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MicroRNAs in the MEF2-regulated *Gtl2-Dio3* Noncoding RNA Locus Promote Cardiomyocyte Proliferation by Targeting the Transcriptional Co-activator Cited2*

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*Running title: miR-410 and miR-495 Regulate Cardiomyocyte Proliferation

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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 regeneration of damaged cardiac tissue.

ABSTRACT

Understanding cell cycle regulation in post-mitotic cardiomyocytes may lead to new therapeutic approaches to regenerate damaged cardiac tissue. Previously, we demonstrated that microRNAs encoded by the *Gtl2-Dio3* noncoding RNA locus function downstream of the MEF2A transcription factor in skeletal muscle regeneration. We 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, we found these miRNAs target 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. Thus, 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 post-mitotic, 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 one 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, miRs) as alternate regulatory pathways to promote cardiomyocyte proliferation (2,3).

miRNAs are a class of small, evolutionarily conserved, non-coding RNAs that are important regulators of post-transcriptional 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 was demonstrated in a high throughput screen which identified over 200 miRNAs capable of promoting proliferation in cultured primary myocytes and in 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 PTEN (10). miR-302-367 was recently shown 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 recently showed 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 were found to modulate the WNT signal transduction cascade in skeletal muscle differentiation and regeneration (12). In addition to their expression in skeletal muscle, we 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 have 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*. These studies reveal a novel miRNA-transcriptional coactivator pathway in the control of cardiomyocyte proliferation.

EXPERIMENTAL PROCEDURES

Isolation of neonatal rat ventricular cardiomyocytes (NRVMs) – Ventricles from neonatal rats were isolated from approximately ten 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 pre-chilled 1X HBSS. Ventricles were transferred to 1X HBSS/0.025% Trypsin and incubated overnight at 4ºC. The following day, digestion was performed by adding 10 mg/mL collagenase II (Worthington Biochemical) to isolate individual cardiomyocytes. Cells were pre-plated on uncoated 100-mm plates to remove fibroblasts. Cells were plated in antibiotic-free growth media at a density of 4 x 10^6 cells/10 cm dish on gelatinized dishes. After 24 hours in culture, cells were washed with 1X PBS and switched to 0.5X Nutridoma-SP (Roche) in DMEM, a low serum media.

Plasmids and miRNA mimics – For 3'UTR reporter assays, the 3'UTR (676bp) 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 and inhibitors were purchased from Dharmacon.

miRNA transfection – miRNA mimics and inhibitors were transfected in NRVMs using a standard reverse transfection protocol at a final concentration of 25nM. Briefly, Lipofectamine RNAiMAX transfection reagent (Life Technologies) was diluted in OPTI-MEM (Life

Technologies) and added to the miRNAs. Cells were seeded 30 minutes later.

Short hairpin RNA (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 (MOI) 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 one hour at room temperature. Cells were incubated with primary antibodies diluted in antibody dilution buffer (1X PBS/1% BSA/0.3% Triton X-100) overnight at 4°C. For immunofluorescence, primary antibodies included: anti-α-actinin (1:500; Sigma), anti-Ki67 (1:200; Abcam), and Click-IT EdU 555 imaging kit (Life Technologies). The following day, cells were washed in 1X PBS and incubated with fluorochrome-conjugated secondary antibodies diluted in antibody dilution buffer. Secondary antibodies included AlexaFluor 488 donkey anti-mouse H+L (1:200; Invitrogen) and AlexaFluor 555 donkey anti-rabbit H+L (1:500; Invitrogen). Cells were washed in 1X PBS and mounted on slides with VECTASHIELD Mounting Medium with DAPI (Vector Labs). Slides were sealed with nail polish and stored at 4°C protected from light. Immunofluorescence images were taken with an Olympus DSU Spinning Disc confocal microscope.

EdU assays – For EdU assays, cells were plated as described. 24 hours after miRNA transfection, media was replaced with fresh media. 28 hours later, the culture media was replaced with media containing 5uM 5-ethynyl-2'deoxyuridine (EdU, Life Technologies) for 20 hours. 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 hours after plating and processed for immunofluorescence or RNA was harvested for analysis.

Luciferase assays – Cells were harvested for luciferase activity 48 hours after transfection. Cells were lysed in 1X passive lysis buffer (Promega). To measure Firefly Luciferase activity, 5uL cell lysate was mixed with 30uL 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 ($n \geq 3$).

qRT-PCR – RNA from cardiac muscle or NRVM experiments ($n \geq 3$) was used to synthesize cDNA using reverse transcriptase (M-MLV) with random hexamers according to the manufacturer's instructions (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 the 7900HT Sequence Detection System (Applied Biosystems). The primers used were 5S rRNA stem loop forward 5'-GTTGGCTCTGGTGCA-GGGTCCGAGGTATTCGCACCAGAGCCAA CAAAGCC; miR-410 stem loop 5'-GTTGGCT-CTGGTGCAGGGTCCGAGGTATTCGCACC-AGAGCCAACACAGGC; miR-495 stem loop 5'-GTTGGCTCTGGTGCAGGGTCCGAGGT-ATTCGCACCAGAGCCAACAAGAAG; 5S rRNA forward 5'- GAATACCGGGTGCTGTA-GGC; miR-410 forward 5'-CCGCCAATATAA-CACAGATGGCC; miR-495 forward 5'-GCC-AAACAAACATGGTGCACTT; *Gapdh* forward 5'-TGGCAAAGTGGAGATTGTTGC-C, and reverse 5'-AAGATGGTGATGGGCTT-CCCG; *Sfrp2* forward 5'-CCCCTGTCTGTCT-CGACGA, and reverse 5'-CTTCACACACCT-TGGGAGCTT; *Axin2* forward 5'-TGACTCTC-CTTCCAGATCCCA, and reverse 5'-TGCCCA-CACTAGGCTGACA; *Cited2* forward 5'-TGGGCGAGCACATACAC-TAC, and reverse 5'-GGGTGATGGTTGAAATACTGGT; *Nr3c1* forward 5'-TCTCAGGCAGATTCCAAGCA, and reverse 5'-TGGACAGTGAAACGGCTTT-G; *Errfi1* forward 5'-GCACAATGTCAACAG-CAGGA, and reverse 5'-TCCAGAGATGGGT-CCTCAGA; *Ppp1cb* forward 5'-GAGTGTGCT-

