MicroRNA (miRNA) are important regulators of many biological processes, but the targets for most miRNA are still poorly defined. In this study, we profiled the expression of miRNA during myogenesis, from proliferating myoblasts through to terminally differentiated myotubes. Microarray results identified six significantly differentially expressed miRNA that were more than 2-fold different in myotubes. From this list, miR-26a (miR-26a), an up-regulated miRNA, was further examined. Overexpression of miR-26a in murine myogenic C2C12 cells induced creatine kinase activity, an enzyme that markedly increases during myogenesis. Further, myoD and myogenin mRNA expression levels were also up-regulated. These results suggest that increased expression of miR-26a promotes myogenesis. Through a bioinformatics approach, we identified the histone methyltransferase, Enhancer of Zeste homolog 2 (Ezh2), as a potential target of miR-26a. Overexpression of miR-26a suppressed the activity of a luciferase reporter construct fused with the 3′-untranslated region of Ezh2. In addition, miR-26a overexpression decreased Ezh2 mRNA expression.

These results reveal a model of regulation during myogenesis whereby the up-regulation of miR-26a acts to post-transcriptionally repress Ezh2, a known suppressor of skeletal muscle cell differentiation.

MicroRNA (miRNA) are a class of small, non-coding RNA important in post-transcriptional gene silencing. They are thought to target the expression of approximately one-third of all mammalian genes (1, 2). miRNA are derived from endogenous transcribed RNA hairpin structures found within introns, exons, or intergenic regions of the genome (3). The biogenesis of the 19–24-nucleotide mature miRNA involves a two-step cleavage process by two ribonuclease III enzymes (4). In the nucleus, Drosha releases the precursor miRNA from its primary transcript, and the precursor, a hairpin-type structure, is then exported from the nucleus into the cytoplasm by the Exportin-5/Ran complex (5, 6). Once in the cytoplasm, Dicer cleaves the hairpin structure to release the double-stranded mature miRNA, which possesses a 3′-hydroxyl and 5′-phosphate overhang (7, 8). This cleavage product is then detected by the miRNA ribonucleoprotein complex, where the less stable strand is incorporated into the complex and guided to its target sequence (9). Sequence homology between the miRNA and its target, typically located in the 3′-untranslated region (3′-UTR) of an mRNA transcript, directs the fate of the target transcript such that identical base pairing results in RNA-induced silencing complex-mediated mRNA cleavage (10), whereas imperfect base pairing results in post-initiation translational repression of the mRNA target (11–13). However, exceptions are increasingly evident as several studies have demonstrated that imperfect pairing of a miRNA to an mRNA target can also result in mRNA cleavage (14, 15).

As a result of the imperfect complementarity between a miRNA and its mRNA target, it is predicted that most miRNA can potentially post-transcriptionally repress a large number of mRNA. More recently, it has also been suggested that miRNA can increase turnover of target mRNA by directing their rapid deadenylation (16) and decapping (17). Regardless of the mechanism by which post-transcriptional gene silencing occurs, the continuous identification of bioinformatically predicted and experimentally validated miRNA suggests that they play important and diverse biological roles in most eukaryotic species. Such roles include developmental timing (18), insulin secretion (19), oncogenesis (20–24), and cellular differentiation including hematopoietic cell differentiation (25), adipogenesis (26), and myogenesis (15, 27–29).

The potential involvement of miRNA in the regulation of myogenesis was first suggested by muscle-specific expression of miR-1, miR-133a, and miR-206 (3, 30, 31). Subsequent studies demonstrated that miR-1 overexpression in HeLa cells caused the direct or indirect down-regulation of more than 100 mRNA transcripts and altered the global expression profile of these cells to resemble that of muscle (32). Interestingly, although miR-1 Drosophila knock-out larvae developed normal musculature, they died as small second instar larvae due to severe muscular deformity, indicating that miR-1 is not involved in muscle formation but rather in the maintenance of differentiation (31). In the mouse myoblast cell line C2C12, miR-1, miR-133a, and miR-206 promote myoblast differentiation (15, 27). Recently, it was also demonstrated that Dicer plays an essential role in skeletal muscle development (33). These results suggest that miRNA regulate myogenesis at various stages by silencing genes that repress the differentiation process.

