Cross-talk between miR-29c and transforming growth factor-β3 is mediated by an epigenetic mechanism in leiomyoma

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Objective: To determine the expression of miR-29c and its target gene transforming growth factor-β3 (TGF-β3) in leiomyoma and the mechanisms of their reciprocal regulation.
Design: Experimental study.
Setting: Academic research laboratory.
Patient(s): Women undergoing hysterectomy for leiomyoma.
Intervention(s): Overexpression and underexpression of miR-29c; blockade of DNA methyltransferase 1 (DNMT1).
Main Outcome Measure(s): The miR-29c and its target gene TGF-β3 in leiomyoma and the effects of TGF-β3 and blockade of DNMT1 on miR-29c expression.
Result(s): Leiomyoma expressed significantly lower levels of miR-29c, but higher expression of TGF-β3 compared with matched myometrium. The expression of TGF-β3 and miR-29c were independent of race/ethnicity. Using 3′ untranslated region luciferase reporter assay we confirmed that TGF-β3 is a direct target of miR-29c in leiomyoma smooth muscle cells (LSMCs). Gain-of-function of miR-29c in LSMCs inhibited the expression of TGF-β3 at protein and messenger RNA levels, whereas loss-of-function of miR-29c had the opposite effect. Treatment of LSMCs with TGF-β3 inhibited the expression of miR-29c, whereas it stimulated DNMT1 expression. Knockdown of DNMT1 through transfection with small interfering RNA significantly decreased the expression of TGF-β3, and induced miR-29c expression. Knockdown of DNMT1 also attenuated the inhibitory effect of TGF-β3 on miR-29c expression. Furthermore, we demonstrated that TGF-β3 increased the methylation level of miR-29c promoter in LSMCs.
Conclusion(s): There is an inverse relationship in the expression of TGF-β3 and miR-29c in leiomyoma. The TGF-β3 is a direct target of miR-29c and inhibits the expression of miR-29c through an epigenetic mechanism. The cross-talk between miR-29c and TGF-β3 provides a feed forward mechanism of fibrosis in leiomyoma. (Fertil Steril® 2019;112:1180–9. ©2019 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Leiomyoma, miR-29c, TGF-β3, fibrosis, epigenetics

Leiomyomas are benign fibrotic tumors afflicting a significant number of women. It is the most common indication for all hysterectomies performed (1). These tumors, whose growth is dependent on ovarian steroids, are characterized by an excess accumulation of extracellular matrix (ECM), inflammation, and increased angiogenesis (2, 3). In addition, in leiomyomas the altered expression of some nonprotein coding genes, including microRNAs (miRNAs), which target the expression of protein coding genes, has been documented (4, 5). The pathogenic mechanisms for leiomyoma initiation and progression have been under intense investigation (6–8). Our laboratory has focused on the mechanism underlying ECM accumulation and we have identified miR-29c, a member of small noncoding RNAs as being pivotal to the fibrosis associated with leiomyoma tumors (9). The levels of miR-29c are suppressed in fibroids compared with myometrium and are under ovarian steroid control, transcription factors SP1 (specificity

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protein 1), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and epigenetic regulation (9). The miR-29c has a number of targets, including collagens (9–12), elastin (10, 11, 13), and matrix metalloproteinases (11, 14), many of which have been demonstrated to play a role in fibroid pathogenesis (2), and previously published reports (9, 15, 16) demonstrated an increased expression of miR-29c targets in leiomyomas, including collagen I, collagen III, matrix metalloproteinases, and epigenetic enzymes DNMTs (DNA methyltransferases). Some investigators (16, 17) have reported other members of the miR-29 family, namely miR-29a and miR-29b, to also be lower in fibroids compared with myometrium. Furthermore, overexpression of miR-29b in an animal model for fibroid resulted in shrinkage of tumors (17). Collectively, these studies point to the highly significant role of the miR-29 family in fibroid pathogenesis.

Transforming growth factor-β3 (TGF-β3) is a profibrotic cytokine that interacts with type 1 and 2 receptors, propagating its effect through activation of signaling pathways such as MAPK/ERK (mitogen activated protein kinase/extracellular-signal-regulated kinase) (18), Smad2/3 (SMAD family member 2/3) (19), and PI3K/Akt (phosphatidylinositol 3-kinases/protein kinase B) (20), the end result of which is increased synthesis of collagen I, CTGF (connective tissue growth factor), fibronectin (FN1), and versican V0 (VCAN V0), all of which are critical to leiomyoma progression (18–22). Furthermore, TGF-β3 inhibits the expression of matrix metalloproteinases, thus resulting in ECM accumulation (22). Treatment of leiomyoma smooth muscle cells (LSMCs) with TGF-β3 but not TGF-β1 stimulated DNA synthesis (23). Other investigators (24) have shown an increase in cell proliferation in response to TGF-β1. Some factors have been shown to reduce the levels of TGF-β3 and fibrotic genes regulated by TGF-β3 in in vitro studies, including tranilast (15), vitamin D3 (18), and selective P receptor (PR) modulators (25–27), whereas activation of β-catenin (Catenin beta-1), which is critical in fibroid pathogenesis induces TGF-β3 (28). The ovarian steroid dependence of the TGF-β family and their influence on smooth muscle cell proliferation underscore the significance of TGF-β and its receptors in fibroid pathophysiology. Because TGF-β3 is a predicted target of miR-29c (Targetscan, http://www.targetscan.org) and both miR-29c and TGF-β3 are critical in regulation of fibrosis, which is the hallmark of fibroids, we hypothesized that miR-29c might exert its effects on the ECM through modulation of TGF-β3 levels, and TGFβ3 in turn through a feedback loop could influence miR-29c transcription. This hypothesis was tested in an in vitro primary cell culture obtained from fibroids removed at the time of hysterectomy from women on no hormonal medications 3 months before surgery.

