after culture in DM, myotubes were observed at day 3, and by day 5 most cells had differentiated and were labelled by immunostaining with MF20 (Fig. 4B). First we examined the effects of antimir-206 transfection, which prevents

effective downregulation of all target genes (Fig. 3C). This led to impaired myogenic differentiation, evidenced by fewer MF20 positive fibres (Fig. 4B) and by determining the fusion index and the total myotube area, which were both reduced. The cells transfected with antimir-206 were more similar to cells grown in GM (Fig. 4C,D).

The antimir-206 prevents downregulation of all miR-1/miR-206 targets. Next we determined the effect of a single target on myogenic differentiation and expression plasmids were transfected individually. In most cases sustained target gene expression led to delayed myogenic differentiation of C2C12 cells as shown by MF20 staining. Representative examples are shown (Fig. 4A). Following transfection with CLCN3, MAP4K3, Fzd7, SH3BGRL3, Smarcd2, Smarcb1 and NFAT5 only very few MF20 positive myotubes were detected after 3 days in DM (Fig. 4A and not shown) when compared to mock-transfected cells (Fig. 4B). After 5 days in DM myotubes were present in

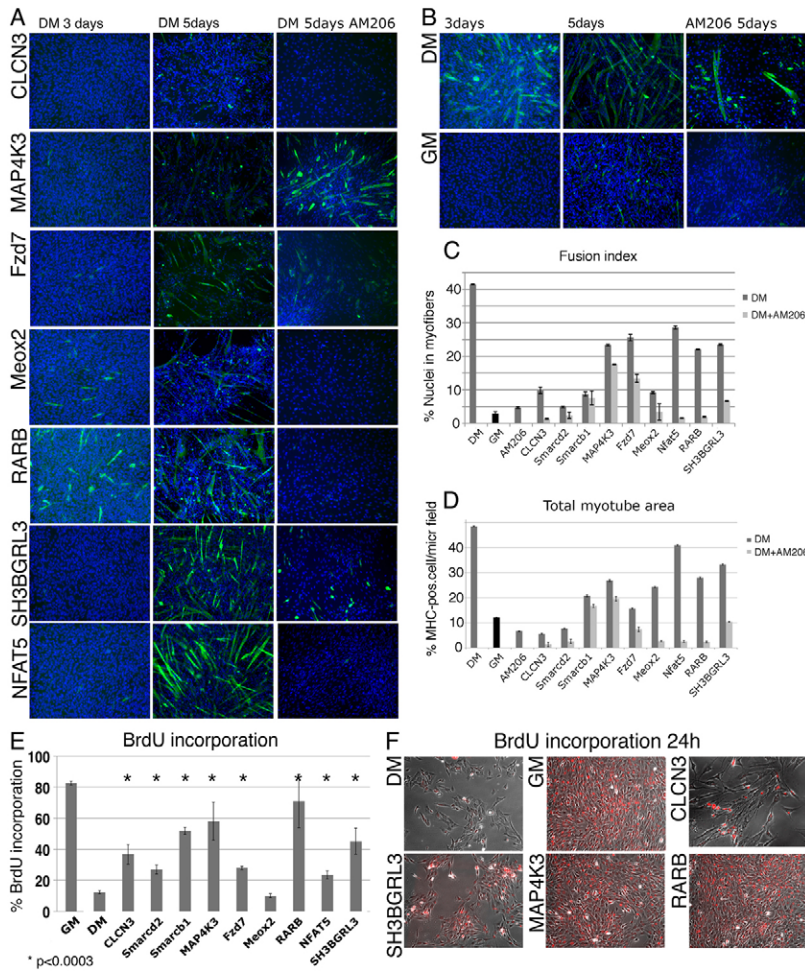


Fig. 4. Downregulation of target genes by miR-1 and miR-206 is required for myogenic differentiation. C2C12 cells were transfected with expression constructs encoding for target genes, as indicated. Following transfection, C2C12 cells were cultured in differentiation medium (DM), as indicated.

(A) C2C12 cells transfected with target genes or co-transfected with AM206 and target genes were cultured in DM and immunostained for MHC after 3 and 5 days of culture (MF20, green; DAPI, blue). (B) The staining obtained was compared with control mock-transfected cells cultured in DM, which express MHC (green) at day 3 and at day 5. By contrast, only few differentiated fibres (green) are present in cells cultured in GM for 5 days. Cells transfected with anti-miR-206 (AM206) and cultured in DM show delayed myogenesis.