To further investigate this hypothesis, we profiled miRNA expression during myogenesis of the mouse myoblast cell line, C2C12. These cells have been extensively studied and differentiated into myotubes under specific culture conditions (34).
From the identified differentially expressed miRNA, we further examined the role of miR-26a in regulating myogenesis. Using a specific bioinformatics approach, we predicted and then experimentally tested a potential target of miR-26a, Enhancer of Zeste homolog 2 (Ezh2), a negative regulator of myogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The C2C12 mouse myoblast cell line, first derived from adult mouse dystrophic muscle (34), was acquired from the American Type Culture Collection (ATCC). Myoblasts were maintained in growth medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 units/ml penicillin/streptomycin/glutamine. Once confluent, the medium was changed to Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 10 units/ml penicillin/streptomycin/glutamine to promote myoblast differentiation into myocytes and subsequent fusion into multinucleated myotubes.

**Immunoblotting and Immunofluorescence Localizations**—Total cellular protein (30 μg) was subjected to SDS-PAGE electrophoresis and standard immunoblotting techniques (35). The following primary antibodies and titers were used: anti-desmin (antibody D1033, Sigma-Aldrich, 1/5000) and anti-Ezh2 (antibody 4905, Cell Signaling, 1/500). Immunoreactive bands were detected using species-specific horseradish peroxidase-conjugated secondary antibodies (Chemicon, 1/3000) and visualized using the Supersignal West Pico chemiluminescent substrate (Pierce, Quantum Scientific).

For immunofluorescence localization studies, C2C12 cells grown on cover slips were fixed in cold methanol at various differentiation time points (36). Immunolocalizations used antibodies against desmin (Sigma-Aldrich, diluted 1/100) and Ezh2 (catalog number 36-6300, Invitrogen, diluted 1/50). Desmin localization was visualized using an anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (AQ326F, Chemicon, diluted, 1/150), and Ezh2 localization was visualized using an antirabbit AlexaFluor 555-conjugated antibody (A21429, Invitrogen, diluted 1/100). The nuclei of the cells were visualized using DAPI staining.

**Creatine Kinase Activity Assay**—C2C12 cells were harvested in 50 mM Tris-Mes buffer (pH 7.8) containing 1% v/v Triton X-100 and stored at −80 °C. To measure creatine kinase enzyme activity, 100 μl of N-acetyl cysteine (TR14010, Thermo Electron Corp.) was added to 5 μl of cell extracts and absorbance read at 340 nm every 20 s over 5 min. The rate of reaction over this period was linear and used as the measure of enzyme activity normalized to total protein concentration (i.e. specific enzyme activity; units/mg).

**Isolation of Total RNA**—Total RNA was isolated from C2C12 cells at the following time points: proliferating (70–80% confluence), confluent (100% confluence), 1 day (+1d), 2 days (+2d), and 4 days (+4d) (see Figs. 1 and 4) after induction of differentiation. Total RNA was isolated using TRizol (Invitrogen) as per the manufacturer's instructions. The purity and concentration of the RNA was determined by measuring its absorbance at 260 and 280 nm.

**miRNA Microarray**—The miRCURY™ locked-nucleic acid array version 8.1 (Exiqon) consists of probes for the mature forms of all miRNA present in the miRBase 8.1 release of the miRNA registry (37, 38). The microarray contains probes for 1488 mature miRNA, each represented twice on the microarray. Since the mature sequence of an miRNA is highly conserved between species, it is possible that cross-species hybridization could occur. However, the locked-nucleic acid miRNA probes are highly sensitive and optimized to minimize cross-hybridization between similar mature miRNA (39). The Adelaide Microarray Facility (University of Adelaide, South Australia) printed the microarray slides and hybridized the samples. The reference RNA (see Fig. 1, reference) consisted of a pool of total RNA isolated from four independent proliferating C2C12 samples. Each time point was represented by four biological replicates, of which two were labeled with Cy3 and two were labeled with Cy5 according to a standard protocol (39). For each sample, 1 μg of total RNA was labeled and hybridized to the microarray. Microarrays were scanned on a ScanArray 4000 XL scanner (Packard BioChip Technologies), and raw data were extracted using Spot software (40). Data were subsequently analyzed using GeneSpring® version 7.2 (Agilent Technologies), and statistical analysis was performed using background-corrected mean signal intensities from each dye channel. Microarray data were normalized using intensity-dependent global normalization (Lowess). Differentially expressed miRNA were identified using one-way analysis of variance (p < 0.05) and the Benjamini and Hochberg false discovery rate with Tukey’s honestly significant differences post hoc test to minimize selection of false positives. Of the significantly differentially expressed miRNA, only those with greater than 2-fold increase or 2-fold decrease in expression at any time point when compared with the proliferating sample were used for further analysis. This conservative approach focused on significantly differentially expressed miRNA that showed the greatest changes in expression during myogenesis. All microarray data presented in this manuscript are in accordance with Minimum Information About a Microarray Experiment (MIAME) guidelines and have been deposited in the National Center for Biotechnology Information (NCBI) GEO data base (accession number: GSE9449). To further analyze differentially expressed miRNA, hierarchical gene-tree clustering and K-means clustering analysis, both using default parameters, were used to group miRNA with similar expression profiles (41).

