Published in final edited form as:

J Mol Med (Berl). 2018 October ; 96(10): 1095–1106. doi:10.1007/s00109-018-1682-1.

Oxidative stress-induced miRNAs modulate AKT signaling and promote cellular senescence in uterine leiomyoma

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Abstract

Uterine leiomyomas (ULM) grow under high oxidative stress due to a hypoxic microenvironment and defects in redox metabolism. AKT is one major pathway activated by reactive oxygen species (ROS) that maintains ULM growth and survival. We previously reported that AKT inactivated by AKT inhibitors can significantly induce cellular senescence in ULM cells. Since some miRNAs are induced by AKT inhibitors in an ROS-dependent manner, we proposed that these miRNAs may modulate AKT function and cellular senescence in ULM. We therefore established *ex vivo* models of three dimensional ULM spheroid culture system to study the role of miRNAs in cellular senescence. Four miRNAs, *miR-29b*, *miR-181a*, *miR-182* and *miR-200c*, were found to induce cellular senescence in primary ULM and MM spheroid cultures when stably overexpressed.

MiR-181a and *miR-182* were found to repress *AKT3* and *CCND2*, respectively. Correspondingly, RNAi of *AKT3* or *CCND2* also induced cellular senescence and G0/G1 arrest. Thus, *miR-181a* and *miR-182* may drive cellular senescence in ULM by repressing *AKT3* and *CCND2* activity, respectively. We further demonstrated that senescent ULM cells can be effectively removed by BH3 mimetics ABT263, which provides a new therapeutic venue for the treatment of ULM. Our findings suggest that miRNAs are potent modulators in regulating ROS-AKT-cell cycle axis in uterine leiomyoma.

Keywords

Leiomyoma; senescence; miR-181a; miR-182; AKT3; CCND2

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Conflict of interest: Authors have nothing to disclose.

INTRODUCTION

Uterine leiomyomas (ULM) are smooth-muscle tumors of the myometrium that occur in 77% of women in the United States¹. ULM are generally slow growing tumors driven by sex steroid hormones and growth factors. Due to poor vasculature and excessive collagen production, ULMs grow in an unfavorable hypoxic microenvironment^{2, 3}. Such hypoxic conditions increase levels of reactive oxygen species (ROS)^{4, 5}. Recently, we demonstrated that ULM has dysfunctional MnSOD activity leading to accumulation of ROS which can activate AKT signaling⁶. AKT may play a major role in maintaining ULM growth and survival under oxidative stress^{6, 7}. Previous studies have shown that inactivation of AKT by the AKT inhibitor MK2206 in ULM cells enhances ROS production and induces cellular senescence at low doses⁸ or cell death at high doses⁷. Therefore a balance of ROS levels and AKT activity is vital for ULM growth and survival. The underlying cellular and molecular mechanisms remain to be fully characterized and deserve further investigation. Deciphering these molecular mechanisms can be valuable for the development of targeted therapies.

MiRNAs, which are small non-coding RNAs, are early response molecules to cellular stresses. It has been shown that a subset of miRNAs can be induced by oxidative stress^{9, 10}. Induced miRNAs react to oxidative stress through modulating expression of their target genes at the levels of transcription and translation. These target genes may redirect the cells from proliferation to cellular arrest to prevent further cellular damage¹¹. Since hypoxia and increased ROS production in cells can elicit an adaptive response that involves activation of the AKT pathway⁶ and induction of miRNAs that serve as downstream effectors and upstream regulators of AKT activity¹², identification of the key miRNAs that are induced under these conditions will help elucidate the functional relationship between miRNAs and stress response in ULM. ULM are under increased oxidative stress and AKT serves as a major pathway for ULM cell growth and survival. Inactivation of AKT by AKT inhibitors can induce cellular senescence. Many miRNAs can be induced by ROS and we speculated that these miRNAs may modulate AKT function and cellular senescence in ULM. Review of the literature and our previous studies demonstrated that a subset of miRNAs were dysregulated with oxidative exposure. In this study, we studied the miRNAs induced by ROS and AKT inhibitor. We demonstrated that *miR-181a* and *miR-182* repress AKT3 and cyclin D2 and function to promote senescence.

