

MicroRNA-214 Promotes Myogenic Differentiation by Facilitating Exit from Mitosis via Down-regulation of Proto-oncogene *N-ras**

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Vertebrate muscle differentiation is coordinated by an intricate network of transcription factors requiring proliferating myogenic precursors to withdraw irreversibly from the cell cycle. Recent studies have implicated a large number of microRNAs exerting another layer of control in many aspects of muscle differentiation. By annealing to short recognition sequences in the 3'-untranslated region, microRNAs attenuate target gene expression through translation repression or mRNA degradation. Here, we show that miR-214 promotes myogenic differentiation in mouse C2C12 myoblasts at a step preceding the induction of p21 and myogenin. Blocking miR-214 function with a 2'-O-methylated double-stranded inhibitor maintained C2C12 cells in the active cell cycle, thereby inhibiting the myogenic differentiation. By global gene expression profiling, we identified the proto-oncogene *N-ras* as one of miR-214 targets. Furthermore, manipulating the N-Ras level with small interfering RNA or adenovirus-mediated forced expression either augmented or attenuated the effect of miR-214, respectively. Thus, our data uncovered a novel microRNA-mediated mechanism that controls myogenic differentiation.

The vertebrate skeletal muscle is developed from mesodermal stem cells that are committed to a muscle fate within paraxial somites, giving rise to immature myoblasts (1–4). Committed myoblasts are proliferating progenitors (5) that must exit from the cell cycle before myogenic regulatory factors such as *MyoD* and *Myf5* activate myocyte enhancer factors and other downstream muscle-specific genes to drive the formation of multinucleated myotubes and eventually the contractile muscle fibers (6–9). This process can be recapitulated *in vitro* with cell culture systems, the most widely used of which is the mouse C2C12 cell (10, 11). In proliferating myoblasts, the *MyoD* targets of myogenic genes are repressed through chromosome remodeling and epigenetic histone modifications (12, 13). Under the influence of extracellular signals in developing muscle tissues or upon serum depletion in culture systems, *MyoD*

orchestrates an orderly exit from the cell cycle, involving concerted interplays between myogenic regulatory factors and the cell cycle machinery (7, 9, 13, 14). The retinoblastoma gene product Rb and its homologue p107 (15–17) are essential in initiating an irreversible withdrawal from the cell cycle by up-regulating cyclin-dependent kinase inhibitor p21 (18, 19). Certain oncogene products including H- and N-Ras exhibit potent inhibition on myogenic differentiation by blocking Rb function (20).

In addition to the complex network of protein-encoding genes, recent molecular and genetic studies have uncovered a myriad of microRNAs (miRNAs)² that exert a different layer of control over myogenic differentiation (21–23). miRNAs are a class of small noncoding RNAs that are synthesized as pri-miRNAs from either dedicated transcription units or introns of protein-encoding genes (24–26). Pri-miRNAs are processed in the nucleus by the RNase Drosha, yielding precursor miRNAs, which have a characteristic “stem-loop” structure and are ~70 nucleotides in length. Precursor miRNAs are then exported by Exportin 5 to the cytoplasm to be further processed by the RNase Dicer into mature miRNAs of ~22 nucleotides (25, 26). Mature miRNAs are incorporated into the RNA-induced silencing complex, where they anneal to their recognition sequences in the 3'-UTR of mRNA genes to attenuate gene expression through translation repression or mRNA degradation (27). Of many miRNAs that are involved in the regulation of muscle biology, miR-1 (28), miR-133 (29), and miR-206 (30) are specifically expressed in the muscles where they superimpose on the intricate network of transcription factors and other regulatory proteins to control growth and myogenesis. Another muscle-specific microRNA, miR-208, is involved in myosin heavy chain production (31).

Here we investigate the mechanism of mouse miR-214, which was first shown to play an essential role in specifying slow muscle development in zebrafish (32). In a mouse model of cardiac hypertrophy, miR-214 was found to be expressed at an elevated level, but forced expression of miR-214 did not lead to increased muscle growth (33). Using the *in vitro* differentiation of mouse C2C12 cells as a model, we report here that miR-214

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² The abbreviations used are: miRNA, microRNA; UTR, untranslated region; RT, reverse transcription; MHC, myosin heavy chain; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; 214mi, miR-214 mimic; 214in, miR-214 inhibitor; qPCR, quantitative PCR; FACS, fluorescence-activated cell sorter; MAP, mitogen-activated protein.

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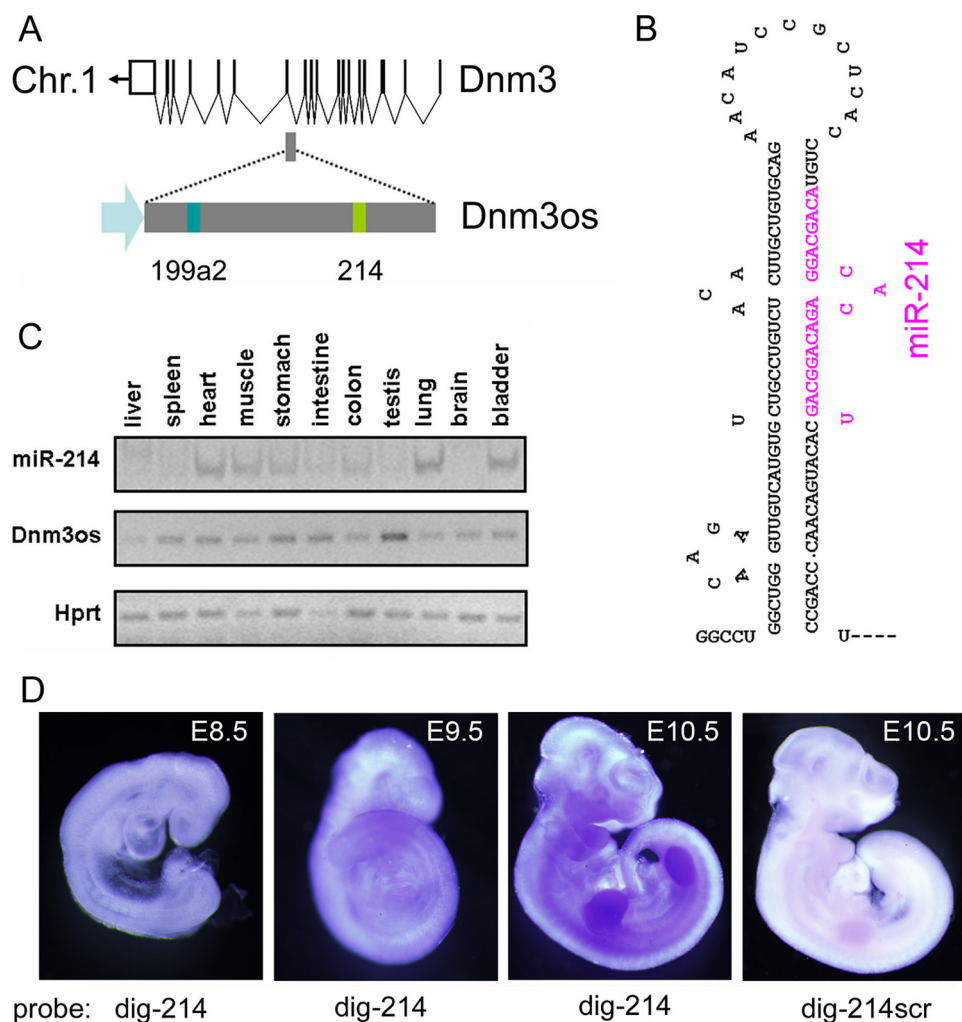


FIGURE 1. Genomic localization and distribution of miR-214 in adult and embryonic mouse tissues. *A*, genomic structure of dynamin 3 gene and the primary transcription unit (Dnm3os) for miR-199a-2 and miR-214 embedded in the 14th intron. *B*, the stem-loop structure of pre-miR-214. The 22-nucleotide mature miR-214 is labeled in pink. *C*, RT-PCR detection of mature miR-214 and the primary transcript, Dnm3os, in adult mouse tissues. Hprt was used as the loading control. *D*, whole mount *in situ* detection of miR-214 with a digoxigenin-labeled antisense probe (*dig-214*) in mouse embryos isolated at embryonic days (E) 8.5, 9.5, or 10.5. Expression of miR-214 was seen in frontal nasal ectoderm, branchial arch, and somite and at the base of limb buds. A scrambled miR-214 probe (*dig-214scr*) was used as a negative control. *chr.1*, chromosome 1.

promotes myogenic differentiation by facilitating the exit from the cell cycle, and we identified *N-ras* as a target of this function through microarray analysis.