**Taqman® miRNA Expression Assays**—RNA was reverse-transcribed using specific miRNA stem-loop primers (42) and the Taqman® miRNA reverse transcription kit (Applied Biosystems). Mature miRNA expression was measured with Taqman® microRNA assays (Applied Biosystems) according to the manufacturer's instructions. The expression of the miRNA was normalized against the expression level of the house-keeping gene, RpPO (NM_007475) or GAPDH (NM_008084), and presented as the mean normalized expression.

**Construction of Expression Plasmids**—The primer pair was used to clone the mouse miR-26a precursor into the pSilencer™ 4.1-CMV vector (Ambion, Geneworks) according to the manufacturer’s instructions: miR-26a 5’-GGATCCGCA-GAAACTCCAGAGAGAAGGA-3’; 3’-AAGCTTGGCCTTTAG-CAGAAAGGGAGTT-5’. Primers contained 5’-BamHI and
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3′-HindIII restriction sites (underlined) to facilitate cloning into the vector. The following primers were used to amplify and clone the 3′-UTR of mouse Ezh2 into the pMIR-REPORT™ luciferase vector (Ambion, Geneworks) according to manufacturer’s instructions: 5′-ATAGCCGCTTCTGACATCTACTACCTCT-3′; 3′-ATAGGTTACAGTTTCAATGATTCTTTA-5′. The forward primer includes a MluI restriction site, and the reverse primer has a HindIII site (underlined) to facilitate ligation into the vector.

The predicted miR-26a binding site (TACTTGAA, italics) located in the 3′-UTR of Ezh2 was mutated by a single base pair change (underlined) using DpnI mediated site-directed mutagenesis (43). Primers used for this procedure were as follows: 5′-GAATTAAGAGGAGCCGGATCTCTCTAAT-3′; 3′-CAGGTTCATTCCCTTAT-5′. The inserts from all cloned plasmids were sequenced by the Australian Genome Research Facility (Queensland, Australia) to verify identities.

Transfections—For miRNA overexpression studies, C2C12 myoblasts were seeded at a cell density of 2.0 × 10^5 cells/well in 6-well plates with OptiMEM® I medium (Invitrogen) 1 day prior to transfection. The following day, cells were transfected with expression plasmids using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Each reaction consisted of 5 μg of expression plasmid DNA (pSilencer™ expression plasmid expressing miR-26a or a scrambled siRNA hairpin control with no homology to any known sequences in the human, mouse, or rat genomes) and 12.5 μl of Lipofectamine™ 2000. To visualize the transfection, an expression plasmid coding for red fluorescent protein was co-transfected in a 1:4 ratio to the pSilencer™ expression plasmid. The transfection reagents were removed after 4 h and replaced with growth medium. Cells were harvested for subsequent assays 48 h after transfection. Transfections of C2C12 myoblasts were also performed in a 96-well plate for creatine kinase activity assays using 0.3 μg of pSilencer™ expression plasmid encoding miR-26a or a scrambled control and 0.75 μl of Lipofectamine™ 2000. In addition, C2C12 myoblasts transfected with these plasmids were also induced to differentiate by changing the growth medium to Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and 10 units/ml penicillin/streptomycin/glutamine. The cells were then visualized by immunofluorescence staining of desmin.

For luciferase reporter assays, 5 × 10^3 cells were initially plated in a 96-well plate. Constructs were transfected into the C2C12 myoblasts at the following concentrations: 0.3 μg of reporter gene (Ezh2-Luc or Ezh2mut-Luc), 0.1 μg of β-galactosidase control plasmid, and 0.1 μg of pSilencer™ expression plasmid encoding miR-26a or a scrambled control. Transfections were performed in triplicate, and the experiment repeated three times. Twenty-four hours after transfection, luciferase and β-galactosidase activities were measured using the Dual-Light™ assay system (Applied Biosystems). Luciferase activity was normalized for transfection efficiency by measuring β-galactosidase control activity according to the manufacturer’s instructions.