(C,D) Quantification of fusion index and total myotube area following transfection and culture of C2C12 cells is shown. Overexpression of all target genes inhibited C2C12 differentiation significantly ($P < 0.05$) with the exception of NFAT5. Cells overexpressing RARB and SH3BGLR3 differentiated efficiently compared with the DM control, probably due to their increased proliferation and consequent growth factor depletion from the culture medium. C2C12 co-transfected with target genes and AM206 showed a greater delay in myogenesis. GM, growth medium; DM, differentiation medium. (E,F) Proliferation of C2C12 cells following transfection with target genes and culture in DM was determined by anti-BrdU antibody (red). (E) The percentage of BrdU-positive cells is shown. Error bars indicate s.d. from three independent experiments. (F) C2C12 overexpressing selected target genes and cultured in GM showed similar proliferation rate to C2C12 cells cultured in GM.

transfected C2C12 cells, however their number was reduced, either strongly (MAP4K3, Fzd7, Meox2, RARB, Smarcb1) or weakly (SH3BGLR3, NFAT5), when compared to control cells (Fig. 4A,B). The negative effect on myogenic differentiation was confirmed by determining the fusion index and the total myotube area (Fig. 4C,D). The fusion index, determined by the number of nuclei per fiber divided by the number of total nuclei, was significantly reduced for most targets compared to control cells in DM (Fig. 4C). In addition, in most cases the percentage of total image area covered by myotubes (total myotube area) was significantly reduced ($P = 0.05$) with the exception of NFAT5 ($P = 0.1$). Co-transfection of anti-miR-206 with the target genes enhanced the negative effect on myogenic differentiation (Fig. 4A,C,D), although the inhibition was sometimes not as great as that seen with anti-miR-206 alone (see Discussion).

The removal of growth factors from the culture leads to cell cycle exit and activation of the C2C12 myogenic differentiation program. To investigate whether miR-1/miR-206 targets affected C2C12 cell proliferation we measured BrdU incorporation (Fig. 4E,F). When cultured in GM 80% of cells incorporated BrdU compared to 12% of cells cultured in DM. Transfection of miR-1/miR-206 targets significantly increased the number of C2C12 cells cultured in DM that incorporated BrdU, CLCN3 (38%), Smarcd2 (28%), Smarcb1 (52%), MAP4K3 (58%), Fzd7 (29%), RARB (70%), NFAT5 (25%), SH3BGLR3 (46%) (Fig. 4E). The exception was Meox2, which showed

no significant increase in the number of BrdU-positive cells (Fig. 4E); representative images are shown (Fig. 4F). Together, these data show that downregulation of miR-1/miR-206 target genes in committed myoblasts is critical and failure to do so inhibits C2C12 myogenic differentiation due to effects on different cellular processes such as proliferation and/or myoblast fusion.

We next asked whether inhibition of miR-1/miR-206 activity or overexpression of individual microRNA targets after the onset of C2C12 differentiation could affect myogenesis. C2C12 cells were cultured in DM and transfected on day 3 with either anti-miR-206 or with target gene expression constructs (transfection efficiency was 10–15%). After a further 3 days of culture in DM myogenic differentiation was analysed by staining with MF20 antibody and determination of fusion and differentiation indices (Fig. 5A–C). C2C12 cells transfected with anti-miR-206, which inhibits miR-1/miR-206 activity and affects all targets, resembled cells cultured in GM and fewer MF20 positive myotubes were seen compared to control cells cultured in DM (Fig. 5A). The fusion index and total myotube area were both reduced and were similar to cells cultured in GM (Fig. 5B,C). Next we determined the effect of individual target genes. Transfection of expression constructs on day 3 led to impaired myogenesis to varying degrees by day 6. The most dramatic effects were observed with CLCN3 and Meox2; however, expression of all other targets after the onset of

