Mechanisms underlying aberrant expression of miR-29c in uterine leiomyoma

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Objective: To determine the expression of miR-29c and its target genes in leiomyoma and the role of NF-κB, specific protein 1 (SP1), and DNA methylation in its regulation.
Design: Experimental study.
Setting: Academic research laboratory.
Patient(s): Women undergoing hysterectomy for leiomyoma.
Intervention(s): Over- and underexpression of miR-29c; blockade of transcription factors.
Main Outcome Measure(s): MiR-29c and its target gene levels in leiomyoma and the effects of blockade of transcription factors on miR-29c expression.
Result(s): Leiomyoma as compared with myometrium expressed significantly lower levels of miR-29c, with an inverse relationship with expression of its targets, COL3A1 and DNMT3A. Gain of function of miR-29c inhibited the expression of COL3A1 and DNMT3A at protein and mRNA levels, secreted COL3A1, and rate of cell proliferation. Loss of function of miR-29c had the opposite effect. E_2, P, and their combination inhibited miR-29c in leiomyoma smooth muscle cells (LSMC). Phosphorylated NF-κB (p65) and SP1 protein expression were significantly increased in leiomyoma. SiRNA knockdown of SP1 and DNMT3A or their specific inhibitors significantly increased the expression of miR-29c, accompanied by the inhibition of cellular and secreted COL3A1 in siRNA-treated cells. Knockdown of p65 also induced miR-29c expression but had no effect on COL3A1 expression.
Conclusion(s): MiR-29c expression is suppressed in leiomyoma, resulting in an increase in expression of its targets COL3A1 and DNMT3A. The suppression of miR-29c in LSMC is primarily mediated by SP1, NF-κB signaling, and epigenetic modification. Collectively, these results indicate a significant role for miR-29c in leiomyoma pathogenesis. (Fertil Steril® 2016;105:236–45. ©2016 by American Society for Reproductive Medicine.)
Key Words: Leiomyoma, miR-29c, COL3A1, DNMT3A, SP1

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Uterine leiomyoma (fibroids) are benign tumors with fibrotic characteristics that develop during the reproductive years. Although their etiology is unknown, leiomyoma are dependent on ovarian steroids for their growth (1). As a fibrotic disorder, leiomyoma as compared with myometrium have excess accumulation of extracellular matrixes (ECM), a hallmark of various fibrotic disorders throughout the body (2). The promoter regions of many fibrotic-related genes are enriched with a family of transcription factor binding sites, including ubiquitously expressed specific protein 1 (SP1) and NF-κB (3–7). SP1 and NF-κB also regulate the expression of genes involved in cell proliferation, apoptosis. and ECM, as well as estrogen (ER) and progesterone receptors (PR) (8–10). The ER and PR promoters contain multiple SP1 binding sites, and SP1 is required for their promoter activities (11, 12). An active SP1 binding site that mediates PRs promoter activity has also been reported (13). In addition, PR-mediated actions in some tissues, including the myometrium, have been reported to involve NF-κB activity (14).

Epigenetic mechanisms involving molecular modification of DNA (e.g., methylation) or histones (e.g., acetylation, phosphorylation, and methylation) also play an important role in regulation of genes and miRNAs (15, 16). DNA methylation catalyzed by

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specific DNA methyltransferases (DNMTs) is the most common and well-characterized epigenetic process (17). DNA hypermethylation is frequently associated with tissue fibrosis and tumorigenesis (18). Recent studies in leiomyoma have demonstrated altered expression of DNMTs and DNA methylation patterns as compared with myometrium (19, 20). Another epigenetic mechanism is post-translational modification, which in the case of SP1 and NF-κB alters their transcriptional activities, thereby affecting their DNA binding ability and transactivation of their target genes (21, 22).

MicroRNAs (miRNAs), a member of short noncoding RNA, have also emerged as important post-transcriptional negative regulators of more than half of protein-coding genes (23, 24). miRNAs play a key role in many aspects of normal cellular activities and functions, and their altered expression or function has been associated with a wide range of disorders, including tissue fibrosis and tumorigenesis (25, 26). Recent studies have provided support for a role of miRNAs in leiomyoma pathogenesis. Altered expression of several miRNAs including let7, miR-25/93/106 cluster, miR-21, miR-200c, and miR-29b as well as validation of some of their specific target genes have been demonstrated in leiomyoma as compared with matched myometrium, and their expression is correlated with the stage of the menstrual cycle, tumor size, and ethnicity (27–31). The miR-29 family, which consists of miR-29a, miR-29b, and miR-29c, shares a common seed sequence with largely overlapping sets of predicted target genes; however, their differential expression and regulation suggest unique functional activities (32). More specifically, miR-29c expression has been found to be downregulated in various fibrotic disorders, and many ECM genes, including collagen subtypes and elastin, are targets of miR-29c regulatory function (33, 34). A recent report demonstrated suppression of miR-29b in leiomyoma as compared with matched myometrium, and restoring miR-29b expression in isolated leiomyoma smooth muscle cells (LSMC) implanted in subrenal xenograft in a mouse model resulted in inhibition of ECM accumulation and rate of cell proliferation (28). In addition, several miRNAs, including miR-29, have been demonstrated to regulate the expression of SP1, NF-κB, and DNMTs in different cell types (35–37). Since miRNA expression and regulatory functions have been reported to occur in a cell- and tissue-dependent manner (38, 39), the objective of our study was [1] to explore the expression of miR-29c and its predicted target genes, specifically COL3A1 and DNMT3A, in leiomyoma; [2] determine the regulatory function of miR-29c on COL3A1 and DNMT3A expression; and [3] assess the influence of ovarian steroids as well as NF-κB, SP1, and DNMT3A on miR-29c expression in isolated LSMC.