For miR-26a inhibition assays, 5 × 10^4 C2C12 myoblasts were plated in a 24-well plate. Cells were transfected with either the miRIDIAN™ microRNA inhibitor designed against miR-26a or the scrambled sequence negative control (C-310118-04 or IN-001000-01, respectively; Dharmacon, Millennium Science). The inhibitor or control were both transfected into cells (250 ng) using Lipofectamine 2000, as per the manufacturer’s instructions. Cells were harvested 48 h after transfection and assayed for creatine kinase activity, myogenin, and myoD mRNA expression. Direct transfection of the miRIDIAN™ microRNA inhibitor into C2C12 myotubes was not possible. Statistical significance of results was calculated using the Student’s t test, where p < 0.05 was considered significant.

Identification of Putative mRNA Targets for miRNA Up-regulated during Myogenesis—mRNA targets for miRNA significantly up-regulated during myogenesis were predicted using the computer programs PicTar (44), miRanda (45), and Targetscan (46). To minimize the number of false predictions, only mRNA targets predicted using all three programs were considered. Predicted mRNA targets of miRNA were then compared with mRNA that were significantly down-regulated during C2C12 differentiation (47).

Quantitative Real-time PCR (qRT-PCR)—The qRT-PCR procedure has been previously described (48). Expression was normalized to the housekeeping gene, GAPDH, which remained constant throughout the experiment (49). The primers used for qRT-PCR to quantify miRNA expression in C2C12 cells were as follows: Ezh2, 5′-GAAAAAGGACGGCTTCCTCTAA-3′, 3′-CATGGACAACCTTGGTGTTG-5′; GAPDH, 5′-CCTGGAGAAAACCTGCAAGT-3′, 3′-AGCCGGTATCATGTCATACCA-5′; myoD, 5′-CATCCCTAAGGCACACAGAC-3′, 3′-AGCCCTGTAAATCGCATGG-5′; and myogenin, 5′-CGAGGGCTCAAGAAGTGAAATG-3′, 3′-TAGCCGCTCAATGCTCGAT-5′. Four technical replicates were used for each assay.

RESULTS

miRNA Are Differentially Expressed during Myogenesis—Expression profiles of miRNA during C2C12 differentiation were examined using a commercial miRNA microarray that contained all miRNA present in the miRBase miRNA registry release 8.1 (37, 38). Expression patterns of miRNA at confluence, 1, 2, and 4 days after induction of differentiation were compared with proliferating myoblasts (reference myoblasts) (Fig. 1a). Each time point was represented by four independent biological samples. Microarray analysis revealed that six miRNA were significantly differentially expressed by greater than 2-fold during the course of myogenesis (Fig. 1b). Five miRNA (miR-133a/miR-133b, mml-miR-133a (Macaca mulatta miR133a), miR-206, miR-26a, and miR-422b) were up-regulated, and one was down-regulated (miR-222). Using the default K-means clustering analysis in GeneSpring® version 7.2 (Agilent Technologies), the differentially expressed miRNA were grouped into three clusters (Fig. 1c). Cluster I represents a group of three miRNA that were rapidly up-regulated as C2C12 cells committed to myogenesis. This cluster of miRNA contains miR-206, miR-133a/miR-133b, and mml-miR-133a, a shorter form of miR-133a from M. mulatta that differs in sequence by one base. miR-133a and miR-133b are located at different genetic loci and differ in their mature sequences by one nucleotide in their
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3’-ends. These miRNA were previously shown to be highly expressed in skeletal muscle (30, 50). The miRNA in Cluster II (miR-422b, miR-26a) were up-regulated more gradually during the course of myogenesis. The third cluster (Cluster III) contains miR-222, which was down-regulated during myogenesis. Its decline in expression began after the cells committed to differentiation (confluent) and was most prominent 2 and 4 days after induction of differentiation.

Using RT-PCR Taqman® assays, the expression of miR-133a and miR-26a was shown to be up-regulated during myogenesis (Fig. 1d). These results corroborate the microarray analysis. Furthermore, the up-regulation of miR-133a and miR-206 in our data confirms previous reports in the same cellular model of myogenesis (3, 30). miR-1 has also been demonstrated to be highly expressed in skeletal muscle (30). Although miR-1 was up-regulated during myogenesis in our data set, it did not pass our stringent statistical analysis.

miR-26a Overexpression Promotes Myogenesis—miR-26a is ubiquitously expressed in tissues including thymus, testes, placenta, and kidney (30, 50, 51). We report for the first time that miR-26a expression is up-regulated during myogenesis, suggesting that it may play a role in the differentiation process. The related isoiorm, miR-26b, which differs from the miR-26a mature sequence by three nucleotides, was not differentially expressed.