AGCATCAACCG, and reverse 5'-GTCAAAC-TCGCCGCAGTAAT; *Smad7* forward 5'-AGC-ATCTTCTGTCCCTGCTT, and reverse 5'-CT-CCTCGAATTCTGTGCACG; *Rere* forward 5'-TCATGTACTTGAGGGCAGCA, and reverse 5'-CACTTCTCGATCAGCTTGG; *Stat3* forward 5'-TCAGTGAGAGCAGCAAGGAA, and reverse 5'-TTTCCGAATGCCTCCTCCTT; *Gad1* forward 5'-ATGTGTGCAGGCTACCT-CTT, and reverse 5'-TCGGAGGCTTTGTGGT-ATGT; *p57* forward 5'-GACTGAGAGCAAGCGAACAG, and reverse 5'-CAGCGAGAAAGAAGGGAA-CG; and *Vegfa* forward 5'-TTCCTGTAGACA-CACCCACC, and reverse 5'-TCCTCCCAAC-TCAAGTCCAC.

Western blot analysis – Western blots were performed as previously described (15). Antibodies included: anti-GAPDH (1:1000; Santa Cruz) and anti-PCNA (1:2000; Cell Signaling). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000; Sigma) and reacted with Western Lightning Chemiluminescent Reagent (Perkin Elmer).

microRNA target analysis – Potential microRNA targets were analyzed using miRANDA, TargetScan, and miRDB.

Statistical analysis - All numerical quantification is representative of the mean±S.E.M. of at least three independently performed experiments. Statistically significant differences between two populations of data were determined using Student's *t*-test. *P*-values of ≤ 0.05 were considered to be statistically significant.

RESULTS

miR-410 and miR-495 are expressed in the heart and downregulated in MEF2A-deficient cardiomyocytes. We previously reported that expression of the MEF2-regulated *Gtl2-Dio3* miRNAs is enriched in 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 we had shown modulate the activity of the WNT signaling pathway (12). Initially, we examined cardiac expression of two of these *Gtl2-Dio3* miRNAs, miR-410 and miR-495, in perinatal and adult hearts. Our prior studies revealed low but detectable expression levels of several *Gtl2-Dio3* miRNAs in the adult mouse heart. As shown in Fig. 1A, 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 post-natal heart (16).

Given the above result, we then examined miR-410 and miR-495 expression in perinatal MEF2A knockout hearts. Previously, we reported 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 significantly downregulated in perinatal MEF2A knockout hearts (Fig. 1B). To determine if miR-410 and miR-495 are specifically downregulated in cardiac muscle and are dependent on MEF2A we examined their expression in neonatal rat ventricular myocytes (NRVMs) in which we have 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. 1C).

To determine if transcription of this locus is dependent on MEF2, we analyzed the activity of the *Gtl2* promoter in NRVMs. Previously, we demonstrated that the proximal promoter region of the *Gtl2-Dio3* locus is directly regulated by MEF2 in skeletal muscle and required for 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. 1D). 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. 1D). Moreover, activity of the *Gtl2* promoter was significantly reduced in NRVMs depleted of MEF2A (Fig. 1E). These results indicate the *Gtl2-Dio3* noncoding RNA locus is dependent on MEF2 activity, particularly MEF2A, in perinatal cardiac muscle.

Based on the established role of MEF2 in regulating the muscle cytoarchitecure we were interested in determining whether expression of sarcomere genes is dependent on these *Gtl2-Dio3* miRNAs (18). As expected, acute knockdown of MEF2A in NRVMs resulted in significant downregulation of sarcomere genes (Fig. 1F). Using hairpin inhibitors (antimiRs) we knocked down miR-410 and miR-495 in NRVMs. Similar to the MEF2A depletion, knockdown of miR-495, but not miR-410 (data not shown), caused a significant reduction in sarcomere gene expression (Fig. 1G). Although sarcomere genes were downregulated, 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. 1H, overexpression of these miRNAs resulted in a modest but significant upregulation of sarcomere gene expression compared to MEF2A-depleted NRVMs alone. Furthermore, upregulation of BIM expression, a pro-apoptotic marker gene, was significantly reduced compared to 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.

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 and abundance of α-actinin positive cardiomyocytes compared to the control (miR-NC) mimic (Fig. 2A). The apparent increase in cardiomyocytes suggested an effect on proliferation. To determine if 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. 2A).

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. 2B). Quantification revealed an increase in EdU^+ NRVMs upon addition of miR-410 or miR-495 (Fig. 2B). 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 2.0-fold or greater increase in PCNA upon overexpression of miR-410 or miR-495 (Fig. 2C). 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 if WNT signaling was also affected in MEF2A-deficient cardiomyocytes, we examined expression of *Sfrp2* and *Axin2*, a WNT responsive target gene. We found no significant dysregulation of these WNT signaling components in MEF2A-depleted NRVMs (Fig. 3A). Moreover, we found no significant difference in TOPflash activity, a WNT-sensitive luciferase reporter, in MEF2A-depleted NRVMs (Fig. 3B). 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. 3C), but did not affect the expression of *Axin2* (Fig. 3D). These results indicate that while *Sfrp2* expression is downregulated by these *Gtl2-Dio3* miRNAs in cardiomyocytes, unlike skeletal muscle, 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.

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 1,388 targets for miR-410 and miR-495, respectively (Fig. 4A). 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. 4A). Because this was still a relatively large number of potential targets for each miRNA, we narrowed down this list further 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 ten genes that fulfilled these criteria (Fig. 4B) (19-31). To validate these predictions we examined expression of eight of these candidate target genes in NRVMs overexpressing miR-410 or miR-495. As shown in Fig. 4C, the majority of the eight predicted common targets were significantly downregulated 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. 5A). 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. Co-transfection 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. 5B). 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 MUT or miR-495 MUT reporters, respectively (Fig. 5B). 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, was shown to be positively regulated by Cited2 in hematopoeitic stem cells (33). Based on the reduction in *Cited2* expression, we would expect downregulation 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 downregulation of *Cdkn1c/p57/Kip2* expression (Fig. 5C). We also examined expression of vascular endothelial growth factor A (*Vegfa*), as previous studies have shown that the *Vegf* promoter is repressed by Cited2 (34). In addition, delivery of VEGF to the injured heart has been shown to induce cardiomyocyte proliferation (35,36). As shown in Fig. 5D, 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. 6A). 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. 6B).