MATERIALS AND METHODS

Tissue Collection and LSMC Isolation

Portions of uterine leiomyoma and matched myometrium were collected from patients (n = 17) undergoing hysterectomy at Harbor-UCLA Medical Center with prior approval from the Institutional Review Board (no. 036247). The patients' age ranged from 38 to 55 years (median, 45.5 ± 4.9). Patient ethnicity was representative of the patient population at our institution. Patients were not taking any hormone medications for at least 3 months before surgery. All leiomyoma used in this study ranged in size from 2 to 5 cm in diameter. Tissues were snap frozen and stored in liquid nitrogen for further analysis or used for isolation of LSMC as described elsewhere (30, 31). Briefly, LSMC were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum until reaching confluence with a change of media every 2–3 days. Cells at passages p1 to p3 were used for all experiments. Cell culture experiments were performed at least 3 times using LSMC obtained from different patients. All supplies for isolation and cell culture were purchased from Sigma-Aldrich, Invitrogen, and Fisher Scientific.

Gain or Loss of Function of miR-29c

LSMC were seeded at a cell density of 3.5×10^4/well in 6-well plates and at subconfluence transfected with 50 nM of pre-miR-29c (miR-29c), anti-miR-29c (a-miR-29c), pre-miR negative control (NC), anti-miR negative control (aNC), or pre-miR-1, which served as a secondary control (Applied Biosystems) for 48 to 96 hours using PureFection transfection reagent (System Biosciences) according to the manufacturer's protocol.

siRNA Transfection

LSMC were cultured as above and at subconfluence transfected with 50 nM of siRNA negative control (siNC), siRNA against SP1 (siSP1), p65 (siP65), and DNMT3A (siDNMT3A; Santa Cruz Biotechnology) for 72–96 hours using PureFection transfection reagent (System Biosciences) according to the manufacturer's protocol. Total RNA, protein, and culture-conditioned media were isolated and subjected to quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and immunoblot analysis, respectively.

Ovarian Steroids Treatment

LSMC were seeded in 6-well plates as above until reaching subconfluence. The cells were washed and incubated in phenol red-free media with charcoal-stripped fetal bovine serum for 24 hours and then treated with 17β-estradiol (E_2), P, or E_2 plus P_4 (Sigma-Aldrich) at 10^{-8} M concentration for 24 hours. The dose of E_2 or P selected is within physiological range and has been used in prior publications (40, 41). Total RNA was isolated and subjected to quantitative RT-PCR as described above.

RNA Isolation and qRT-PCR Analysis

Total RNA was extracted from paired tissues and cell cultures using Trizol (Invitrogen), and their quantity and quality were determined (ND-1000 Spectrophotometer, NanoDrop Technologies) as described elsewhere (42). Subsequently, 10 ng (for miRNA) or 2 μg was reverse transcribed using specific stem-loop primer for miR-29c or random primers for COL3A1 and DNMT3A according to the manufacturer's

guidelines (Applied Biosystems). Quantitative RT-PCR was carried out using TaqMan or SYBR gene expression master mix, TaqMan miRNA expression assays (Applied Biosystems). Reactions were incubated for 10 minutes at 95°C followed by 40 cycles for 15 seconds at 95°C and 1 minute at 60°C. The level of mRNA and miRNA expression was determined using the Invitrogen StepOne System with 18S and RNU6B used for normalization, respectively. All reactions were run in triplicate, and relative expression was analyzed with the comparative cycle threshold method ($2^{-\Delta\Delta}$CT) according to the manufacturer (Applied Biosystems). Values were expressed as fold changes compared with the control group. The primer sequences used were as follows: COL3A1 (sense, 5′- ATTATTTTGGCACAACAGGAAGCT -3′; antisense, 5′- TCCGCATAGGACTGACCAAGAT -3′), DNMT3A (sense, 5′-GGTTCGGAGACGGCAAATT -3′; antisense, 5′- GGAACGCACTGCAAAACGA-3′) and 18S (sense, 5′- CGAGCCGCCTGGATACC -3′; antisense, 5′- CAGTTCCGAAAACCAACAAAATAGA-3′).

Immunoblotting

Total protein isolated from leiomyoma and paired myometrium as well as LSMC transfected with pre-miR-29c, anti-miR-29c, pre-miR negative, anti-miR NC, and pre-miR-1, as well as siRNA NC, siRNA against SP1, p65, and DNMT3A was subjected to immunoblotting as described elsewhere (34). Specific antibodies generated against COL3A1 (Proteintech Group), DNMT3A (Santa Cruz Biotechnology), p65 (Cell Signaling Technology), p-p65 (Ser 536; Cell Signaling Technology), SP1 (Proteintech Group), and p-SP1 (Thr 453; Assay Biotech) were used to detect specific protein expression. The membranes were also stripped and probed with GAPDH antibody (Proteintech Group) serving as the loading control. Culture-conditioned medium from LSMC transfected with miR-29c or SP1 and DNMT3A siRNAs was also collected for COL3A1 detection. A Ponceau S-stained protein band on the nitrocellulose membrane was used as a loading control for media samples. The band densities were determined using image J program (http://imagej.nih.gov/ij/), normalized to GAPDH and expressed as a ratio relative to the control group designated as 1.

Luciferase Reporter Assays

LSMC were seeded in 6-well plates until reaching subconfluence and transiently cotransfected with 50 nM pre-miR-29c oligonucleotides (miR-29c), pre-miR-1 (miR-1), which does not target COL3A1 and DNMT3A as a control miRNA, pre-miR NC, and a luciferase reporter plasmid (1 μg/well) containing 3′ UTR sequences for COL3A1 or DNMT3A (GeneCopoeia) using PureFection transfection reagent (System Biosciences). Firefly and Renilla luciferase activities were measured after 48 hours of transfection using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity, and the level of induction was reported as the mean ± SEM of three experiments performed in duplicate and compared with cells transfected with preNC independently set as 1.