Morphological analysis of C2C12 cells overexpressing miR-26a suggested an increased number of myotubes 2 days after the induction of differentiation, indicating possible acceleration of myogenesis in these cells (Fig. 2a). Indeed, overexpression of miR-26a in C2C12 myoblasts significantly increased creatine kinase enzyme activity when compared with the control 48 h after transfection (Fig. 2b). Moreover, this was accompanied by significant increase in mRNA expression levels of the muscle-specific genes, myogenin (Fig. 2c) and myoD (Fig. 2d). Thus, overexpression of miR-26a in C2C12 myoblasts was able to induce molecular markers of muscle differentiation. Taken together, these data suggest that increased expression of miR-26a during myogenesis is involved in the promotion of myogenesis. Direct transfection of an inhibitor of miR-26a into C2C12 myotubes was not possible. However, inhibition of the relatively low level of miR-26a in C2C12 myoblasts with the miRIDIANTM inhibitor significantly suppressed creatine kinase activity (Fig. 2e) and the expression of myogenin (Fig. 2f) but not myoD (Fig. 2g). This suggests that miR-26a has additional targets in myoblasts.

The Histone Methyltransferase Enhancer of Zeste homolog 2 (Ezh2) Is a Target of miR-26a—Three miRNA target prediction programs were used to identify the potential miRNA targets of the miRNA that were up-regulated during C2C12 differentiation i.e. TargetScan, PicTar, and miRanda (44–46). Each of these programs use a different algorithm to predict miRNA targets; thus, their predictions are varied. In a conservative approach, only targets predicted by all three programs for each miRNA were considered. This approach minimized the number of false positives that could have arisen from use of...
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FIGURE 2. Overexpression of miR-26a promotes myogenesis. a–d, effect of miR-26a overexpression in C2C12 cells transfected with an expression plasmid encoding miR-26a or a scrambled control sequence. a, morphology of control and miR-26a-overexpressing cells 1 day (+1d), 2 days (+2d), or 4 days (+4d) after transfection and induction of differentiation. The cells were visualized by immunofluorescence localization of desmin. b, creatine kinase enzyme activity in C2C12 myoblasts overexpressing miR-26a, normalized such that the value of the control is 100. Samples were taken 48 h after the transfection. c and d, mRNA expression of myogenin (c) and myoD (d) in control and miR-26a-overexpressing myoblasts 48 h after transfection. e–g, effect of miR-26a inhibition on creatine kinase activity (e) and mRNA expression of myogenin (f) and myoD (g) in C2C12 myoblasts 48 h after transfection. Data are presented as mean normalized expression (MNE) ± S.E. of triplicate determinations from three independent experiments. ***, p < 0.001.

individually targeted. It is noteworthy that some miRNA did not have any predicted targets that were common to all three programs. To predict mRNA targets that are biologically relevant during myogenesis, the list of potential targets for each miRNA was compared with a list of genes that were significantly down-regulated during C2C12 differentiation (Fig. 3a) (47). Using this method, we identified three genes that were: (i) predicted to be targets of the significantly upregulated miRNA by all three programs; and (ii) down-regulated during myogenesis (Fig. 3b). This approach presumes that the action of the miRNA suppresses target mRNA levels. As mentioned previously, even mRNA with imperfect complementarity to their target mRNA can cause mRNA degradation. Using this method, Gap junction protein α1 (Gja1 or Connexin 43) was a predicted target of miR-206. This has been experimentally confirmed by others (15, 52) and lends validity to this approach. The predicted targets for miR-26a included Enhancer of Zeste Homolog 2 (Ezh2) and EPH receptor A2 (Epha2) (Fig. 3b). Fig. 3c illustrates the predicted miRNA binding sites in the 3′-UTR of Ezh2. The role of Epha2 in skeletal myogenesis is unknown and was not further investigated. An analogous approach was also used to screen for mRNA targets of the significantly down-regulated miRNA, miR-222; however, there were no mRNA targets that were up-regulated during C2C12 myogenesis and a predicted target of miR-222.