MATERIALS AND METHODS

Collection of tissue samples and culture of primary cells

Fresh surgical specimens of uterine leiomyoma (ULM) and myometrium (MM) were obtained from consented premenopausal women undergoing hysterectomy at the Northwestern University Prentice Women's Hospital (Chicago, IL, USA) and the protocol for acquisition of specimens was approved by Northwestern University's Institutional Review Board (IRB). Study tissue pool include 28 cases with mean age of 44.9 years old (range of 37–55 years old) and tumor size of 9.2±2.7 cm. Among them, 46.4% (13/28) were African American, 32.1% (9/28) were Caucasian, and 21.4% (6/28) of the ethnic group (Supplementary Table 1). Subjects included in the study were not taking hormonal contraceptives or had hormonal treatment for at least 3 months. Tissue was minced into small

pieces and digested with collagenase A and DNAase (Sigma Aldrich) for 5hrs on a 37 °C tissue shaker. The digested material was filtered to obtain a single-cell suspension. The primary cells were cultured in smooth muscle cell basal medium (SmBM; Clonetics, Lonza Group, Switzerland) at 37°C and 5% CO₂ atmosphere no longer than 10 days.

Three dimensional leiomyoma spheroid cultures

Primary cells were suspended and seeded in 6-well (coated with 1.5% agarose gel) or 96-well ultra low attachment culture plates (Corning). Cells were cultured with mesenchymal stem cell medium (Lonza, cat#190632 or cat#PT-3001), and incubated at 37°C in the humidified incubator with 5% CO₂ for at least 48 hrs for formation of spheroids. The spheroids were evaluated with the inverted microscope. Spheroid cultures were maintained for up to 5–7 days and further processed for β -galactosidase staining.

Treatment of Cells

Primary ULM and MM cells or spheroids were treated with 0 (DMSO), 2 and 5 μ M MK-2206 (Selleckchem) for 24hrs; with 0 (3DW), 100, 200 μ M Paraquat (PQ) (Sigma-Aldrich) in serum-free medium for 6hrs; with 0 (DMSO), 5 μ M ABT263 and ABT199 (provided by AbbVie Inc, North Chicago, IL) for 0, 2, 4 and 6 days.

siRNA transfection

ULM and MM cells in monolayers were transfected with ON-TARGET plus SMART pool siRNAs (GE Dharmacon) for CCND2 and AKT3 using Lipofectamine RNAiMAX (ThermoFisher Scientific) according to the manufacturer's instructions. A non-targeting siRNA, (siCTR; GE Dharmacon) was used in parallel as a control for each assay. After 48 hrs, cells were harvested or fixed for further analysis. For spheroids, cells transfected with test and control siRNA in monolayer cultures first and then transferred to three dimensional culture for spheroid formation.

Quantitative real-time RT-PCR

Total RNA was isolated using the mirVana™ RNA Isolation Kit (Ambion, Austin, TX). The detailed method was described previously¹³. Then cDNA was prepared by a Mir-X™ miRNA First Strand Synthesis Kit (Clontech, Mountain View, CA). The entire sequence of mature miRNA was used as miRNA-specific, 5' primer (Suppl Table 2). The 3' primer for qPCR is the mRQ 3' Primer supplied with the kit. For real-time PCR, cDNA was synthesized by PowerUp™ SYBR® Green Master Mix (Life Technologies) using The Applied Biosystems® StepOnePlus™ Real-Time PCR Systems with sequence specific miRNA primers. Relative mRNA levels were calculated using the 2- $\Delta\Delta$ Ct method. The miRNA levels were normalized to U6. Results were obtained from at least three independent experiments performed in triplicate.

Western blotting

Cultured cells were harvested and lysed on ice in RIPA lysis and extraction buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific). The concentration was determined by using BCA Protein Assay kit (Thermo Fisher Scientific). Total protein (30 μ g)

was separated by SDS-PAGE and electro-transferred onto polyvinylidene fluoride membrane (Invitrogen, Grand Island, NY). The membranes were blocked with 5% BSA in TBST, then incubated with primary antibodies overnight at 4°C. The specific horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) was used to blot the target proteins (Suppl Table 3), and the secondary antibody was detected by an enhanced chemiluminescence ECL detection kit (Bio-Rad, Hercules, CA).