Cell Proliferation Assay

LSMC were seeded at 1,000 cells/well in 96-well plates and cultured for 48 hours. The cells were then transfected with 50 nM pre-miR-29c oligonucleotides (miR-29c) or pre-miR NC as described above. The rate of cell proliferation was determined using the MTT assay, and cells were photographed. Briefly, MTT (Sigma) was added to the culture medium at a final concentration of 1 mg/mL and incubated for 2 hours at 37°C. The medium was aspirated, the formazan product was solubilized with dimethyl sulfoxide, and the absorbance at 570 nm was determined and subtracted from the absorbance at 630 nm (background) for each well. The assay was performed in six replicates per condition and repeated 3 times.

Statistical Analysis

Throughout the text, results are reported as mean ± SEM and analyzed by PRISM software (Graph-Pad). Data set normality was determined by the Kolmogorov-Smirnoff test. Comparisons involving two groups were analyzed using unpaired Student's *t*-tests. For comparisons involving multiple groups, one way analysis of variance was used with Tukey's honest significant difference (HSD) for post hoc analysis. $P<.05$ was considered statistically significant.

RESULTS

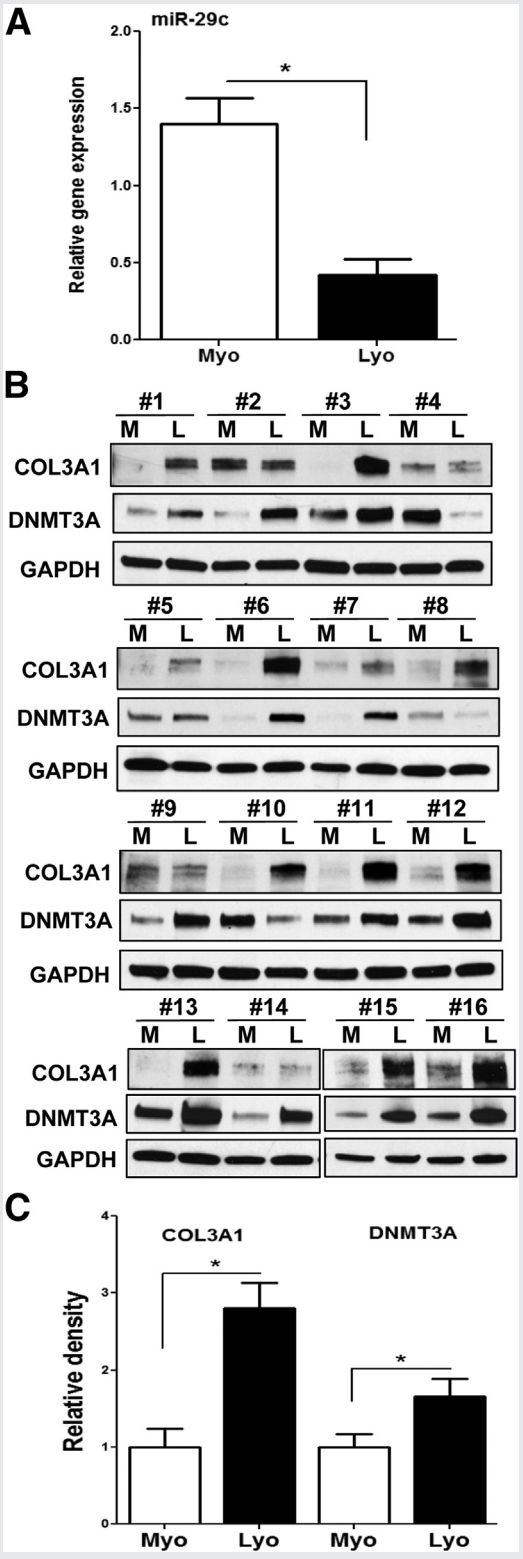
MiR-29c Expression in Leiomyoma Is Suppressed and Inversely Correlated with COL3A1 and DNMT3A Expression

To decipher the expression, regulation of specific target genes, and molecular mechanisms of miR-29c expression in leiomyoma, we first determined its expression in leiomyoma and paired myometrium. Using qRT-PCR, the results indicated that leiomyoma expressed significantly lower levels of miR-29c (100%, 17/17 pairs) as compared with myometrium ($P<.05$; Fig. 1A), and this was independent of patient ethnicity. Since miR-29c is predicted to regulate the expression of several ECM and DNMTs, we selected COL3A1 and DNMT3A as miR-29c specific target genes and through immunoblot analysis found that the expression of COL3A1 (75%, 12/16 pairs) and DNMT3A (81.3%, 13/16 pairs) protein was significantly increased in leiomyoma as compared with in matched myometrium ($P<.05$; Fig. 1B and C).

COL3A1 and DNMT3A Are Direct Targets of miR-29c

Using luciferase reporter assay we showed that COL3A1 and DNMT3A are direct targets of miR-29c in isolated primary LSMC (Fig. 2A and B). Figure 2A shows the predicted 3′UTR COL3A1 and DNMT3A binding site for miR-29c. Figure 2B shows that following transfection of pre-miR-29c in LSMC the relative luciferase activity of COL3A1 and DNMT3A is significantly inhibited, indicating that these genes are targets of miR-29c in LSMC. We next overexpressed (gain of function) miR-29c in LSMC by pre-miR-29c transfection. This resulted in a significant ($P<.05$) suppression of COL3A1 and DNMT3A at protein (Fig. 2C and D) and mRNA levels (Fig. 2E), while

FIGURE 1



(**A**) The relative expression of miR-29c in paired (n = 17) myometrium (MYO) and leiomyoma (LYO) (**A**). *$P<.05$. (**B**) Western blot analysis of COL3A1 and DNMT3A in paired (n = 16) myometrium (M) and leiomyoma (L) with a bar graph (**C**) showing their relative band densities in myometrium (MYO) and leiomyoma (LYO). *$P<.05$.