MATERIALS AND METHODS

Tissue Collection and Primary Cell Isolation

Leiomyomas and paired myometrium were obtained from patients (N = 42) not on hormonal treatments for at least 3 months before surgery at Harbor-UCLA Medical Center with prior approval obtained from LA BioMed at Harbor-UCLA Medical Center Institutional Review Board (#036247). Informed consent was obtained before surgery from all the patients participating in the study. The paired tissues were obtained from White Hispanics (n = 14), African Americans (n = 14), and Whites (n = 14) aged 35–56 years (mean, 45 ± 5.1 years). Among the White group 6 pairs were kindly provided by Dr. Al-Hendy (University of Chicago). The tissues were either snap frozen and stored in liquid nitrogen for further analysis, or used for isolation of LSMCs as previously described (29). Briefly, LSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum until reaching confluence with a change of media every 2–3 days. Cells at passages p1–p3 were used for all experiments. Cell culture experiments were performed at least three times using LSMCs 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

The LSMCs were seeded in six-well plates and at subconfluence transfected with 50 nM of pre-miR-29c, anti-miR-29c, pre-miR negative, or anti-miR negative control (Applied Biosystems) for 72–96 hours using PureFection transfection reagent (System Biosciences, Inc.) according to the manufacturer's protocol.

Small Interfering RNA Transfection

The LSMCs were cultured as previously and at subconfluence transfected with 50 nM of small interfering RNA negative control or small interfering RNA against DNMT1 (Santa Cruz Biotechnology) for 72–96 hours using PureFection transfection reagent (System Biosciences, Inc.) according to the manufacturer's protocol.

RNA Isolation and Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis

Total RNA was extracted from LSMCs using Trizol (Thermo Fisher Scientific) and their quantity and quality was determined (ND-1000 Spectrophotometer; NanoDrop Technologies) as previously described (30–32). Subsequently, RNA sample of 1 μg each was reverse transcribed using random primers for TGF-β3. The miR-29c primer design and polymerase chain reaction (PCR) conditions have been described previously (33). Quantitative reverse transcriptase–PCR was carried out using SYBR gene expression master mixes (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. Levels of messenger RNA and miRNA were quantified using the Invitrogen StepOne System and normalized to FBXW2 (34) and RNU6B, respectively. All reactions were run in triplicate and relative expression was determined using the comparative cycle threshold method ($2-\Delta\Delta CT$), as recommended by the supplier (Applied Biosystems). Abundance values were expressed as fold changes compared with the corresponding control group. The primer sequences used were as follows: TGF-β3 (sense, 5′-CGGGCTTTGGACACCAATTA-3′; antisense, 5′-GGGCGCACACAGCAGTTC-3′); and FBXW2 (sense, 5′-CCTCGTCTCTAAACAGTGGAATAA-3′; antisense,

5′- GCGTCCTGAACAGAATCATCTA-3′). miR-29c (sense, 5′-GCAGTAGCACCATTTGAAATC-3′; antisense, 5′-GGTCCAGTTTTTTTTTTTTTTAACC-3′); and RNU6B (sense, 5′-ATTGGAACGATACAGAGAAGATTAG-3′; antisense, 5′-AATATGGAACGCTTCACGAAT-3′).

Immunoblotting

Total protein isolated from paired tissue samples and LSMCs after treatment conditions was subjected to immunoblotting as previously described (10, 35). Briefly, samples were suspended in RIPA buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) (Boston BioProducts) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and a complete protease inhibitor mixture (Roche Diagnostics), sonicated, and centrifuged at 4°C for 10 minutes at 14,000 rpm. The concentration of protein was determined using the BCA Protein Assay Kit (Thermo Scientific Pierce). Equal aliquots (50 μg) of total protein for each sample were denatured with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and separated by electrophoresis on an SDS polyacrylamide gel. After transferring the samples to a nitrocellulose membrane, the membrane was blocked with tris-buffered saline (TBS)-Tween + 5% milk, and probed with the following primary antibodies: TGF-β3 and DNMT1 (Santa Cruz Biotechnology). The membranes were washed with TBS containing 0.1% Tween-20 wash buffer after each antibody incubation cycle. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce) was used for detection, and photographic emulsion was used to identify the protein bands, which were subsequently quantified by densitometry. The membranes were also stripped and probed with GAPDH antibody (Santa Cruz Biotechnology) serving as the loading control. The densities of the specific protein bands were quantified with a scanning densitometer (Bio-Rad GS-800), and the results were expressed as means ± SEM normalized to GAPDH.

Luciferase Reporter Assays

The LSMCs were seeded in six-well plates and at 70%–80% subconfluence were transfected with 50 nM pre-miR-29c oligonucleotides or a negative control using the PureFection transfection reagent as previously described (36). At the same time, the cells were co-transfected with a luciferase reporter plasmid (1 μg/well) containing a 3′ untranslated region sequence of TGF-β3 (GeneCopoeia). After 48 hours of transfection Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. The level of induction was reported as mean ± SEM of three experiments performed in triplicates and compared with a ratio in cells transfected with negative control independently set as 1.