β-Galactosidase staining

Senescence-associated β-Gal staining was done as previously described⁸. In brief, monolayer cells were seeded in 6-well plates overnight. After appropriate treatments (siRNA, lentiviral transfection, MK2206, PQ), the cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS at room temperature for 15 minutes. After washing with PBS, cells were stained with staining solution containing 1 mg/mL X-gal and incubated in a CO₂-free incubator at 37°C for 16 hrs. Blue cells were counted under the microscope and statistically analyzed. Five randomly selected fields were captured, and the percentage of cells that were positive for β-galactosidase was calculated and averaged. For spheroids, cells were transfected with miRNA lentivirus for 48 hrs and then change medium, after 5 days, all the spheroids were collected in tubes, then fixed with formaldehyde and 0.2% glutaraldehyde in PBS at room temperature for 15 minutes. After washing with PBS, cells were stained with staining solution containing 1 mg/mL X-gal and incubated in a CO₂-free incubator at 37°C for 3 hrs. The reactions were terminated when the cells were stained blue-green. Spheroids were resuspended in 100 μL 70% glycerinum, and seeded onto glass slide, then visualized under an inverted bright-field microscope.

Lentiviral packaging and transduction

Lentiviral pre-miRNA constructs and viral particles were prepared as previously described¹⁴. Briefly, lentiviral vectors with and without pre-miRNAs were cotransfected with psPAX2 and pMD2G vectors into HEK293T cells. Supernatants were collected at 24 hrs and 48 hrs after transfection and stored in -80°C. For transduction, 5×10⁴ cells were plated in each well of the six-well plates along with 2 mL of medium without antibiotics. After 48 hrs' incubation, the medium was removed and replaced with 1 mL medium containing lentivirus. 48 hrs later, fresh medium containing 1 μg /mL puromycin was added to each well.

Analysis of cell cycle by flow cytometry

Cells were serum-starved for 24 hrs before treatment. Control and treated cells were harvested using 0.25% Trypsin, and washed once with cold PBS. The pellets were resuspended in ice-cold 70% ethanol and stored at -20°C. Samples were incubated with 20 mg/mL propidium iodide 0.1% Triton X-100 staining solution with 0.1 mg/mL RNase A. Cell-cycle distribution was determined using the BD Biosciences FACSCanto II Analyzer. At least 20,000 cells were collected. For these studies, all experiments were repeated three times.

WST-1 viability assay

Primary ULM and MM cells were cultured in 96-well plates. At the end of treatments, WST-1 (BioVision) was added to each well (1:10 dilution) for 4 hrs at 37°C, and absorbance was read at 420 nm with reference wavelength set at 650 nm. Data were analyzed following the manufacturer's manual.

3'UTR of AKT3 vector construction and luciferase reporter assay

The 3' untranslated region (UTR) of human AKT3 containing two putative *miR-181a* binding sites was amplified from human genomic DNA and cloned into psiCHECK2 vector (Promega, Madison, WI, USA) using the XhoI and NotI sites. The psiCHECK2-AKT3-mut construct (replacing the nucleotide at the *miR-181a* 'seed' sequence binding sites¹⁵, Suppl Table 2) was mutated by using the QuickchangeXL Mutagenesis Kit (Stratagene, La Jolla, CA)¹⁶. For the luciferase assay, HEK293T cells were seeded at 1×10^4 cells per well in a 96-well plate. 24 hrs later, cells were transfected with 100 ng of psiCHECK2 control, psiCHECK2-AKT3-WT, or psiCHECK2-AKT3-mut plasmid, and for each well, 20 nM of *miR-181a* mimic or miR-NC was co-transfected with the reporter construct as indicated. 24 hrs later, luciferase activity was measured using the Dual-Luciferase Reporter System (Promega).

Statistical analysis

Results are presented as mean $\pm$ SEM. GraphPad Prism was used for statistical analysis. Student's t-test (two groups) and ANOVA (multiple groups) was used to determine significance. $P < 0.05$ was considered statistically significant, and levels of significance were presented at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, respectively. Data from each patient were considered as an independent experiment.

RESULTS

Oxidative stress-induced miRNA expression in ULM and matched myometrium (MM).