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 co-silenced miR-410 and miR-495 along with Cited2. As shown in Figure 6C, 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 upregulated, whereas *Vegfa* was significantly downregulated in MEF2A-depleted NRVMs (Fig. 7A). In a similar fashion, *Cited2* and *p57* were significantly upregulated in perinatal MEF2A knockout hearts (Fig. 7B). The above gene expression pattern is opposite of that observed in miR-410 and miR-495 induced cardiomyocyte proliferation but entirely consistent with the downregulation 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. Previously, we reported 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 downregulation 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 post-mitotic, 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 molecularly characterized 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 was shown to be upregulated 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 since 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 arch malformations (19,20). 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 co-activator 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). Whereas 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 above findings, we show that depletion of Cited2 resulted 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 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 upregulated 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 the 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. While 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 analysis of the *Gtl2-Dio3* miRNAs in models of cardiac disease is likely to provide valuable information on 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* miRs may be a potential therapeutic target to stimulate cardiomyocyte proliferation and reduce cardiac damage post-injury in the postnatal heart.

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Conflict of Interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
ALC and FJN designed the study and wrote the paper. ALC performed and analyzed the experiments in Figure 1 through 7 and Supplemental Tables S1 and S2. All authors reviewed the results and approved the final version of the manuscript.

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Abbreviations

The abbreviations used are miRNA, microRNA; miR, microRNA; MEF2, Myocyte enhancer factor 2; *Gtl2*, gene trap locus 2; NRVMs, neonatal rat ventricular myocytes; shRNA, short hairpin RNA; Sfrp2,

secreted frizzled-related protein 2; Cited2, CREB binding protein/p300-interacting transactivator with glutamic acid/aspartic acid-rich carboxy-terminal domain 2; NR3C1, nuclear receptor subfamily 3, group C, member 1; ERRFI1, ERBB receptor feedback inhibitor 1; PPP1CB, protein phosphatase 1, catalytic subunit, beta isozyme; SMAD7, SMAD family member 7; RERE, arginine-glutamic acid peptide repeats; STAT3, signal transducer and activator of transcription 3; GAD1, glutamate decarboxylase 1; ADM, adrenomedullin; DPYSL2, dihydropyrimidinase-like 2; VEGFA, vascular endothelial growth factor A; TFAP2A, transcription factor AP2; HIF1-α, hypoxia-inducible factor 1-α.

FIGURE LEGENDS

FIGURE 1. ***miR-410 and miR-495 are expressed in the heart and downregulated 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 wild-type (WT) and MEF2A knockout (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 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 promoter activity is dependent on MEF2A. *F,* Quantitative RT-PCR analysis of cardiac sarcomere genes in control (sh*lacZ*) and MEF2A knockdown (sh*MEF2A*) NRVMs. *G,* Quantitative RT-PCR analysis of cardiac sarcomere genes in control (miR-NC) and miR-495 overexpression NRVMs. *H*, Overexpression of miR-410 and miR-495 in MEF2A-depleted NRVMs upregulates sarcomere gene expression and reduces BIM expression. *Error* bars represent S.E.M. *, $p < 0.05$; **, $p < 0.01$.

FIGURE 2. ***Overexpression of miR-410 and miR-495 promotes cardiomyocyte proliferation.*** *A,* Representative images of Ki67 immunostaining. miR-NC (top), miR-410 (middle), miR-495 (bottom). α-actinin staining in green, Ki67 staining in red, Dapi staining in blue. Relative fold change of $Ki67^+$ NRVMs in miR-410 and miR-495 overexpression compared to miR-NC. *B,* Representative images of EdU assay. miR-NC (top), miR-410 (middle), miR-495 (bottom). α-actinin staining in green, EdU staining in red, Dapi staining in blue. Relative fold change of EdU^+ NRVMs in miR-410 and miR-495 overexpression compared to miR-NC. *C,* Western blot analysis of proliferating cell nuclear antigen (PCNA) in NRVMs overexpressed with miR-NC, miR-410, and miR-495. Densitometry of PCNA western blot. Error bars represent S.E.M. *, $p < 0.05$; **, $p < 0.01$.

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 S.E.M. n.s., not significant; **, $p<0.01$; ***, $p<0.001$.

