Atractylodes Lancea Volatile Oils Target ADAR2-miR-181a-5p Signaling to Mesenchymal Stem Cells Chondrogenic Differentiation

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Research Article

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Posted Date: January 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-133036/v1

Atractylodes lancea volatile oils target ADAR2-miR-181a-5p signaling to mesenchymal stem cells chondrogenic differentiation

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Abstract

Background： The Rhizoma Atractylodis has long been recommended for the treatment of different diseases in traditional Chinese medicine. The main component of Rhizoma Atractylodis is Atractylodes lancea volatile oils which possess anti-microorganism, anti-tumour, cognitive protection and immunoregulation. The study aimed to elucidate the mechanism of Atractylodes lancea volatile oils promoting mesenchymal stem cells (MSCs) chondrogenic differentiation.

Method： Atractylodes lancea volatile oils were extracted from Chinese medicine Cangzhu by volatile oil extractor. MSCs culture were treated with Atractylodes lancea volatile oils medium**.** Real-time reverse transcription PCR was conducted to verify the candidate microRNAs discovered by microarray analysis. Western-blot analyzed the expressions of mark genes. Sanger sequences identified the changes of the base pairs, which would be edited by ADAR2 enzyme. Toluidine blue staining identified the changes in cells chondrogenic differentiation.

Result： Treatment of Atractylodes lancea volatile oils increased the chondrogenic cells

differentiation of MSCs. Atractylodes lancea volatile oils promoted the expression of ADAR2 enzyme, which may edit the precursor of miR-181a-5p. A dual-luciferase reporter system assay verified that transcription factors yingyang1(YY1) was targeted by miR-181a-5p which was downregulated in MSCs chondrogenic differentiation.

Conclusion： This work demonstrates the mechanism of Atractylodes lancea volatile oils, promoting MSCs to chondrogenic differentiation. It may provide an alternative strategy for treatment purposes and diagnosis in the clinic.

Keywords: Atractylodes lancea volatile oils; MSCs; ADAR2 enzyme; chondrogenic differentiation; miR-181a-5p; YY1

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Background

Osteoarthritis (OA)is the most common joint disorder and the most common arthritis in the world[1], mainly manifesting by cartilage injury. OA would lead to loss of functions in the patients, mainly manifesting as articular cartilage injury, narrowing space, synovitis and dyskinesia[2]. The pathological changes occur in the final stage of the disease, and at the beginning of the disease, possibly before the cartilage degenerations[3]. The main clinical features are pain and loss of function, and treatment methods include non-drug, drug and surgical methods[4]. OA manifested as cartilage alterations, which mainly concern an imbalance in tissue remodelling due to defection in chondrocyte behaviour[5]. Moreover, the previous study has reported that an increased proliferation activity by chondrocytes that produce inflammatory mediators was one of the features of OA[6]. Clusters of chondrocytes form and the concentration of growth factors in the matrix rises, which attempted to repair the injury areas at an early stage[7, 8].

Mesenchymal stem cells (MSCs) differentiated into chondrocytes, so it has been the most extensively explored as a new therapeutic medium in OA's cells therapy [9]. In animal models of OA, the Molecular mechanisms involved in MSC-based regeneration of injured cartilage and attenuation of joint inflammation has been confirmed. During the developing limb and the synthesis of extracellular matrix components by articular chondrocytes, it was the transforming growth factor-beta took good part in[10]. MSCs-multipotent precursors of connective tissue cells that can be isolated from many tissues, including those of the diarthrodial joint-have emerged as a potential therapy for joints

disease and repair [11]. MSCs express various chemokines and cytokines that could repair the degraded tissue, restore normal tissue metabolism and especially counteract inflammation[12].

The Atractylodes lancea volatile oils are one of the major bioactive components of Rhizoma atractylodis which widely distributed in north Asia[13], named Cangzhu in Chinese medicine. These components have been demonstrated to exhibited a series of benefits, including anti-microorganism, anti-tumour, cognitive protection and immunoregulation[14-16]. Atractylodes lancea volatile oils have been shown to have an inhibitory effect on inflammation due to a sesquiterpene lactone in Atractylodes macrocephala Koidz[17]. For now, there has no research about the Atractylodes lancea volatile oils for treating OA, and the mechanism of anti- osteoarthritis is unclear. We hypothesized that Atractylodes lancea volatile oils could promote chondrogenic differentiation of MSCs to achieve cartilage repair.

Recently, more and more studies focused on epigenetic mechanisms and the associated role of microRNAs in regulating gene expression in OA cartilage, and several miRs were also identified as regulators of chondrocyte signalling pathways[18]. It has reported that more than 25 miRNAs have been implicated in chondrogenesis and OA. In particular, chondrogenic differentiation, chondrocyte proliferation, chondrocyte hypertrophy, endochondral ossification, and proteolytic enzyme regulation are targeted or facilitated by more than one miRNA[19]. The recent evidence in OA research suggested that the transformation of microRNA(miRNA) may affect the development of such disease, which based on the treatment with MSCs.

Pri-miRNAs can be recognized by Adenosine deaminases acting on RNA(ADAR) with a double-stranded structure as the substrates. ADAR enzymes mediate one of the most prevalent forms of post-transcriptional RNA modification exhibited by the conversion of adenosine-to-inosine (A-to-I). RNA editing may transform the processing and final activity of miRNA in different ways. The editing of miRNA precursors might prevent their processing by Drosha/DGCR8 or Dicer, leading to degradation by the nuclease Tudor-SN, which recognizes the inosine residues generated editing[20]. Adenosine to inosine (A-to-I) editing represents a post-transcriptional modification of double-stranded RNA, including miRNA precursors.[21]It was verified that ADARs edit specific adenosine residues of certain miRNA precursors[22]. The 3' UTR of mRNAs edited by ADARs, further increasing the interplay between mRNA targets and miRNAs[23]. Furthermore, the presence of human diseases related to A-to-I RNA editing has recently become known[24-26].

