Decreased expression of microRNA-29 family in leiomyoma contributes to increased major fibrillar collagen production

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Objective: To determine the expression and function of the microRNA-29 family (miRNA-29a, miRNA-29b, miRNA-29c) in human leiomyoma and myometrium.
Design: Basic science experimental design.
Setting: Academic medical center.
Patient(s): Women undergoing surgery for symptomatic uterine fibroids.
Intervention(s): Overexpression and knockdown of miRNA-29a, miRNA-29b, and miRNA-29c in primary leiomyoma and myometrial cells.
Main Outcome Measure(s): [1] Expression of the miRNA-29 family members in vivo in leiomyoma versus myometrium; [2] Major fibrillar collagen (I, II, III) expression in leiomyoma and myometrial cells with manipulation of miRNA-29 species.
Result(s): Members of the miRNA-29 family (29a, 29b, 29c) are all down-regulated in leiomyoma versus myometrium in vivo. The expression of the miRNA-29 family can be successfully modulated in primary leiomyoma and myometrial cells. Overexpression of the miRNA-29 family in leiomyoma cells results in down-regulation of the major fibrillar collagens. Down-regulation of the miRNA-29 species in myometrium results in an increase in collagen type III deposition.
Conclusion(s): The miRNA-29 family is consistently down-regulated in leiomyoma compared to matched myometrial tissue. This down-regulation contributes to the increased collagen seen in leiomyomas versus myometrium. When miRNA-29 members are overexpressed in leiomyoma cells, protein levels of all of the major fibrillar collagens decrease. The miRNA-29 members are potential therapeutic targets in this highly prevalent condition. (Fertil Steril® 2016;106:766–72. ©2016 by American Society for Reproductive Medicine.)
Key Words: Leiomyoma, fibroids, microRNA, collagen, extracellular matrix

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Leiomyomas, or fibroids as they are more commonly known, are benign uterine smooth muscle tumors that represent the most common tumor in reproductive-aged women. By the age of 50 years, these tumors have a prevalence of 60%–65% in white women and >80% in African-American women in the United States. Although they are clinically symptomatic in only 20%–40% of women who have them, most women who have symptoms from fibroids have multiple symptoms, which include heavy uterine bleeding, pelvic pain, infertility, and recurrent pregnancy loss (1). Due to the prevalence and the sequelae of these tumors, they continue to be the leading cause of hysterectomy in the United States and annually represent ≤$34 billion dollars in cost nationally (2). Despite the public health impact of these tumors, we are still early in our understanding of their pathogenesis.

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The primary distinguishing factor between these tumors and their adjacent normal myometrial tissue is the abundance of extracellular matrix. Multiple gene and tissue microarrays have demonstrated that the matrix consists largely of the major fibrillar collagens (types I, II, and III) (3). Although advances have been made in understanding the pathophysiology of the growth of these tumors, there is still not a good understanding of the molecular basis of the extracellular matrix dysregulation seen in leiomyomas (4).

MicroRNAs (miRNAs) have been found to be novel regulators of fibrosis in many disease processes including liver fibrosis, lung fibrosis, and cardiac fibrosis. MicroRNAs are 20–25 nucleotide long noncoding RNAs that are involved in regulation of gene expression by translational repression (5). This repression is the result of either destruction of messenger RNA or destabilization and prevention of translation of messenger RNA. More than 3,000 miRNAs have been identified (6) and bioinformatic estimates place the number of miRNA target sites in the human genome at >45,000 (7). Furthermore, it is now speculated that >60% of human protein coding genes are regulated by miRNAs (7). We and other investigators have demonstrated that, in addition to differential gene expression between leiomyoma and myometrium, there is also a differential expression of miRNAs (8–10), suggesting that miRNAs play a role in gene regulation in these tumors. Although several studies have demonstrated that hormonal and growth factor regulation of miRNAs in leiomyomas alters cell proliferation (11), few have demonstrated a functional role for them in terms of extracellular matrix overproduction (12).