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum at 37 °C and 5% CO₂. Myogenic differentiation was induced after the cells reached confluence by replacing the growth medium with the differentiation medium consisting of Dulbecco's modified Eagle's medium supplemented with 2% horse serum. Osteogenic differentiation was induced by treating the confluent cells in the differentiation medium with 300 ng/ml recombinant human BMP-2.

Oligonucleotides and Transfection—Inhibitor, mimic, and nonspecific control oligonucleotides for miR-214 were purchased from Dharmacon Inc. Specific small interfering RNA against mouse *N-ras* was purchased from Qiaagen. In each case,

200 μM oligonucleotides were used in transient transfection with Oligofectamine (Invitrogen) as a delivery agent.

Microarray—To prepare RNA samples for microarray analysis, C2C12 cells were transfected with miR-214 mimic (214mi) or a nonspecific control oligonucleotide, and the total RNA was isolated 48 h later after transfection using RNAiso reagent (Takara). Subsequent steps for the hybridization to Agilent mouse whole genome 4x44K array were done according to standard Agilent protocols. The array data were analyzed with Agilent feature extraction and GeneSpring GX v7.3.1 software. The gene expression profiles were compared between control and 214mi-transfected samples in each replicate. A gene is considered to be differentially expressed when the change in gene expression between experimental (214mi-transfected sample) and base-line (control transfected sample) conditions was greater than 1.5-fold in the same direction in both replicate experiments.

RT-PCR and Real Time RT-PCR—Total RNA from C2C12 cells or mouse tissues was extracted using the RNAiso reagent. Reverse transcription was carried out using the PrimeScript RT reagent kit (TaKaRa). Standard RT-PCR primers were synthesized by Invitrogen. Real time PCR was carried out in a SYBR Premix Ex Taq (Takara) on a 7500 real

time PCR system (ABI). Expression of mature miRNA was determined using the miRNA-specific TaqMan microRNA assay kit (Applied Biosystems). The U6 RNA was used for normalization. The cycling condition was 95 °C for 5 min, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. The experiments were repeated at least three times, and the samples were analyzed in triplicate. Primers used for RT-PCR analyses of *Sufu* and *Hprt* expression were described (34). Primer sequences for other genes are: *N-ras*: 5'-ACTGAGTACAACTGGTGGTGG-3', 5'-TCGGTAAGAATCCTCTATGGTGG-3'; *c-Fos*: 5'-CCCATCCTTACGGACTCCCCACC-3', 5'-CTGCTCTACTTTGCCCTTCTGC-3'; and *Dnm3os*: 5'-CACAGGTCCCTTCCCTATTTCTC-3', 5'-CAGGCTTAACACCATGTCTTCATAC-3'.

Immunoblotting—For immunoblotting experiments, the cells were lysed in the radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). After clearing out cell