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knockdown (loss of function) of miR-29c by anti-miR-29c transfection significantly ($P<.05$) increased the expression of COL3A1 and DNMT3A at protein (Fig. 2C and D) and mRNA levels (Fig. 2E). To confirm that the effects observed were due to miR-29c, we transfected LSMC with pre-miR-1, which does not target COL3A1 and DNMT3A, and measured its regulatory function on the expression of COL3A1 and DNMT3A. The results indicated that overexpression of miR-1 in LSMC did not interact with 3′UTR of COL3A1 and DNMT3A (Supplemental Fig. 1A) or altered their expression at the mRNA and protein levels (Supplemental Fig. 1B and C).

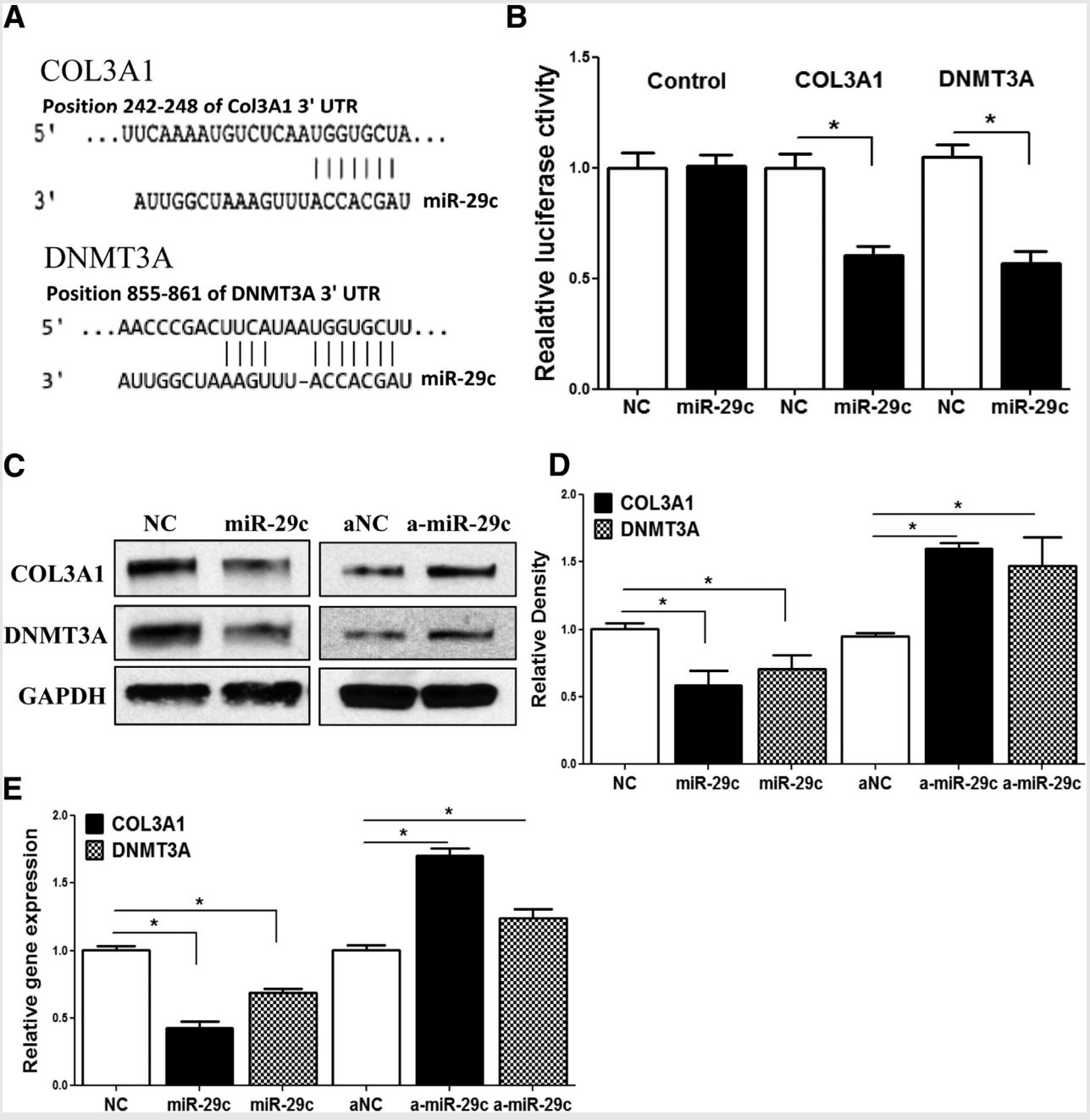
MiR-29c Is Hormonally Regulated and Inhibits LSMC Proliferation

We next analyzed the influence of ovarian steroids on the expression of miR-29c. As shown in Figure 3A, treatments with E_2, P, and E_2+P significantly decreased the expression of miR-29c ($P<.05$). Additionally, gain of function of miR-29c significantly inhibited the rate of LSMC cell proliferation ($P<.05$; Fig. 3B and Supplemental Fig. 1D).

MiR-29c Expression Is Epigenetically and Transcriptionally Regulated in Leiomyoma