A previous study has reported that the histone methyltransferase, Ezh2, is a negative regulator of muscle differentiation, and its expression is down-regulated during myogenesis (53). Our results showed that both Ezh2 mRNA and protein expression decreased as C2C12 cells formed myotubes (Fig. 4, a and b, respectively) and that Ezh2 co-localized with DAPI in the nucleus (Fig. 4c). To determine whether miRNA may be directly involved in suppressing Ezh2, we cloned the 3′-UTR of Ezh2 into a vector fused to a luciferase reporter gene (Ezh2-Luc) and determined the change in luciferase activity when this construct was co-transfected with the miR-26a expression plasmid into C2C12 myoblasts. Overexpression of miR-26a significantly suppressed Ezh2-Luc activity (Fig. 4d). Mutation of a single nucleotide in the predicted miR-26a binding site (Ezh2mut-Luc) abolished the suppression of the luciferase activity by miR-26a, indicating a specific suppressive effect of miR-26a on Ezh2 (Fig. 4d). These data demonstrate that Ezh2 is indeed a direct target of miR-26a and imply the involvement of this miRNA in suppression of Ezh2 during myogenesis.

MicroRNA are able to mediate post-transcriptional gene silencing by either mRNA degradation or translational inhibition. In the latter case, only decreased protein expression levels of the target should be observed. To determine whether miR-26a acts on Ezh2 at the mRNA level, miR-26a was transiently transfected into C2C12 myoblasts, and endogenous Ezh2 mRNA and protein expression was measured 48 h after transfection. In the presence of miR-26a, Ezh2 mRNA levels decreased significantly by 1.6-fold when compared with the control (Fig. 4e), and Ezh2 protein levels decreased by 1.5-fold (Fig. 4f). This result indicates that miR-26a is able to decrease Ezh2 mRNA levels.

DISCUSSION

Myogenesis is a process orchestrated by complex transcriptional networks involving pivotal transcription factors such as myoD, myf5, mrf4, and myogenin (54). Recent studies reveal that muscle-specific miRNA can also play important and varied roles in myogenesis (15, 27, 28, 33, 55–57). These include: promotion of myoblast proliferation by miR-133-mediated suppression of serum response factor (27); initiation of cell cycle withdrawal by miR-206-mediated inhibition of the p180 sub-
unit of DNA polymerase α (15); and promotion of muscle differentiation by miR-1-mediated inhibition of the known transcriptional repressor of muscle-gene expression, HDAC4 (27). These miRNA act in partnership with the transcriptional network to suppress the expression of genes and proteins that act as negative regulators during the tightly controlled process of myogenesis.

Our study further contributes to this model and reports for the first time that miR-26a expression levels are up-regulated during myogenesis. This coincides with the decline in expression of the chromatin-modifying enzyme, Ezh2, a known negative regulator of myogenesis (53). Prior to myogenesis, Ezh2 (a histone methyltransferase), Y1 (a transcriptional co-activator), and HDAC1 (a class 1 histone deacetylase) bind to the E-box region of muscle-specific gene promoters (53), causing silencing of muscle-specific genes primarily through H3K27 methylation. Upon activation of myogenesis, the Ezh2-containing complex dissociates from chromatin due to H3K27 hypomethylation and is replaced by positive transcriptional regulators of muscle transcription (53). We propose that the increased level of miR-26a during myogenesis serves to post-transcriptionally repress Ezh2 such that it is no longer able to elicit its suppressive effects on myogenesis (Fig. 5). Although it is common that miRNA act on their targets by translational inhibition, there is increasing evidence indicating that miRNA can target mRNA through RNA-induced silencing complex-mediated cleavage or transcript decay. Our data indicate that increased expression of miR-26a is able to suppress Ezh2 mRNA expression, although we are yet to decipher the mechanism. It is also unclear whether miR-26a-mediated Ezh2 mRNA suppression causes the decrease in Ezh2 protein expression observed in myogenesis or whether the decline in Ezh2 protein acts as a negative feedback causing decreased Ezh2 mRNA expression.
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In our model, as cells commit to terminal differentiation, the up-regulation of miR-26a acts to post-transcriptionally silence Ezh2. Although it is unlikely that miR-26a is solely responsible for complete suppression of Ezh2, it may be involved in dampening its expression during myogenesis so that myogenic transcription factors can bind to muscle-specific promoters. This model is similar to miR-206-mediated mRNA cleavage of the p180 subunit of DNA polymerase α, which serves to eliminate its expression during cell cycle withdrawal and commitment to terminal differentiation (15). Failure to repress Ezh2 during myogenesis could result in prolonged proliferation and delayed differentiation, which may lead to aberrant changes in myogenesis. Consistent with this proposal, inappropriate overexpression of Ezh2 is evident in tumorigenesis, including prostate, breast, and epithelial cancers, where elevated Ezh2 expression is associated with high incidence of tumor formation and metastasis (58–62). miR-26a-mediated repression of Ezh2 may be an important regulatory mechanism that is aberrant in these tumors that show abnormal cell proliferation and differentiation.