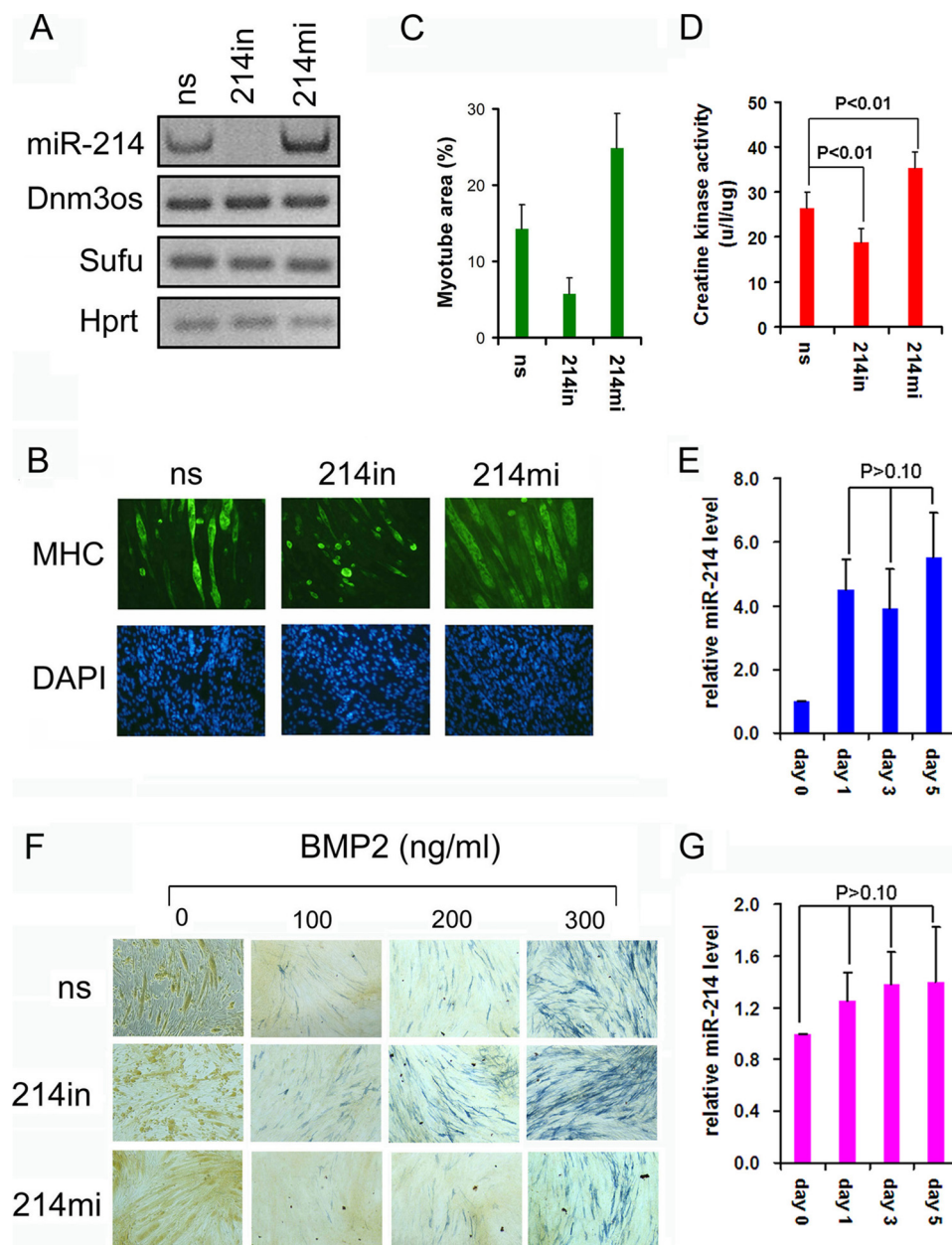


FIGURE 2. miR-214 promotes myogenic differentiation of C2C12 cells. *A*, RT-PCR detection of mature miR-214 and Sufu levels in C2C12 cells that were transfected with a nonspecific double-stranded control oligonucleotides (*ns*), miR-214 inhibitor (214in), or mimic (214mi). The experiment was done 48 h after transfection. *B*, effects of 214in and 214mi on myotube formation assayed by immunofluorescence staining for MHC. 48 h after transfection with microRNA oligonucleotides, myogenic differentiation was induced by switching the cells to the differentiation medium, and the immunofluorescence staining was done 5 days thereafter. *C*, quantification of myotube areas as in *B*. Each data point represents the fluorescence intensity calculated from a minimum of eight randomly chosen fields from three individual plates. *D*, muscle creatine kinase assays for myogenic differentiation. *E*, qPCR quantification of miR-214 levels with a specific TaqMan probe. Days indicate the time the cells were in the differentiation medium. The error bars are the standard deviations among three repeated experiments. *F*, histochemistry staining of alkaline phosphatase after induction of osteogenic differentiation by 300 ng/ml recombinant human BMP-2 for 5 days. Prior to induction, the cells were transfected with nonspecific miRNA, 214in, or 214mi for 48 h. *G*, TaqMan qPCR as in *E* for miR-214 levels during osteogenic differentiation. The error bars are the standard deviations among three repeated experiments. DAPI, 4',6-diamidino-2-phenylindole.

debris by centrifugation, 30 μ g of total protein was loaded onto each lane of a 10% SDS-polyacrylamide gel. Following electrophoresis, the resolved protein was transferred to a polyvinylidene difluoride membrane and subjected to Western blot analysis. The source and dilution for each antibody were: N-Ras (Santa Cruz Biotechnology), 1:200; and Sufu (Santa Cruz

Biotechnology), 1:400; and glyceraldehyde-3-phosphate dehydrogenase (Kangchen), 1:10000.

Immunofluorescence Staining—Immunofluorescence staining of C2C12 cells was carried out with antibodies specific for MHC (1:200) (Sigma), myogenin (1:50) (Santa Cruz Biotechnology), and p21 (1:50) (Santa Cruz Biotechnology).

FACS Analysis—After various treatments as indicated, C2C12 cells were incubated with 3 μ M BrdUrd for 3 h at 37 $^{\circ}$ C, harvested by trypsinization, and fixed overnight with 70% ethanol at 4 $^{\circ}$ C. After one round of wash, the cells were treated with 2 M HCl (freshly prepared) for 20 min at room temperature. Next, the cells were treated with 0.1 M borate buffer (pH 8.5) and washed with PBS containing 0.25% Tween 20 and 1% BSA. Incubation with anti-BrdUrd antibodies (Sigma) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG was carried out for 1 h in 1% bovine serum albumin/PBS. The RNase (100 μ g/ml) treatment was carried out for 10 min at room temperature. The cells were then analyzed on a fluorescence-activated cell sorter (Becton Dickinson) using CellQuest software.

Luciferase Reporter Assay—The PCR fragments containing 3'-UTR of N-ras (NM_010937) gene were subcloned into the XbaI site of the luciferase pGL3-promoter vector (Promega). Site-directed mutagenesis (QuikChange kit; Stratagene) was used to generate the miR-214 M1, M2, and 2M mutants. For luciferase assay, C2C12 cells were transfected with the above reporter constructs and the Renilla luciferase (pRL-TK) construct as an internal control. The cells were collected 48 h after transfection, and the luciferase activities were measured with a Dual-Luciferase reporter system (Promega).

Alkaline Phosphatase Assay—Histochemical detection of the alkaline phosphatase activity was performed on cells that were fixed for 10 min in 3.7% formaldehyde at room temperature. After washing with PBS, the cells were incubated for 30 min in a mixture of 0.1 mg/ml Naphthol AS-MX phosphate, 0.5% *N,N*-dimethylformamide, 2 mM MgCl₂, and 0.6 mg/ml of fast blue BB salt (Sigma) in 0.1 M

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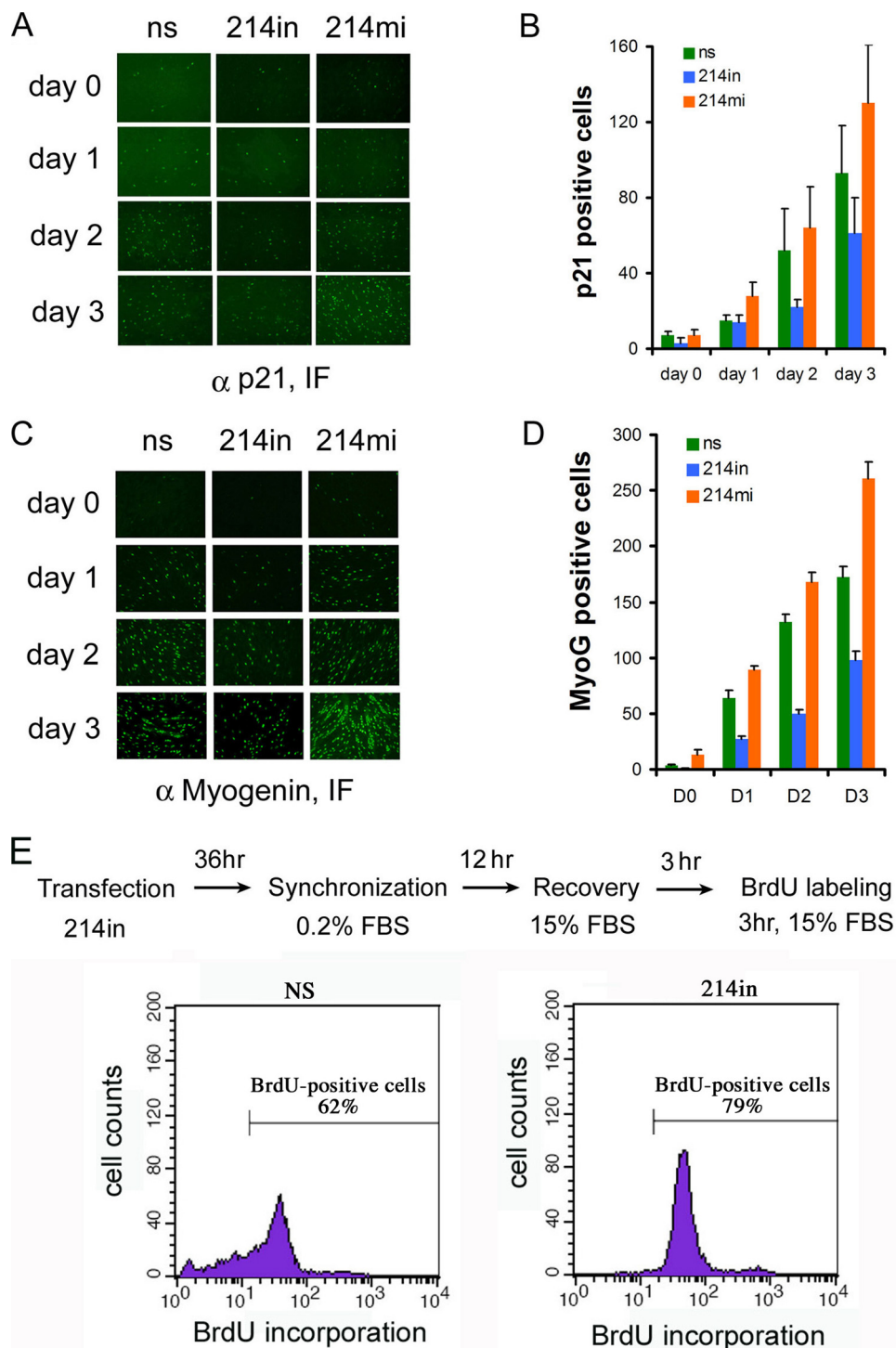


FIGURE 3. miR-214 accelerates the induction of p21 and myogenin and promotes the exit from the cell cycle. A and C, transfection of 214in hampered but 214mi enhanced the induction of p21 (A) and myogenin (C). B and D, corresponding bar graphs for the quantification of p21- (B) or myogenin-positive (D) cells. Each bar represents cell counts from a minimum of eight randomly chosen fields from three individual wells. The error bars are the standard deviations. E, BrdUrd incorporation assay. C2C12 cells were transfected with nonspecific (ns) or miR-214in and kept in growth medium for 36 h before culturing in 3 μ g/ml BrdUrd, and then cells were trypsinized from the plate for FACS. For synchronization of cell cycle, 36 h after transfection the cells were transferred to 0.2% fetal bovine serum for 12 h, released for 3 h, cultured in 3 μ g/ml BrdUrd for 3 h, and then harvested for FACS.