Methylation-specific PCR

The LSMCs at subconfluence were treated with TGF-β3 (5 ng/mL) for 48 hours and genomic DNA was extracted and used for bisulfite conversion (EZ DNA Methylation Kit, Zymo Research Corporation) as previously described (37). Because methylated cytosine residues remained unchanged after bisulfite conversion, this approach allows distinguishing DNA sequences that are methylated or unmethylated in specific genomic regions using sequence-specific PCR primers (38). The pair of primers used to detect the methylated sequence of miR-29c promoter were 5′-GATGGGATTAAATTTTGGAATATTC-3′ and 5′-AAATCCTAAAACCCGTCGAA-3′ and for the unmethylated sequence of miR-29c promoter were 5′-GGGATTAAATTTTGGAATATTTGG-3′ and 5′-ATCAAATCCTAAAACCCATCAAA-3′. DNA amplification was carried out following manufacturer's protocol (HotStarTaq Plus PCR reagent, Qiagen). The PCR condition was 5 minutes at 95°C, followed by 40 cycles of amplification at 94°C for 30 seconds, 30 seconds at 55°C, and 1 minute at 72°C. The PCR amplified products were electrophoresed on 2% agarose gel and visualized under ultraviolet illumination.

Statistical Analysis

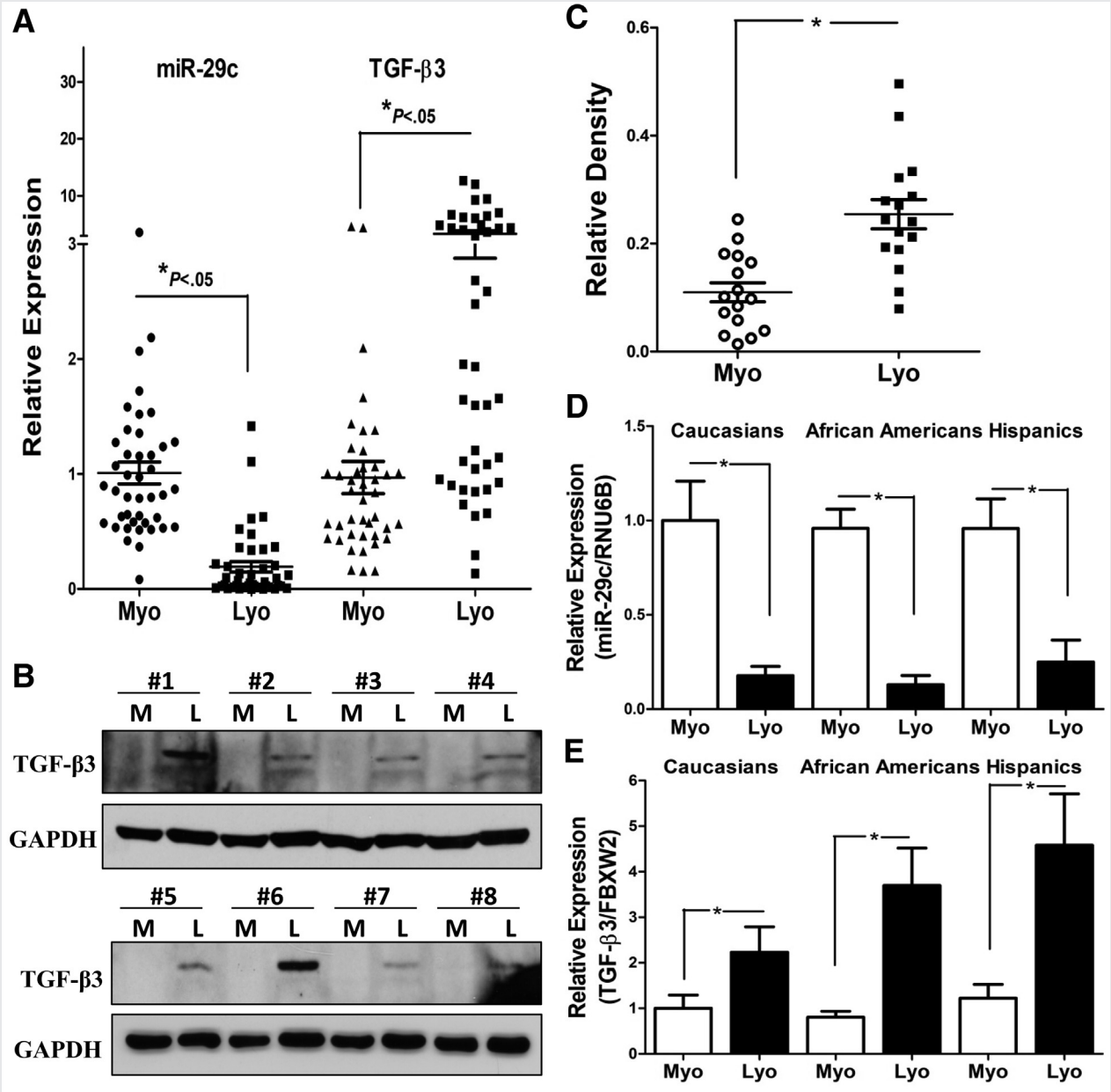
Power calculation indicated that the sample size estimates for studies have been based on a power of 80% to detect 30% changes between the two groups (myometrium vs. leiomyoma) (assuming an expected SD of 20% of mean values). This analysis results in a requirement for 10 patients in each group. Throughout the text, all data are presented as mean ± SEM and analyzed by PRISM software (Graph-Pad). Dataset normality was determined by the Kolmogorov-Smirnov test. Comparisons involving two groups were analyzed using paired or unpaired Student's *t*-tests as appropriate. One-way analysis of variance (ANOVA) was used for comparisons involving multiple groups. Statistical significance was established at $P < .05$.