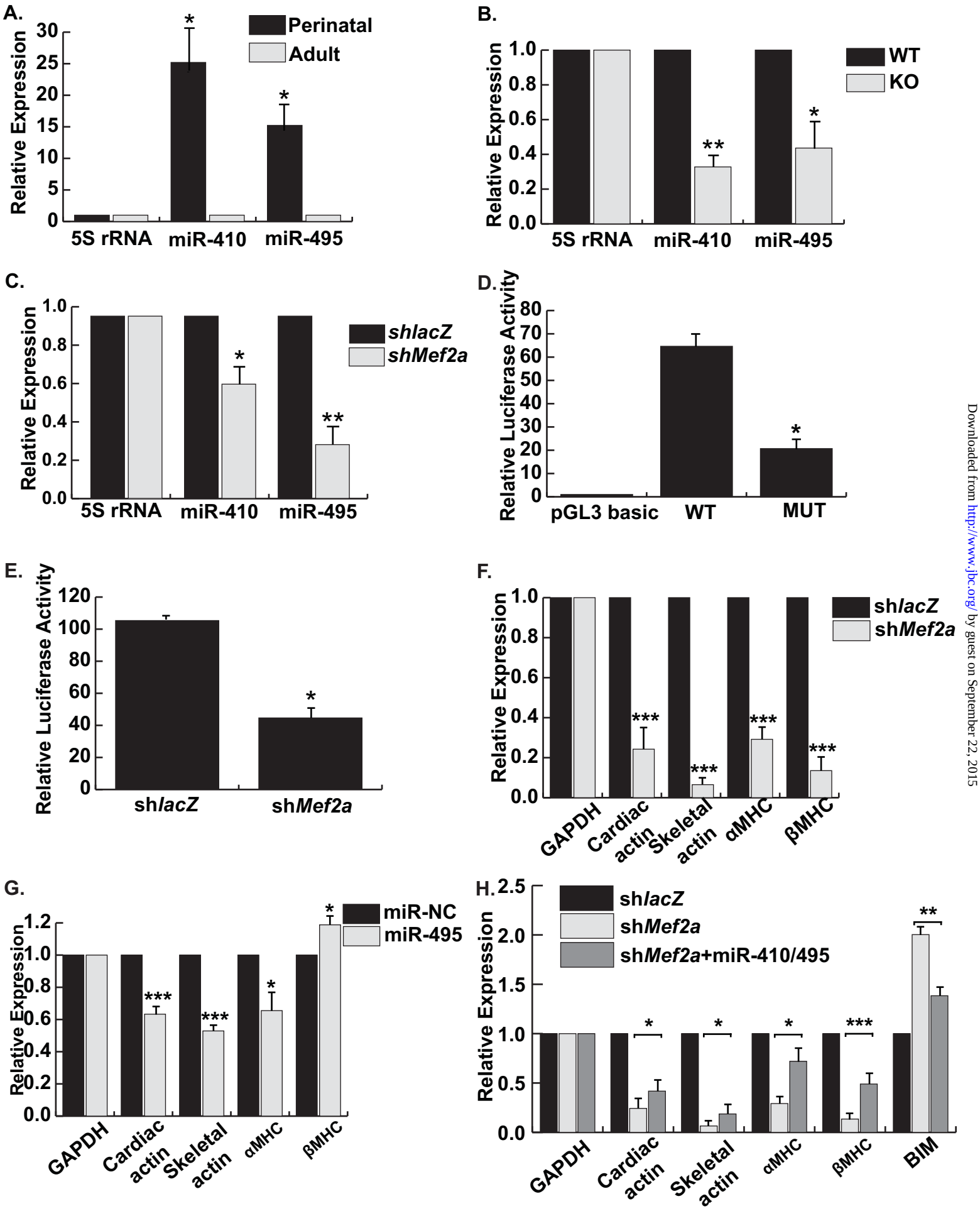
FIGURE 4. ***Identification and validation of predicted target genes of miR-410 and miR-495.*** *A,* Venn diagrams of potential targets for miR-410 and miR-495 according to miRANDA, TargetScan, and miRDB. Overlap of the three algorithms reveals 64 and 148 predicted targets for miR-410 and miR-495, resepectively. *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 8 of the top 10 predicted targets for miR-410 and miR-495. Error bars represent S.E.M. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

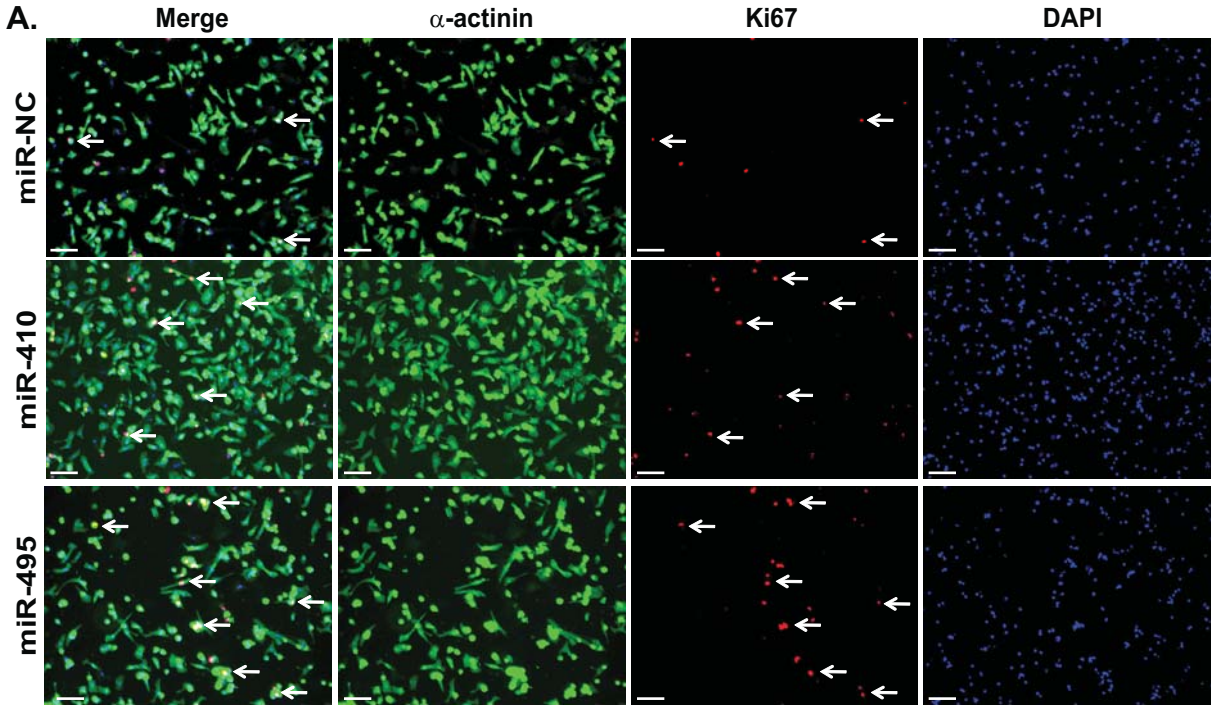
FIGURE 5. ***miR-410 and miR-495 directly target the 3'UTR of Cited2.*** *A,* Sequence alignments of miR-410 and miR-495 seed sequences and predicted target sites in the 3'UTR of human and mouse Cited2. *B,* Luciferase analysis of wild type (WT) and mutant (MUT) pMIR-REPORT-3'UTR-CITED2 co-

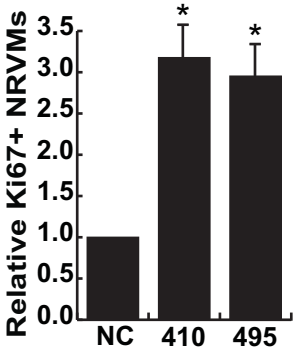
transfected with miR-410 and miR-495 mimics (150nM, Dharmacon) in NRVMs compared with a non-specific control (miR-NC). *C,* Quantitative RT-PCR of *p57* expression levels when overexpressing miR-410 and miR-495. *D,* Quantitative RT-PCR of *Vegfa* expression levels when overexpressing miR-410 and miR-495. Error bars represent S.E.M. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***.