Among those miRNAs regulated by oxidative stress (45 miRNAs were upregulated and 24 downregulated (Suppl Table 4)), we chose 11 miRNAs that were also highly dysregulated in ULM^{17, 18}, including *let-7c*, *miR-22*, *miR-29b*, *miR-96*, *miR-99a*, *miR-145*, *miR-155*, *miR-181a*, *miR-182*, *miR-195*, *miR-200a*, and *miR-200c*. In order to determine which miRNAs were induced by both ROS and AKT inhibitors, primary ULM cells and MM cells were treated with the superoxide anion generator, paraquat (PQ) as well as the AKT inhibitor, MK2206. Among three independent cases, miRNAs (*miR-29b*, *miR-181a*, *miR-182* and *miR-200c*) were repeatedly induced by both PQ and MK2206 in primary ULM cells which was dose dependent (Figure 1B and 1D). Similar results were observed in some but not all MM cells (Figure 1A and 1C). The findings show that these specific miRNAs are inducible under oxidative stress or AKT inactivation.

Establish stable miRNA expression in primary ULM and MM cells

To evaluate the functional role of ROS/MK2206-induced miRNAs in ULM and MM, stable miRNA expression was established using lentiviral constructs which contain pre-miRNAs of

miR-29b, *miR-181a*, *miR-182* or *miR-200c*, by method described previously¹⁴. The efficacy of lentiviral infection was illustrated by bright and diffuse green fluorescence in over 80% of culture cells (Suppl Figure 1). To confirm miRNA overexpression in primary culture ULM and MM cells, miRNA expression was examined by miRNA RT-PCR (Figure 2A). Of note, the fold increase of miRNA expression varied in different miRNA constructs, suggestive of different efficacy in miRNA maturation processes. To evaluate the miRNA function in primary ULM and myometrial cells, we overexpressed miRNAs in the spheroid cultures. After miRNA overexpression was confirmed, the primary cells in monolayers were cultured as spheroids. Upon spheroid formation after 3–5 days, stable lentiviral infection was confirmed by the presence of green fluorescence (Figure 2B).

Overexpression of stress-induced miRNAs resulted in cellular senescence in ULM and MM

To determine whether the selected miRNAs induced senescence, ULM and MM cells overexpressing the miRNAs and cultured as spheroids were fixed and stained for SA- β -galactosidase. As illustrated in Figure 3A and 3B, 3D spheroid MM and ULM showed global senescence pattern and significant increase of cellular senescence was observed in both ULM and MM spheroids with overexpressing *miR-29b*, *miR-181a*, *miR-182*, and *miR-200c* compared to vector controls. The level of senescence in matched MM was relatively lower than in ULM (Figure 3A and 3B). Of note, *miR-181a* and *miR-182* overexpression resulted in an intense and diffusive pattern of β -galactosidase staining in ULM. *miR-29b* overexpression could also increase senescence but not *miR-200c* (Figure 2A and 2B). These data were confirmed in three cases (Supplementary Figure 2). The monolayer of ULM and MM cells with miRNA overexpression were also examined by β -galactosidase staining and were found to be positive (Supplementary Figure 3). In the 2D cultures of primary MM and LM cells, senescence were evaluated in individual cells which showed that *miR-181a* and *miR-182* overexpression resulted in nearly 1.5 fold increase of cellular senescence as detected by β -Gal staining (Supplementary Figure 3B,C). Senescent cells are usually arrested in G0 phase of the cell cycle. Cell cycle analysis showed that about 50% of cells were in G0/G1 phase in primary culture (day 5–7) of both myometrial and leiomyoma cells (Figure 3C and 3D). In contrast, the same primary culture of ULM and MM cells with overexpression of *miR-181a* and *miR-182* had significantly high percentages of G0/G1 cells (69.43% in MM and 79.9% in LM with overexpression of *miR-181a*; 86.96% in MM and 76.84% in LM with overexpression of *miR-182*; $p < 0.05$). These findings further support that *miR-181a* and *miR-182* overexpression can drive smooth muscle cells into cellular arrest or senescence.