We next examined whether suppression of miR-29c levels in leiomyoma is under specific regulatory mechanisms involving epigenetic modification or transactivation of transcriptional factors, namely, NF-κB and SP1. To accomplish this we treated LSMC with Zebularine, Bay 11-7082, or Mithramycin A, which are specific inhibitors of DNMTs, NF-κB signaling, and SP1, respectively. The dose of Zebularine (100 μM), Bay 11-7082 (5 μM), and Mithramycin A (1 μM) chosen for this study was based on a preliminary dose-response relationship in leiomyoma cells in our laboratory and adopted from other studies using these drugs at these concentrations in other cell types (43–45). As shown in Figure 3C, treatment with these pharmacologic inhibitors resulted in a significant ($P<.05$) induction of miR-29c expression in LSMC. Consistent with these results, we also demonstrated that knockdown of DNMT3A, NF-κB (p65), and SP1 using their respective siRNAs also induced miR-29c expression in LSMC (Fig. 3D). Since knockdown of DNMT3A, NF-κB (p65), and SP1 in LSMC resulted in miR-29c induction, we next examined their influence on COL3A1 expression, a direct target of miR-29c regulatory function. The results indicated that knockdown of DNMT3A and SP1, but not NF-κB (p65), resulted in a significant reduction in COL3A1 expression (Fig. 3E). Knockdown of NF-κB (p65) also resulted in a significant reduction of SP1 expression in LSMC (Fig. 3E). Gain of function of miR-29c and/or knockdown of SP1 and DNMT3A by siRNAs also inhibited COL3A1 secreted into culture-conditioned media of LSMC (Fig. 3F). Since phosphorylation of p65 and SP1 is critical for their activation, we next determined whether leiomyoma expressed higher levels of phosphorylated forms of these transcription factors. As shown in Figure 4A and B, although total SP1 and p65 were not significantly different in leiomyoma as

FIGURE 2



(**A**) Complementary sequences between miR-29c and 3′UTR of COL3A1 and DNMT3A. (**B**) Relative luciferase activity in isolated LSMC cotransfected with Renilla and Firefly luciferase reporter carrying a 3′UTR fragment of COL3A1 or DNMT3A, pre-miR-29c or control oligonucleotides (NC) for 48 hours. The relative luciferase activity is presented as the ratio of Firefly:Renilla as compared with NC, which was independently set as 1. (**C**) Western blot analysis of COL3A1 and DNMT3A after transfection of LSMC with pre-miR-29c or anti-miR-29c (a-miR-29c) oligonucleotides for 96 hours with their relative band densities shown as a bar graph (**D**). (**E**) QRT-PCR analysis of COL3A1 and DNMT3A mRNA expression in LSMC after transfection with pre-miR-29c or anti-miR-29c oligonucleotides for 72 hours. The results are presented as mean ± SEM of at least three independent experiments with *P* values (*$P<.05$) indicated by corresponding lines.

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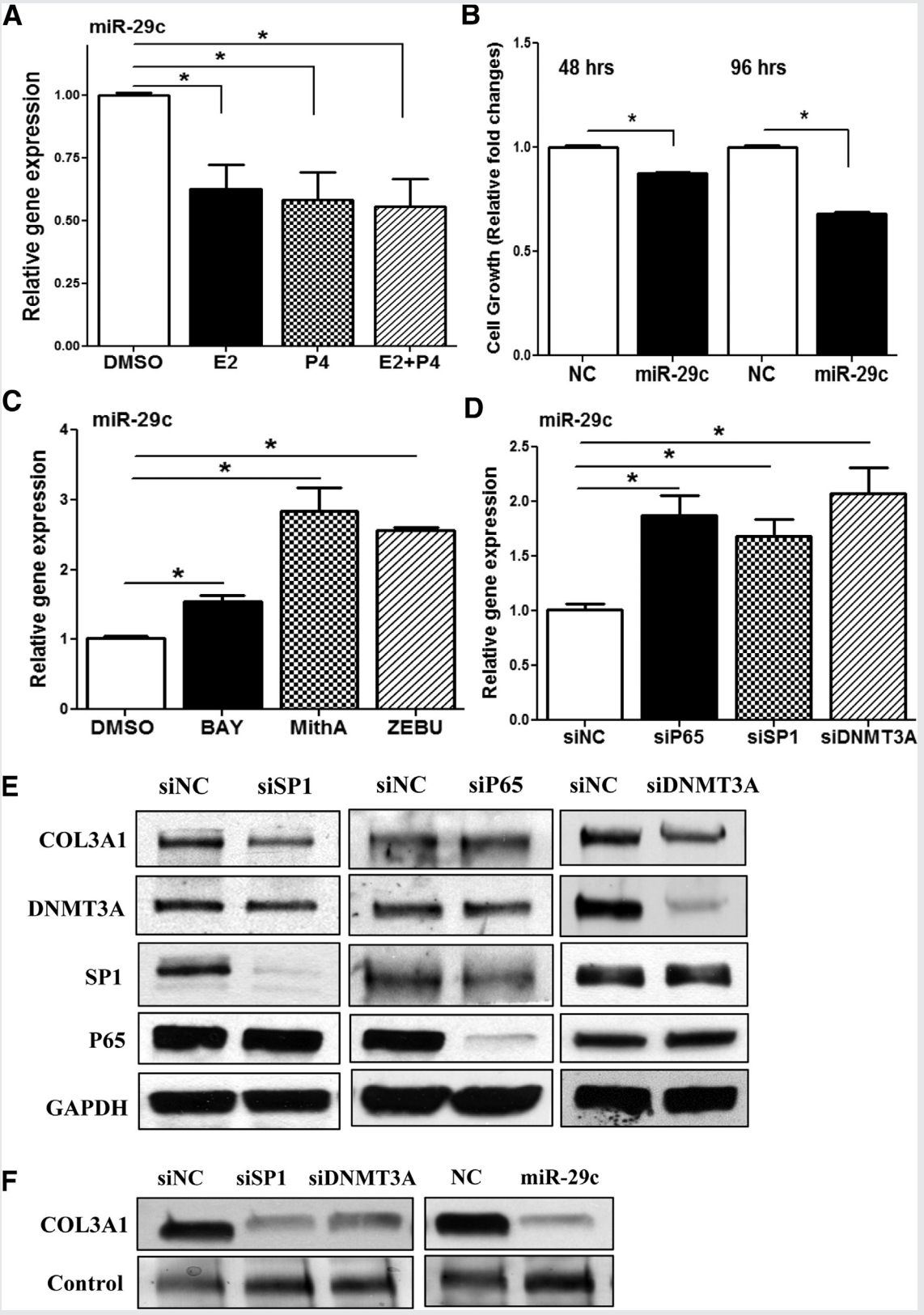
compared with matched myometrium, the levels of phosphorylated p65 at serine 536 and phosphorylated SP1 at threonine 453 were significantly ($P<.05$) higher in leiomyoma as compared with in matched myometrium.