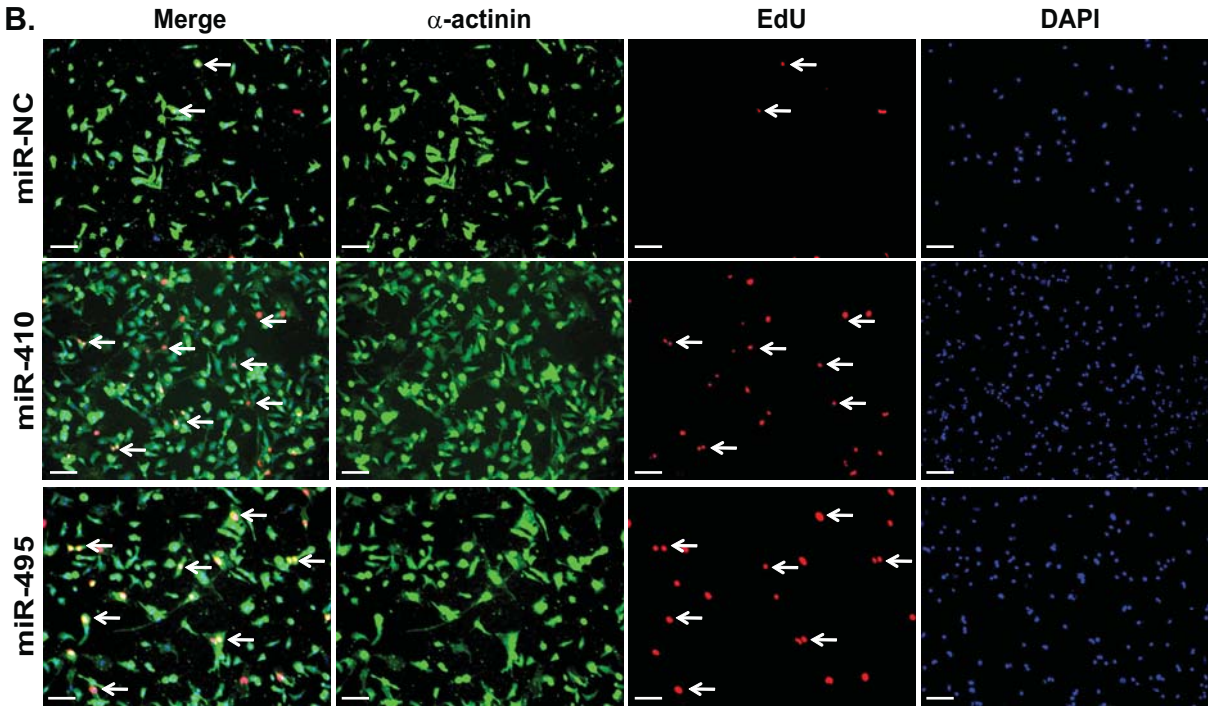
FIGURE 6. ***Knockdown of Cited2 and co-silencing of Cited2, miR-410, and miR-495 in neonatal cardiomyocytes.*** *A*, Knockdown of Cited2 results in increased cardiomyocyte proliferation. Representative images of EdU incorporation assay when knocking down *Cited2*. siControl (top), siCited2 (bottom). α-actinin staining in green, EdU staining in red, Dapi staining in blue. Relative fold change of EdU^+ NRVMs in siCited2 compared to siControl (siCtl). *B,* Quantitative RT-PCR of *Cited2, p57,* and *Vegfa* expression levels when knocking down *Cited2* using siCited2 in NRVMs. *C,* Co-silencing of Cited2, miR-410, and miR-495 prevent cardiomyocyte proliferation. Representative images of EdU incorporation assay. siControl (top), siCited2+antimiR-NC, siCited2+antimiR-410, siCited2+antimiR-495 (bottom). α-actinin staining in green, EdU staining in red, Dapi staining in blue. Relative fold change of EdU+ NRVMs shows combinatorial knockdown of Cited2 and miR-410 and miR-495 results in normal cardiomyocyte proliferation. Error bars represent S.E.M. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

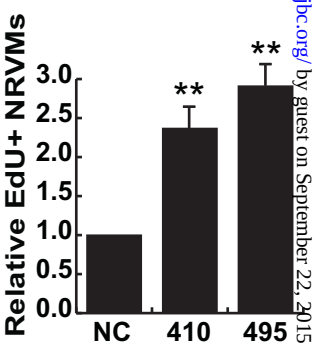
FIGURE 7. ***Dysregulated Cited2, p57, and Vegfa expression in MEF2A-deficient cardiomyocytes.*** *A,* Quantitative RT-PCR of *Cited2, p57,* and *Vegfa* expression levels in MEF2A-deficient NRVMs. *B,* Quantitative RT-PCR of *Cited2, p57,* and *Vegfa* expression levels in perinatal MEF2A knockout cardiac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