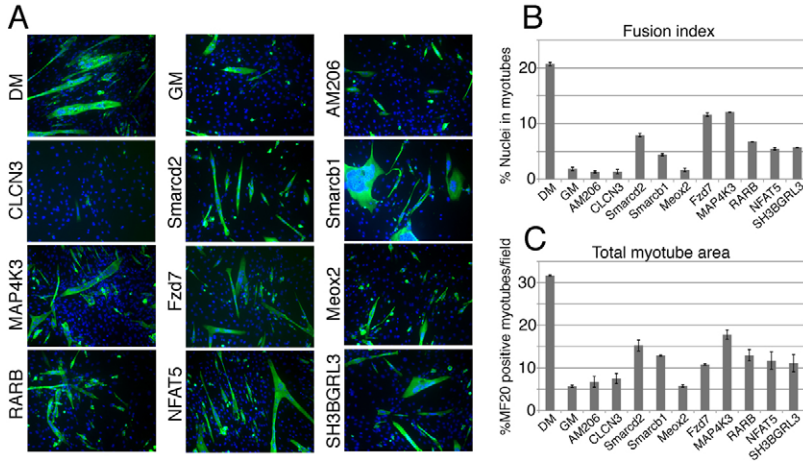


Fig. 5. Downregulation of target genes by miR-1 and miR-206 is required for terminal myogenic differentiation.

C2C12 cells were transfected with expression constructs encoding for the indicated target genes at day 3 of differentiation when myotubes started to form. (A) Following transfection, C2C12 cells were cultured in differentiation medium (DM) until day 6 and then immunostained for MHC (MF20, green; DAPI, blue). (B,C) Quantification of fusion index and total myotube area is shown. Overexpression of all target genes inhibited terminal differentiation of committed C2C12 cells significantly ($P < 0.05$). Cells transfected with Smarcb1 also displayed unusual morphology.

C2C12 cell myogenesis led to reduced fusion and total myotube area.

C2C12 myoblasts activate alternative differentiation programs after sustained expression of miR-1/miR-206 target genes

C2C12 and primary myoblasts can transdifferentiate into osteogenic or adipogenic cells following treatment with bone morphogenic proteins (BMPs) or adipogenic inducers, respectively (Asakura et al., 2001; Nakajima et al., 2006). To investigate whether miR-1/miR-206 targets can promote

alternative differentiation programs, we used quantitative RT-PCR and histological stains to examine osteogenesis, adipogenesis and chondrogenesis (Fig. 6). Osteogenic markers included early and late markers, osterix and osteocalcin, as well as alkaline phosphatase staining. Collagen 10A (Col10A) and the matrix metalloproteinase, MMP13 as well as Alcian Blue staining were used to indicate chondrogenic differentiation. TM4SF1 and leptin were used to assay for adipogenesis.

None of the microRNA-regulated genes reported here were able to induce adipogenesis-related gene expression in C2C12 cells (data not shown). In contrast, osterix and osteocalcin were detected