In previously published microarray analysis, we identified 81 differentially expressed miRNAs between leiomyomas and myometrial tissue. The miRNA-29b and miRNA-29c were identified as being among the most significantly down-regulated in leiomyoma versus myometrium (8). The down-regulation of these particular miRNAs is special as the miRNA-29 family of miRNAs have been implicated in fibrosis and in other disease processes including fibrosis after myocardial infarction (13), pulmonary fibrosis (14), and systemic sclerosis (15). Based on recent studies in other fibrotic diseases (16–20), we believe that miRNAs may play a functional role in the aberrant extracellular matrix components found in leiomyomas. Although a previous study has investigated miRNA-29b, to our knowledge, the entire miRNA-29 family has not been considered (21).

The goal of this project is to validate the differential expression of the entire miRNA-29 family (miRNA-29a, miRNA-29b, and miRNA-29c) in leiomyoma versus myometrium and to determine whether these miRNAs have a functional role in leiomyoma extracellular matrix pathogenesis. Based on miRNA microarray studies done by this laboratory and others, as well as studies done in other fibrotic diseases, we hypothesize that all the members of the miRNA-29 family will be down-regulated in leiomyoma versus myometrium. We further hypothesize that this down-regulation contributes to the increased collagen production in these tumors and that overproduction of the miRNA-29 species will lead to decreased collagen production in leiomyoma cells.

MATERIALS AND METHODS

Study Subjects

Uterine leiomyoma and matched myometrial tissue were collected from subjects (n = 20) undergoing hysterectomy for symptomatic uterine leiomyoma, and leiomyoma alone was collected from an additional 10 subjects. The subjects were all premenopausal women 27–49 years old, who were on no hormonal medications within 3 months of surgery and who were nonsmokers. All of the subjects gave written informed consent for participation in the study. The study protocol was approved by the Institutional Review Board of Northwestern University and all surgeries were performed at Northwestern Memorial Hospital.

Tissue Specimens

The resected tissue was collected in the operating room and taken directly to the pathology department where samples were provided to the research team within 1 hour of being removed from the subject. Leiomyoma ranged from 4–12 cm in greatest dimension and samples were routinely obtained at 1–2 cm from the outer capsule of the leiomyoma to avoid variation of findings due to location within the tumor. All of the fibroids were either subserosal or intramural. No submucosal fibroids were used in this study. Myometrium was collected from within 2 cm of the excised leiomyoma. The tissues were rinsed in cold phosphate-buffered saline (PBS) three times and were either flash frozen and stored at -80°C, cut into 2–3 mm^3 pieces, and placed in vials containing RNALater (Ambion) for nucleic acid preservation, or were immediately digested for primary cell isolation.

Nucleic Acid Isolation

Ribonucleic acid was isolated from either tissue or cells using the protocol previously described (8). Briefly, flash frozen tissue specimens were homogenized using a mortar and pestle and liquid nitrogen. The crushed tissue was allowed to incubate in Tri-Reagent (Sigma) for 5 minutes then mixed with one-fifth volume of chloroform. This mixture was then kept on ice for 15 minutes and then centrifuged at 14,000 rpm for 20 minutes. The resultant clear aqueous layer was transferred and mixed with an equal volume of isopropanol. The mixture was then incubated at 4°C for 10 minutes and then centrifuged at 14,000 rpm for 20 minutes. The liquid was removed from the vial, and the formed pellet was resuspended and 1 mL of 75% ethanol was added and centrifuged at 8,000 rpm for 10 minutes. The liquid was again removed and the pellet was allowed to air dry at room temperature for 5 minutes at which time it was resuspended in diethylpyrocarbonate-treated sterile water. The RNA purity and concentration were determined by spectrophotometry using the NanoDrop ND-1000 (NanoDrop Technologies).

Cell Culture

Surgical tissue specimens were rinsed in PBS and placed in a mixture of Hanks' balanced salt solution (HBSS) containing DNase at a concentration of 150 mg/mL and collagenase at

a concentration of 1.5 mg/mL. The tissue in the HBSS mixture was placed in an agitating incubator for 5–8 hours at 37°C. The digested tissue solution was then filtered with a 100-μm cell filter to remove any undigested debris and the resultant solution was centrifuged to collect isolated cells. The digestion mixture was removed from the pelleted cells and the cells were rinsed in fresh cell media (Dulbecco's minimum essential medium [DMEM]/F12 with 10% fetal bovine serum and 1% antibiotic-antimycotic solution) and then plated in 15-cm cell culture plates.