RESULTS

Using paired leiomyoma and matched myometrium (N = 42) the co-expression of miR-29c and TGF-β3 in the same specimens was determined. The analysis indicated that miR-29c (Fig. 1A) expression is reduced, whereas TGF-β3 expression at both messenger RNA (Fig. 1A) and protein (Fig. 1B,C) levels is elevated in leiomyomas compared with paired myometrium. The analysis further indicated no significant racial/ethnic differences in expression of miR-29c and TGF-β3 (Fig. 1D,E). Collectively, these data confirmed previous results from our laboratory and others with regard to miR-29c and TGF-β3 expression in leiomyomas, demonstrating an inverse pattern of expression (9, 21, 23).

Because TGF-β3 is a predicted target of miR-29c based on Targetscan, we assessed such regulatory function of miR-29c on TGF-β3 expression in isolated LSMCs. As shown in Figure 2A,B, luciferase reporter assay revealed that miR-29c directly interacts with the 3′ untranslated region of TGF-β3 thereby regulating its expression in LSMCs. Transfection of LSMCs with pre-miR-29c down-regulated the expression of TGF-β3, whereas knockdown of miR-29c with anti-miR-29c, which mimics the expression pattern of miR-29c in leiomyoma, increased the expression of TGF-β3 at mRNA (Fig. 2C) and protein (Fig. 2D,E) levels. We also demonstrated

FIGURE 1



(A) The relative expression of miR-29c and transforming growth factor-$\beta 3$ (TGF-$\beta 3$) in paired (N = 42) myometrium (Myo) and leiomyoma (Lyo). *$P<.05$. **(B)** Western blot analysis of TGF-$\beta 3$ in paired (N = 16) myometrium (M) and leiomyoma (L) with dot plot **(C)** showing their relative band densities in myometrium (Myo) and leiomyoma (Lyo). *$P<.05$. Relative (mean ± SEM) expression of miR-29c **(D)** and TGF-$\beta 3$ **(E)** in Lyo and matched Myo from untreated group based on ethnicity in Whites (Caucasians) (n = 14), African Americans (N = 14), and Hispanics (N = 14). The relative expression was determined by setting the averaged expression value of each gene in myometrium from Whites as 1. *$P<.05$.

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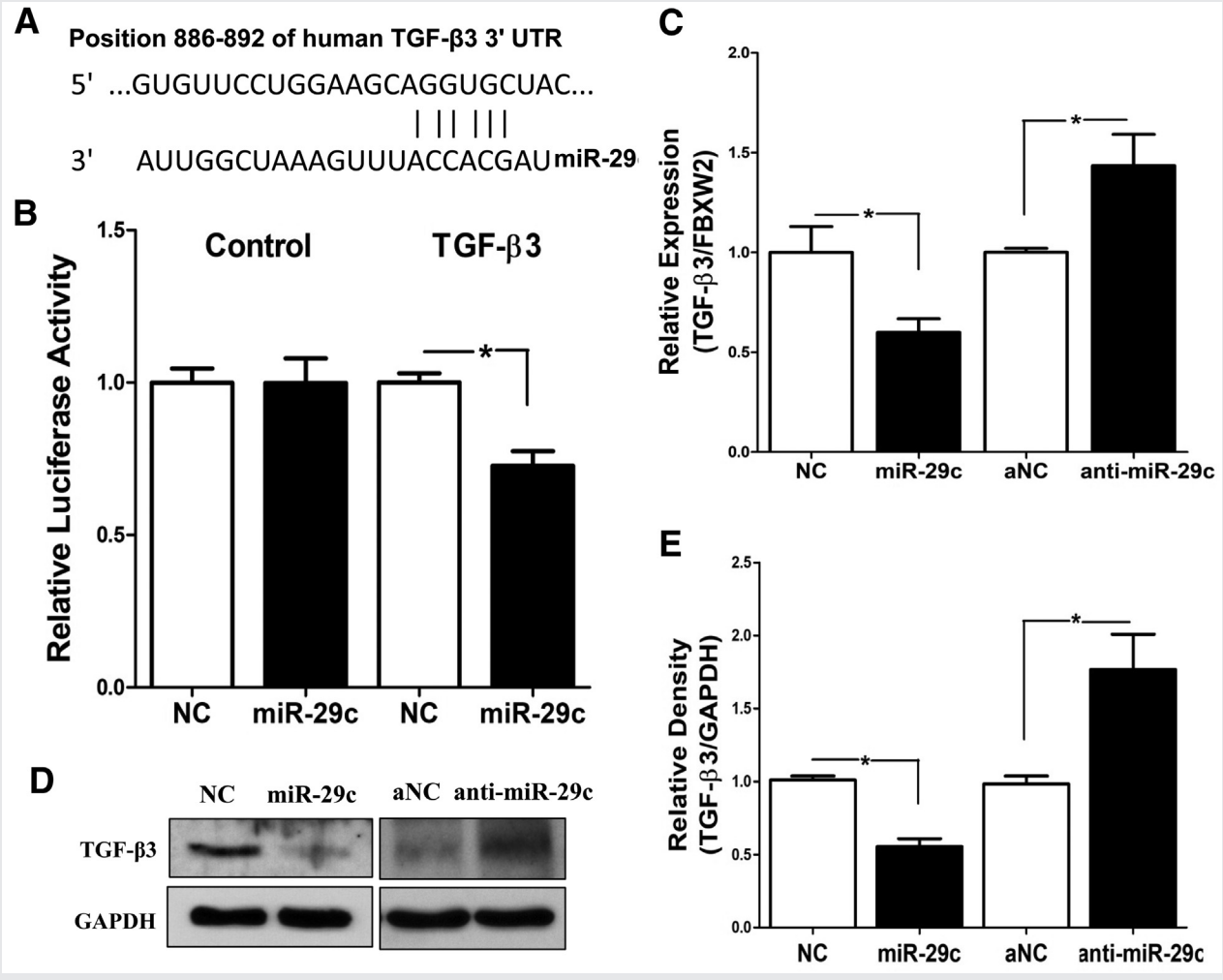
that treatment of LSMCs with TGF-$\beta 3$ (5 ng/mL) resulted in inhibition of miR-29c expression (Fig. 3A). Because miR-29c expression in LSMCs is under epigenetic control (15, 37, 39), we determined whether TGF-$\beta 3$-mediated inhibition of miR-29c expression is mediated through an epigenetic mechanism. As such we determined the effect of TGF-$\beta 3$ on epigenetic enzymes namely, DNMT1 and DNMT3A, which catalyze DNA methylation and enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), which is involved in histone methylation. The analysis indicated that treatment of LSMCs with TGF-$\beta 3$ had no significant effect on DNMT3A and EZH2 expression (data not shown); however, TGF-$\beta 3$ induced the expression of DNMT1 protein levels (Fig. 3B,C). To further establish the role of DNMT1 in mediating the effects of TGF-$\beta 3$ on miR-29c expression, LSMCs were treated with DNMT1 small interfering RNA, which effectively inhibited DNMT1 (Fig. 3D,E). Inhibition of DNMT1 in LSMCs resulted in suppression of TGF-$\beta 3$ at both messenger RNA and