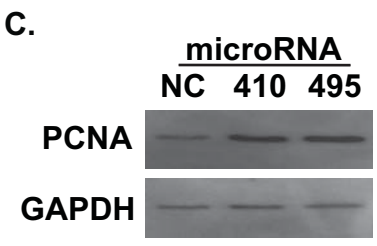


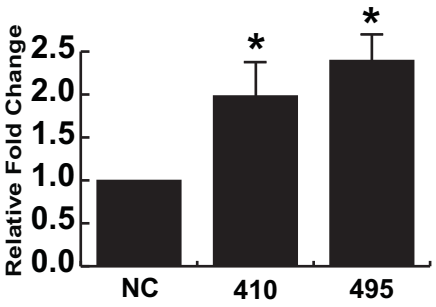


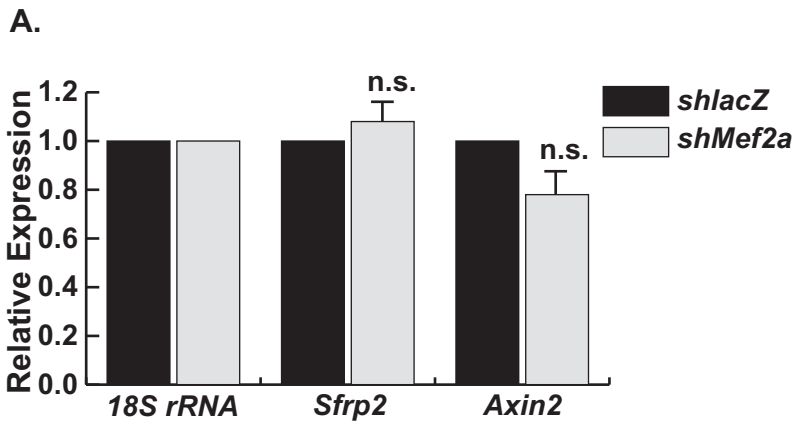


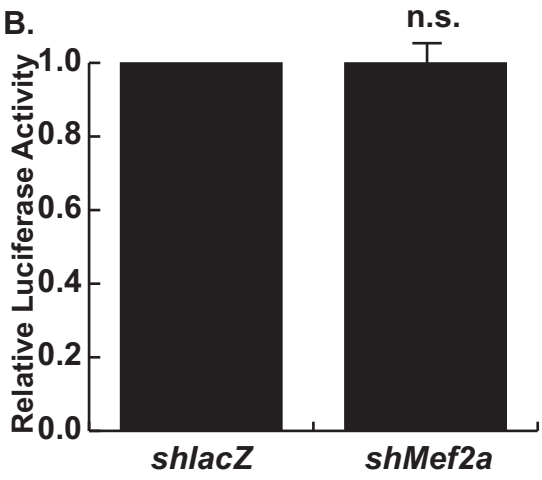


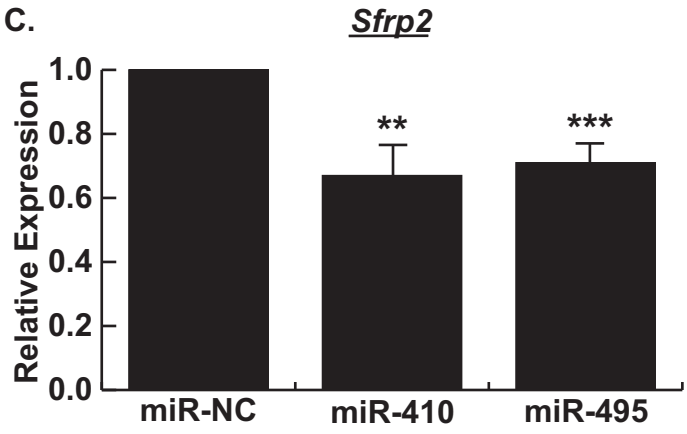


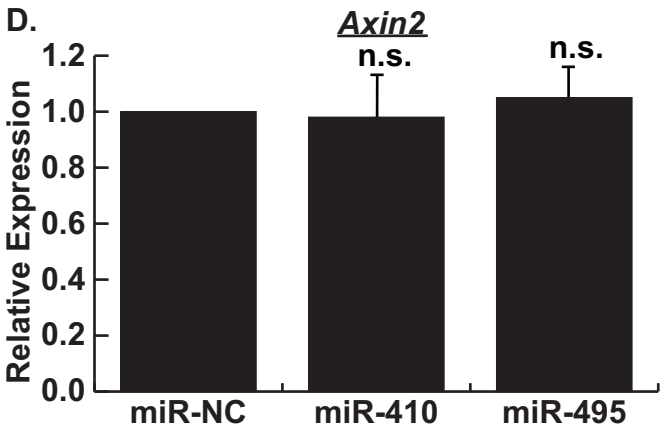




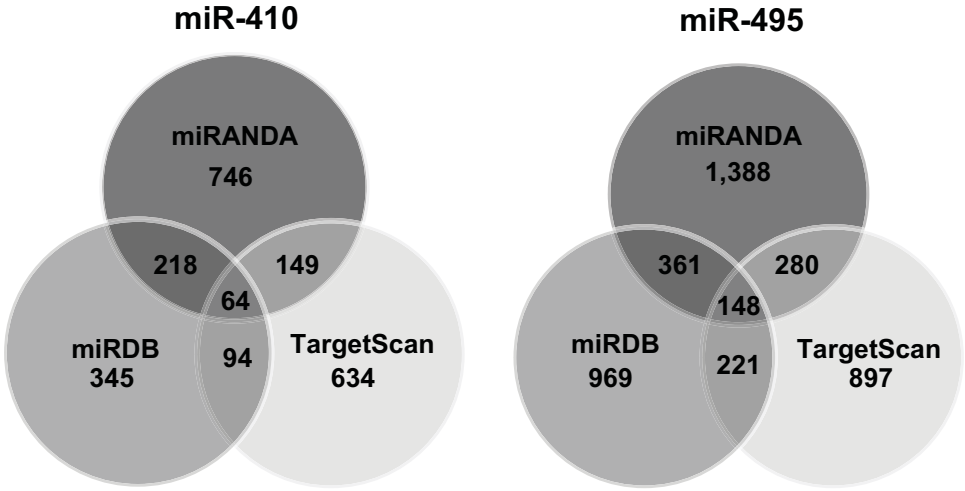








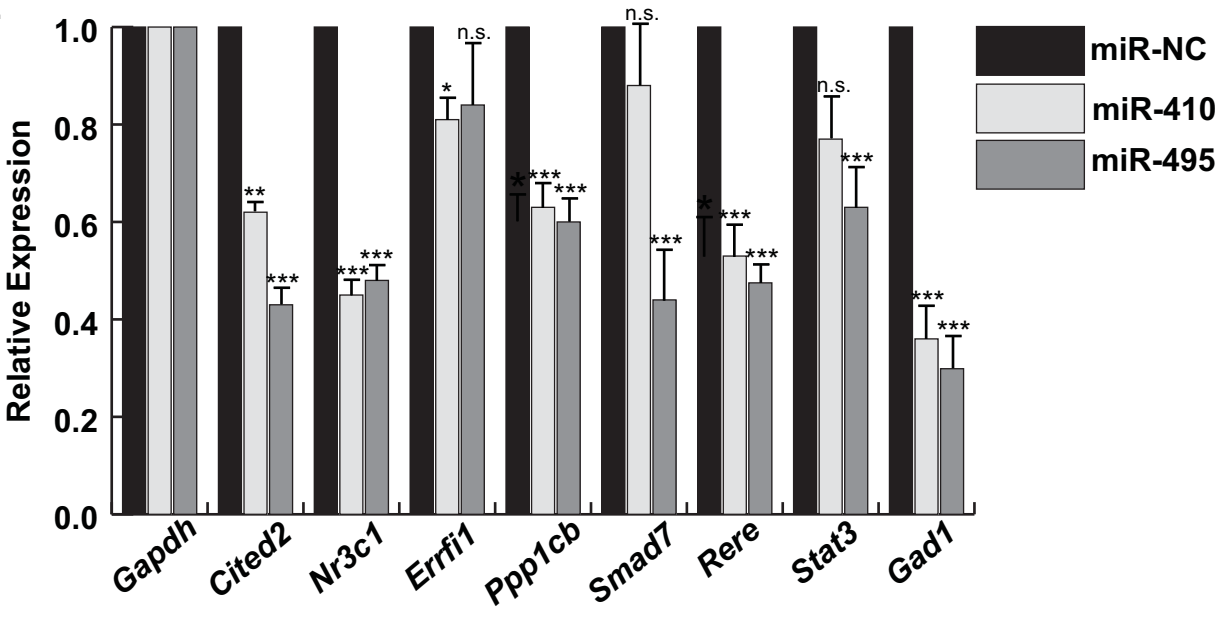
A.



B.

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