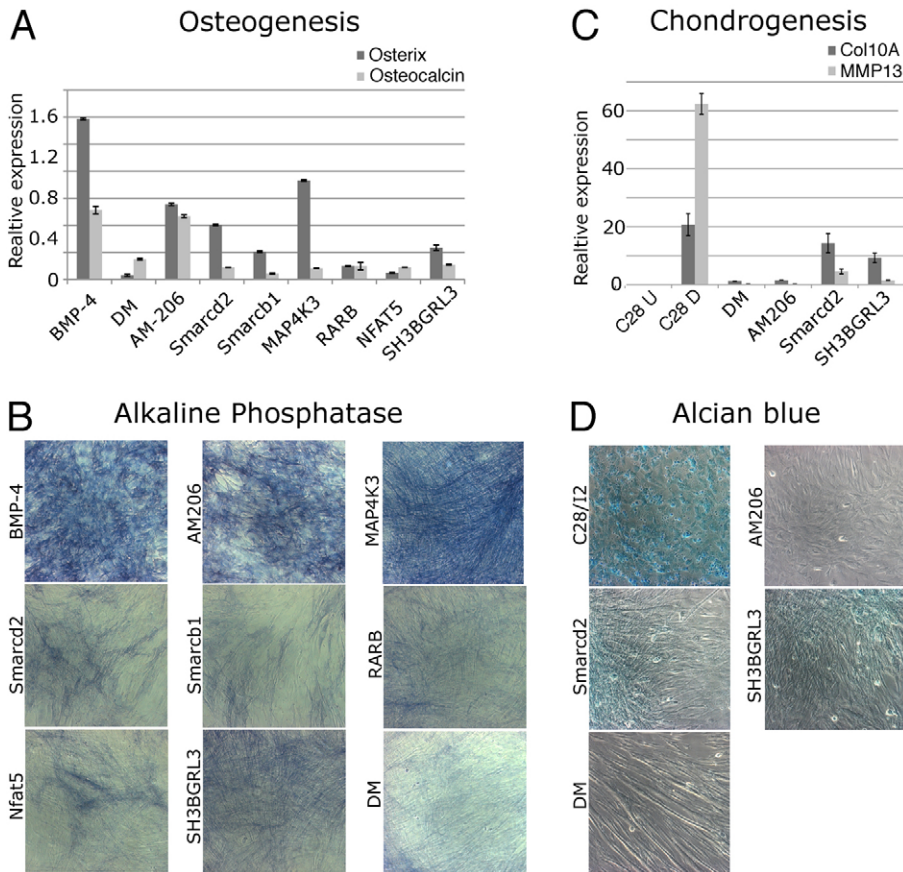


Fig. 6. Overexpression of target genes in C2C12 cells leads to expression of chondrogenic and/or osteogenic markers.

C2C12 cells were transfected with target genes and cultured in DM. (A,C) q-PCR showed that expression of AM206 or selected target genes led to increased expression of osteogenic (A) and chondrogenic (C) markers, compared with cells cultured in DM. (A) Expression of AM206 and all target genes shown led to significantly increased expression ($P < 0.05$) of the early osteogenic marker osterix compared with mock-transfected control cells and some genes also increased expression of the late osteogenic marker, osteocalcin. (B) C2C12 cells transfected with AM206 or the target genes indicated showed positive alkaline phosphatase staining compared with controls. C2C12 cells treated with BMP-4 served as a positive control for osteogenic differentiation. Smarcd2, Smarcb1, RARB and NFAT5 were less efficient in inducing osteogenesis compared with AM206- or MAP4K3-transfected cells and only ~10% of cells stained positive for alkaline phosphatase. (C) Relative expression levels of chondrogenic markers: Col10A and MMP13 in cells transfected with Smarcd2 or SH3BGRL3 were significantly higher ($P < 0.05$) than in control C2C12 cells or undifferentiated C28/I2 cells. Transfection of AM206 did not result in increase of chondrogenic markers. Differentiated C28/I2 cells served as a positive control. (D) Alcian Blue staining indicating chondrogenic differentiation. Approximately 20% of cells displayed positive Alcian Blue staining after transfection with Smarcd2 or SH3BGRL3.

in C2C12 cells cultured in DM and transfected with anti-miR-206 or expression plasmids encoding Smarcd2, Smarcb1, MAP4K3 or SH3BGRL3 (Fig. 6A). Transfection of RARB and NFAT5 plasmids also led to expression of osteogenic markers, however, at relatively low levels. Histological staining for alkaline phosphatase resulted in a strong signal, comparable to that in control cells cultured in BMP-4, or in cells transfected with anti-miR-206 or MAP4K3 (Fig. 6B). The chondrogenic markers MMP13 and Cola10A were significantly increased in C2C12 cells transfected with Smarcd2 or SH3BGRL3 (Fig. 6C) and these cells also stained positive for Alcian Blue (Fig. 6D). We used differentiated chondrocytes, C28/I2, as control.

Finally, we investigated whether transfection of C2C12 cells on day 3 of differentiation could activate osteogenic or chondrogenic gene expression after a further three days culture (supplementary material Fig. S3). Surprisingly, some alkaline phosphatase positive cells were detected after transfection of anti-miR-206 or transfection of expression plasmids encoding Smarcd2 or SH3BGRL3. Alcian Blue signal was seen in very small patches of C2C12 cells after transfection with SH3BGRL3. These data indicate that inappropriate expression of at least some miR-1/miR-206 target genes can promote cell fates closely related to but distinct from skeletal muscle.

