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MicroRNAs in the Myocyte Enhancer Factor 2 (MEF2)-regulated *Gtl2-Dio3* Noncoding RNA Locus Promote Cardiomyocyte Proliferation by Targeting the Transcriptional Coactivator Cited2*[S]

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Background: MicroRNAs have recently emerged as key regulatory molecules in cardiomyocyte proliferation.
Results: miR-410 and miR-495 are regulated by MEF2 in cardiomyocytes, and their overexpression results in increased cardiomyocyte proliferation.
Conclusion: miR-410 and miR-495 potently induce cardiomyocyte proliferation by directly inhibiting the coactivator Cited2.
Significance: These findings reveal novel microRNAs that can be modulated to stimulate the regeneration of damaged cardiac tissue.

Understanding cell cycle regulation in postmitotic cardiomyocytes may lead to new therapeutic approaches to regenerate damaged cardiac tissue. We have demonstrated previously that microRNAs encoded by the *Gtl2-Dio3* noncoding RNA locus function downstream of the MEF2A transcription factor in skeletal muscle regeneration. We have also reported expression of these miRNAs in the heart. Here we investigated the role of two *Gtl2-Dio3* miRNAs, miR-410 and miR-495, in cardiac muscle. Overexpression of miR-410 and miR-495 robustly stimulated cardiomyocyte DNA synthesis and proliferation. Interestingly, unlike our findings in skeletal muscle, these miRNAs did not modulate the activity of the WNT signaling pathway. Instead, these miRNAs targeted Cited2, a coactivator required for proper cardiac development. Consistent with miR-410 and miR-495 overexpression, siRNA knockdown of Cited2 in neonatal cardiomyocytes resulted in robust proliferation. This phenotype was associated with reduced expression of *Cdkn1c/p57/Kip2*, a cell cycle inhibitor, and increased expression of *VEGFA*, a growth factor with proliferation-promoting effects. Therefore, miR-410 and miR-495 are among a growing number of miRNAs that have the ability to potently stimulate neonatal cardiomyocyte proliferation.

Mature cardiomyocytes are postmitotic, differentiated cells with a limited capacity to proliferate. Improving cardiac function of the diseased adult heart by way of stimulating cardiomyocyte proliferation proves difficult because the underlying molecular mechanisms that lead to postnatal cell cycle exit are not well understood. In mammals, cardiomyocytes are able to regenerate shortly after birth, but, by 1 week, they exit the cell cycle. Initial attempts to reactivate the cell cycle primarily relied on overexpressing cell cycle activators or suppressing cell cycle inhibitors (1). Current investigations, however, have focused on mitogenic signal transduction cascades, transcription factors, and microRNAs (miRNAs)[2] as alternate regulatory pathways to promote cardiomyocyte proliferation (2, 3).

miRNAs are a class of small, evolutionarily conserved, noncoding RNAs that are important regulators of posttranscriptional gene expression (4). A number of miRNAs are firmly established, important modulators in mammalian cardiac development and stress remodeling pathways (5). There is increasing evidence that miRNAs are also central regulators of mammalian cardiomyocyte proliferation. Deletion of the muscle-specific miR-1–2 or miR-133 resulted in cardiac defects associated with increased cardiomyocyte proliferation (6, 7). Most recently, direct involvement of miRNAs in proliferation has been demonstrated in a high-throughput screen that identified over 200 miRNAs capable of promoting proliferation in cultured primary myocytes and intact hearts (8). Additional miRNAs have been shown to play a role in cardiomyocyte cell cycle regulation. miR-195, a member of the miR-15 family, regulates cell cycle genes, and its inhibition resulted in an increased number of cardiomyocytes (9). The miR-17–92 cluster regulates cardiomyocyte proliferation through its modulation of phosphate and tensin homolog (10). miR-302–367 has been shown recently to promote cardiomyocyte proliferation through activation of the Hippo pathway (11). These findings make it clear that miRNAs regulate cardiomyocyte proliferation but do so by targeting a variety of pathways.

We have shown recently that the myocyte enhancer factor 2A (MEF2A) transcription factor regulates the imprinted *Gtl2-Dio3* noncoding RNA locus in skeletal muscle. MiRNAs encoded in this locus have been found to modulate the WNT signal transduction cascade in skeletal muscle differentiation

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[S] This article contains supplemental Tables S1 and S2.

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[2] The abbreviations used are: miRNA, microRNA; NRVM, neonatal rat ventricular myocyte; EdU, 5-ethynyl-2′deoxyuridine; PCNA, proliferating cell nuclear antigen.

and regeneration (12). In addition to their expression in skeletal muscle, we have reported expression of these miRNAs in the heart. However, the role of these miRNAs in the heart is not fully understood. Therefore, we were interested in characterizing their function in cardiomyocytes.

Here we investigated two miRNAs generated by the *Gtl2-Dio3* noncoding RNA locus in cardiac muscle, miR-410 and miR-495. Initially, we examined expression of these miRNAs in MEF2A knockout hearts and found a significant decrease of these miRNAs in mutant perinatal hearts as well as in MEF2A-deficient neonatal rat ventricular myocytes (NRVMs). We then overexpressed them using miRNA mimics in NRVMs and observed a dramatic increase in cardiomyocyte proliferation. Target prediction analysis of miR-410 and miR-495 found that they commonly target and repress Cited2, a transcriptional coactivator. Significantly, its inhibition also triggered cardiomyocyte proliferation. Proliferation induced by miRNA overexpression or Cited2 knockdown was associated with reduced expression of the cell cycle inhibitor *Cdkn1c/p57/Kip2* and elevated *VEGFA*. This study reveals a novel miRNA transcriptional coactivator pathway in the control of cardiomyocyte proliferation.

Experimental Procedures

Isolation of NRVMs—Ventricles from neonatal rats were isolated from approximately 10 1-day-old SASCO Sprague-Dawley neonatal rats (Charles River Laboratories). Briefly, whole hearts were harvested, and ventricles were isolated from the atria and transferred to prechilled 1× Hanks' balanced salt solution. Ventricles were transferred to 1× Hanks' balanced salt solution/0.025% trypsin and incubated overnight at 4 °C. The following day, digestion was performed by adding 10 mg/ml collagenase II (Worthington) to isolate individual cardiomyocytes. Cells were preplated on uncoated 100-mm plates to remove fibroblasts. Cells were plated in antibiotic-free growth medium at a density of 4×10^6 cells/10-cm dish on gelatinized dishes. After 24 h in culture, cells were washed with 1× PBS and switched to 0.5× Nutridoma-SP (Roche) in DMEM, a low-serum medium.