MiR-181a represses AKT3 in fibroids

Findings of *miR-181a* and *miR-182* overexpression driving cellular senescence prompted us to investigate the underlying mechanism. We speculated that some *miR-181a* target genes may play an important role in protecting ULM cells from ROS mediated DNA damage and drive ULM cell into senescence. Among the target genes predicted to be targeted by *miR-181a* by PicTar and Target Scan, AKT3 was one of the top predicted target genes. AKT activity is critical for ULM cell survival and proliferation. AKT3 was moderately expressed in both MM and ULM, but varied from case to case (Figure 4A). Transient transfection of *miR-181a* in MM and ULM cells consistently reduced AKT3 expression (Figure 4A). *miR-181a*

mediated repression of phosphorelated AKT were also observed in MM and ULM cells (Figure 4B). Cilicon analysis revealed that there are two highly reserved *miR-181a-5p* binding sites at 3'UTR of AKT3 (Figure 4C). To confirm that *miR-181a-5p* specifically repress AKT3 expression, two *miR-181a-5p* binding sites at 3'UTR of AKT3 were mutated by CRISPR/CAS9 techniques at 3rd and 6th nucleotides complementary to *miR-181a-5p* seed sequence (Figure 4C). Luciferase activity was blocked in wild type 3'UTR, but not in mutant 3'UTR, by *miR-181a-5p* in HEK293 cell line (Figure 4D). Consistently, *miR-181a* overexpression can reduce the luciferase activity when transfection of luciferase with AKT3 3'UTR in myometrial cells (Figure 4E). These results indicate that *miR-181a-5p* can specifically inhibit AKT3 expression.

While *miR-182* has several direct and indirect target genes involved in DNA repair, several cell cycle regulators are also regulated by *miR-182*, including p21 and CCND2¹⁹. When *miR-182* was overexpressed in MM and ULM cells, upregulation of p21 was observed in some but not all cases (data not shown). In contrast, *miR-182* overexpression consistently represses CCND2 expression in all MM and LM cells tested (Figure 4F). Taken together, ROS induced *miR-181a* and *miR-182* result in significant downregulation of AKT3 and CCND2.

Silencing of AKT3 and CCND2 expression drives ULM cell into cellular senescence and cycle arrest.

To further evaluate the functional role of AKT3 in cultured ULM and MM cells, AKT3 expression was silenced by siRNA. Upon AKT3 silencing (Fig 5A), levels of β -galactosidase increased, demonstrating an increase in senescent cells in ULM and MM spheroids (Fig 5C). Moreover, AKT3 silencing increased the percentage of cells in G0/G1 of the cell cycle (Fig 5E). Similarly, silencing CCND2 (Fig 5B), showed increases in β -galactosidase staining and the number of cells in the G0/G1 phase (Fig 5D, F). These data suggest that, in particular, *miR181a* and *miR-182* serve as regulators of AKT3 and CCND2 that eventually drive cells into permanent arrest/senescence.

BH3 mimetics are effectively in reducing viability of senescent cells in ULM.

Recent studies showed that a novel class of therapeutic molecules, BH3-mimetics, can reduce or clear senescent cells through targeting at bcl-2 family²⁰. ABT263 is one of such compounds developed by Abbvie. To evaluate whether ABT263 can be used in reduction or removal of senescent cells in ULM, we examined the level of cellular senescence and cell viability in ULM cells. *miR-181a* and *miR-182* was overexpressed by transduction of *miRNA* lentivirus in fresh collected primary ULM cells. Cells were transferred to spheroid culture and miRNA overexpression were evaluated by fluorescent scope (Figure 6A and B). Then ULM spheroids with *miRNA* overexpression were treated with control (DMSO) and senolytic compound (ABT263). After ABT263 treatment for four days, there was significant increase of cell death under light microscope, demonstrating a ring of diassociated cell debris around spheroids (Figure 6A and 6B). These spheroids were then fixed briefly and further examined by β -galactosidase stain. Notably spheroids treated by ABT263 showed minimal β -galactosidase positive cells in comparison to DMSO control (Figure 6A and 6B). These findings indicate that ABT263 can effectly kill senescence ULM cells induced by

miR-181a and *miR-182*. To further confirm the findings, the viability of ULM cells with miRNA overexpression were examined liquid base assay. As showed in Figure 6 C and D, significantly reduced cell viability could only be seen in ULM cells with *miR-181a* or *miR-182* overexpression when treated by ABT263. The findings suggest that highly senescent ULM cells can be effectively removed by senolytic therapies such as ABT263.