Discussion

Skeletal myogenesis serves as an important paradigm for cell differentiation and the muscle differentiation program is recapitulated in C2C12 cells. To determine the role of miR-1/miR-206 during skeletal myogenesis, we identified potential targets based on mRNA profiling following manipulation of miR-206 activity in C2C12 cells, predictions by multiple algorithms and prior knowledge of genes known to be involved in myogenesis. This approach has helped to avoid false positive hits; however, it is likely that there are additional targets that have yet to be confirmed. We focused on targets that were more likely to be biologically relevant and most of the targets selected for further study have been implicated in skeletal or cardiac myogenesis. Using a number of complementary assays we validated nine novel miR-1/miR-206 targets from our screen.

Interestingly, inhibiting miRNA function by anti-miR-206 affected the expression of a greater number of genes in C2C12 cells than overexpression of miR-206 (Fig. 1A,B). This may indicate that many miR-206 targets are not yet expressed on day one of differentiation and therefore increasing the level of miR-206 had a limited effect. Conversely, some target genes involved in embryonic myogenesis may no longer be expressed in C2C12 myoblasts, which are derived from adult satellite cells. An example of this is Pax3, which we showed recently, is targeted by miR-206 during the progenitor to myoblast transition (Goljanek-Whysall et al., 2011) but was not identified in this new screen, although it has been reported that Pax3 and the highly related Pax7 are regulated by miR-1/miR-206 during satellite cell activation (Chen et al., 2010; Hirai et al., 2010).

The finding that the expression profile obtained from the D2-anti-miR-206 sample was similar to D1 control suggests that repression of miR-206 partially restores day 1 expression profiles (Table 1). Functional annotation of genes differentially expressed between D2-control and D2-anti-miR-206 showed that genes downregulated in the D2-anti-miR-206 group, so-called anti-targets, were enriched for terms related to muscle (supplementary material Table S1). This suggests that miR-206 directly targets,

and negatively regulates a relatively small number of genes, which would otherwise repress a larger number of muscle-related genes (supplementary material Table S2 for the list containing these genes). This is consistent with previous findings, which reported that after introduction of miR-1/miR-206 a significant number of upregulated genes were related to muscle structure and function. Similar to our observations the authors suggested that this is most likely due to indirect effects after the primary differentiation-inducing stimuli of the miRNAs (Kim et al., 2006).

We validated candidate genes using *in vitro* and cell based approaches. miR-1 and miR-206 downregulated luciferase sensor constructs whilst introduction of specific mutations into the potential miR target site showed that this was dependant on the presence of these conserved target sites in the 3'UTR of the respective genes (Fig. 2A). Notably not all confirmed target sites are equally regulated by miR-1 and miR-206. For example, the target site we identified in CLCN3 is regulated by miR-206 but not miR-1 while the Meox2 site is more strongly affected by miR-1 than miR-206. Furthermore, transfection of miR-206 and miR-1 led to a significant reduction of the putative target proteins in NIH3T3 cells, whilst co-transfection with anti-miRs restored protein expression to normal levels (Fig. 2B). This indicates that miR-1/miR-206 can interact with endogenous mRNAs in a cellular context.

During C2C12 cell myogenic differentiation miR-206 and miR-1 are highly expressed from day 2 onwards (Sweetman et al., 2008; Chen et al., 2006; Kim et al., 2006; Rao et al., 2006) and, consistent with previous observations, inhibition of miR-1/miR-206 activity led to impaired myogenesis (Fig. 4B) (Chen et al., 2006). Increased microRNA expression was associated with reduced expression of the novel targets in C2C12 cells (Fig. 3A,C) and suggests that downregulation of these genes may be required to facilitate myogenic differentiation. This was confirmed by sustained expression of target genes in C2C12 cells undergoing differentiation following plasmid transfection. All expression plasmids led to a significant increase of protein levels compared to normal endogenous levels (supplementary material Fig. S2) and these increased protein levels were still seen at day 5 of differentiation. Transfection of validated miR-1/miR-206 targets had a negative effect on myogenesis, which was further enhanced by co-transfection of anti-miR-206 in all cases. However, co-transfection of both target gene expression plasmid plus anti-miR-206 was sometimes less effective than transfection with anti-miR-206 alone. There are different possible explanations for this. The transfection of plasmid DNA plus anti-miR may lead to less efficient uptake of anti-miR. We also show that sustained overexpression of target genes increased proliferation (Fig. 4E,F). Therefore cells reached confluency faster and cultures were depleted of growth factors, which can then promote spontaneous differentiation. This would ameliorate the negative effects on myogenesis.