miRNA Expression Polymerase Chain Reaction

MicroRNA expression was determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR). Complementary DNA (cDNA) was made from 10 ng of total RNA from each sample using the TaqMan microRNA reverse transcription kit (Applied Biosystems) and miRNA specific primers for miRNA-29a, miRNA-29b, and miRNA-29c (Applied Biosystems). These cDNA samples, along with RNU48 as a loading control, were then amplified using the ABI TaqMan MiRNA PCR kit (Applied Biosystems) and the ABI Prism 7900HT sequence detection system (Applied Biosystems). The real-time PCR was performed in triplicate in a minimum of three subjects per experiment. Values for each miRNA were normalized to the expression levels of RNU 48 using $2^{-\Delta\Delta CT}$ methodology (22).

Oligonucleotide transfection

To modify the miRNA-29 expression levels in leiomyoma and myometrial cells, we used the Life Technologies PremiRs to overexpress the target miRNAs in leiomyoma cells, and AntimiRs to down-regulate the targeted miRNAs in myometrial cells (Life Technologies). PremiRNA and antimiRNA transfection controls were used for the respective experiments. For the premiRNA transfections of leiomyoma cells, the passage one primary cells were plated in 6-cm dishes and grown to 50% confluency in standard media (DMEM/F12 with 10% fetal bovine serum and 1% antimycotic/antibiotic solution). The cells were then incubated in OptiMEM Reduced Serum Medium (Life Technologies) and transfected with premiRNAs using FuGENE HD (Promega) as the transfection agent, or antimiRNAs using RNAiMAX (Life Technologies) as the transfection agent.

Protein Isolation

After 48 hours of transfection, cell protein lysates were collected with M-PER (ThermoFisher) using the manufacturer's protocol and protein was isolated. The lysates were transferred to fresh tubes and the protein was quantified with the Pierce BCA Colorometric kit (ThermoFisher). The proteins were stored at −80°C until ready for immunoblotting.

Immunoblotting

Isolated proteins were loaded onto 3%–12% Novus Bis-Tri gels (Life Technologies) at 30 mg per lane. The gels were run on the Power Ease 500 per protocol. The separated proteins were transferred to a 20-μm nitrocellulose membrane. Transfer was confirmed with Ponceau S staining of the membrane which was rinsed off with 10% acetic acid. The membranes were blocked with 1% milk in 0.1% TBS-Tween for 2 hours at room temperature and then incubated overnight at 4°C in either COL1A1, COL2A1, or COL3A1 primary antibodies (Santa Cruz Biotechnology). The following morning, the membranes were rinsed in fresh TBS-Tween and incubated in horseradish peroxidase conjugated secondary antibody (Sigma-Aldrich) for 2 hours at room temperature. The secondary antibody was removed and the membranes were rinsed with fresh TBS-Tween. Chemiluminescent detection was done with ECL Prime (GE Life Sciences). After collagen detection, the membranes were stripped with Restore Western Blot Stripping Buffer (ThermoFisher) and incubated in anti-human β-actin (Sigma Aldrich) as a loading control according to the manufacturer's protocol. To better quantify the differences seen after antimiRNA and premiRNA treatments, Image Studio Lite Western Blot Analysis Software (LI-CORE Biosciences) was used per the developer's guidelines. Semiquantitative protein data are presented as a Western blot and quantitative protein data are presented on a mean-fold difference of treated cells relative to their respective controls, corrected for the β-actin internal control.

Statistical Analysis

Real-time RT-PCR data were assessed using $2^{-\Delta\Delta CT}$ calculations normalized to RNU48 (22). The data are reported as mean ± SEM. Means of paired samples were compared using Student's paired two-tailed t test. A $P<.05$ was considered statistically significant.