FIGURE 2



(A) Complementary sequences between miR-29c and 3′ untranslated region (UTR) of transforming growth factor-β3 (TGF-β3). **(B)** The relative luciferase activity in isolated leiomyoma smooth muscle cells (LSMCs) co-transfected with Renilla and Firefly luciferase reporter carrying a 3′ UTR untranslated region fragment of TGFβ3, pre-miR-29c, or control oligonucleotides (NC) for 48 hours. The relative luciferase activity is presented as the ratio of Firefly:Renilla compared with NC, which is independently set as 1. **(C)** Quantitative reverse transcriptase–polymerase chain reaction (PCR) analysis of TGF-β3 messenger RNA expression in LSMCs after transfection with pre-miR-29c or anti-miR-29c oligonucleotides (aNC) for 72 hours. **(D)** Representative Western blot analysis of TGF-β3 after transfection of LSMCs with pre-miR-29c or anti-miR-29c oligonucleotides for 96 hours. Bar graph **(E)** shows the relative band densities of TGF-β3. 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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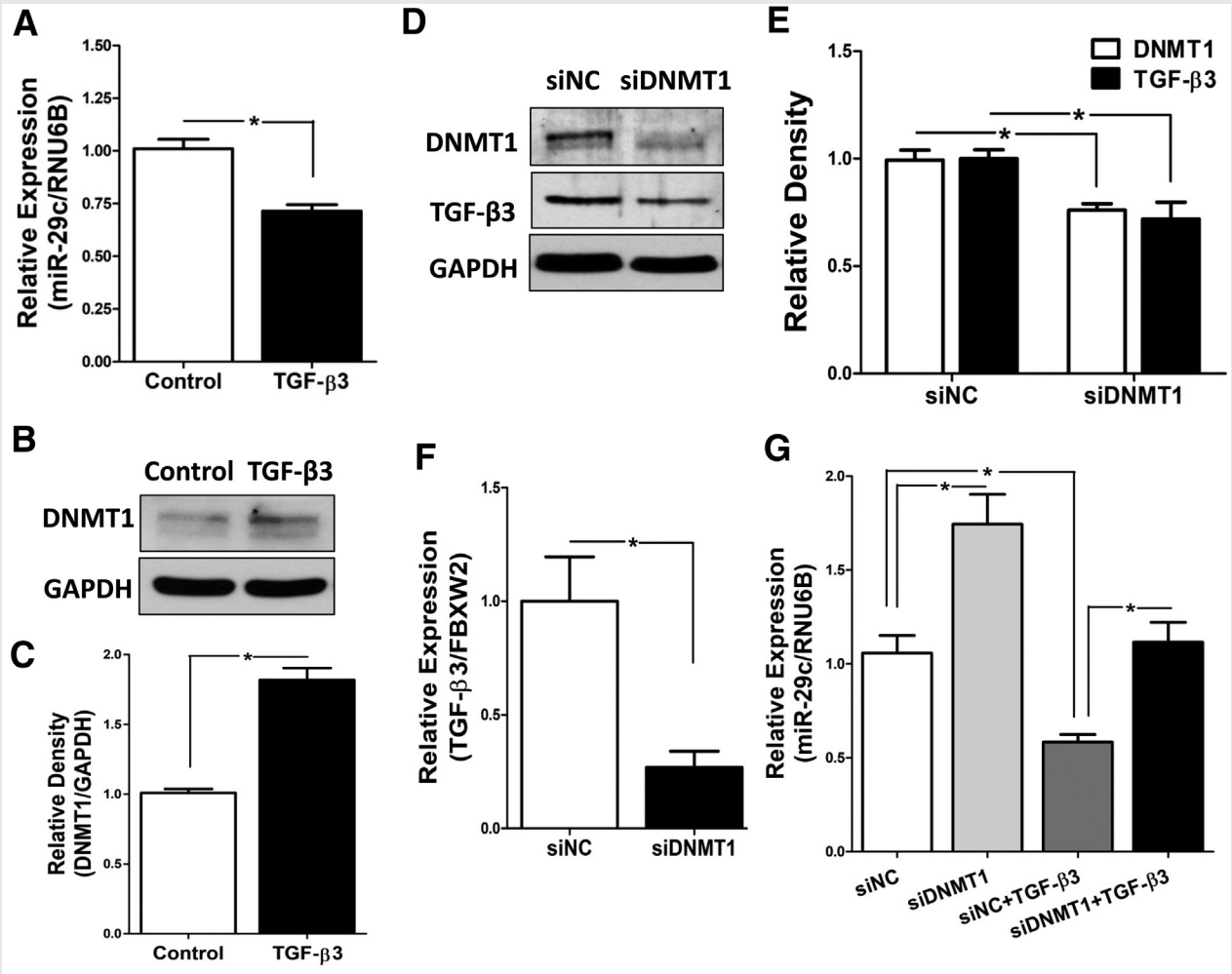
protein levels (Fig. 3D–3F), whereas it increased the expression of miR-29c (Fig. 3G). Furthermore, treatment of LSMCs with DNMT1 small interfering RNA attenuated the inhibitory effect of TGF-β3 on miR-29c expression, indicating that the effect of TGF-β3 on miR-29c is mediated by DNMT1 (Fig. 3G). To provide additional support that TGF-β3-induced inhibition of miR-29c is epigenetically mediated, LSMCs were treated with TGF-β3 and the methylation status of miR-29c promoter was determined by methylation-specific PCR. As shown in Figure 4A,B, TGF-β3 increased the methylation level of miR-29c promoter, thus highlighting the importance of an epigenetic mechanism for TGF-β3-induced inhibition of miR-29c.