Plasmids and miRNA Mimics—The mouse Gtl2 proximal promoter (0.5 kb) containing the MEF2 binding site was cloned into pGL3-Basic (Promega) as previously described (12). For 3′UTR reporter assays, the 3′UTR (676 bp) of Cited2 was cloned into pMIR-REPORT (Ambion). The mutant 3′UTR-Cited2 constructs were generated by mutating the miR-410 seed sequence binding site GTTATATT to GGGGGGGG and the miR-495 seed sequence binding site TGTTTGTT to GGGGGGGG. pMIR-REPORT-β-galactosidase (Ambion) was also used for luciferase assays. miRNA mimics, anti-miRs, and siCited2 were purchased from Dharmacon.

miRNA Transfection—miRNA mimics and inhibitors were transfected into NRVMs using a standard reverse transfection protocol at a final concentration of 25 nM. Briefly, Lipofectamine RNAiMAX transfection reagent (Life Technologies) was diluted in Opti-MEM (Life Technologies) and added to the miRNAs. Cells were seeded 30 min later.

shRNA Design and Knockdown in NRVMs—Adenoviruses carrying shRNAs specific for *lacZ* or *MEF2A* were generated as described previously (13). Adenoviruses were used at a multiplicity of infection of 25 for all assays.

Cell Culture Immunofluorescence—Cells were cultured on sterilized coverslips coated with Matrigel. Phase-contrast images of NRVMs were taken using an Olympus MX50 microscope. Cells were fixed in 4% paraformaldehyde. Cells were blocked in 3% BSA (Promega) for 1 h at room temperature. Cells were incubated with primary antibodies diluted in antibody dilution buffer (1× PBS/1% BSA/0.3% Triton X-100) overnight at 4 °C. For immunofluorescence, primary antibodies included anti-α-actinin (1:500, Sigma) and anti-Ki67 (1:200, Abcam). The Click-IT EdU 555 imaging kit (Life Technologies) was also used. The following day, cells were washed in 1× PBS and incubated with fluorochrome-conjugated secondary antibodies diluted in antibody dilution buffer. Secondary antibodies included Alexa Fluor 488 donkey anti-mouse heavy and light chains (H+L) (1:200, Invitrogen) and Alexa Fluor 555 donkey anti-rabbit H+L (1:500, Invitrogen). Cells were washed in 1× PBS and mounted on slides with Vectashield mounting medium with DAPI (Vector Laboratories). Slides were sealed with nail polish and stored at 4 °C protected from light. Immunofluorescence images were taken with an Olympus disk scanning unit spinning disk confocal microscope.

EdU Assays—For 5-ethynyl-2′deoxyuridine (EdU) assays, cells were plated as described. 24 h after miRNA transfection, the medium was replaced with fresh medium. 28 h later, the culture medium was replaced with medium containing 5 μM EdU (Life Technologies) for 20 h. Cells were fixed in 4% paraformaldehyde and processed for immunofluorescence.

siRNA Transfection—For the siRNA transfections, siRNAs were transfected as described previously for the miRNA mimics. Cells were fixed 72 h after plating and processed for immunofluorescence, or RNA was harvested for analysis.

Luciferase Assays—Cells were harvested for luciferase activity 48 h after transfection. Cells were lysed in 1× passive lysis buffer (Promega). To measure firefly luciferase activity, 5 μl of cell lysate was mixed with 30 μl of luciferase assay reagent (Promega), and readings were taken on a luminometer. Results were normalized by β-galactosidase assay. All luciferase assays were performed in triplicate ($n \geq 3$).

Quantitative RT-PCR—RNA from cardiac muscle or NRVM experiments ($n \geq 3$) was used to synthesize cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase with random hexamers according to the instructions of the manufacturer (Promega). cDNAs were synthesized using the TaqMan miRNA reverse transcriptase kit (Applied Biosystems) for detection of mature miRNAs as described by Chen *et al.* (14). miRNA and 5S rRNA sequences were amplified using forward-specific primers and a universal reverse primer. Quantitative RT-PCR was performed in triplicate using Power SYBR Green Master Mix (Applied Biosystems) with a 7900HT sequence detection system (Applied Biosystems). The primers used were 5S rRNA stem loop forward 5′-GTTGGCTCTGGTGCAGGG-TCCGAGGTATTCGCACCAGAGCCAACAAAGCC, miR-410 stem loop 5′-GTTGGCTCTGGTGCAGGGTCCGAGG-TATTCGCACCAGAGCCAACACAGGC, miR-495 stem loop 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC-CAGAGCCAACAAGAAG, 5S rRNA forward 5′-GAATACC-

GGGTGCTGTAGGC, miR-410 forward 5′-CCGCCAATAT-AACACAGATGGCC, miR-495 forward 5′-GCCAAACAAA-CATGGTGCACTT, *Gapdh* forward 5′-TGGCAAAGTGGA-GATTGTTGCC and reverse 5′-AAGATGGTGATGGGCTT-CCCG, *Sfrp2* forward 5′-CCCCTGTCTGTCTCGACGA and reverse 5′-CTTCACACACCTTGGGAGCTT, *Axin2* forward 5′-TGACTCTCCTTCCAGATCCCA and reverse 5′-TGCCC-ACACTAGGCTGACA, *Cited2* forward 5′-TGGGCGAGCA-CATACACTAC and reverse 5′-GGGTGATGGTTGAAATA-CTGGT, *Nr3c1* forward 5′-TCTCAGGCAGATTCCAAGCA and reverse 5′-TGGACAGTGAAACGGCTTTG, *Errfi1* forward 5′-GCACAATGTCAACAGCAGGA and reverse 5′-TCCAGAGATGGGTCCTCAGA, *Ppp1cb* forward 5′-GAGT-GTGCTAGCATCAACCG and reverse 5′-GTCAAACTCGC-CGCAGTAAT, *Smad7* forward 5′-AGCATCTTCTGTCCC-TGCTT and reverse 5′-CTCCTCGAATTCTGTGCACG, *Rere* forward 5′-TCATGTACTTGAGGGCAGCA and reverse 5′-CACTTCTCGATCAGCTTGG, *Stat3* forward 5′-TCAGT-GAGAGCAGCAAGGAA and reverse 5′-TTTCCGAATGCC-TCCTCCTT, *Gad1* forward 5′-ATGTGTGCAGGCTACC-TCTT and reverse 5′-TCGGAGGCTTTGTGGTATGT, *p57* forward 5′-GACTGAGAGCAAGCGAACAG and reverse 5′-CAGCGAGAAAGAAGGGAACG, *Vegfa* forward 5′-TTCCT-GTAGACACACCCACC and reverse 5′-TCCTCCCAACTCA-AGTCCAC, and Bim forward 5′-TCGTCCACCCAATGT-CTGACTC and reverse 5′-CTCCTGTCTTGCGATTCT-GTCTGT.

Western Blot Analysis—Western blots were performed as described previously (15). Antibodies included anti-GAPDH (1:1000, Santa Cruz Biotechnology) and anti-PCNA (1:2000, Cell Signaling Technology). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000, Sigma) and reacted with Western Lightning chemiluminescent reagent (PerkinElmer Life Sciences).

microRNA Target Analysis—Potential microRNA targets were analyzed using miRANDA, TargetScan, and miRDB.

Statistical Analysis—All numerical quantifications are representative of the mean ± S.E. of at least three experiments performed independently. Statistically significant differences between two populations of data were determined using Student's *t* test. $p \leq 0.05$ was considered to be statistically significant.