C.



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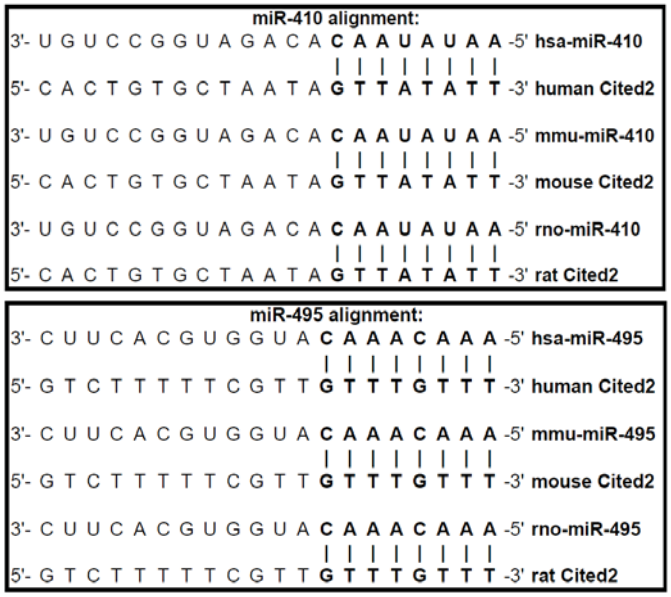

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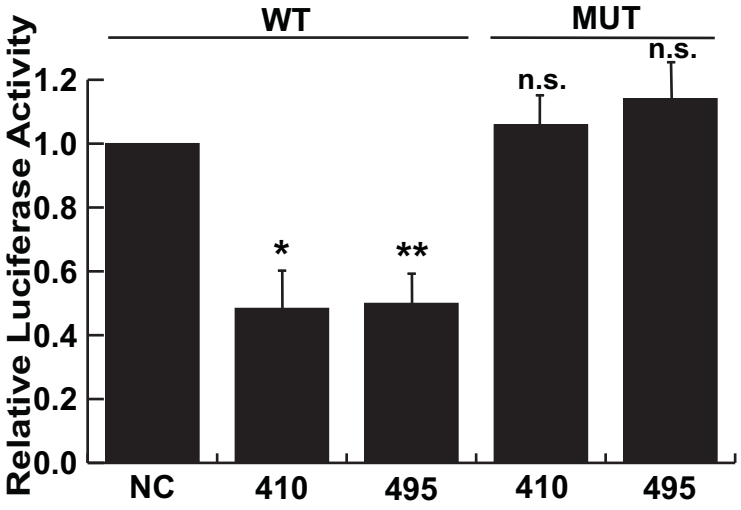

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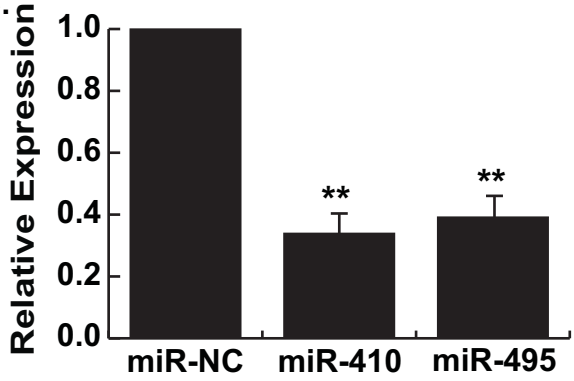

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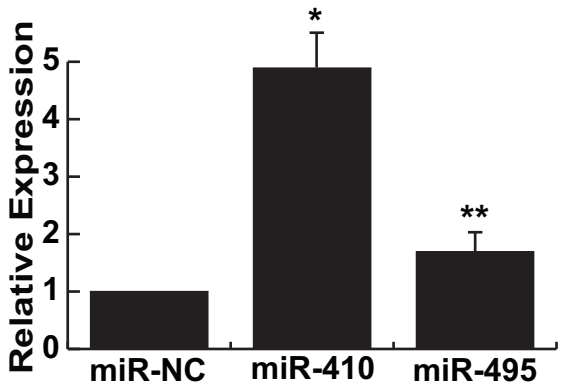