DISCUSSION

Leiomyomas require pro-survival mechanisms to adapt to an unfavorable hypoxic microenvironment. Emerging evidence point to AKT as a major survival pathway in ULM^{6, 7}. Activation of AKT signaling in ULM is in part caused by oxidative stress due to hypoxia and defects of ROS metabolism⁶. Inhibiting the AKT pathway results in a dynamic and transient regulation of specific pathways that ultimately navigate cells into various downstream fates, including decreased proliferation and increased cellular senescence. Direct inactivation of AKT by AKT inhibitors (MK2206) in ULM resulted in significant cellular senescence in vitro⁸. The mechanisms of AKT-mediated cellular senescence are unclear in ULM. Previous studies showed that AKT can either induce or prevent senescence depending on the cellular context. In cells with RAS mutations, activation of AKT can significantly suppress RAS induced senescence and promote malignant tumor cell growth²¹. In ULM and other cell types, increase of ROS exposure can trigger senescence in vitro⁸, but elevated oxidative exposure also activates AKT signaling through depleting PTEN functions⁶. It has been reported that in glioblastoma cells with PTEN mutations, AKT may directly drive cellular senescence²². These findings suggest that AKT may act differently during senescence depending on cell types and functional status. Our findings indicate that miRNAs are important members of the ROS-AKT axis during cellular senescence.

MiRNAs are important regulators of cellular response to ROS and oxidative DNA damage. A subset of miRNAs can be induced by ROS exposure^{8, 23}. ROS-induced miRNAs play a major role in protecting cells from DNA damage²⁴, mitochondria dysfunction²⁵. Since MK2206-induced cellular senescence occurs in a ROS-dependent manner⁸, we proposed that some ROS induced miRNAs may modulate AKT activity and cell cycle genes. We found a subset of miRNAs can be induced by both ROS or AKT inhibitor MK2206 in primary ULM and matched myometrial cells. Overexpression of these miRNAs (*miR-29b*, *miR-181a*, *miR-182*, and *miR-200c*) significantly increased cellular senescence and cell cycle arrest at G0/G1 in both ULM and myometrial cells (Figure 3). Among them, *miR-181a* and *miR-182* overexpression resulted in a strong induction of cellular senescence in all cases of ULM and myometrial cells, observed in both monolayer and spheroid cultures (Figure 3, Suppl Figure 3).

MiR-182 targets multiple pathways. It was shown to negatively regulate BRCA1^{14, 26} and positively regulate P21(CDKN1A) in cells with normal p53 status²⁷. In addition, *miR-182* can regulate cell cycle in response to DNA damage²⁴. A recent study showed *miR-182* overexpression induced G1-phase arrest via inhibition of AKT/FOXO3a signaling²⁸. Interestingly, Cyclin D2 (CCND2) is a major cell cycle regulator downstream of AKT. AKT promotes CCND2 activity through its negative regulation of GSK-3 β ²⁹. CCND2 and

CDK4/6 form a complex to promote cell proliferation at G1/S transition³⁰. The findings of *miR-182* repression of CCND2 in ULM in this study (Figure 4) and other cell types¹⁹ suggest ROS induced G1-phase arrest or senescence in ULM is related to downregulation of CCND2 by *miR-182*.

MiR-181a is recognized as a senescence associated miRNA and is modulated by ROS³¹ and highly expressed in senescent cells^{32, 33}. MiRNA expression profiling revealed continuous changes in the expression of *miR-181a* during the whole senescence process³⁴. Importantly, pathway analysis revealed many *miR-181a* target genes are related to replicative senescence and cell cycle progression, both G1/S and G2/M cell cycle phase transitions and telomere maintenance³⁵. We found *miR-181a* is significantly upregulated in ULM when exposed to ROS. Silicon target gene analysis revealed that several functional genes in the AKT pathway may be directly regulated by *miR-181a*, including IGF2BP2 and AKT3. IGF2BP2 was recently confirmed to be *miR-181a* target³⁶. In this study, we demonstrated that AKT3, a AKT subunit overexpressed in ULM, is the target gene of *miR-181a*. Two conservative binding sites of *miR-181a* at the 3'UTR of AKT were identified. Introducing *miR-181a* overexpression in ULM cells *in vitro* resulted in AKT3 downregulation. When generating specific mutations of *miR-181a* binding sites, *miR-181a* repression of AKT3 was lost. Collectively, these findings confirmed that AKT3 is the direct target of *miR-181a* (Figure 5). This along with its upstream target of IGF2BP2 make *miR-181a* a potent negative regulator for AKT pathway. Taken together, ROS-induced *miR-181a* may promote cellular senescence through its negative regulation of cell cycle and AKT pathway genes.