Results

miR-410 and miR-495 Are Expressed in the Heart and Down-regulated in MEF2A-deficient Cardiomyocytes—We have reported previously that expression of the MEF2-regulated *Gtl2-Dio3* miRNAs is enriched in the brain, skeletal muscle, and heart (12). Given their expression in the heart and the established role of MEF2 in cardiac development and disease, we aimed to investigate the MEF2-*Gtl2-Dio3* miRNA pathway in this tissue. We chose to focus on a subset of *Gtl2-Dio3* miRNAs that, as we have shown, modulate the activity of the WNT signaling pathway (12). Initially, we examined the cardiac expression of two of these *Gtl2-Dio3* miRNAs, miR-410 and miR-495, in perinatal and adult hearts. Our prior studies have revealed low but detectable expression levels of several *Gtl2-Dio3* miRNAs in the adult mouse heart. As shown in Fig. 1*A*, miR-410 and miR-495 are expressed in both the perinatal and adult heart, but their expression was significantly higher in perinatal hearts, suggesting a role in perinatal cardiac function. Moreover, the temporal expression pattern of these *Gtl2-Dio3* miRNAs is consistent with MEF2 transcriptional activity in the postnatal heart (16).

Given the above result, we then examined miR-410 and miR-495 expression in perinatal MEF2A knockout hearts. We have reported previously that the majority of MEF2A knockout mice die in the perinatal period, with severe structural abnormalities in cardiomyocytes (17). We found that miR-410 and miR-495 are down-regulated significantly in perinatal MEF2A knockout hearts (Fig. 1*B*). To determine whether miR-410 and miR-495 are specifically down-regulated in cardiac muscle and are dependent on MEF2A, we examined their expression in NRVMs in which we depleted MEF2A using shRNA adenovirus (13). shRNA-mediated knockdown of MEF2A in NRVMs resulted in a significant decrease in miR-410 and miR-495 expression (Fig. 1*C*).

To determine whether transcription of this locus is dependent on MEF2, we analyzed the activity of the *Gtl2* promoter in NRVMs. We have demonstrated previously that the proximal promoter region of the *Gtl2-Dio3* locus is directly regulated by MEF2 in skeletal muscle and required for the proper expression of miRNAs encoded by this locus (12). Similar to our results in C2C12 skeletal myoblasts, the wild-type *Gtl2* promoter was active in NRVMs (Fig. 1*D*). A mutation in the MEF2 binding site in the *Gtl2* promoter significantly reduced its activity, demonstrating that transcription of the *Gtl2-Dio3* locus is dependent on endogenous MEF2 in cardiomyocytes (Fig. 1*D*). Moreover, activity of the *Gtl2* promoter was reduced significantly in NRVMs depleted of MEF2A (Fig. 1*E*). These results indicate that the *Gtl2-Dio3* noncoding RNA locus is dependent on MEF2 activity, particularly MEF2A, in perinatal cardiac muscle.

On the basis of the established role of MEF2 in regulating the muscle cytoarchitecture, we were interested in determining whether the expression of sarcomere genes is dependent on these *Gtl2-Dio3* miRNAs (18). As expected, acute knockdown of MEF2A in NRVMs resulted in significant down-regulation of sarcomere genes (Fig. 1*F*). Using hairpin inhibitors (anti-miRs), we knocked down miR-410 and miR-495 in NRVMs. Similar to MEF2A depletion, knockdown of miR-495, but not miR-410 (data not shown), caused a significant reduction in sarcomere gene expression (Fig. 1*G*). Although sarcomere genes were down-regulated, transient knockdown of either miR-410 or miR-495 in NRVMs did not cause an overt morphological phenotype (data not shown). Finally, to determine whether these miRNAs are involved in the structural and cell death phenotype in MEF2A-deficient NRVMs (13), we overexpressed miR-410 and miR-495 in MEF2A-depleted NRVMs. As shown in Fig. 1*H*, overexpression of these miRNAs resulted in a modest but significant up-regulation of sarcomere gene expression compared with MEF2A-depleted NRVMs alone. Furthermore, up-regulation of Bcl2-Like 11 (BIM) expression, a proapoptotic marker gene, was reduced significantly compared with MEF2A-depleted NRVMs alone. Taken together, these results strongly suggest that the *Gtl2-Dio3* noncoding RNAs function downstream of MEF2A and play a role in cardiomyocyte differentiation and/or maturation.