Ezh2 belongs to the polycomb group of proteins that are known to suppress gene transcription through histone methylation. Due to the mechanism by which it represses genes, silencing of Ezh2 provides an efficient mechanism for the muscle cell to relieve repression of multiple differentiation-specific genes. Similarly, down-regulation of Ezh2 mRNA expression in human primary fibroblasts is associated with inactivation of Rb/E2F pathways, which leads to cell cycle arrest and commitment to differentiation (63).

Interestingly, inhibition of miR-26a in myoblasts suppressed two markers of myogenesis, creatine kinase activity and myogenin mRNA expression, but not myoD expression, which are normally present at relatively low levels in these cells prior to differentiation. This suggests that miR-26a has additional targets in myoblasts before commitment to terminal differentiation.

In conclusion, we demonstrate that six miRNA are differentially expressed during myogenesis and are thus likely to play important roles in this tightly regulated process. Indeed, this result is consistent with a number of studies demonstrating the involvement of specific miRNA in myogenesis (15, 27). Furthermore, we propose that up-regulated expression of miR-26a is required during terminal differentiation in order that the negative regulator of myogenesis, Ezh2, is rapidly and efficiently silenced, thereby promoting myogenesis and terminal differentiation. This regulatory model suggests that miRNA may have broad capability of targeting negative regulators of cellular differentiation. This concept may explain the observation that many miRNA show strong tissue-specific patterns (30, 51).

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REFERENCES

1. Kim, V. N. (2005) Nat. Rev. Mol. Cell. Biol. 6, 376–385
2. Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005) Cell 120, 15–20
3. Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., and Tuscher, T. (2003) RNA (Cold Spring Harbor) 9, 175–179
4. Lee, Y., Jeon, K., Lee, J. T., Kim, S., and Kim, V. N. (2002) EMBO J. 21, 4663–4670
5. Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V. N. (2003) Nature 425, 415–419
6. Lund, E., Gutttinger, S., Calaldo, A., Dahlberg, J. E., and Kutay, U. (2004) Science 303, 95–98
7. Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. (2001) Genes Dev. 15, 2654–2659
8. Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuscher, T., and Zamore, P. D. (2001) Science 293, 834–838
9. Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002) Genes Dev. 16, 720–728
10. Yekta, S., Shih, I. H., and Bartel, D. P. (2004) Science 304, 594–596
11. Kim, H. K., Lee, Y. S., Sivaapradas, U., Malhotra, A., and Dutta, A. (2006) J. Cell Biol. 174, 677–687
12. Wu, L., Fan, J., and Belasco, J. G. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 4034–4039
13. Esau, C., Kang, X., Peralta, E., Hanson, E., Marcusson, E. G., Ravichandran, K. D., Hannon, G. J., and Plasterk, R. H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9779–9784
14. Olsen, P. H., and Ambros, V. (1999) Dev. Biol. 216, 671–680
15. Doench, J. G., and Sharp, P. A. (2004) Genes Dev. 18, 504–511
16. Carrington, J. C., and Ambros, V. (2003) Science 301, 336–338
17. Yekta, S., Shih, I. H., and Bartel, D. P. (2004) Science 304, 594–596
18. Kim, H. K., Lee, Y. S., Sivaapradas, U., Malhotra, A., and Dutta, A. (2006) J. Cell Biol. 174, 677–687
19. Calin, G. A., Demitriu, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., and Croce, C. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15524–15529
20. Calin, G. A., Sevignani, C., Demitriu, C. D., Hyslop, T., Noch, E., Yендamuri, S., Shimizu, M., Ratan, S., Bullrich, F., Negrini, M., and Croce, C. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2999–3004
21. Calin, G. A., Liu, C. G., Sevignani, C., Ferracin, M., Felli, N., Demitriu, C. D., Shimizu, M., Cimmino, A., Zupo, S., Dono, M., Dell’Aquila, M. L., Alder, H., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., and Croce, C. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 11755–11760
22. He, L., Thomson, J. M., Hemann, M. T., Hernando-Munge, E., Mu, D., Goddson, S., Powers, S., Cordon-Cardo, C., Lowe, S. W., Hannon, G. J., and Hammond, S. M. (2005) Nature 435, 828–833
23. Hannon, G. J., and Ruvkun, G. (2002) Science 296, 103–105
24. Ruan, J., Zhou, Q., and Han, J. (2004) EMBO J. 23, 6564–6574
25. He, L., Thomson, J. M., Hemann, M. T., Hernando-Munge, E., Mu, D., Goddson, S., Powers, S., Cordon-Cardo, C., Lowe, S. W., Hannon, G. J., and Hammond, S. M. (2005) Nature 435, 828–833
26. Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B. L., Mak, R. H., Ferrando, A. A., Downing, J. R., Jacks, T., Horvitz, H. R., and Golub, T. R. (2005) Nature 435, 834–838
27. Chen, C.-Z., Li, L., Lobink, H. F., and Bartel, D. P. (2004) Science 303, 83–86
28. Esau, C., Xiang, X., Peralta, E., Hanson, E., Marcuss, E. G., Ravichandran, L. V., Sun, Y., Koo, S., Perera, R. J., Jain, R., Dean, N. M., Freier, S. M., Bennett, C. F., Lolbo, L., and Griffey, R. (2004) J. Biol. Chem. 279, 52361–52365
29. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Ham mond, S. M., Conlon, F. L., and Wang, D. Z. (2006) Nat. Genet. 38, 228–233
30. Nakajima, N., Takahashi, T., Kitamura, R., Isodono, K., Asada, S., Ueyama, T., Matsubara, H., and Oh, H. (2006) Biochem. Biophys. Res. Commun. 350, 1006–1012
31. Rao, P. K., Kumar, R. M., Farkhondeh, M., Baskerville, S., and Lodish, H. F. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 8721–8726
32. Sempere, L. F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E.,
miR-26a Targets Ezh2 during Myogenesis