RESULTS

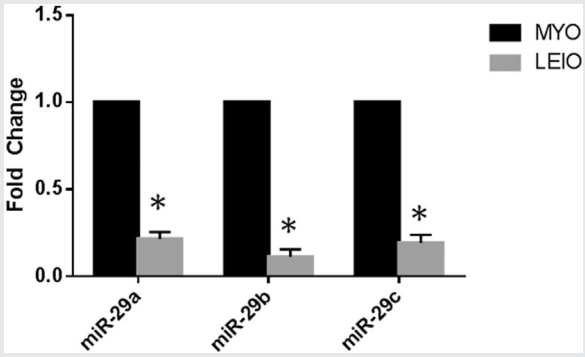
Differential miRNA-29 Expression in Leiomyoma versus Myometrium In Vivo

MicroRNA-29 expression was assessed in matched leiomyoma and myometrial tissue from 20 subjects who had undergone hysterectomy (Fig. 1). Using quantitative RT-PCR, we found that all of the miRNA-29 species (miRNA-29a, miRNA-29b, and miRNA-29c) were significantly down-regulated in leiomyoma versus myometrium in vivo (n = 20, $P<.01$).

Modification of miRNA in Leiomyoma and Myometrial Cells

To assess the function of miRNA-29 in leiomyoma and myometrial cells, we had to first confirm that their expression could be manipulated. Using the Ambion miRNA precursors (premiRNAs), we were able to successfully overexpress all three miRNA-29 members in leiomyoma cell culture as determined by quantitative RT-PCR (n = 5) (Fig. 2A). Also using antimiRNA miRNA inhibitors, we were able to successfully inhibit expression of the miRNA-29 species in cultured myometrial cells (n = 5) as determined by quantitative RT-PCR (Fig. 2B).

FIGURE 1



Real-time polymerase chain reaction (PCR) of miRNA-29 (miR-29) family species in matched myometrium (MYO) and leiomyoma (LEIO) tissue pair (n = 20). MicroRNA-29a, miRNA-29b, and miRNA-29c all show significantly decreased expression in leiomyoma relative to myometrium. *$P<.001$ relative to matched control.

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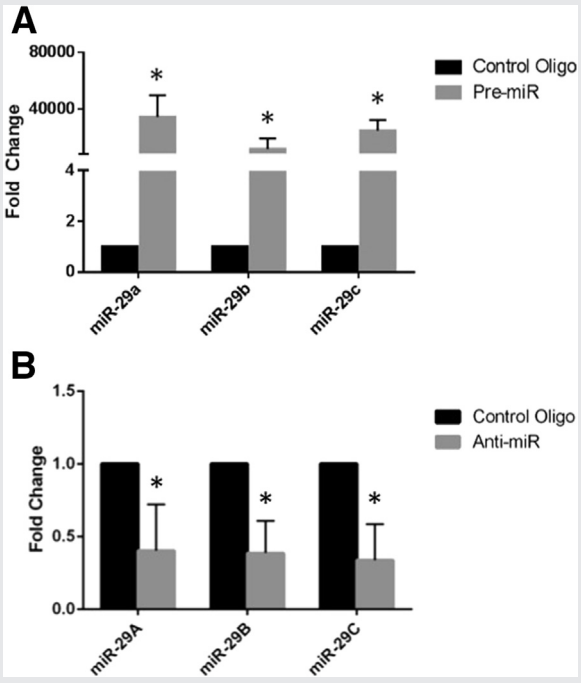
Overexpression of miRNA-29 Leads to Decreased Collagen Production in Leiomyoma Cells

The functional significance of miRNA-29 differential expression in relation to collagen production was determined by overexpressing miRNA-29a, miRNA-29b, and miRNA-29c in leiomyoma cells, using the aforementioned miRNA-29 precursors. The overexpression of the miRNA-29 family members lead to a significant reduction in the major collagens (I, II, and III) relative to the control premiRNA. The experiment was performed in cells from four subjects. A representative experiment is shown in Figure 3. Overexpression of miRNA-29b and miRNA-29c showed a more robust suppression of the major collagens than miRNA-29a.

FIGURE 2



(**A**) Transfection of leiomyoma smooth muscle cells with miRNA-29. PremiRNAs resulting in overexpression versus control. *$P<.001$. (**B**) Transfection of myometrial smooth muscle cells with miRNA-29. AntimiRNAs resulting in decreased expression versus control. *$P<.05$.

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Inhibition of miRNA-29 in Myometrial Cells Leads to Increased Collagen Production

The knockdown of miRNA-29 family members in myometrial cells was assessed in cells from four subjects. A representative experiment is shown in Figure 4. The knockdown of miRNA-29 had no impact on collagen type I or collagen type II, but resulted in increased collagen type III relative to the control antimiRNA.