Tris-HCl, pH 8.5, at room temperature, followed by observation under a microscope.

Creatine Kinase Assay—C2C12 cells grown in 12-well plates were washed twice in cold PBS and scraped from the plates in

70 μ l of PBS. After sonication with two 30-s bursts at 4 $^{\circ}$ C, the cells were centrifuged at 14,000 rpm for 10 min. Creatine kinase activity was measured with the CK-NAC kit (Roche Applied Science) according to the manufacturer's suggestion. Briefly, 40 μ l of the cell lysate was mixed with 1 ml of reaction buffer and incubated for 3 min at room temperature; after which the optical density reading at 340 nm was recorded at 1-min intervals over a period of 3 min. The creatine kinase activity was expressed as units over protein concentration, which was determined by BCA assay using bovine serum albumin as the standard.

Adenovirus Infection—Recombinant adenovirus for green fluorescent protein and N-ras were purchased from Vector Biolabs, and infections of C2C12 cells were conducted as described (35).

N-ras-3'-UTR Suppression Experiment—Mouse N-ras cDNA was amplified from total RNAs of C2C12 cells and subcloned into the pRK5 vector between ClaI and BamHI sites. The cognate 3'-UTR of N-ras or the 2M mutant form was subcloned from the pGL-Nras-3'-UTR or pGL-Nras-3'-UTR(2M) reporter constructs into the XbaI site to generate pRK5-Nras-3'-UTR and pRK5-Nras-3'-UTR(2M). These constructs and the pRK5 vector control were transfected to C2C12 cells with nonspecific or 214mi double-stranded RNA. Transfected cells were then induced to differentiate along the myogenic lineage for 5 days before assaying for differentiation by immunofluorescence staining for MHC.

Statistic Analysis—All of the data are presented as the means \pm S.D. ($n \geq 3$). We performed statistical analysis by *t* test, and $p < 0.05$ was considered statistically significant.

RESULTS

Expression of miR-214 in Muscle Tissues—Mouse miR-214 is encoded by a bi-cistronic transcript along with miR-199 in Dynamin 3 opposite (Dnm3os) that is embedded in the opposite strand within an intron of Dynamin 3 (Fig. 1, A and B). This genomic organization is conserved from zebrafish to humans.

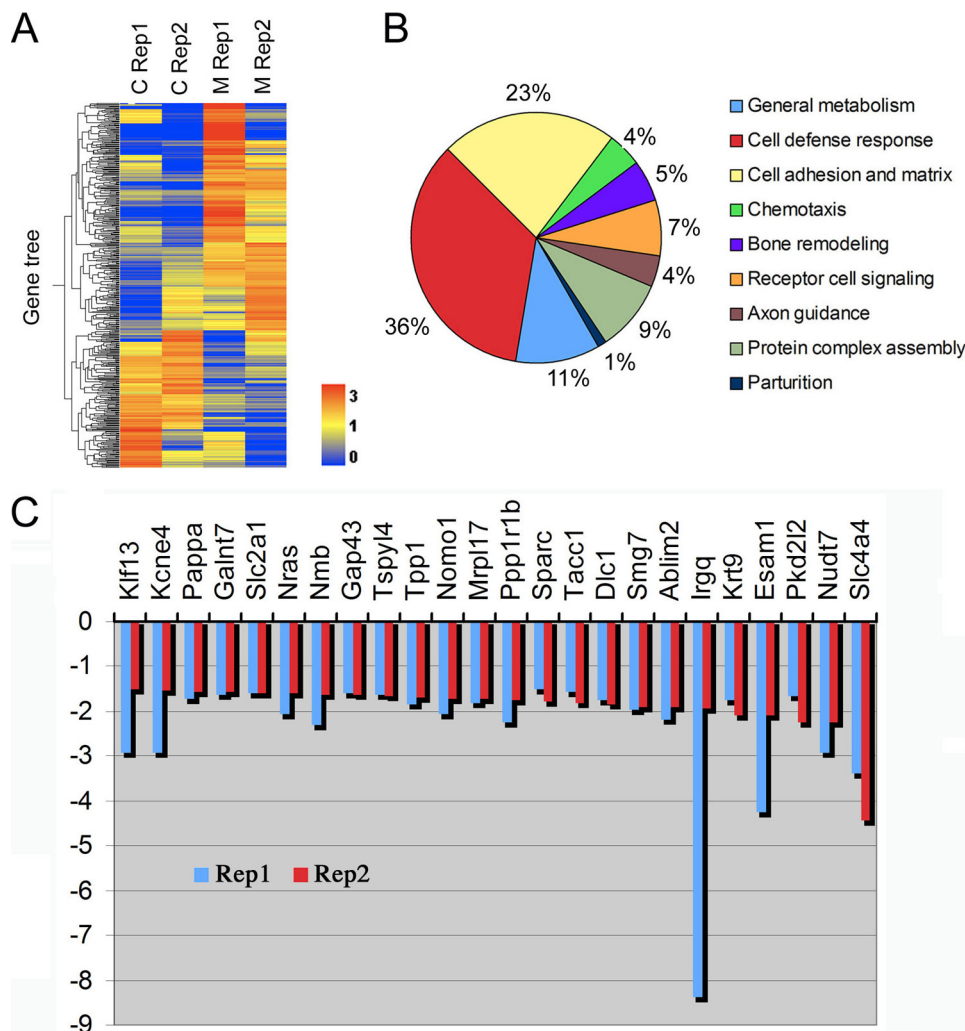


FIGURE 4. Global gene expression profiling of miR-214 targets. A, heat map representation of 487 differentially expressed genes in undifferentiated C2C12 cells transfected with 214mi or the control oligonucleotides. Total RNA was isolated 48 h after transfection. Each row represents a single gene in the microarray. B, distribution of matches to gene ontology categories. C, the list of 214mi repressed genes with at least one miR-214 recognition sequence identified in the 3'-UTR. Ratios of gene expression between cells transfected with 214mi and control oligonucleotides in two replicates are shown.