DISCUSSION

In the present study we demonstrated that leiomyoma expresses significantly lower levels of miR-29c as compared

FIGURE 3



(**A**) The effect of DMSO (control), E_2, P, and E_2+P after 24 hours of culture on the expression of miR-29c in LSMC. The results are presented as mean ± SEM of three independent culture experiments. *P<.05. (**B**) The rate of cell proliferation determined by MTT assay after transfection of LSMC with

FIGURE 3 Continued

pre-miR-29c and control oligonucleotides (NC) for 48 and 96 hours. The results are presented as mean ± SEM of six independent experiments. *$P<.05$. (**C**) Relative expression of miR-29c after 24-hour treatment of LSMC with Bay 11-7082 (BAY, 5 μM), Mithramycin A (MithA, 1 μM), and Zebularine (ZEBU, 100 μM). *$P<.05$. (**D**) Effect of transfection with siRNA against p65, SP1, and DNMT3A for 72 hours on miR-29c levels in isolated LSMC. *$P<.05$. (**E**) Western blot analysis of COL3A1, DNMT3A, SP1, and p65 after transfection of LSMC with siRNA against p65, SP1, and DNMT3A for 96 hours. (**F**) Western blot analysis of COL3A1 in LSMC culture-conditioned media after transfection with SP1 and DNMT3A for 96 hours or pre-miR-29c or NC oligonucleotides (NC) for 72 hours. An equal volume of conditioned media was used, and a Ponceau S-stained protein band on the nitrocellulose membrane was used as a loading control. The results are representative of three sets of independent experiments.

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with myometrium. Our study confirmed previous microarray observations that have illustrated aberrant expression of a number of miRNAs, including miR-29c in leiomyoma (46, 47). Furthermore, we demonstrated that miR-29c inhibition results in up-regulation of its targets, namely, COL3A1 and DNMT3A. Both COL3A1 and DNMT3A were verified to be targets of miR-29c in leiomyoma cells by the luciferase assay. Our data indicate that several mechanisms are potentially involved in maintaining lowered expression of miR-29c in leiomyoma. These include estrogen, P, and their combination, which inhibit miR-29c expression in LSMC. A second mechanism is through activation of the transcription factors NF-κB and SP1, which are known key regulators of a number of genes (3–7), and a third mechanism is epigenetic, involving the miR-29c promoter. Evidence for these mechanisms consisted of our demonstration of activation of SP1 and NF-κB through phosphorylation in leiomyoma and our data showing that blockade of these transcription factors by siRNA or pharmacologic agents prevented the suppression of miR-29c in LSMC. Evidence that miR-29c was under epigenetic modification was based on our data that show that blockade of DNMT3A by siRNA or Zebularine prevented the down-regulation of miR-29c in leiomyoma cells. The latter suggests the presence of an auto-feedback mechanism by which inhibition of miR-29c in leiomyoma leads to activation of its target DNMT3A, which in turn could cause further inhibition of miR-29c expression. Additional evidence for the relevance of miR-29c in leiomyoma pathogenesis is our data demonstrating that overexpression of miR-29c inhibits LSMC proliferation; thus the lower expression of miR-29c in leiomyoma could be a contributing mechanism for excess cell proliferation in these tumors. On the basis of our data, we have proposed a model for the role of miR-29c in leiomyoma pathogenesis in Figure 4C.

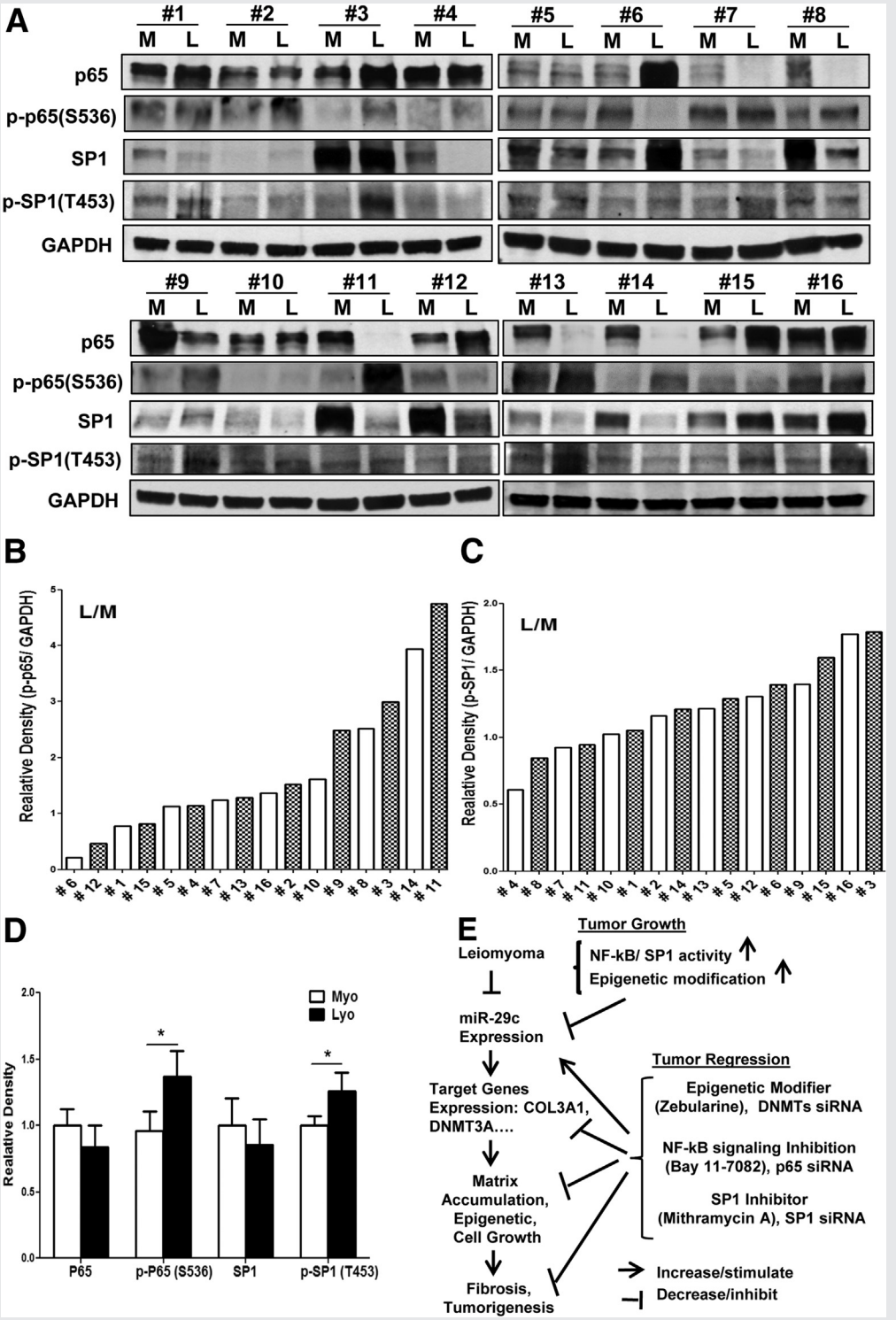
Our study confirmed previous microarray and next-generation sequencing analyses that have illustrated aberrant expression of a number of miRNAs, including miR-29 family, in leiomyoma (46–49). Since COL3A1 and DNMT3A have been identified as direct targets of miR-29c regulatory function (34, 50), we found an inverse relationship between their expression and the expression of miR-29c in leiomyoma. We verified that both COL3A1 and DNMT3A are targets of miR-29c in isolated LSMC. Previous reports have also demonstrated elevated expression of several ECM proteins, including COL3A1 (51, 52), and altered expression of DNMTs in leiomyoma as compared with myometrium (19, 20). Our data are in agreement with these prior studies and further demonstrate that suppression of miR-29c in leiomyoma plays a significant regulatory function in cellular events leading to excess deposition of ECM and altered genomic methylation status of leiomyoma.