The inhibitory effects varied from almost complete inhibition of myotube formation (e.g. CLCN3) to only slight phenotypic changes, such as formation of thinner fibres containing a smaller number of nuclei (e.g. Fzd7, RARB) (Fig. 4A,B). This was quantified by determination of fusion index and total myotube area (Fig. 4C,D) using established approaches (Ricotti et al., 2011). To examine possible effects of miR-1/miR-206 inhibition after the onset of differentiation, we modified miRNA levels, by anti-miR-206 transfection at day 3 (in DM). At this point C2C12

myoblasts have elongated and begun to fuse and express sarcomeric myosin, detectable with MF20 immunostaining. They also express high levels of miR-1/miR-206 (e.g. Chen et al., 2006; Sweetman et al., 2008) (Fig. 3D; Fig. 4B). However, after anti-miR-206 transfection both fusion index and total myotube area were dramatically reduced and were similar to that of cells kept in growth medium (Fig. 5B,C). Despite low transfection efficiency, transfection of individual target genes at day 3 also negatively affected C2C12 cell myogenesis, with the strongest effects apparent for the CLCN3 chloride channel (Fig. 5A). For RARB, NFAT5 and SH3BGRL3 the negative effect on myogenesis appeared to be stronger when cells were transfected at day 3 compared to transfection at day 0. At present we cannot explain the reasons for this observation. However, it is possible that this is related to specific functions of these genes, which are currently not well understood.

Together our results showed that modifying miRNA activity or the levels of single targets had a negative effect on myogenesis, even when performed after the onset of differentiation. This illustrates that miRNA function is pivotal for the successful execution and maintenance of the differentiation program. In addition, most of the genes investigated enhanced cell proliferation when introduced into C2C12 cells cultured in differentiation medium (Fig. 4E,F).

To establish whether any of the validated miR-1/miR-206 targets are associated with alternative differentiation programs we examined the expression of adipogenic, chondrogenic and osteogenic markers. miR-206 has been shown to be expressed in osteoblasts; however, it needs to be downregulated to facilitate their differentiation (Inose et al., 2009). This is consistent with data from C2C12 cells, which no longer express miR-1 after induction of osteoblastic differentiation (Nakajima et al., 2006). Whilst the importance of miR-1/miR-206 for myogenic differentiation and maturation is widely recognised, it has been suggested that miR-1/miR-206 are less important for cell fate determination (Chen et al., 2006; Nakajima et al., 2006). Interestingly, we show here that inhibition of miR-1/miR-206 activity or sustained expression of a number of novel target genes not only interferes with myogenesis (Figs 4,5) but can also lead to the activation of osteogenic and chondrogenic markers (Fig. 6, supplementary material Fig. S3). These findings confirm that miR-1 and miR-206 activity is integral to myogenesis at both the early commitment stages (Goljanek-Whysall et al., 2011) and during the subsequent differentiation process. We propose a dual function whereby miRNA mediated downregulation of genes, which would enhance non-myogenic programs and/or promote proliferation, is crucial for both the complete cellular commitment as well as efficient execution of the differentiation program. This is similar to our recent observations in developing embryos, where we showed that miR-206 contributes to the complete silencing of Pax-3, a pro-myogenic gene expressed in skeletal muscle progenitor cells (Goljanek-Whysall et al., 2011). This downregulation facilitates the transition of progenitor to committed myoblast, and similar regulatory interactions are also important during the activation of satellite cells (Chen et al., 2010; Hirai et al., 2010).

Thus, miR-1 and miR-206 represent a core mechanism ensuring efficient cell fate commitment and myogenic differentiation, in developing embryos, satellite cells and in C2C12 myoblasts. Our study identifies a series of novel targets for miR-1/miR-206, and demonstrates that their coordinated downregulation is required to prevent continued myoblast proliferation and the expression of genes associated with alternative cell fates.