Transcription of *Dnm3os* is powered by a dedicated promoter, thus appearing unrelated to *Dynamin 3*. To begin to address the function of mouse miR-214, we first examined its expression pattern in adult tissues by RT-PCR analysis. High expression of mature miR-214 was found in all three types of muscle tissues, heart, stomach, bladder, and skeletal muscle, as well as in those internal organs that are rich in smooth muscle fibers; these organs included intestine, colon, and lung (Fig. 1C). In contrast, little expression was found in the liver, brain, spleen, and testis (Fig. 1C). Interestingly, the 7.9-kb pri-miR-214, *Dnm3os*, was universally expressed in all tissues examined (Fig. 1C), implying a tissue-specific control of its processing. During embryonic development, miR-214 was not expressed until embryonic day 10.5. At that point, high expression was detected in nasal ectoderm, branchy arch, somites, along the length of neural tube, and at the bases of limb buds (Fig. 1D). Thus, the pattern of miR-214 expression suggests a muscle-specific function.

miR-214 Promotes Myogenic Differentiation—To investigate a possible role of miR-214 in myogenesis, we elected to

use the *in vitro* differentiation of mouse C2C12 myoblasts as a model in which expression of mature miR-214 can be readily inhibited or augmented with chemically synthesized 2'-O-methylated miR-214 inhibitor (214in) or 214mi, respectively (Fig. 2A). In zebrafish, miR-214 was reported to regulate slow muscle fiber differentiation through targeting *Sufu* (32), a critical negative downstream regulator in the Hedgehog pathway (34, 36). However, we found that the level of *Sufu* message in C2C12 myoblasts did not change following transfection of either 214in or 214mi (Fig. 2A). Upon serum withdrawal, C2C12 cells that were grown to confluence irreversibly withdrew from the cell cycle and underwent a spontaneous differentiation along the myogenic lineage (10, 11). After 5 days, many large, multinucleated myotubes consisting of fused myocytes were readily detectable by immunofluorescence staining against MHC (Fig. 2B). Relative to the C2C12 cells that were transfected with a nonspecific double-stranded RNA, however, fewer myotubes were formed in those cells in which miR-214 expression was blocked by 214in, whereas more were formed in those cells whose miR-214 expression was augmented by 214mi (Fig. 2, B and C). We also measured the levels of creatine kinase activity, a differentiated myo-

cyte-specific marker, and found that its level was altered similarly in the C2C12 cells by 214in or 214mi, respectively (Fig. 2D). Using qPCR analysis with a specific TaqMan probe, we found that mature miR-214 was already expressed in proliferating C2C12 cells, but its level was significantly up-regulated 24 h following the induction of differentiation and stayed high throughout the rest of the course of differentiation (Fig. 2E). Because the C2C12 myoblast is a progenitor cell that has dual differentiation potential, we further assayed the role of miR-214 along the osteogenic lineage. Under the influence of 300 ng/ml BMP, C2C12 cells differentiated into osteoblasts after 5 days of culturing as evident by staining for alkaline phosphatase (Fig. 2F). Transfection of 214in into C2C12 cells prior to induction of differentiation augmented the BMP-induced osteogenic differentiation, whereas 214mi blocked it (Fig. 2F). However, BMP treatment suppressed the expression of miR-214 to a level comparable with that of undifferentiated cells during this process (Fig. 2G). Thus, the above data indicate that miR-214 has a specific role in promoting myogenic while inhibiting osteogenic differentiation.

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TABLE 1
miR-214-down-regulated genes with recognition sites in the 3'-UTR

Gene symbol	GenBank™ accession number	miR-214/control ratio		Description
		Replicate 1	Replicate 2	
Klf13	AK002926	0.34	0.66	Kruppel-like factor 13
Kcne4	NM_021342	0.34	0.66	Potassium voltage-gated channel, Isk-related gene 4
Pappa	NM_021362	0.58	0.64	Pregnancy-associated plasma protein A
Galnt7	NM_144731	0.61	0.63	polypeptide N-acetylgalactosaminyltransferase 7
Slc2a1	NM_011400	0.62	0.63	Solute carrier family 2 (facilitated glucose transporter)
N-ras	NM_010937	0.49	0.62	Neuroblastoma <i>ras</i> oncogene
Nmb	NM_026523	0.43	0.62	Neuroedin B
Gap43	NM_008083	0.62	0.62	Growth-associated protein 43
Tspy14	NM_030203	0.61	0.60	TSPY-like 4
Tpp1	NM_009906	0.54	0.59	Tripeptidyl peptidase 1
Nomo1	NM_153057	0.48	0.58	Nodal modulator 1
Mrpl17	NM_025301	0.55	0.58	Mitochondrial ribosomal protein L17
Ppp1r1b	NM_144828	0.44	0.57	Protein phosphatase 1, regulatory subunit 1B
Sparc	NM_009242	0.66	0.56	Secreted acidic cysteine rich glycoprotein
Tacc1	NM_177089	0.64	0.55	Transforming, acidic coiled-coil containing protein 1
Dlc1	AK162413	0.57	0.54	Deleted in liver cancer 1
Smg7	NM_001005507	0.50	0.53	Nonsense mediated mRNA decay factor
Ablim2	NM_177678	0.45	0.52	Actin-binding LIM protein 2
Irgq	NM_153134	0.12	0.52	Immunity-related GTPase family, Q
Krt9	NM_201255	0.57	0.48	Keratin 9
Esam1	NM_027102	0.23	0.48	Endothelial cell-specific adhesion molecule
Plkd2l2	NM_016927	0.60	0.45	Polycystic kidney disease 2-like 2
Nudt7	NM_024437	0.34	0.44	Nucleoside diphosphate linked moiety X-type motif 7
Slc4a4	NM_018760	0.30	0.22	Solute carrier family 4 (anion exchanger), member 4

miR-214 Promotes Exit from Cell Cycle during C1C12 Cell Differentiation—In many aspects, C2C12 cells resemble the committed muscle precursors, which already express the muscle regulatory factors such as MyoD. When cultured in the differentiation medium that lacks fetal bovine serum, proliferative C2C12 cells exited from the mitosis as marked by a gradual up-regulation of the cyclin-dependent kinase inhibitor p21 (Fig. 3, A and B) and began to express myocyte enhancer factor, myogenin (Fig. 3, C and D). To determine at which step miR-214 exerts its role in myogenic differentiation, we repeated these experiments with C2C12 cells that had been transfected with 214in or 214mi, respectively. As with MHC, blocking mature miR-214 with 214in decreased the expression of p21 and myogenin (Fig. 3, A–D). Likewise, augmenting miR-214 with 214mi enhanced the expression of both markers (Fig. 3, A–D). Because both up-regulation of p21 and induction of myogenin are early post-mitotic events, we reasoned that miR-214 may play a role in exiting from the cell cycle. To investigate this possibility, we arrested normal as well as 214in-transfected C2C12 cells in low serum medium for 12 h. After a brief period of recovery in high serum medium, we pulse-labeled the cells with BrdUrd and then subjected them to FACS analysis (Fig. 3E). At this early stage of the cell cycle, a significant portion of mock transfected C2C12 cells trailed in incorporating BrdUrd (Fig. 3E), most likely because the cell population was not completely synchronized by serum starvation. Despite this, more 214in-transfected cells incorporated BrdUrd (Fig. 3E), implying an ability to force re-entry into the cell cycle. We observed that 64% of mock transfected C2C12 cells compared with 79% of 214in-transfected cells were in the active S phase (BrdUrd-positive) (Fig. 3E). Thus, blocking miR-214 function helped C2C12 cells maintain an active cell cycle progression, indicating that miR-214 normally has a role in promoting the exit from the cell cycle.