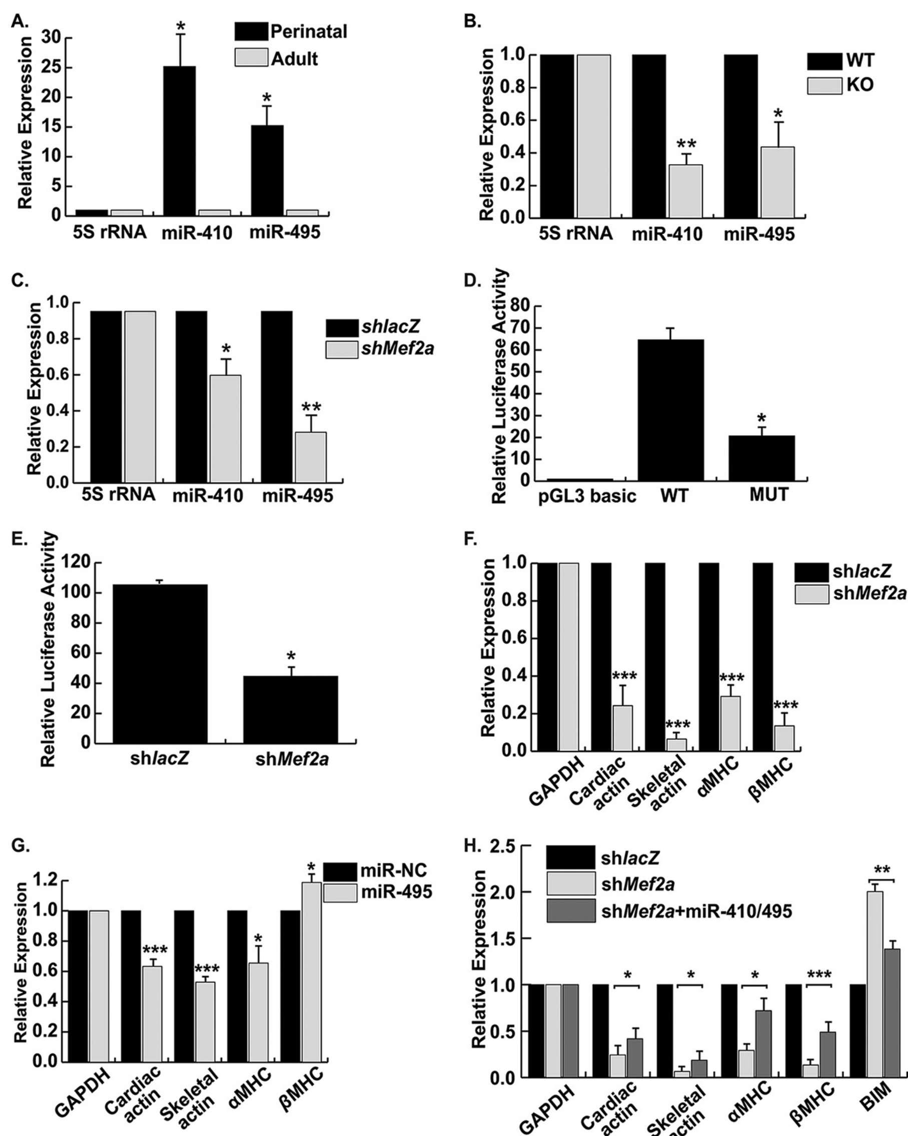


FIGURE 1. **miR-410 and miR-495 are expressed in the heart and down-regulated in MEF2A-deficient cardiomyocytes.** *A*, quantitative RT-PCR analysis of miR-410 and miR-495 in perinatal and adult wild-type cardiac muscle. *B*, quantitative RT-PCR analysis of miR-410 and miR-495 in perinatal WT and MEF2A KO cardiac muscle. *C*, quantitative RT-PCR analysis of miR-410 and miR-495 in control (*shlacZ*) and MEF2A knockdown (*shMef2a*) NRVMs. *D*, luciferase analysis of the *Gtl2* promoter showing that muscle-specific activity (*WT*) and mutation of the MEF2 site (*MUT*) results in decreased reporter activity. *E*, luciferase analysis of the *Gtl2* promoter in MEF2A-deficient NRVMs showing that promoter activity is dependent on MEF2A. *F*, quantitative RT-PCR analysis of cardiac sarcomere genes in control and MEF2A knockdown NRVMs. *G*, quantitative RT-PCR analysis of cardiac sarcomere genes in control (*miR-NC*) and miR-495 knockdown NRVMs. *H*, overexpression of miR-410 and miR-495 in MEF2A-depleted NRVMs up-regulates sarcomere gene expression and reduces BIM expression. *Error bars* represent mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Overexpression of miR-410 and miR-495 Promotes Cardiomyocyte Proliferation—Given the expression of miR-410 and miR-495 in NRVMs and perinatal hearts and the effect of miR-495 knockdown on sarcomere genes, we asked whether overexpression of these miRs alters cardiomyocyte maturation and growth. Toward this end, we overexpressed miR-410 and miR-495 in NRVMs using miRNA mimic oligonucleotides. Upon overexpression of miR-410 or miR-495 mimics, we noticed an abundance of α-actinin-positive cardiomyocytes compared with the control (miR-NC) mimic (Fig. 2*A*). The apparent increase in cardiomyocytes suggested an effect on proliferation. To determine whether these miRNAs were inducing cell cycle activity in cardiomyocytes, we performed Ki67 immunostaining on NRVMs in which we overexpressed miR-410 or miR-495 mimics. Quantification of Ki67$^+$ NRVMs revealed a significant 3-fold increase in Ki67 immunofluorescence upon addition of miR-410 or miR-495 mimics (Fig. 2*A*).

In a complementary set of experiments, we asked whether increased cell cycle activity was associated with increased DNA synthesis. We performed an EdU incorporation assay and found that overexpression of miR-410 or miR-495 caused a noticeable increase in EdU immunofluorescence in NRVMs (Fig. 2*B*). Quantification revealed an increase in EdU$^+$ NRVMs upon addition of miR-410 or miR-495 (Fig. 2*B*). Furthermore, as an independent means of verifying the increase in DNA synthesis, we examined the expression of proliferating cell nuclear antigen (PCNA), an essential cofactor in DNA replication. Western blot analysis revealed a 2-fold or

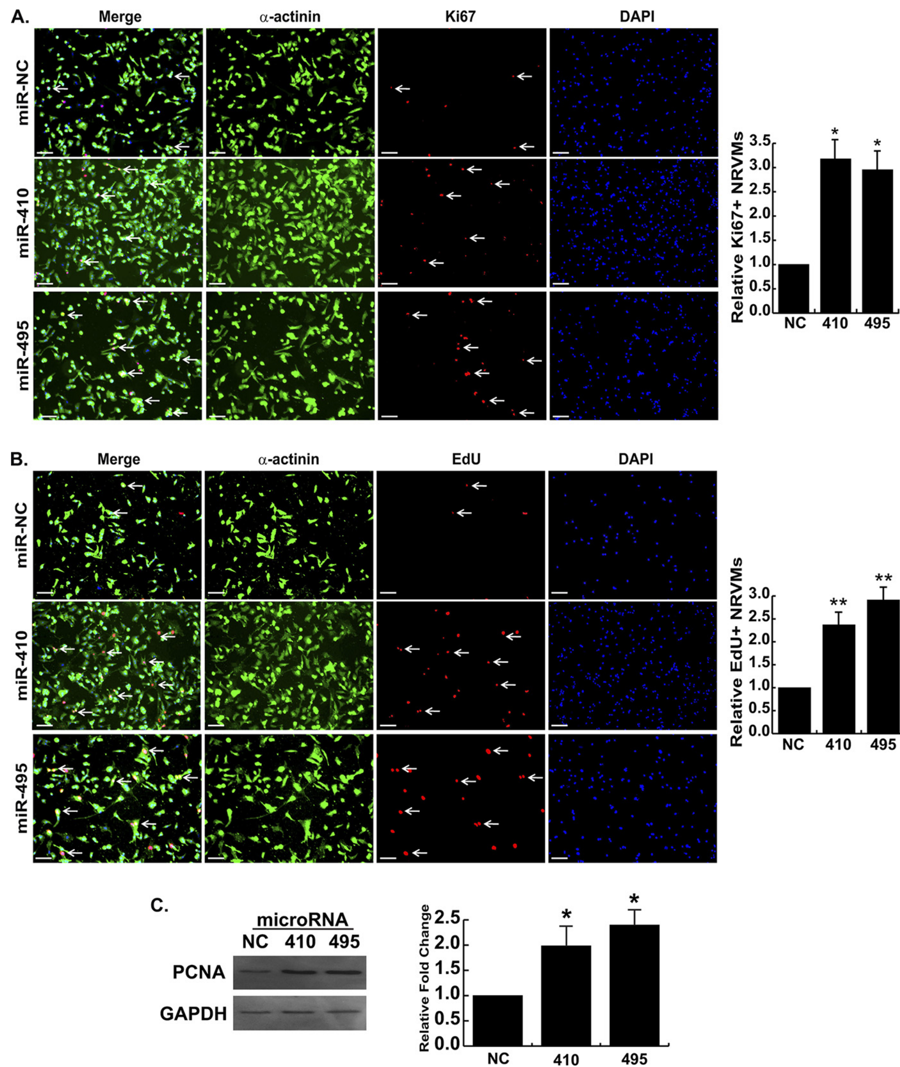


FIGURE 2. **Overexpression of miR-410 and miR-495 promotes cardiomyocyte proliferation.** *A*, representative images of Ki67 immunostaining: miR-NC (*top row*), miR-410 (*center row*), and miR-495 (*bottom row*). α-Actinin staining is shown in *green*, Ki67 staining in *red*, and DAPI staining in *blue*. The relative -fold change of Ki67$^+$ NRVMs in miR-410 and miR-495 overexpression is compared with miR-NC (*NC*). For each miR mimic overexpression, a total of 1500 cardiomyocytes were counted. *B*, representative images of the EdU assay: miR-NC (*top row*), miR-410 (*center row*), and miR-495 (*bottom row*). α-Actinin staining is shown in *green*, EdU staining in *red*, and DAPI staining in *blue*. The relative -fold change of EdU$^+$ NRVMs in miR-410 and miR-495 overexpression is compared with miR-NC. For each miR mimic overexpression, a total of 1500 cardiomyocytes were counted. *Scale bars* are 20 μm. *C*, Western blot analysis of PCNA in NRVMs overexpressed with miR-NC, miR-410, and miR-495. Shown is the densitometry of the PCNA Western blot. *Error bars* represent mean ± S.E. *, $p < 0.05$; **, $p < 0.01$.

greater increase in PCNA upon overexpression of miR-410 or miR-495 (Fig. 2*C*). Taken together, these results indicate a role for miR-410 and miR-495 in promoting neonatal cardiomyocyte proliferation.