Liu, C. G., Calin, G. A., Meloon, B., Gamliel, N., Sevignani, C., Ferracin, M., Dumitru, C. D., Shimizu, M., Zupo, S., Dono, M., Alder, H., Bullrich, F., Negrini, M., and Croce, C. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9740–9744

Barad, O., Meiir, E., Avniel, A., Aharonov, R., Barzilai, A., Bentwich, I., Einav, U., Gilad, S., Hurban, P., Karov, Y., Lobenhofer, E. K., Sharon, E., Shibolet, Y. M., Shtutman, M., Bentwich, Z., and Einat, P. (2004) Genome Res. 14, 2486–2494

Anderson, C., Catoe, H., and Werner, R. (2006) Nucleic Acids Res. 34, 5863–5871

Caretti, G., Di Padova, M., Micales, B., Lyons, G. E., and Sartorelli, V. (2004) Genes Dev. 18, 2627–2638

Tapscott, S. J. (2005) Development (Camb.) 132, 2685–2695

Naguibneva, I., Ameyar-Zazoua, M., Poleskaya, A., Ait-Si-Ali, S., Grosman, R., Souidi, M., Cuvellier, S., and Harel-Bellan, A. (2006) Nat. Cell Biol. 8, 278–284

Horstein, E., Mansfield, J. H., Yekta, S., Hu, J. K., Harfe, B. D., McManus, M. T., Baskerville, S., Bartel, D. P., and Tabin, C. J. (2005) Nature 438, 671–674

Rosenberg, M. I., Georges, S. A., Asawachaicharn, A., Analau, E., and Tapscott, S. J. (2006) J. Cell Biol. 175, 77–85

Tonini, T., Bagella, L., D’Andrilli, G., Claudio, P. P., and Giordano, A. (2004) Oncogene 23, 4930–4937

Visser, H. P., Gunster, M. J., Kluij-Nelemans, H. C., Manders, E. M., Raaphorst, F. M., Meijer, C. J., Willemse, R., and Otte, A. P. (2001) Br. J. Haematol. 112, 950–958

Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumas-Sinha, C., Sandra, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., Rubin, M. A., and Chinnaiyan, A. M. (2002) Nature 419, 624–629

Klee, C. G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S. A., Ghosh, D., Sewalt, R. G., Otte, A. P., Hayes, D. F., Sabel, M. S., Livant, D., Weiss, S. I., Rubin, M. A., and Chinnaiyan, A. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11606–11611

Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003) EMBO J. 22, 5323–5335

Tang, X., Milyavsky, M., Shats, I., Erez, N., Goldfinger, N., and Rotter, V. (2004) Oncogene 23, 5759–5769