DISCUSSION

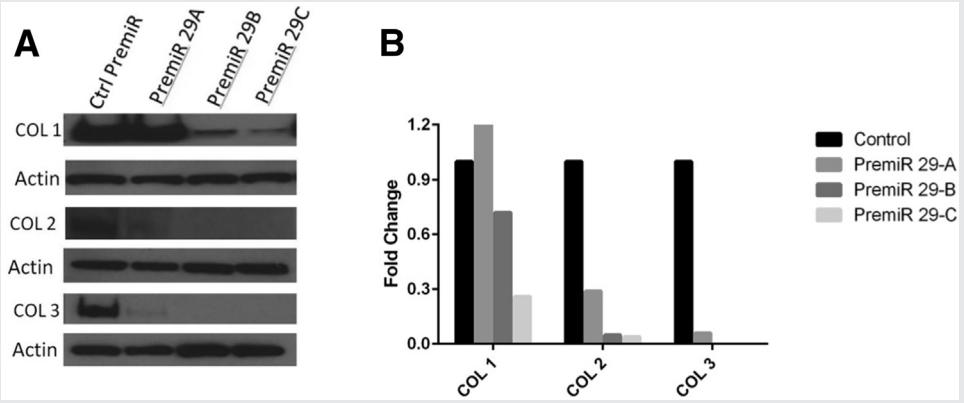
In the present study, we demonstrate that the entire miRNA-29 family is down-regulated in leiomyoma versus myometrium. Using a primary cell culture model, we further demonstrated that the members of the miRNA-29 family are able to regulate the expression of the major fibrillar collagens in both leiomyoma and myometrial cells. To our knowledge, this is one of the first studies to report a functional role of miRNA in the extracellular matrix production in leiomyomas.

Although the etiologies of fibrotic disease can vary significantly, several features are shared by all fibrotic disorders, including activation of myofibroblast cells and excess extracellular matrix production (23). Through their post-transcriptional effect on gene expression, miRNAs have been demonstrated to regulate this process of collagen production in a number of diseases. Cardiac fibrosis was one of the first fibrotic diseases in which miRNAs were implicated as having a contributory role. Since that time, miRNAs have been implicated in many fibrotic disease states including systemic sclerosis (24), renal fibrosis (25), pulmonary fibrosis (26), and liver fibrosis (27). For example, Duisters et al. (28) found that miRNA-30 targets connective tissue growth factor, whereas Thum and colleagues (29) demonstrated that miRNA-21 targets sprout1 and regulated fibroblast survival.

The miRNA-29 family, which is composed of miRNA-29a, miRNA-29b, and miRNA-29c, has specifically been heavily implicated in fibrotic disease (8). This family of miRNAs, differing only in two or three bases, are encoded and transcribed in tandem by two genes located on chromosome 7 and chromosome 1, respectively (30). MicroRNA-29b, in particular, has been suggested to play an especially significant role in fibrotic disease (15). It was first implicated in cardiac fibrosis by van Rooij et al. (31). Maurer et al. (15) found that it was down-regulated and contributed to the progression of systemic sclerosis. Other studies have demonstrated that down-regulation of miRNA-29 contributes to the progression of liver fibrosis (32, 33) and renal fibrosis (25, 34); however, the role of the miRNA-29 family in leiomyoma is largely unexplored.

In 2008, our first generation microarray analysis (8) suggested that miRNA-29b and miRNA-29c are down-regulated

FIGURE 3



(**A**) Western blot of leiomyoma cells transfected with premiRNAs to induce overexpression of miRNA-29 family results in down-regulation of the major fibrillar collagens (COL). (**B**) Quantitative premiRNA data presented as a mean-fold difference of treated cells relative to their respective controls (Ctrl), corrected for the β-actin internal control.