WNT Activity Is Not Dysregulated in NRVMs Depleted of MEF2A or Overexpressing miR-410 and miR-495—In skeletal muscle, both of these MEF2-regulated miRNAs were predicted to target *Sfrp2*, an inhibitor in the WNT signaling pathway, and we subsequently showed that miR-410 directly repressed *Sfrp2* expression (12). In the same study, we demonstrated impaired WNT signaling in MEF2A-deficient skeletal muscle. Therefore, we were interested in determining whether MEF2A and miR-410 and miR-495 modulate WNT signaling in cardiomyocytes and whether the WNT pathway is involved in proliferation induced by these miRNAs. Initially, to determine whether WNT signaling was also affected in MEF2A-deficient cardiomyocytes, we examined the expression of Sfrp2, a WNT inhibitor, and Axin2, a WNT-responsive target gene. We found no significant dysregulation of these WNT signaling components in MEF2A-depleted NRVMs (Fig. 3*A*). Moreover, we found no significant difference in TOPflash activity, a WNT-sensitive luciferase reporter, in MEF2A-depleted NRVMs (Fig. 3*B*). Subsequently, we asked whether WNT signaling is perturbed upon overexpression of miR-410 or miR-495. Overexpression of miR-410 or miR-495 significantly repressed *Sfrp2* expression (Fig. 3*C*) but did not affect the expression of *Axin2* (Fig. 3*D*). These results indicate that although *Sfrp2* expression is down-regulated by these *Gtl2-Dio3* miRNAs in cardiomyocytes, unlike skeletal muscle, the reduction of *Sfrp2* expression is not sufficient to attenuate WNT activity in neonatal cardiomyocytes. Taken together, these results suggest that WNT signaling is not a major pathway through which miR-410 and miR-495 stimulate proliferation in neonatal cardiomyocytes.

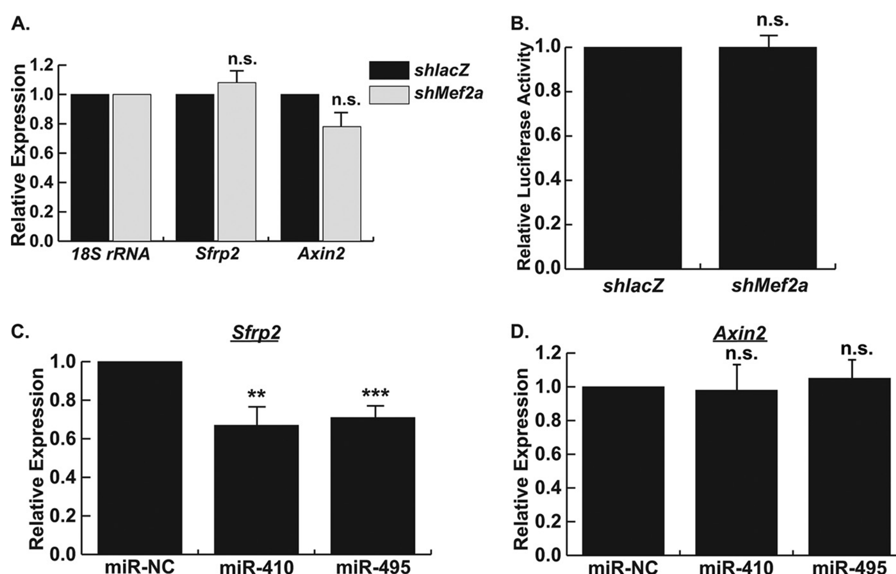
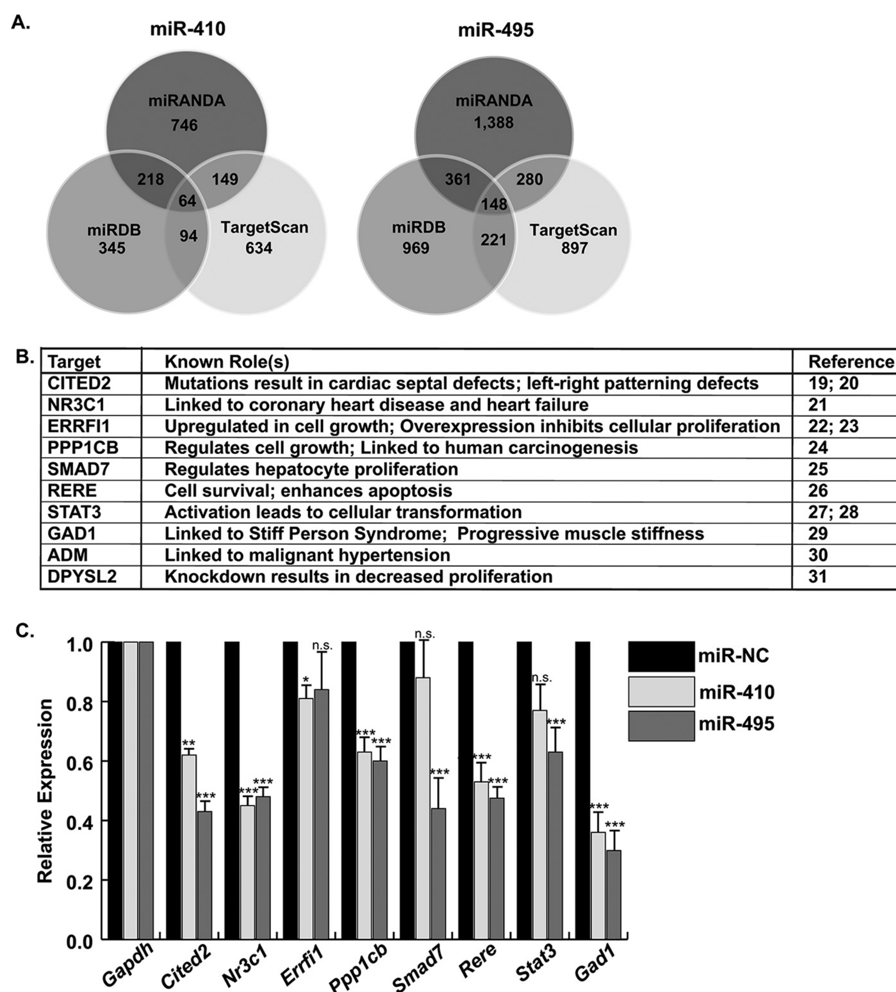


FIGURE 3. **WNT signaling is not dysregulated in MEF2A-deficient NRVMs or upon overexpression of miR-410 and miR-495.** *A*, expression of *Sfrp2* and *Axin2* in MEF2A-deficient NRVMs shows no significant dysregulation. *B*, luciferase analysis of TOPflash reporter activity in MEF2A-deficient NRVMs shows no significant difference in activity. *C*, expression of *Sfrp2* upon overexpression of miR-410 and miR-495 in NRVMs. *D*, expression of *Axin2* upon overexpression of miR-410 and miR-495 in NRVMs. *Error bars* represent mean ± S.E. *n.s.*, not significant; **, $p < 0.01$; ***, $p < 0.001$.