Materials and Methods

DNA constructs and luciferase assay

The modified pGL3 vector (Promega) (Tuddenham et al., 2006) contained 3' UTR regions that were PCR amplified from chick embryo cDNA (HH stage 17–26). To mutate the predicted target sites a BamHI site (miR-1/miR-206 target sites) was introduced, which led to point mutations and facilitated identification of clones. A list of primers used is available in supplementary material Table S3.

DF1 chicken dermal fibroblasts were cultured in 96-well plates and transfected using Lipofectamine 2000 (Invitrogen) with either WT or mutant sensor constructs (200 ng), with or without miR-206/miR-1 (50 nM) (Sigma) as described (Goljanek-Whysall et al., 2011). The empty pGL3 vector or transfection with miR-140, which is not predicted to target any of the genes screened were used as a negative controls. Each experiment had triplicate samples and was carried out four times using two independent plasmid preparations of each reporter. Protein was extracted after 48 hours, luciferase activity was measured using a multilabel counter (Victor2, Perkin-Elmer, MA) and normalised to Renilla luciferase. Relative reporter activity for miR treated cells was obtained by normalisation to non-miR-treated WT or mutant constructs, respectively. *P*-values were calculated by Student's *t*-test.

Cell culture, western blots and analysis of cell differentiation

Mouse NIH3T3 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. NIH3T3 cells were transfected with miR-206 or miR-1 (50 nM, Sigma), with or without anti-miR-206 (100 nM, Ambion) using Lipofectamine 2000. Protein was extracted after 48 hours following standard protocols using lysis buffer with protease and protein phosphatase inhibitors (Roche), 20 µg cell lysate was run on 4–12% polyacrylamide gels and blotted onto nitrocellulose using a semidry blotter (Bio-Rad). Primary antibodies (1:500 or 1:1000) and secondary antibodies coupled to HRP (Jackson Laboratories) were applied for 1 hour each at room temperature. Primary antibodies used: CLCN3 (LifeSpan Biosciences), Meox2, SMARCD2, SMARCB1, NFAT5, RARB, caspase-9 and actin (Abcam, Cambridge, UK).

Mouse C2C12 cells were maintained in growth medium (GM: DMEM, 10% FBS, 1% penicillin-streptomycin). Myogenic differentiation was induced in sub-confluent cells in differentiation medium (DM: DMEM, 2% horse serum, 1% penicillin-streptomycin) as described (Sweetman et al., 2008). After transfection with anti-miRs (50 nM, Ambion), cells were incubated in DM. Whole cell lysates were extracted on the day of transfection and then every 24 hours for 5 days. Osteogenic differentiation of C2C12 cells was induced in DMEM supplemented with 5% horse serum and 400 ng/ml human recombinant BMP-4 for 6 days as described (Katagiri et al., 1994). To examine alkaline phosphatase activity histochemically, cells were fixed and stained as described (Katagiri et al., 1994).

C28/12 cells were cultured and induced to chondrogenic differentiation as described previously (Greco et al., 2011). Alcian Blue staining was performed as previously described (Swingler et al., 2012) and C28/12 cells served as positive control. For adipogenic differentiation, C2C12 cells were grown to confluency in GM and switched to an adipogenic medium as described (Akimoto et al., 2005). Apoptosis was induced by 2 mM H₂O₂ for 6 hours as described (Salucci et al., 2010).

To determine the C2C12 fusion index we counted the number of nuclei in myotubes and divided this by the total number of nuclei, after multiplication by 100 the *y*-axis in Fig. 4C, Fig. 5B represents percent of nuclei within myotubes. The total myotube area represents the percent of total image area covered by myotubes. Both were established using six different microscopic fields from three different experiments using ImageJ.

Microarray samples and annotation methods

We chose two time points that showed the highest differences in mRNA levels of a known miR-206 target (GJA1). Control C2C12 cells were cultured in DM and harvested at day 1 or day 2 (D1 control, D2 control). To activate miR-206 prematurely, cells were transfected with miR-206 and RNA was harvested at day 1 (D1 miRNA group). To inhibit miR-206, C2C12 cells were treated with anti-miR-206 and harvested at day 2 of differentiation (D2 anti-miR-206 group). Three replicate samples of RNA were harvested and Illumina's MouseWG-6 V2 BeadChip microarrays were probed. Pre-processing of microarray data was performed using R and Bioconductor. Normalisation was performed using the lumi package (Du et al., 2008). Microarray probes were annotated using the lumiMouseIdMapping package. 3'UTR sequences were downloaded from Ensembl. Functional annotation was performed using the DAVID webserver (Huang et al., 2008). The data was submitted to GEO (accession number GSE34069) and meets the MIAME requirements.