Global Expression Profiling of miR-214 Target Genes—To understand the mechanism by which miR-214 regulates cell

cycle exit and leads to myogenic differentiation, we generated a global expression profile of miR-214 target genes in 214mi-transfected C2C12 cells on Agilent mouse whole genome 4x44K arrays. Comparing 214mi-transfected to control transfected C2C12 cells, we found that 487 of 41000 genes or transcripts present in the array showed differential expression commonly in two repeated experiments (Fig. 4A and Table 1). Grouping against the Gene Ontology databases indicated that these genes were distributed in general metabolism, cell defense response, cell adhesion and matrix, chemotaxis, bone remodeling, receptor cell signaling, axon guidance, protein complex assembly, and parturition categories (Fig. 4B). Of these 487 differentially regulated genes, 303 were up-regulated, whereas 184 were down-regulated. Because microRNAs are expected to repress gene expression, the up-regulated genes/transcripts were most likely due to secondary effects resulting from 214mi transfection. Direct targets of miR-214 were most likely in the list of down-regulated genes/transcripts. To identify these targets, we analyzed the 3'-UTRs of all 184 down-regulated genes/transcripts for miR-214 recognition sequences with microRNA target prediction tools (37) and found 24 genes with at least one hit (Fig. 4C). Several genes among them encode functions linked to cell adhesion, extracellular matrix, and cytoskeleton (Fig. 4C), consistent with dramatic changes in cell shape and cell fusion associated with myogenic differentiation (11). The potential miR-214 targets also include those that are involved in cell cycle regulation, transcription, signal transduction, and ion transport (Fig. 4C). One of these was *N-ras*, a well known signaling protein that promotes S phase cell cycle entry and inhibits myogenic differentiation (20, 38). For these reasons, we selected it for further analysis.

N-ras Is a Direct Target of miR-214—To determine whether miR-214 specifically attenuates *N-ras* expression, we measured the endogenous *N-ras* mRNA levels by qPCR after transfection of 214in or 214mi in C2C12 cells. We found that inhibition of miR-214 expression with 214in increased whereas enhancing

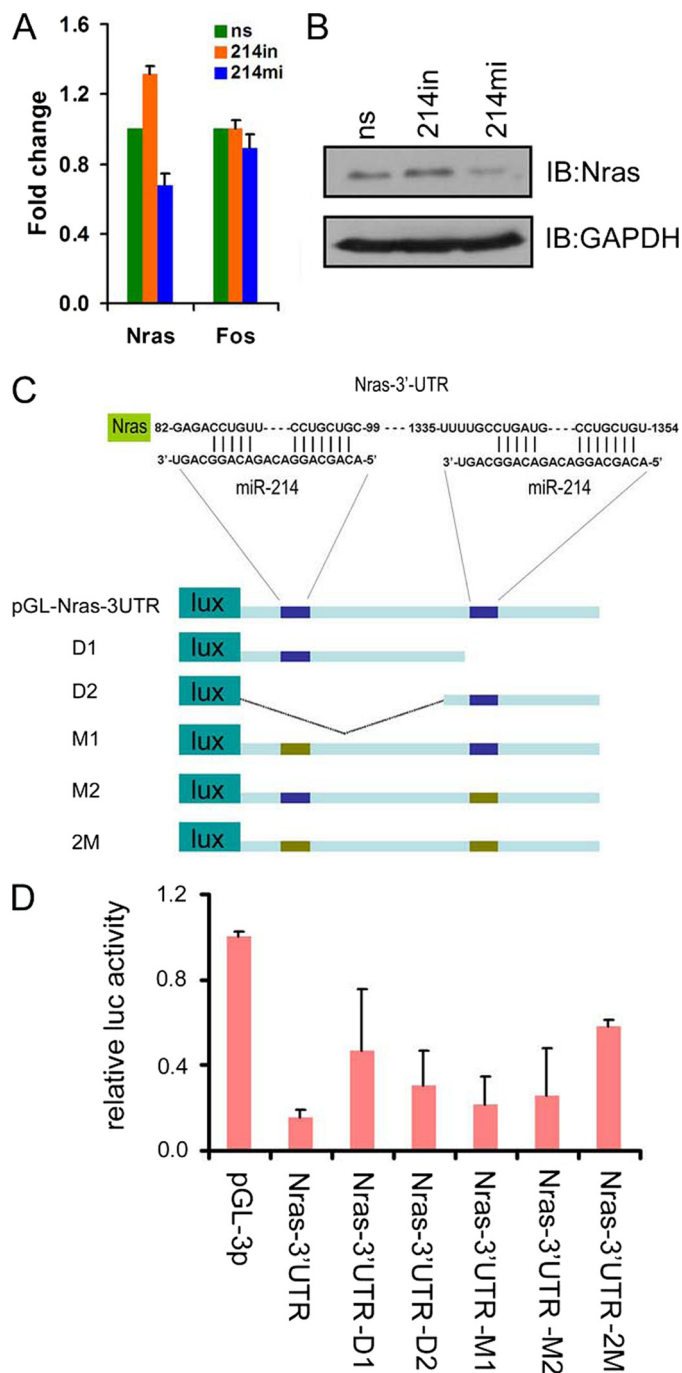


FIGURE 5. Validation of N-ras as a direct target of miR-214. *A*, effects of 214in or 214mi on the N-ras and Fos mRNA levels in C2C12 cells determined by real time PCR. *B*, Western analysis of N-Ras protein levels regulated by 214in or 214mi in the C2C12 cells. *C*, predicted miR-214 target recognition sites in the 3'-UTR of mouse N-ras and schematics showing reporter constructs with mutations in the two target sites. The wild type luciferase reporter was generated with a DNA fragment covering ~1500 bp of the entire N-ras 3'-UTR. *D*, luciferase reporter assays for the response of N-ras 3'-UTR and its mutant variants to 214mi in the transfected C2C12 cells. The relative luciferase activity, defined as the ratio of 3'-UTR reporter activity (firefly) to that of the internal control (*Renilla*), was determined 48 h after transfection, and each data point represents the average of three independent experiments done in duplicate. *IB*, immunoblot; *ns*, nonspecific.

miR-214 expression with 214mi decreased N-ras mRNA levels relative to that in the control cells transfected with a nonspecific miRNA (Fig. 5*A*). As a comparison, qPCR showed that

c-fos, which does not contain the miR-214 binding site in its 3'-UTR, did not change by either 214in or 214mi (Fig. 5*A*). Consistent with the changes in mRNA levels, the level of N-Ras protein was also altered by 214in and 214mi (Fig. 5*B*). These results confirmed the ability of miR-214 to down-regulate N-ras first seen in the microarray study. To determine whether regulation of N-ras by miR-214 is specifically mediated by the microRNA mechanism, we cloned the 3'-UTR of the N-ras gene containing two miR-214 recognition sites and inserted it downstream to a luciferase reporter (Fig. 5*C*). Relative to the virgin luciferase reporter, annexation of the N-ras 3'-UTR resulted in an ~5-fold reduction in the luciferase activity (Fig. 5*D*). Deletion and point mutation analyses showed that the proximal miR-214 recognition site had a stronger role in attenuating the expression, whereas mutating both sequences severely curtailed the transcriptional repression activity of N-ras 3'-UTR (Fig. 5, *C* and *D*). Taken together, the above results indicated that N-ras is a direct target of miR-214.