Target	Known Role(s)	Reference
CITED2	Mutations result in cardiac septal defects; left-right patterning defects	19; 20
NR3C1	Linked to coronary heart disease and heart failure	21
ERRFI1	Upregulated in cell growth; Overexpression inhibits cellular proliferation	22; 23
PPP1CB	Regulates cell growth; Linked to human carcinogenesis	24
SMAD7	Regulates hepatocyte proliferation	25
RERE	Cell survival; enhances apoptosis	26
STAT3	Activation leads to cellular transformation	27; 28
GAD1	Linked to Stiff Person Syndrome; Progressive muscle stiffness	29
ADM	Linked to malignant hypertension	30
DPYSL2	Knockdown results in decreased proliferation	31

FIGURE 4. **Identification and validation of predicted target genes of miR-410 and miR-495.** *A*, Venn diagrams of potential targets for miR-410 and miR-495 according to miRANDA, TargetScan, and miRDB. The overlap of the three algorithms reveals 64 and 148 predicted targets for miR-410 and miR-495, respectively. *B*, the top 10 predicted targets in common for miR-410 and miR-495 and their known roles in either cardiac muscle or proliferation. *C*, quantitative RT-PCR analysis of eight of the top 10 predicted targets for miR-410 and miR-495. *Error bars* represent mean ± S.E. *n.s.*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

FIGURE 5. **miR-410 and miR-495 directly target the 3′UTR of Cited2.** *A*, sequence alignments of miR-410 and miR-495 seed sequences and predicted target sites in the 3′UTR of human and mouse Cited2. *B*, luciferase analysis of WT and mutant (*MUT*) pMIR-REPORT-3′UTR-CITED2 cotransfected with miR-410 and miR-495 mimics (150 nM, Dharmacon) in NRVMs compared with a nonspecific control (*miR-NC*). *C*, quantitative RT-PCR of *p57* expression levels when overexpressing miR-410 and miR-495. *D*, quantitative RT-PCR of *Vegfa* expression levels when overexpressing miR-410 and miR-495. *Error bars* represent mean ± S.E. *n.s.*, not significant; *, $p < 0.05$; **, $p < 0.01$.

Identification and Validation of Predicted Target Genes of miR-410 and miR-495—To determine the pathway potentially targeted by these miRNAs, we compared the predicted targets from three prediction algorithms: miRANDA, TargetScan, and miRDB. Because miRANDA generated thousands of predicted targets, we only analyzed targets with a miRSVR score of −1.00 or higher. This resulted in 746 and 1388 targets for miR-410 and miR-495, respectively (Fig. 4*A*). Then, we compared these miRANDA targets with those identified in the TargetScan and miRDB algorithms, with each predicting several hundred candidate genes (supplemental Tables S1 and S2). This comparative analysis resulted in a total of 64 and 148 overlapping predicted targets for miR-410 and miR-495, respectively (Fig. 4*A*). Because this was still a relatively large number of potential targets for each miRNA, we further narrowed down this list by looking for genes that were in common in the miR-410 and miR-495 target prediction sets and involved in either cell proliferation or cardiac muscle. Using this approach, we identified and selected 10 genes that fulfilled these criteria (Fig. 4*B*) (19–31). To validate these predictions we examined the expression of eight of these candidate target genes in NRVMs overexpressing miR-410 or miR-495. As shown in Fig. 4*C*, the majority of the eight predicted common targets were down-regulated significantly in NRVMs overexpressing either miR-410 or miR-495.

miR-410 and miR-495 Directly Target the 3′ UTR of Cited2—Of the validated candidate target genes, we chose to focus on Cited2. Cited2 interacts with the p300/CBP coactivator and its deficiency in mice results in cardiac septal defects and other cardiac morphological abnormalities (19, 20, 32). To determine whether miR-410 and miR-495 could directly repress Cited2, we examined their seed sequences and the target sequence in Cited2. The seed sequences of miR-410 and miR-495 and their target sequences in *Cited2* are conserved between human, mouse, and rat (Fig. 5*A*). We cloned the 3′UTR of Cited2 into the pMIR-REPORT vector (Ambion) and used this construct in transient transfection assays to examine the ability of these miRNAs to repress this reporter. Cotransfection of the pMIR-REPORT-3′UTR-CITED2 with either miR-410 or miR-495 mimics in NRVMs resulted in significant inhibition of the reporter (Fig. 5*B*). Mutation of the miR-410 or the miR-495 binding site in Cited2 reduced the ability of these miRNAs to repress the reporter, resulting in no significant difference between the miR-NC and miR-410 mutant or miR-495 mutant reporters, respectively (Fig. 5*B*). These results demonstrate that miR-410 and miR-495 are capable of directly inhibiting Cited2 in cardiomyocytes.

Dysregulated Expression of p57 and Vegfa Is Associated with miRNA-induced Neonatal Cardiomyocyte Proliferation—To better understand the mechanism by which miR-410 and miR-495 promote cardiomyocyte proliferation, we reasoned that target genes of Cited2 would be misregulated in this process. Specifically, we searched for Cited2 target genes that have been linked to cellular proliferation. *Cdkn1c/p57/Kip2*, a cell cycle inhibitor, has been shown to be positively regulated by Cited2 in hematopoietic stem cells (33). On the basis of the reduction in *Cited2* expression, we would expect down-regulation of *Cdkn1c/p57/Kip2* expression in NRVMs overexpressing miR-410 or miR-495. As predicted, overexpressing miR-410 or miR-495 mimics in NRVMs resulted in a significant down-regulation of *Cdkn1c/p57/Kip2* expression (Fig. 5*C*). We also examined the expression of *Vegfa* because previous studies have shown that the *Vegf* promoter is repressed by Cited2 (34). In addition, delivery of VEGF to the injured heart has been