DISCUSSION

In the present study we provide evidence for a novel feed forward cross-talk between miR-29c and TGF-β3. We demonstrate an inverse pattern of expression between miR-29c and TGF-β3 in fibroids that is race independent. We confirm that TGF-β3 is a direct target of miR-29c in LMSCs and knockdown of miR-29c results in overexpression of TGF-β3, whereas its overexpression has the opposite effect in LMSCs. The TGF-β3 in turn influences miR-29c expression through an epigenetic mechanism by increasing the expression of DNMT1, resulting in increased methylation of miR-29c promoter. Blockade of DNMT1 by small interfering RNA

FIGURE 3



(A) The effect of transforming growth factor-$\beta 3$ (TGF-$\beta 3$; 5 ng/mL) after 48 hours of culture on the expression of miR-29c in leiomyoma smooth muscle cells (LSMCs). The results are presented as mean ± SEM of three independent culture experiments. *$P<.05$. **(B)** and **(C)** Western blot analysis of DNMT1 in LSMCs after 24 hours of TGF-$\beta 3$ (5 ng/mL) treatment and the relative band densities is shown in **(C)**. **(D–F)** The effect of DNMT1 knockdown through transfection of LSMCs with small interfering RNA (siRNA) against DNMT1 for 72 hours on protein levels of DNMT1 and TGF-$\beta 3$ determined by Western blot **(D)** along with the relative band densities analysis **(E)**. The messenger RNA level of TGF-$\beta 3$ determined by quantitative reverse transcriptase–polymerase chain reaction (PCR) is shown in **(F)**. NC = pre-miR negative. The results shown in **(B)** and **(D)** are representative of three sets of independent experiments. **(G)** The effect of transfection with siRNA against DNMT1 for 96 hours along with TGF-$\beta 3$ (5 ng/mL) treatment in the last 48 hours on miR-29c levels in isolated LSMCs (n = 4). *$P<.05$.