N-ras Mediates the Inhibition of Myogenic Differentiation by miR-214—Western analysis showed that the expression of N-Ras was progressively diminished during myogenic differentiation (Fig. 6*A*), with a time course that inversely correlated with the up-regulation of cyclin-dependent kinase inhibitor p21 and myogenin (Fig. 3, *A–D*). This observation is consistent with the possibility that miR-214 may down-regulate N-ras to allow for myogenic differentiation. To determine whether this was actually the case, we infected C2C12 cells with adenoviruses expressing N-ras to augment the endogenous level of N-Ras. Because the N-ras expression unit carried on this viral vector does not contain the 3'-UTR, it should be refractory to miR-214 regulation. Relative to the control cells infected with Ad-GFP, Ad-N-ras-infected C2C12 cells showed a lesser degree of myogenic differentiation, and this trend was true even in 214mi-transfected samples (Fig. 6, *B* and *C*). To further demonstrate the role of N-ras as a miR-214 target, we used small interfering RNA to knock down N-ras expression before the induction of myogenic differentiation. In this experiment, formation of multinucleated myotubes in N-ras small interfering RNA-transfected C2C12 cells was ostensibly increased, but most strikingly, small interfering RNA against N-ras completely neutralized the role of 214in in blocking the myogenic differentiation (Fig. 6, *D* and *E*). Finally, we isolated a cDNA fragment covering the mouse N-ras open reading frame by RT-PCR from the total RNA of C2C12 cells and subcloned it along with its cognate 3'-UTR or the 2M mutant form into the pRK5 expression vector. Transfection of these Nras-3'-UTR and Nras-3'-UTR(2M) constructs into C2C12 cells strongly inhibited the myogenic differentiation, but if this experiment was repeated in the presence of 214mi, the level of inhibition by Nras-3'-UTR was reduced, whereas that of the inhibition by Nras-3'-UTR(2M) was still unabated (Fig. 6, *F* and *G*). This result indicated that the N-ras expression construct with mutant miR-214 recognition sites just like the one without its cognate 3'-UTR was refractory to miR-214-mediated gene silencing. Based on these analyses, we conclude that N-ras lies downstream in the gene network that controls progenitor cell differentiation and therefore is a functional target of miR-214.

miR-214 Targets N-ras in Muscle Differentiation

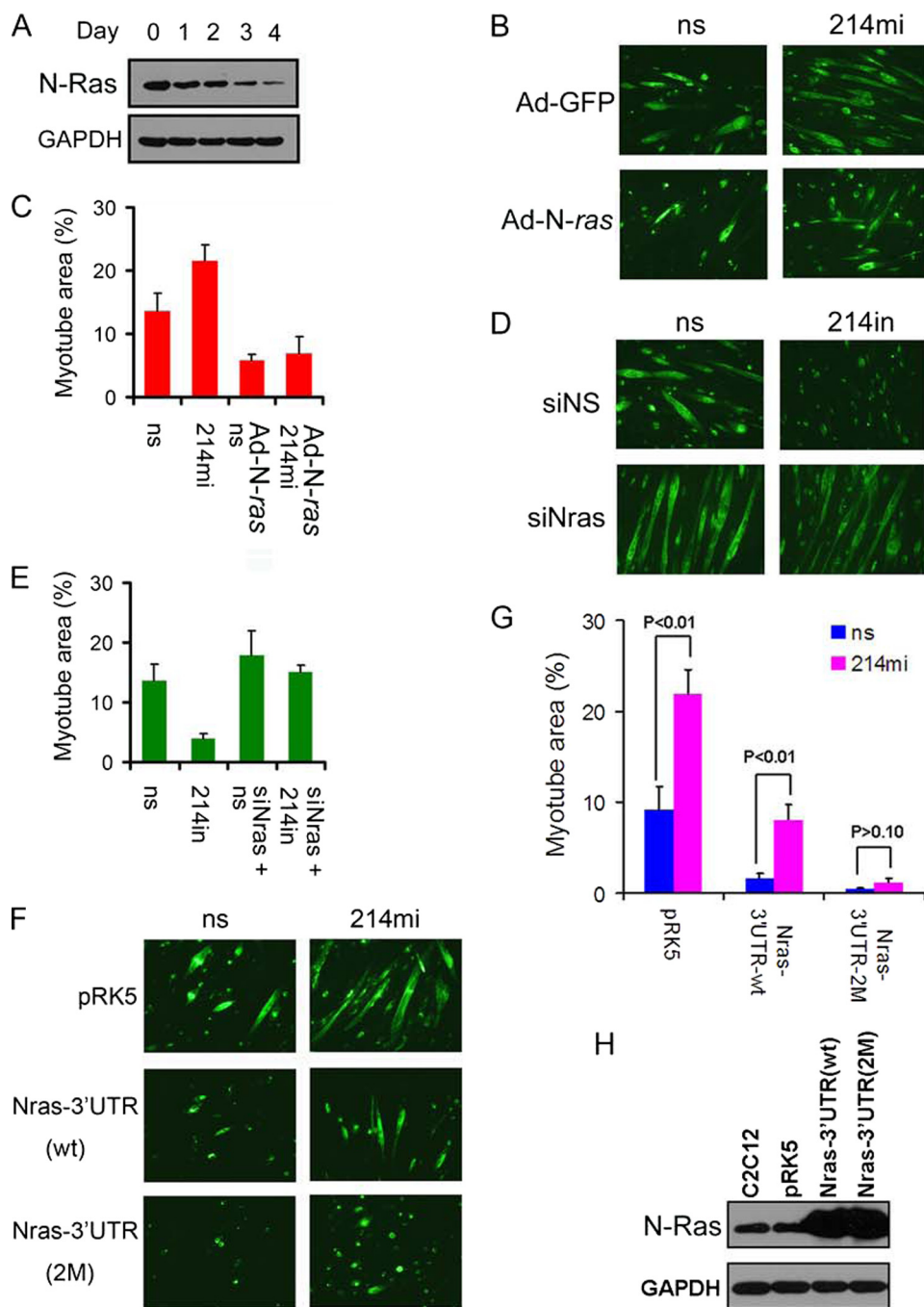


FIGURE 6. Down-regulation of N-ras accounts for the role of miR-214 in promoting myogenic differentiation. A, Western analysis showing that the N-Ras level decreases as a result of myogenic differentiation. B, forced expression of N-Ras by adenovirus infection blocked the promoting effect of 214mi on myogenic differentiation. Ad-N-ras infection and 214mi transfection were carried out at the same time, and the immunofluorescence staining of MHC was done 5 days after the induction of differentiation. C, quantification of myotube areas from six randomly chosen fields in three separate wells for experiments described in B. D, silencing N-ras expression bypassed the inhibition of 214in on myogenic differentiation. E, experimental conditions and the quantification of myotube areas were as in B and C, respectively. F and G, immunofluorescence staining of MHC (F) and quantification of myotube areas (G) showing the resistance of mutant N-ras 3'-UTR to 214mi-mediated silencing effect. The results showed that 214mi was able to overcome the inhibition by N-ras 3'-UTR but not N-ras 3'-UTR(2M) of myogenic differentiation. (H) Western analysis showing the expression levels of exogenous N-Ras. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; wt, wild type; ns, nonspecific.

DISCUSSION

In the current study, we investigated the mechanism by which miR-214 promotes myogenic differentiation and uncovered one aspect of miR-214 functions in facilitating the cells

exiting from the cell cycle, a prerequisite to differentiation of all cell lineages. However, we were puzzled by the strong inhibition of miR-214 on BMP-induced osteogenic differentiation (Fig. 2F), which suggested that the role of miR-214 was not simply limited to promoting differentiation *per se*; rather it showed a specific preference for generating myocytes. This dichotomy was finally resolved by our global gene expression profiling studies, which identified N-ras as one of the functional targets of miR-214. We subsequently confirmed this. As a proto-oncogene, N-ras is not only a strong cell growth promoter, it also has a proven role in inhibiting muscle differentiation (20, 38). So, by targeting N-ras, miR-214 forced the cells out of the cell cycle, but in a way that shepherded them down the pathway toward muscle formation.