Despite elevated levels of phosphorylated p65 (at serine 536) and SP1 (at threonine 453) in leiomyoma, knockdown of p65 had a limited effect on COL3A1 expression in LSMC. These results in LSMC are in contrast with other studies that have demonstrated the regulatory function of p65 on collagen expression in fibroblasts (5, 53). Although NF-κB is known to regulate the expression of many genes, its effect on the expression of COL3A1 in LSMC may occur through mechanisms independent of miR-29c (5, 53) or by regulation of other transcription factors such as SP1 (shown by our results in Fig. 3E) or a combination of these mechanisms such that the end result would be no effect on COL3A1 expression.

In addition to ECM and DNA methylation enzymes, the miR-29 family, including miR-29c, also regulate the expression of genes involved in cell proliferation, apoptosis, and differentiation (54–57). Our results indicate that gain of function of miR-29c led to significant inhibition of LSMC proliferation, which support the in vivo study in mice that demonstrated that miR-29b overexpression also inhibits xenografted leiomyoma cell proliferation (28). Thus one potential mechanism by which leiomyoma cells proliferate and grow in size may in part be due to reduced expression of miR-29c.

The significance of miR-29c in leiomyoma pathogenesis is further demonstrated by our data showing that both E_2 and P and their combination inhibit the expression of miR-29c in LSMC. These results suggest that ovarian steroids could contribute to the maintenance of the low expression of miR-29c in leiomyoma. Our in vitro findings are in agreement with the in vivo study of Qiang et al. (28) who reported that E_2+P administration in mice inhibited miR-29b levels in xenografted leiomyoma cells. As with collagens (3, 58) and DNMT3A (59), multiple SP1 binding sites are present in ER and PR promoters (11, 13), and PR-mediated actions in the myometrium have been reported to involve NF-κB activity (14). Since ovarian steroids through ER and PR regulate the expression of a large number of profibrotic and ECM-related genes as well as miRNAs in leiomyoma (2), our data would suggest that NF-κB and SP1 through ER/PR activation may regulate miR-29c and its target ECM genes. The regulation and intrinsic nuclear trafficking of NF-κB (p65), which is necessary for promoter activation of NF-κB target genes and miRNAs, has been shown to be regulated by ECM (60). As with

FIGURE 4



(**A**) Western blot analysis of p65, p-p65 (S536), SP1, and p-SP1 (T453) in paired (n = 16) myometrium (M) and leiomyoma (L) with a bar graph (**B**) and (**C**) for relative intensity by L/M in each pair of p-p65 (S536) and p-SP1 (T453), respectively. (**D**) Mean average of relative band densities in (**A**). *$P<.05$. (**E**) Schematic diagram representing our working model in which leiomyoma is characterized by low levels of miR-29c expression. MiR-29c inhibition is due to elevated SP1 and NF-κB signaling and epigenetic modification. Inhibition of miRNA-29c leads to overexpression of its target genes, which causes an increase in cell growth and ECM accumulation. Restoration of miR-29c to normal levels in leiomyoma using selective inhibitors of NF-κB signaling (Bay 11-7082), SP1 (Mithramycin A), and epigenetic inhibitor (Zebularine), as well as their respective siRNAs, would be expected to result in inhibition of its target genes, such as COL3A1 and DNMT3A, and leiomyoma regression.

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our current observation of p65 regulation of miR-29c, we have previously reported that miR-200c, whose expression is also suppressed in leiomyoma, regulates the expression of IL-8 through IKBKB (42). Several ECM-related genes such N-cadherin have also been shown to be regulated through p65 activation as well as miRNA action (61). As such, we have reported that miR-200c, through an indirect mechanism, regulated CDH1 expression, which interacted with p65 in cytoplasmic compartment, preventing NF-κB nuclear translocation in LSMC (42).