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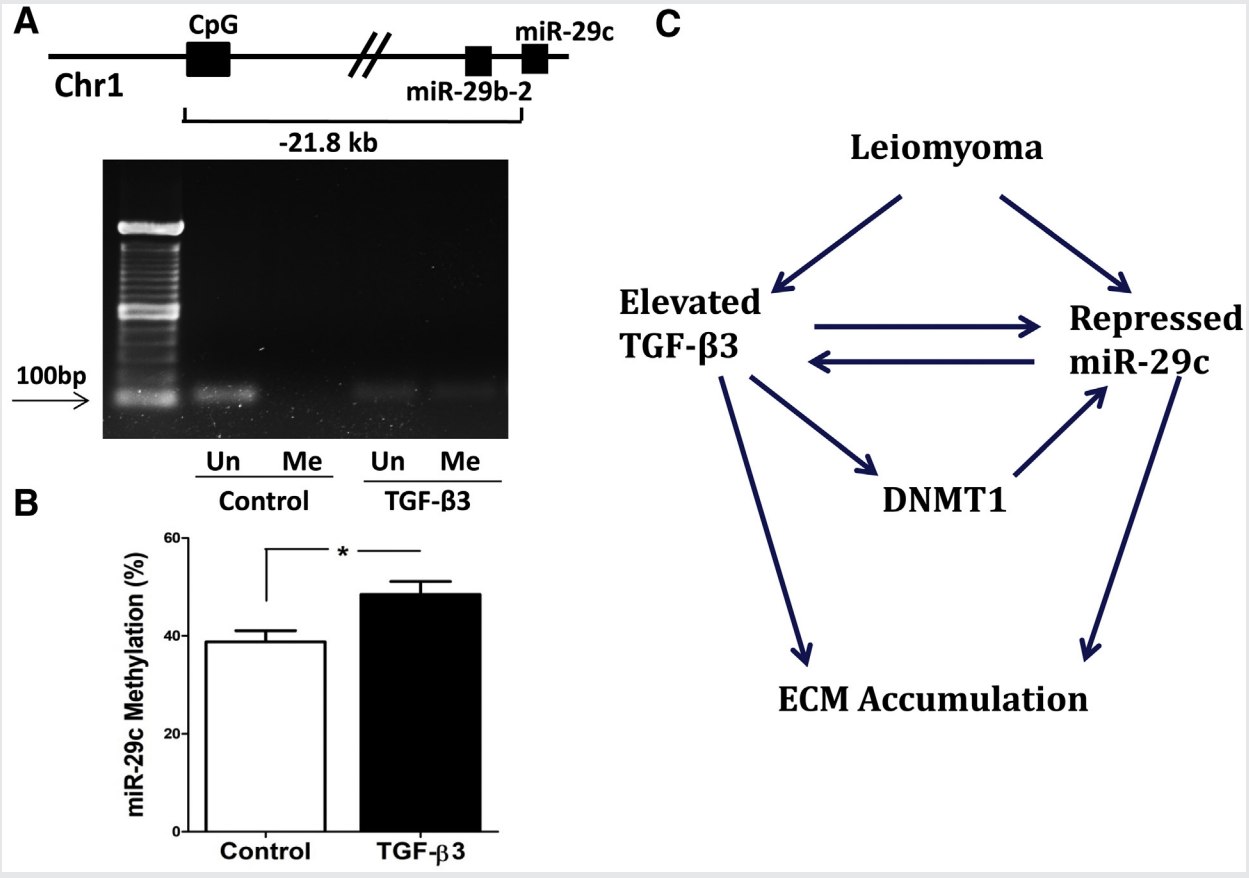
transfection of LMSCs blocked the inhibitory effect of TGF$\beta 3$ on miR-29c expression, thus establishing the importance of this epigenetic mechanism. A schematic demonstrating this cross-talk between miR-29c and TGF-$\beta 3$ is shown in Figure 4C.

Leiomyomas are considered a fibrotic disorder characterized by increased cell proliferation, inflammation, angiogenesis, and excess accumulation of ECM where elevated expression of TGF-β family plays a central role (2, 3,6–8). Driver mutations (mediator complex subunit 12, high mobility group AT-hook 2, fumarate hydratase, collagen type IV, alpha 5) and chromosomal alteration, in addition to widespread alteration in DNA methylation in multiple genes critical to fibroid growth, have been reported (40, 41). The miR-29 family consists of miR-29a, miR-29b, and miR-29c, which are encoded by two gene clusters and are predicted to target many genes functionally associated with cellular transformation, inflammatory responses, and ECM turnover (30, 42–47). It is clear that a low level of antifibrotic miR-29c and elevated level of profibrotic TGF-$\beta 3$ in leiomyoma provides an environment necessary to promote and maintain tumor fibrotic characteristic. The miR-29c directly interacted with the 3′ untranslated region of TGF-$\beta 3$ regulating TGF-$\beta 3$ at both transcriptional and translational levels, which we further confirmed using gain- or loss-of-function of miR-29c. Although our result

FIGURE 4



(A) Graphic depiction of the miR-29b-2/29c with the location of CpG indicated the regions tested for DNA methylation status in miR-29c promoters. Methylation-specific polymerase chain reaction (PCR) shows transforming growth factor-β3 (TGF-β3; 5 ng/mL) mediated increased miR-29c promoter methylation after 48 hours of incubation. The result is a representative of at least three sets of independent experiments using leiomyoma smooth muscle cells (LSMCs) isolated from three patients. **(B)** The bands were quantified with National Institutes of Health Image J software and the percentage of methylation was calculated as follows: Methylation/Methylation + Unmethylation (Me/Me+Un). The results are presented as mean ± SEM of at least three independent experiments using LSMCs isolated from three patients. *$P<.05$. **(C)** Schematic diagram representing our working model in which leiomyoma are characterized by reduced miR-29c and elevated TGF-β3 expression. Lower expression of miR-29c leads to overexpression of its target gene, TGF-β3, which causes an increase in extracellular matrix accumulation, and by induction of DNMT1 causes increased methylation of miR-29c promoter resulting in its decreased expression. ECM = extracellular matrix.

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is the first to confirm the regulatory function of miR-29c on TGF-β3 expression in leiomyoma, in a recent study (48) using a unilateral ureteral obstruction model for renal fibrosis, IM injection of exosome-encapsulated miR-29 was shown to target TGF-β3, thus providing support for our observation. Furthermore, we found that TGF-β3 through an epigenetic mechanism involving DNMT1 expression inhibited miR-29c expression. The regulatory action of TGF-β3-induced DNMT1 occurred by increased methylation of miR-29c promoter, thus further supporting the importance of epigenetic regulatory mechanism in leiomyoma (49). A similar cross-talk involving miR-29b and TGF-β1 and TGF-β2 has been previously reported in trabecular meshwork cells (50), in renal tubular cells where miR-29b was down-regulated by TGF-β1 through Smad3 (51, 52), and in cardiac fibroblast cells where TGF-β1 inhibited the expression of the miR-29 family (46). However, to our knowledge our data are the first evidence demonstrating a feedback interaction between miR-29c and TGF-β3.