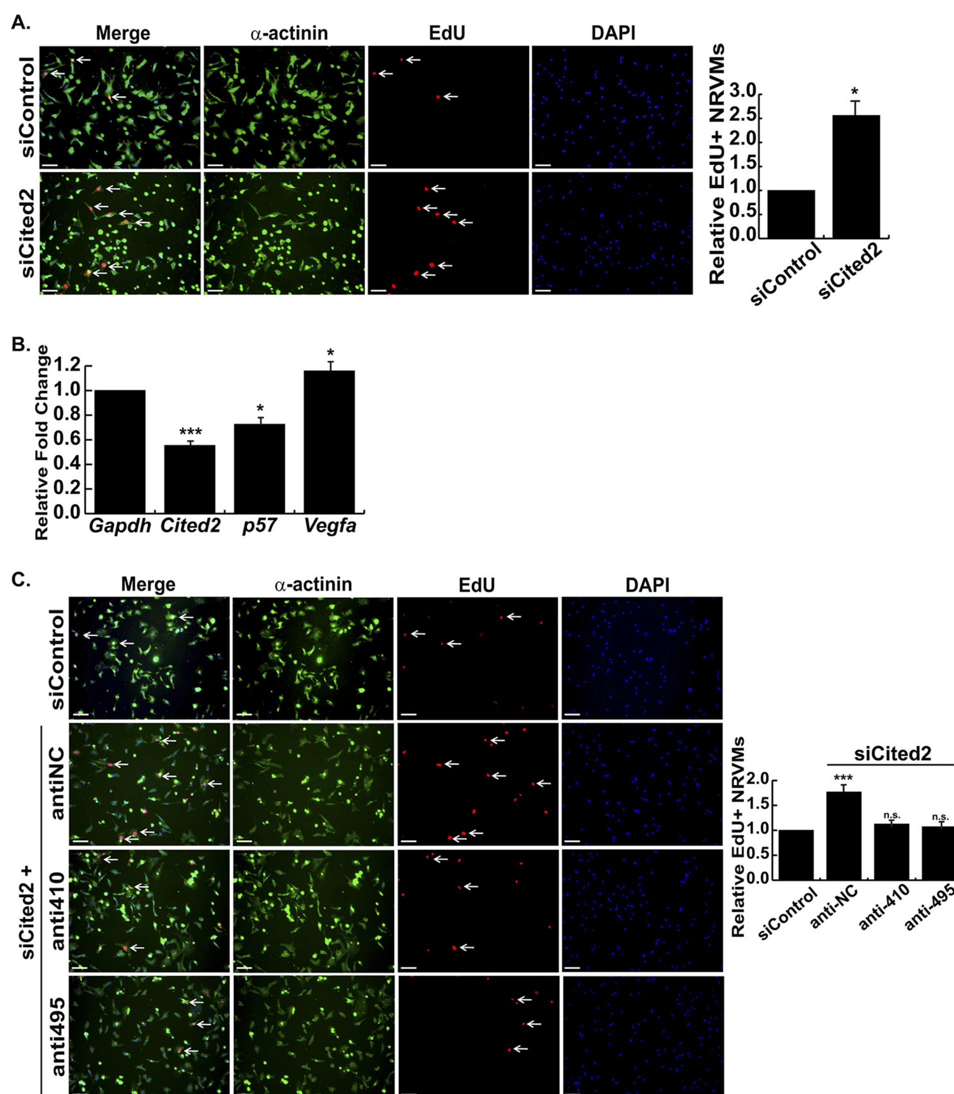


FIGURE 6. **Knockdown of Cited2 and cosilencing of Cited2, miR-410, and miR-495 in neonatal cardiomyocytes.** *A*, knockdown of Cited2 results in increased cardiomyocyte proliferation. Shown are representative images of the EdU incorporation assay when knocking down *Cited2*: siControl (*top row*) and siCited2 (*bottom row*). α-Actinin staining is shown in *green*, EdU staining in *red*, and DAPI staining in *blue*. The relative -fold change of EdU^+ NRVMs in siCited2 was compared with siControl (*siCtl*). For each knockdown, a total of 1400 cardiomyocytes were counted. *B*, quantitative RT-PCR of *Cited2, p57,* and *Vegfa* expression levels when knocking down *Cited2* using siCited2 in NRVMs. *C*, cosilencing of Cited2, miR-410, and miR-495 prevent cardiomyocyte proliferation. Shown are representative images of the EdU incorporation assay: siControl (*first row*), siCited2 + antimiR-NC (*second row*), siCited2 + antimiR-410 (*third row*), and siCited2 + antimiR-495 (*bottom row*). α-Actinin staining is shown in *green*, EdU staining in *red*, and DAPI staining in *blue*. The relative -fold change of EdU+ NRVMs shows that combinatorial knockdown of Cited2 and miR-410 and miR-495 results in normal cardiomyocyte proliferation. For each combinatorial knockdown, a total of 1000 cardiomyocytes were counted. *Error bars* represent mean ± S.E. *n.s.*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Scale bars* are 20 μm.

shown to induce cardiomyocyte proliferation (35, 36). As shown in Fig. 5*D*, overexpression of both miR-410 and miR-495 resulted in a significant increase in *Vegfa*.

miR-410, miR-495, and Cited2 Function in the Same Pathway to Promote Neonatal Cardiomyocyte Proliferation—Initially, to establish that knockdown of *Cited2* is capable of promoting cardiomyocyte proliferation, we inhibited *Cited2* in NRVMs. Neonatal myocytes transfected with siCited2 resulted in a significant increase in EdU incorporation, similar to levels observed in miR-410 and miR-495 overexpression experiments (Fig. 6*A*). Additionally, transfection of siCited2 resulted in a modest but significant decrease in *p57* and increase in *Vegfa* levels, similar to the effect observed upon miR-410 and miR-495 overexpression (Fig. 6*B*).

To demonstrate that miR-410 and miR-495 function in the same genetic pathway as Cited2 and that this gene is a relevant physiological target in cardiomyocytes, we cosilenced miR-410 and miR-495 along with Cited2. As shown in Fig. 6*C*, this combinatorial knockdown prevented NRVMs from proliferating. This result demonstrates that loss of Cited2 is likely responsible for the miR-410 and miR-495-induced cardiomyocyte proliferation.

Dysregulated Expression of Cited2, p57, and Vegfa in MEF2A-deficient Cardiomyocytes—Because the *Gtl2-Dio3* miRNAs function downstream of MEF2A in cardiomyocytes, we asked whether the above genes dysregulated in miR-410 and miR-495 overexpression were also affected in MEF2A deficiency. We found that both *Cited2* and *p57* were up-regulated, whereas

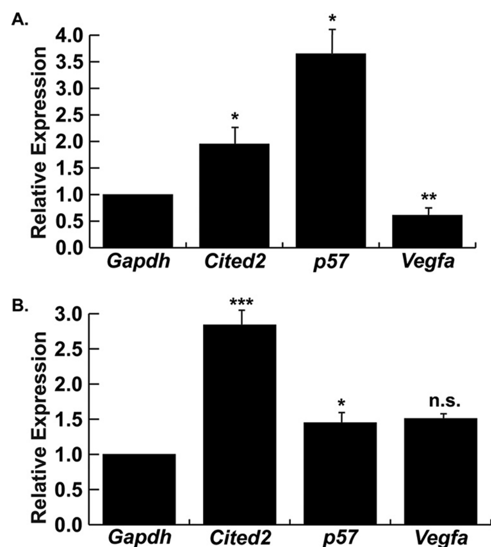


FIGURE 7. **Dysregulated Cited2, p57, and Vegfa expression in MEF2A-deficient cardiomyocytes.** *A*, quantitative RT-PCR of *Cited2, p57,* and *Vegfa* expression levels in MEF2A-deficient NRVMs. *B*, quantitative RT-PCR of *Cited2, p57,* and *Vegfa* expression levels in perinatal MEF2A knockout cardiac muscle. *Error bars* represent mean ± S.E. *n.s.*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Vegfa was down-regulated significantly in MEF2A-depleted NRVMs (Fig. 7*A*). In a similar fashion, *Cited2* and *p57* were up-regulated significantly in perinatal MEF2A knockout hearts (Fig. 7*B*). The above gene expression pattern is contrary to that observed in miR-410- and miR-495-induced cardiomyocyte proliferation but entirely consistent with the down-regulation of these miRNAs in MEF2A deficiency. Collectively, these data support the notion that the MEF2-*Gtl2-Dio3* noncoding RNA pathway regulates proper neonatal cardiomyocyte growth and survival.