MiR-29b and *miR-200c* are two stress-responding miRNAs in regulation of cell proliferation and remodeling signaling. These two miRNAs express in low level in primary ULM^{17, 37–40} and can be induced by ROS and AKT inhibitors and also drive ULM cell into senescence. Based on current study and published data, the selected miRNAs can directly or indirectly regulate many functional genes in AKT pathway and they are summarized in Figure 7. These findings support that in an environment of oxidative stress, miRNAs and activation of AKT and its downstream effectors are intricately connected and balanced in order for leiomyoma cells to grow and survive. If the balance is interrupted, oxidative stress can promote cellular senescence or cell death. Understanding these molecular mechanisms in ULM will help to develop targeted therapies to control ULM growth. One of challenge for such efforts is that the senescent cells may produce the undesirable effects by the acquisition of a senescence-associated secretory phenotype (SASP). SASP may promote non-senescent tumor cell growth⁴¹. Apparently, clearance of senescent cells is desirable. Recent studies discovered that ABT263, an anti-bcl-2 family compound, can effectively kill senescent cells²⁰. In our pilot test, when treated with ABT263 in the senescent ULM cells induced by *in vitro* passage and *miR-182* overexpression, significant cell death of senescent cells was observed (Figure 6). This preliminary results provide a great potential for our future study in which a combined senescence and senolytic approach in treating ULM.

In summary, we identified a subset of miRNAs which were inducible by both oxidative stress and AKT inactivation. Overexpression of these miRNAs resulted in significant cellular senescence in ULM *in vitro* and also in *ex vivo* system. Our findings suggest that under

oxidative stress, an interaction of the stress-induced miRNAs and AKT activation are the major cellular and molecular events for cell survival and growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Drs. Serdar Bulun, Debabrata Chakravarti and Ping Yin for their valuable scientific comments and technical supports. Mrs. Stacy Ann Kujawa for consenting patients and providing all fresh samples for the study. All immunostains and histology were performed in Pathology Core Facility. This study was supported by NIH P01HD57877, NSFC grant 81572785 and China Scholarship Council.

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1. A subset of oxidative stress-induced miRNAs involve in AKT signaling in uterine leiomyoma
2. Overexpression of *miR-181a* and *miR-182* resulted in cellular senescence in leiomyoma through repression of AKT3 and CCND2, respectively.
3. Silencing of AKT3 and CCND2 drive leiomyoma cell into senescence and cycle arrest.
4. Application of our newly developed 3D leiomyoma spheroids can provide a quick and reliable *Ex Vivo* model for cytopathologic and functional analysis.
5. BH3 mimetics can effectively reduce the viability of miRNA mediated senescent cells in leiomyoma.

Figure 1:

The selected miRNA expression by real-time quantitative RT-PCR in primary myometrial (MM) and leiomyoma (ULM) cells induced by oxidative stress (PQ) and AKT inhibitor (MK) (n=3). A-B. The relative miRNA expression in MM (A) and ULM (B) cells treated by control (Con, open box, DMSO), paraquat (PQ, 100μM, gray box and 200μM, black box). C-D. The relative miRNA expression in MM (A) and ULM (B) cells treated by control (open box, DMSO), MK2206 (MK, 2μM, gray box and 4μM, black box). E. Relative expression of the selected miRNAs between primary myometrium (MM) and leiomyoma (ULM) were illustrated. Small T bar stands for standard error. *p<0.05; **p<0.01, ***p<0.001.