RNA isolation and northern analysis

Standard methods were used as described previously (Sweetman et al., 2008).

Quantitative real time PCR

cDNA was generated using random hexamers and Superscript II reverse transcriptase (Invitrogen). TaqMan primers for real-time PCR reactions were

obtained from Applied Biosystems as follows: Meox2: Mm00801881_m1; NFAT5: Mm00467257_m1; RARB: Mm01319674_m1; GJA1: Mm00439105_m1; CLCN3: Mm01348786_m1; Evl1: Mm00514810_m1; SMARCB1: Mm00448776_m1; SMARCD2: Mm00473467_g1. 18S ribosomal RNA was used as an internal control to normalise for differences in the amount of total RNA in each sample. TaqMan 18S primers and a 5'-VIC-labelled probe were used according to the manufacturer's instructions (Applied Biosystems). Each experiment had triplicate samples and the values for day 1–5 were normalised to the value obtained for day 0. Cell fate was analysed using SYBR Green qPCR SuperMix (Invitrogen) and following primers: OsterixF: 5'-GGGTAAAGGGGAGCAAAGTCAGAT-3', OsterixR: 5'-CTGGGGAAAGGAGGCACAAGAAG-3', OsteocalcinF: 5'-CT-GAGTCTGACAAAGCCTTC-3', OsteocalcinR: 5'-GCTGTGACATCCATACTTGC-3', Col10F: 5'-ATTATCCACTGCTCCCTGGGCAGA-3', Col10R: 5'-ATAGATTCTGGTGCTGGGAGATGCG-3', MMP13F: 5'-AAAGACAGATTCTCTGGCGCCTG-3'. Mouse β -actin was used as reference gene.

Expression vectors for validated target genes and BrdU incorporation assay

Expression plasmids encoding target genes were obtained from Source Bioscience Life Sciences (I.M.A.G.E. full length cDNAs). The expression vectors were pCMV.Sport 6 (4396bp) and pCMV.Sport6.1 (4177bp), empty pCMV.Sport6 served as control. The I.M.A.G.E. clone numbers are as follows, Smarcd2: 2647494, Smarcb1: 3154501, CLCN3: 6833732, Fzd7: 6844727, Meox2: 008594, MAP4K3: 6825201, RARB: 30608242, NFAT5: 40083218, SH3BGR3: 341987. C2C12 cell proliferation was assessed by BrdU incorporation as described (Shan et al., 2007). Briefly, C2C12 cells were seeded on glass coverslips and transfected as described (Sweetman et al., 2008). Control cells were treated with lipofectamine only. After 4 h incubation cells were labelled with 10 μ M of BrdU (Sigma-Aldrich) for 3 h and harvested after an additional 24 h. Cells were fixed in 4% paraformaldehyde and stained with BrdU antibody (1:100) and donkey anti-mouse IgG (FITC conjugated, 1:250; Jackson ImmunoResearch Laboratories). BrdU quantification was assessed by analysing 6 different microscopic fields from three different experiments by dividing the number of nuclei stained positive with anti-BrdU antibody by the total number of nuclei. C2C12 cell differentiation was assessed by MF20 staining (Sweetman et al., 2008). Images were analysed using Axiovision and ImageJ. *P*-values were calculated by Student's *t*-test.

Acknowledgements

We thank Nick Redshaw for help with luciferase assays and molecular cloning, Caroline Pennington for help with qPCR (Taqman), Tracey Swinger and Orla Jupp for providing C28/12 cells and protocols, and Vince Moulton and Grant Wheeler for discussions.

Funding

K.G., H.P. and D.S. were funded by the Biotechnology and Biological Sciences Research Council [grant numbers BB/D016444, BB/H019979] to A.M. T.R. was funded by an EU FP6 network [SCIROCCO, LSHG-CT-2006-037900] of which T.D. was a member.

Supplementary material available online at

<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.101758/-/DC1>

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