Discussion

Molecularly defining the mechanisms by which differentiated cardiomyocytes can be induced to proliferate remains an important endeavor given the possibilities of translating this knowledge to stimulate repair of damaged cardiac tissue. In this report, we demonstrate that miR-410 and miR-495, miRNAs transcribed from the *Gtl2-Dio3* noncoding RNA locus, effectively promote proliferation in neonatal cardiomyocytes. Our results also show that expression of miR-410 and miR-495 and regulation of the *Gtl2* promoter in cardiomyocytes are dependent on the MEF2A transcription factor. We have reported previously that miR-410 and miR-495 belong to a subset of miRNAs in the *Gtl2-Dio3* locus that modulate WNT signaling in skeletal muscle differentiation and regeneration (12). By contrast, these miRNAs and MEF2A do not significantly modulate WNT activity in cardiomyocytes. Instead, miR-410 and miR-495 regulate the expression of the transcriptional coactivator Cited2, whose down-regulation induces cardiomyocyte proliferation.

Recently, miRNAs have emerged as key regulators of cardiomyocyte proliferation (37, 38). These small regulatory RNAs have been shown to modulate proliferation in either a positive or negative manner, indicating that cardiomyocytes employ these molecules to tightly control the cell cycle. Indeed, a high-throughput overexpression screen revealed that miR-199a and miR-590 stimulated proliferation of postmitotic, neonatal, and adult cardiomyocytes (8). Interestingly, this study listed miR-495 among a cohort of miRNAs capable of stimulating cardiomyocyte proliferation. However, this miRNA was not characterized molecularly, and the mechanism by which it promotes proliferation was not investigated.

MicroRNAs encoded by the *Gtl2-Dio3* noncoding RNA locus have been linked to cancer in multiple tissue types (39–42). Regarding the individual function of miR-410 and miR-495, a number of reports have linked both of these miRNAs to oncogenic pathways. Some studies have suggested a tumor suppressor role for these miRNAs (43–45), whereas others have indicated a pro-proliferative effect on tumor growth. Along these lines, miR-410 has been shown to be up-regulated in liver cancer and enhanced tumor cell growth (46). MiR-495 has been shown to stimulate proliferation of human umbilical vein endothelial cells (47). Taken together, these findings provide strong evidence that miR-410 and miR-495 have the ability to regulate cell cycle activity and that this function has been conserved in cardiomyocytes.

It is intriguing that Cited2 emerged as the top predicted target for both miR-410 and miR-495 because this transcriptional coactivator has been linked to important developmental processes in the heart. *Cited2* global knockout mice are embryonic lethal due to defects in left-right patterning, septation, outflow tract, and aortic malformations. Cardiomyocyte-specific *Cited2* knockout mice revealed a requirement specifically in cardiomyocytes with defects in normal myocardial thickening and ventricular septation (32). Furthermore, mutations in *Cited2* are associated with congenital heart disease in humans, pointing to an important role for this transcriptional coactivator in cardiac muscle (48, 49).

Cited2 has been shown to interact with a number of transcription factors, including transcription factor AP-2 (TFAP2) and hypoxia-inducible factor 1 α (HIF1-α) (50, 51). Interestingly, TFAP2 mutations have also been linked to congenital heart disease (52, 53). Although Cited2 functions to stimulate TFAP2 activity, it is a negative regulator of HIF1-α (51). Indeed, HIF1-α is increased in *Cited2* knockout mice, resulting in the increase in HIF1-α-responsive genes such as VEGF (32). Moreover, mutations in Cited2 result in dysregulation of VEGF in humans (34).

Regarding cell cycle control, overexpression of Cited2 triggers tumor formation in fibroblasts (54). Cited2 has also been shown to regulate the expression of the cell cycle inhibitor p57 in hematopoietic stem cells, and *p57* levels are decreased in the *Cited2* knockout mouse (20, 21). Furthermore, TFAP2 overexpression results in increased *p57* expression (55). Consistent with the findings above, we showed that depletion of Cited2 results in decreased *p57* expression, leading to increased cell cycle activity and cardiomyocyte proliferation. We also showed that increased cardiomyocyte proliferation in Cited2-depleted NRVMs is associated with increased expression of *Vegfa*. Interestingly, a recent study has reported that miR-410 directly targets human VEGF in osteosarcoma cells (56). These observations suggest that the proliferation phenotype in miR-410-overexpressing NRVMs may be due to a direct effect of this

miRNA on *Vegfa* expression. Because *Vegfa* was up-regulated upon miR-410 and miR-495 overexpression, it is unlikely that miR-410 is directly repressing *Vegfa* in this context and reinforces the notion that *Vegfa* is primarily regulated through Cited2 activity in cardiomyocytes. Therefore, the fine-tuning of Cited2 activity by miR-410 and miR-495 appears to be important for proper perinatal cardiomyocyte maturation and growth control.

In addition to its function in cardiomyocyte proliferation, the possibility that the *Gtl2-Dio3* noncoding RNA locus plays a role in heart disease is suggested by recent reports describing the dysregulated expression of miRNAs from this imprinted locus in mice subjected to myocardial infarction and thyroid hormone-induced cardiac hypertrophy (57, 58). Given the established role of the MEF2 transcription factor in stress signaling in the heart, it is tempting to speculate that dysregulation of the MEF2-*Gtl2-Dio3* pathway plays a central role in a spectrum of cardiac diseases. Although our study focused primarily on the role of the *Gtl2-Dio3* miRNAs in neonatal cardiomyocytes, it would be interesting to examine whether their overexpression is capable of stimulating proliferation in adult cardiomyocytes. Moreover, gain-of-function and loss-of-function analyses of the *Gtl2-Dio3* miRNAs in models of cardiac disease are likely to provide valuable information regarding the relative contribution of these small noncoding RNAs to remodeling pathways in the diseased heart. Our study clearly demonstrates a role for *Gtl2-Dio3* miRNAs in cardiomyocyte proliferation and the potential of these regulatory RNAs to induce regeneration of diseased cardiac muscle *in vivo*. Delivery of the *Gtl2-Dio3* miRNAs may be a potential therapeutic approach to stimulate cardiomyocyte proliferation and reduce cardiac damage post-injury in the postnatal heart.

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