Figure 2: Stable miRNA expression established by transduction of miRNA lentivirus in primary myometrial (MM) and leiomyoma (ULM) cells. A. MiRNA (listed above the bars) expression was examined and quantified by real-time RT-PCR (see Methods) in primary culture MM and ULM cells in vector control and miRNA lentiviral transduction. Small T bar stands standard error. B. Fluorescent photomicrographs illustrated the efficacy of control and each of four miRNA lentiviral infection (green fluorescent color) in primary three dimensional spheroid culture in MM and ULM cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3:

miR181a and *miR182* overexpression resulted in cellular senescence and arrest at G0–1 status in primary myometrial (MM) and leiomyoma (ULM) cells. A-B. Cellular senescence detected by global β -galactosidase stain (left panels) in 3-dimensional spheroid culture cells of ULM (A) and MM (B) in vector control and four miRNA overexpression (indicated above). The relative expression of β -galactosidase (right panels) was measured by the intensity of blue color detected by Image J software. C-D. Cell cycle analysis of percentage of MM and ULM cells with vector control, *miR-181a* (C) and *miR-182* overexpression in G0/G1 (open box), S (gray box) and G2/M (black box) phases. Small T bar stands for standard error. * $p<0.05$; ** $p<0.01$. The results were calculated in three cases ($n=3$).

Figure 4: Analysis of *miR181a* repression of AKT3 and *miR182* repression of CCND2 in myometrial (MM) and leiomyoma (ULM) cells. A. Western blot analysis of AKT3 expression in MM and ULM cells (n=3) with (+) and without (-) *miR-181a* overexpression. Total AKT, GAPDH and β-actin were used as loading controls. B. Western blot analysis of phosphorlated AKT1/2/3, AKT3, total AKT and Actin expression in MM and ULM cells (n=3) with (+) and without (-) *miR-181a* overexpression. C. Two *miR-181a* binding site sequences at 3'UTR of AKT3 with introduced mutant nucleotides (in red) were prepared. D. Luciferase activity in AKT3 3'UTR sequence without (left, UTR-WT) and with (right, UTR- mut) mutant binding sites of *miR-181a* was examined when treated with miRNA mimic control (miRNC) and *miR-181a* in HEK293T cell line. E. Luciferase activity in AKT3 3'UTR sequence with binding sites of *miR-181a* was examined when treated with miRNA mimic control (miR-NC) and *miR-181a* in myometrial cells. F. Western blot analysis of CCND2 expression in MM and ULM cells (n=3) with and without *miR-182*

overexpression. β -actin was used as loading control. β -actin was used as loading control. Small T bar stands for standard error. * $p < 0.05$; ** $p < 0.01$.

Figure 5:

Silencing *AKT3* and *CCND2* expression by siRNA resulted in cellular senescence in myometrium (MM) and leiomyoma (ULM). A-B. Western blot analysis of *AKT3* and *CCND2* expression with and without siRNA treatment in MM and ULM cells. GAPDH is protein loading controls. C-D. Cellular senescence was detected by global β -galactosidase stain in 3-dimensional spheroid culture of ULM and MM in siRNA of *AKT3* (C) and *CCND2* (D). E-F. Cell cycle analysis of percentage of MM and ULM cells with vector control, siRNA of *AKT* and *CCND2* treatments in G0/G1 (open box), S (gray box) and G2/M (black box) phases. Small T bar stands for standard error. * $p < 0.05$; ** $p < 0.01$.

Figure 6:

Treatment of ABT263, a senolytical compound, resulted in cell death of senescent cells in leiomyoma (ULM) cells. A. Phenotype analysis of spheroids of primary ULM cells with *miR-181a* and *miR-182* overexpression followed by ABT263 treatment. Top panel: strong green fluorescence demonstrating miRNA expression induced by lentivirus; Mid panel: bright microscopic examination demonstrating a ring of diassociated cell debris in ABT263 treated spheroids; lower panel: The relative β -galactosidase stain in ULM spheroids treated by control (DMSO) and test (ABT263). C and D. Relative cell viability measurement in primary culture leiomyoma (ULM) cells at 0–6 days. ULM cells with *miR-181a* (C) or *miR-182* (D) overexpression were treated by DMSO (blue line) and ABT263 (red line). *** $p < 0.001$.

Figure 7: Sketch diagram illustrated the target genes by the selected miRNAs in ROS-AKT-cyclin-senescence pathway.