In summary, the results presented in this study demonstrate that miR-29c expression is suppressed in leiomyoma and its expression is inversely correlated with the expression of its target genes, namely, COL3A1 and DNMT3A. This suppression of miR-29c in leiomyoma is mediated through an epigenetic mechanism and by transcriptional regulation by NF-κB and SP1. Since miR-29c target genes regulate various cellular activities, such as cell proliferation, angiogenesis, tissue turnover, and ECM proteins that are central to leiomyoma development, growth, and fibrotic characteristics, and miR-29c is under ovarian steroid control, our results support a key regulatory function of the miR-29 family in the genesis of this disorder. Furthermore, our data suggest that drugs that could target NF-κB, SP1, or DNMT3A through their effects in restoring miR-29c levels to normal levels might have a therapeutic potential for the treatment of leiomyoma.

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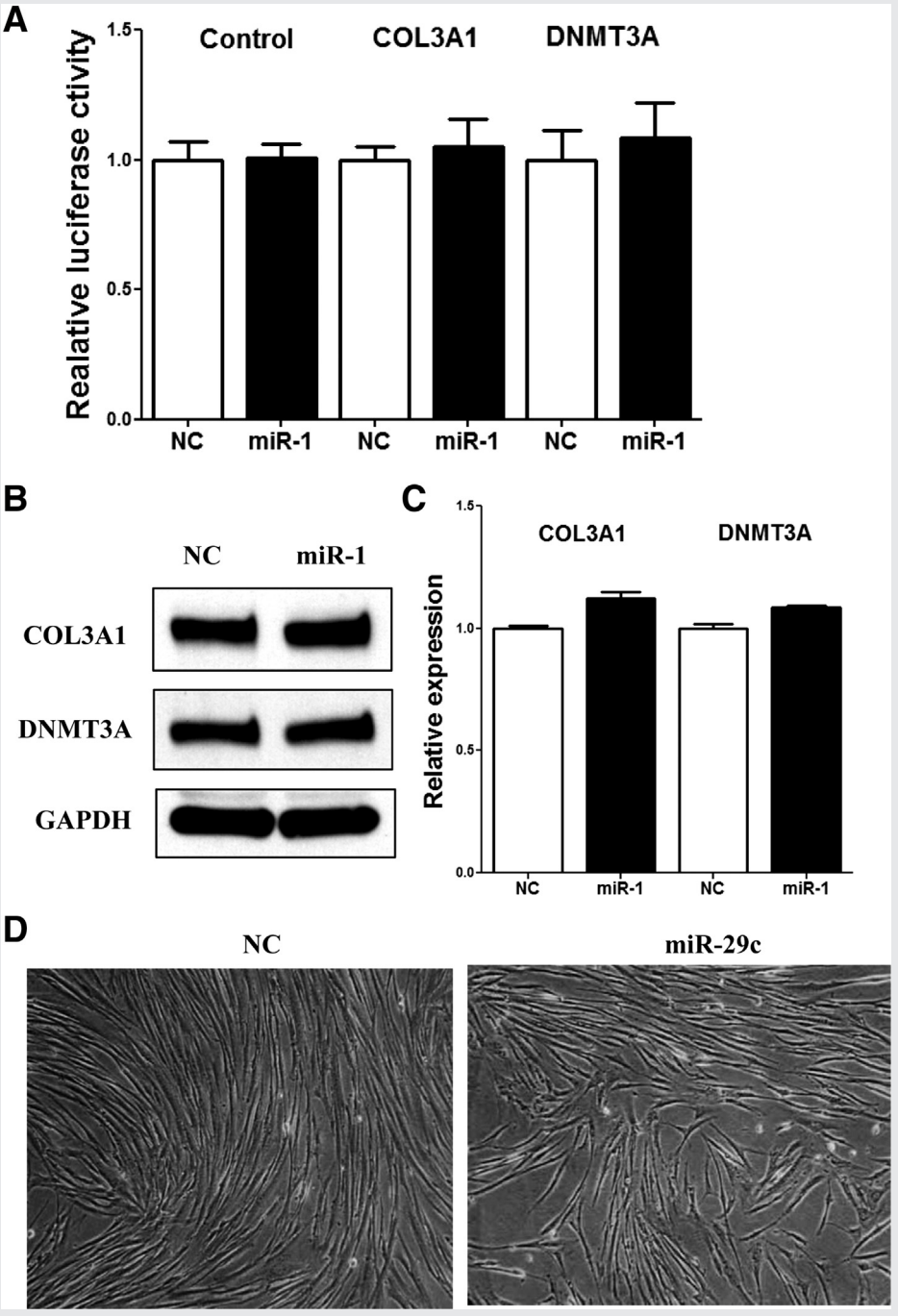
SUPPLEMENTAL FIGURE 1



(**A**) Effect of miR-1 overexpression on 3′UTR of COL3A1 and DNMT3A by luciferase reporter assay. The pre-miR-1 or control oligonucleotides (NC) was cotransfected with luciferase reporter plasmids carrying a 3′UTR fragment of COL3A1 or DNMT3A in isolated LSMC for 48 hours. The relative luciferase activity is presented as the ratio of Firefly:Renilla as compared with NC, which was independently set as 1. (**B**) Western blot analysis of COL3A1 and DNMT3A after transfection of LSMC with pre-miR-1 or NC oligonucleotides for 96 hours. (**C**) QRT-PCR analysis of COL3A1 and DNMT3A mRNA expression in LSMC after transfection with pre-miR-1 or NC oligonucleotides for 72 hours. (**D**) Photomicrographs of LSMC transfected with pre-miR-1 or NC for 96 hours. The results are presented as mean ± SEM of at least three independent experiments with *P* values (**P*<.05) indicated by corresponding lines.

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