Our data indicated that TGF-β3 induced DNMT1 expression in LSMCs, which in turn resulted in methylation of miR-29c promoter and lowering of miR-29c. Interestingly, although DNMT1 is not a direct target of miR-29, recent studies (53, 54) have demonstrated that miR-29b by targeting the expression of transcription factor SP1 regulates DNMT1 expression in acute myeloid leukemia cell lines. In addition, our laboratory and others have reported that leiomyoma express significantly higher DNMT1 compared with matched myometrium (15, 55, 56). In line with our findings, showing a significant decrease in the expression of TGF-β3 after DNMT1 knockdown in LSMCs, zebrafish with a mutation in DNMT1 were reported to have 50% decrease in expression of TGF-β3 in the lens (57). Several studies in other tissues and cells have demonstrated an effect of the TGF-β1 on

DNA methylation, including global changes in DNA methylation of genes critical to epithelial to mesenchymal transition in ovarian cancer cell line through induction of DNMT1, DNMT3A, and DNMT3B (58). In cardiac fibroblast TGF-β1 reduced the expression of DNMT1 and DNMT3A and induced COL1A1 expression (59). Collectively, these studies indicate the tissue/cell-specific effect of TGFβ isoforms on DNA methylation.

Our data further support the regulatory function of miRNAs and epigenetic modification of genes that drive tumorigenesis and tissue fibrosis (60–63), and leiomyoma growth and progression (56, 64–67). The miRNAs have been implicated in the pathogenesis of some gynecologic and obstetrics conditions, including endometriosis (68), polycystic ovary syndrome (PCOS; 69), fetal growth restriction (70), and preeclampsia (71), and as such have been proposed to serve as biomarkers or targets of therapeutics. Our profiling data indicating the inverse pattern of expression between miR-29c and TGF-β3 expression with antifibrotic and profibrotic functions, respectively, implicate their importance in leiomyoma pathogenesis. In addition, the regulatory interaction between miR-29c and TGF-β3 provides a feed-forward mechanism that could explain leiomyoma growth through ECM accumulation and an ideal therapeutic target to interrupt fibroid growth. This aim could be achieved by overexpression of miR-29c or inhibition of TGF-β3 to disrupt the fibrotic cross-talk between TGF-β3 and miR-29c. The strength of our study is the in vitro demonstration of the link between TGF-β3 known to play a key role in fibrosis associated with leiomyoma and the antifibrotic miRNA miR-29c and the limitation of the study is that it is an in vitro study and unknown if it applies to the in vivo setting.

In summary, our data established a novel interaction between miR-29c and TGF-β3 in leiomyoma cells. In this scheme (Fig. 4C) TGF-β3 is a direct target of miR-29c. Elevated levels of TGF-β3 in fibroids induce the expression of DNMT1, which in turn increases the methylation of miR-29c promoter and its reduced expression. This feed-forward axis would result in increased accumulation of the ECM and progression of fibroid growth.

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El diálogo entre miR-29c y el factor de crecimiento transformante β3 está mediado por un mecanismo epigenético en el mioma

Objetivo: Determinar la expresión de miR-29c y su gen diana factor de crecimiento transformante β3 (TGF-β3) en miomas y los mecanismos de su regulación recíproca.

Diseño: Estudio experimental.

Lugar: laboratorio de investigación académica.

Paciente(s): Mujeres sometidas a histerectomía por mioma.

Intervención(es): Sobreexpresión y subexpresión de miR-29c; bloqueo de ADN metiltransferasa 1 (DNMT1).

Principales resultados: El miR-29c y su gen diana TGF-β3 en el mioma y los efectos del TGF-β3 y el bloqueo de DNMT1 en la expresión de miR-29c.

Resultado(s): El mioma expresó niveles significativamente más bajos de miR-29c, pero una mayor expresión de TGF-β3 en comparación con el miometrio compatible. Las expresiones de TGF-β3 y miR-29c fueron independientes de la raza / etnia. Usando 3' regiones no traducidas de la luciferasa mediante un ensayo, confirmamos que TGF-β3 es una diana directa de miR-29c en células de músculo liso de mioma (LSMC). La ganancia de función de miR-29c en LSMC inhibió la expresión de TGF- β3 a niveles de proteína y ARN mensajero, mientras que la pérdida de función de miR-29c tuvo el efecto contrario. El tratamiento de LSMC con TGF-β3 inhibió la expresión de miR-29c, mientras que estimuló la expresión de DNMT1. La eliminación de DNMT1 a través de la transfección con ARN interferente pequeño disminuyó significativamente la expresión de TGF-β3 e indujo la expresión de miR-29c. La eliminación de DNMT1 también atenuó el efecto inhibitorio de TGF-β3 sobre la expresión de miR-29c. Además, demostramos que TGF-β3 aumentó el nivel de metilación del promotor miR-29c en LSMC.

Conclusión(es): Existe una relación inversa entre la expresión de TGF-β3 y miR-29c en el mioma. El TGF-β3 es una diana directa de miR-29c e inhibe la expresión de miR-29c a través de un mecanismo epigenético. El diálogo cruzado entre miR-29c y TGF-β3 proporciona un mecanismo de fibrosis en mioma.