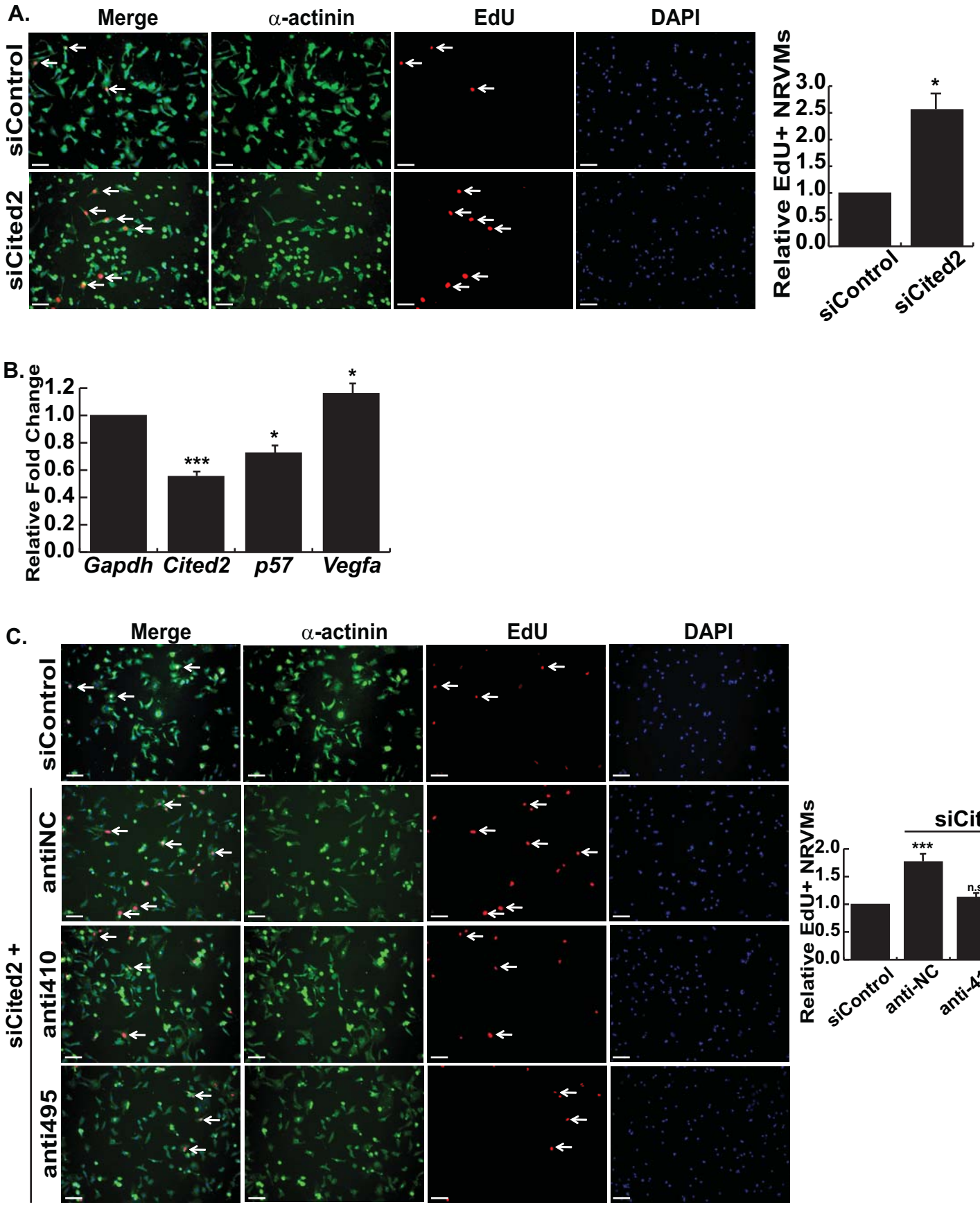

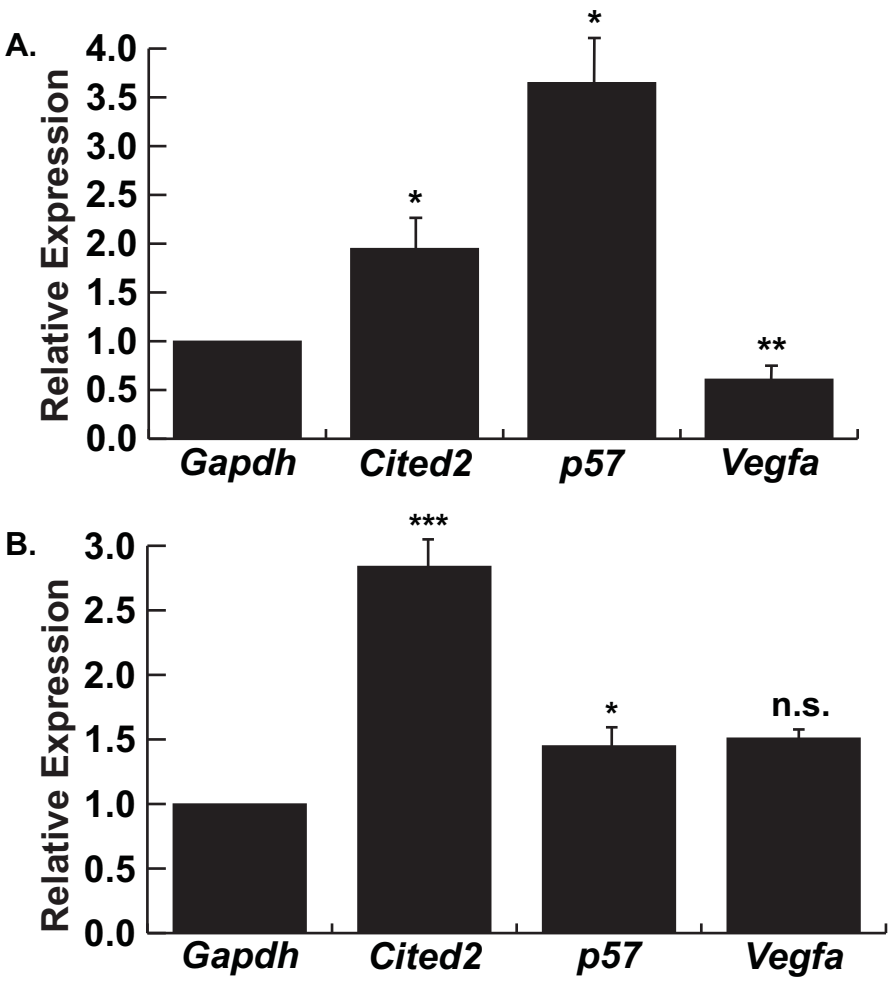

Gene Regulation:

MicroRNAs in the MEF2-regulated Gtl2-Dio3 Noncoding RNA Locus Promote Cardiomyocyte Proliferation by Targeting the Transcriptional Co-activator Cited2

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