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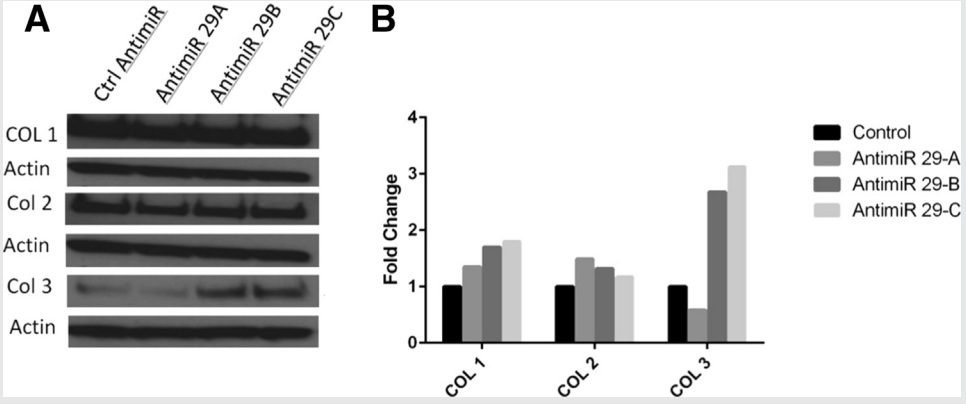
in leiomyoma; a finding supported by the present study. Adding to the findings of Qiang and colleagues (21), which identified a relationship between extracellular matrix production and down-regulation of miRNA-29b, in the present study we considered the entire miRNA-29 family. Beyond reporting the observational finding that the miRNA-29 family was down-regulated in leiomyoma, as it is in other fibrotic diseases, we were able to establish the functional significance of miRNA-29 in relation to collagen production by demonstrating an increase in the major fibrillar collagen expression after down-regulation of all members of the miRNA-29 family. Conversely, we also found that overexpression of the miRNA-29 family members led to a significant reduction in the major collagens—further supporting the functional significance of miRNA-29 differential expression.

Although our primary finding that down-regulation of the miRNA-29 family is associated with an increase in major fibrillar collagen secretion in leiomyoma is novel, the miRNA-29 family, miRNA-29b in particular, has been associated with the fibrotic process in other organs (13, 15, 35). MicroRNA-29 has been found to directly bind to the 3′ untranslated region of collagen type I suggesting in part a direct effect of miRNA-29 on the suppression of collagen translation (36). In addition, the transforming growth factor (TGF)-β/Smad signaling pathway has been implicated in miRNA-29 regulation in renal, pulmonary, and cardiac fibrosis (37). Transforming growth factor-β is known to be dysregulated (i.e., increased) in leiomyoma relative to myometrium (38, 39). This increase, in part, could also explain the down-regulation of miRNA-29 seen in leiomyoma.

These findings are consistent with previous studies from our laboratory and that of other investigators, adding further support to the growing body of research suggesting that the miRNA-29 family plays an important role in fibroid

FIGURE 4



(**A**) Western blot of myometrial cells transfected with antimiRNAs to down-regulate miRNA-29 expression results in increased expression of collagen (COL) type 3. (**B**) Quantitative antimiRNA data presented as a mean-fold difference of treated cells relative to their respective controls (Ctrl), corrected for the β-actin internal control.

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pathogenesis. Although this study may be limited by the inherent potential for intersubject variability when using human samples, the use of human tissue is also a strength as these results are extremely relevant to the clinical disease process at hand. Although only four to five samples were used to perform miRNA overexpression and underexpression studies, miRNA-29 expression was consistently down-regulated in all samples used and the results of the small interfering RNA and overexpression studies were highly consistent. Although the results were highly consistent across all subjects, we did not have menstrual cycle phase data and therefore were not able to comment on possible impact of hormonal status. Though these data indicate a role of miRNA-29 in major fibrillar collagen production, they do not confirm a direct effect of miRNA-29 on the collagen genes. Additional studies are needed to identify the pathway(s) through which the miRNA-29 family exerts its effect on collagen.

In summary, the miRNA-29 family of miRNAs is significantly down-regulated in leiomyoma versus myometrium. This differential expression contributes to the excess extracellular matrix seen in leiomyomas versus myometrium. The disease burden of leiomyomas remains disturbingly high, given that they are benign tumors without direct multisystem sequelae. With limited medical treatment options currently available US Food and Drug Administration approved for leiomyomas, and no long-term medical options are available, the miRNA-29 family potentially represents a novel therapeutic target in the treatment and perhaps prevention of these prevalent morbid tumors. A miRNA-29 mimic, which could be delivered locally, could treat and potentially prevent the development of these collagen-laden tumors.

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