Irreversible withdrawal from the cell cycle is mediated by the retinoblastoma protein, pRB (15, 16). In proliferating myoblasts, pRB is progressively inactivated by phosphorylation in the G₁ phase by concerted actions of multiple cyclin-dependent kinase complexes to allow for proper progression through the cell cycle until late in the mitosis when it is degraded (39). Mitogenic signals through the intracellular MAP kinase pathways initiated from one of the three Ras small GTPases maintain pRB as an inactive hyperphosphorylated protein. At the onset of cell differentiation triggered by serum withdrawal in the case of C2C12 model system, the RAS-MAP kinase pathways are silent, allowing hypophosphorylated pRB to block cell growth by repressing the key cell cycle transcription factor, E2F, through binding. Of the three *ras* genes in the mammalian genomes, N-ras was shown previously to possess a muscle specific function (38). In pRb-deficient mouse embryos, thoracic skeletal muscles showed a dramatic

reduction in fiber density and the length of myotubes; however, removal of both N-Ras and pRb simultaneously restored normal muscle development (37). Our results were consistent with this earlier observation in that down-regulation of N-ras by

miR-214 led to an increase in the myotube formation while reducing the BMP-mediated osteogenic differentiation (Fig. 2). Thus, by controlling the level of N-Ras, miR-214 exerts an additional layer of control of myogenic differentiation in this complex regulatory network.

Promoting cell cycle exit as a way to regulate myogenic differentiation was demonstrated previously with miR-206 (30), one of the three microRNAs that are specifically expressed in the muscle. Also through global expression profiling analysis, several miR-206 targets were identified, including DNA polymerase α , and three other genes. However, the exact roles of miR-214 and miR-206 were likely to be different, because miR-206 was absent in proliferating C2C12 cells, and its expression did not commence until 2 days after induction of differentiation. The late appearance of miR-206 argues that its role probably lies in preventing the reversal of the cell cycle exit, which would result in a catastrophic consequence. In contrast, mi-214 was already expressed in proliferating C2C12 cells, albeit at a level lower than that in the differentiated cells (Fig. 2E). This suggests that miR-214 likely plays a permissive rather than an inductive role in allowing the cells to exit from the cell cycle. Moreover, the fact that forced expression of both 214in and 214mi exerted an effect on the myogenic differentiation argues that the cells are sensitive to fluctuations of the miR-214 level. So, it is possible that miR-214 constitutes a part of a measuring mechanism that senses the extent of the myogenic differentiation in a developing tissue environment and allocating an appropriate number of proliferating muscle precursors accordingly.

Recently, miR-214 was found to target the polycomb group protein, Ezh2, in an independent study (40). The MyoD locus in undifferentiated embryonic stem cells and several MyoD target genes in proliferating myoblasts were known to be occupied by Ezh2, which represses the transcription from these genes via chromatin remodeling. This study identified MyoD and myogenin as the transcription factors that control the pri-miR-214 transcription, thus suggesting a positive feedback loop, in which activation of MyoD and myogenin turns on the expression of miR-214, which in turn reinforces MyoD and myogenin expression by negatively feeding back to Ezh2. These and our current data demonstrate that miR-214 can function through multiple mechanisms to achieve the same goal, a theme that was increasingly proven to be true for all microRNAs.

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REFERENCES

- Rudnicki, M. A., Le Grand, F., McKinnell, I., and Kuang, S. (2008) *Cold Spring Harbor Symp. Quant. Biol.* **73**, 323–331
- Arnold, H. H., and Winter, B. (1998) *Curr. Opin. Genet. Dev.* **8**, 539–544
- Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., and Relaix, F. (2003) *J. Anat.* **202**, 59–68
- Buckingham, M., and Relaix, F. (2007) *Annu. Rev. Cell Dev. Biol.* **23**, 645–673
- Buckingham, M., and Montarras, D. (2008) *Curr. Opin. Genet. Dev.* **18**, 330–336
- Black, B. L., and Olson, E. N. (1998) *Annu. Rev. Cell Dev. Biol.* **14**, 167–196
- Brand-Saberi, B. (2005) *Ann. Anat.* **187**, 199–207
- Tapscott, S. J. (2005) *Development* **132**, 2685–2695
- Walsh, K., and Perlman, H. (1997) *Curr. Opin. Genet. Dev.* **7**, 597–602
- Andrés, V., and Walsh, K. (1996) *J. Cell Biol.* **132**, 657–666
- Yaffe, D., and Saxel, O. (1977) *Nature* **270**, 725–727
- McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001) *Curr. Opin. Genet. Dev.* **11**, 497–504
- Pownall, M. E., Gustafsson, M. K., and Emerson, C. P., Jr. (2002) *Annu. Rev. Cell Dev. Biol.* **18**, 747–783
- Poleskaya, A., and Rudnicki, M. A. (2002) *Dev. Cell* **3**, 757–758
- Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J. D., Phillips, R. A., and Gallie, B. L. (1996) *Genes Dev.* **10**, 3051–3064
- Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994) *Science* **264**, 1467–1471
- Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993) *Cell* **72**, 309–324
- Halevy, O., Novitsch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995) *Science* **267**, 1018–1021
- Guo, K., Wang, J., Andrés, V., Smith, R. C., and Walsh, K. (1995) *Mol. Cell Biol.* **15**, 3823–3829
- Olson, E. N., Spizz, G., and Tainsky, M. A. (1987) *Mol. Cell Biol.* **7**, 2104–2111
- Callis, T. E., Deng, Z., Chen, J. F., and Wang, D. Z. (2008) *Exp. Biol. Med.* **233**, 131–138
- van Rooij, E., Liu, N., and Olson, E. N. (2008) *Trends Genet.* **24**, 159–166
- Brennecke, J., Stark, A., and Cohen, S. M. (2005) *Genes Dev.* **19**, 2261–2264
- Ambros, V. (2004) *Nature* **431**, 350–355
- Bartel, D. P. (2004) *Cell* **116**, 281–297
- Tomari, Y., and Zamore, P. D. (2005) *Genes Dev.* **19**, 517–529
- Carmell, M. A., and Hannon, G. J. (2004) *Nat. Struct. Mol. Biol.* **11**, 214–218
- Zhao, Y., Samal, E., and Srivastava, D. (2005) *Nature* **436**, 214–220
- Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006) *Nat. Genet.* **38**, 228–233
- Kim, H. K., Lee, Y. S., Sivaprasad, U., Malhotra, A., and Dutta, A. (2006) *J. Cell Biol.* **174**, 677–687
- van Rooij, E., Sutherland, L. B., Qi, X., Richardson, J. A., Hill, J., and Olson, E. N. (2007) *Science* **316**, 575–579
- Flynt, A. S., Li, N., Thatcher, E. J., Solnica-Krezel, L., and Patton, J. G. (2007) *Nat. Genet.* **39**, 259–263
- Watanabe, T., Sato, T., Amano, T., Kawamura, Y., Kawamura, N., Kawaguchi, H., Yamashita, N., Kurihara, H., and Nakaoka, T. (2008) *Dev. Dyn.* **237**, 3738–3748
- Yue, S., Chen, Y., and Cheng, S. Y. (2009) *Oncogene* **28**, 492–499
- Ying, S. X., Hussain, Z. J., and Zhang, Y. E. (2003) *J. Biol. Chem.* **278**, 39029–39036
- Cheng, S. Y., and Yue, S. (2008) *Adv. Cancer Res.* **101**, 29–43
- Betel, D., Wilson, M., Gabow, A., Marks, D. S., and Sander, C. (2008) *Nucleic Acids Res.* **36**, D149–D153
- Takahashi, C., Contreras, B., Bronson, R. T., Loda, M., and Ewen, M. E. (2004) *Mol. Cell Biol.* **24**, 10406–10415
- De Falco, G., Comes, F., and Simone, C. (2006) *Oncogene* **25**, 5244–5249
- Juan, A. H., Kumar, R. M., Marx, J. G., Young, R. A., and Sartorelli, V. (2009) *Mol. Cell* **36**, 61–74