Yin Yang 1(YY1) was predicted as the target gene of miR-181-5p in this work. YY1 was first described in 1991, which is a transcription factor. It broadly expressed in mammalian cells.[27] Chen Etc. revealed many putative miR targets of YY1 during skeletal myoblast differentiation into myotubes by combining computational prediction with expression profiling data.[28] The regulatory role of YY1 in vascular smooth muscle cells differentiation from embryonic stem cells in vitro and in vivo has been verified.[29]So, we hypothesized that YY1 might impact the chondrogenic differentiaiton of MSCs.

Material and method

Extraction for Atractylodes lancea volatile oils.

The Atractylodes lancea volatile oils were separated from Chinese herb Cangzhu by using the Volatile oil extractor. The weight of Cangzhu was 500gram, and the final volume of the Atractylodes volatile oil was 500μl. The obtained volatile oils were subsequently dried over anhydrous $MgSO_4$ and store at 4 °C, and then subjected to GC-MS analysis.

MSCs obtaining and cultivating

Bone marrow was obtained from the femur and tibia of four-week-old Sprague-Dawley (SD) rats. The animal experiment was performed with the regulations of the Institutional Animal Ethics Committee in Guangzhou University of Chinese. The rats bone mesenchymal stem cell basal medium (Cyagen, RASMX-01001) was contained 10% fetal bovine serum and 1% penicillin streptomycin. In the experiment, MSCs were seeded at 4×10^6 cells/well into 10cm plates. The medium was replaced every 3 days and non-adherent cells were removed. When the MSCs were cultured to the third generation, they would be divided into different groups. In the control group, MSCs were incubated with stem cell basal medium. The model groups and other sample groups would be incubated in Atractylodes lancea volatile oils with high glucose medium, depending on different stimulation conditions.

Cytotoxicity assay

Cytotoxic effect of Atractylodes lancea volatile oils on the proliferating cells was detected by Cell Counting Kit 8 (CCK8, Dojindo, Japan). Cells were seeded onto 96-well plates at a density of 3 × 104 cells/well and treated with different concentrations of Atractylodes lancea volatile oils (0,0.3, 3, 30μg/ml) for24 hours. Then we added 10μl of CCK8 solution into each well and incubated the cells for another 3 hours. The absorbance was measured by Multifunctional microplate reader (EnVision Xcite/HTS, PerkinElmer, America) at 450 nm. We then, calculated the cell viability as a percentage of the viable cells in the Atractylodes lancea volatile oils treated group compared with the untreated control.

Oligonucleotides

The oligonucleotides used for PCR were designed by Sangon Biotech co., Ltd.miR-181a-5p RT primer:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC,
Forward CGAACATTCAACGCTGTCG, Reverse AGTGCAGGGTCCGAGGTATT;
pre-miR-181a-5p Forward GTGAACATTCAACGCTGTCGGT,
Reverse GGGTACAATCAACGGTCGATGG;
pri-miR-181a-5p Forward AGGATTGGGCTTCCCTCTGC,
Reverse TCCAAACTCACCGACAGCGT;
U6 RT primer
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA,
Forward AGAGAAGATTAGCATGGCCCCTG,

Reverse ATCCAGTGCAGGGTCCGAGG;

SOX9 Forward GACAACTTTACCAGTTTCGGTC,

Reverse GAGGGAAAACAGAGAACGAAAC;

COLLAGEN2 Forward TTGCGTACCTGGACGAGGAGAC,

Reverse AATGGCAGGCGAGATGGCTTATTC;

AGGRECAN Forward TATGATGTCTACTGCTACGTGG,

Reverse GTAGAGGTAGACAGTTCTCACG ;

ADAR2(ADARB1) Forward GAGGAATGTTCACGGAATAAGC,

Reverse ACACGTTTCTTGGCTGAATTAC;

β-actin Forward TTCGCCATGGATGACGATATC,

Reverse TAGGAGTCCTTCTGACCCATAC;

YY1 Forward GAGGAGGAGGACGACGACGAAG;

Reverse TGGTGGTGGTGGTGGTGATGG.

The expression of mature miRNAs was determined using the $2^{-\triangle\triangle Ct}$ method, using U6 as a reference gene. To identify A-to-I changes in the precursor sequences, PCR products from the analysis of pri- and pre-miRNA-181a-5p were tested by Sanger sequences. To assess the regulation and involvement of the ADAR enzyme in the editing of miRNAs in MSCs chondrogenic differentiation, the expression of the ADAR2 genes was quantified by RT-qPCR.

Transfection

Transfection of siRNA‑ADAR2 in MSCs. Three different siRNA-ADAR2 segments

were purchased from Guangzhou RiboBio Co., Ltd., which specifically bound and degraded the ADAR2 mRNA. Using Lipofectamine® 3000, MSCs were transfected with siRNA-ADAR2-1, siRNA-ADAR2-2 and siRNA-ADAR2-3 (100 nM) for 24 h in 6-well plates ($5x10^5$ cells per well). After 24 h transfection, western blotting and PCR were used to select the most efficient segment.

Western blot analysis

Corresponding antibodies tested the expression of chondrocytes genes. GAPDH (cat.no. ab8245, 1:5000; Abcam); SOX9 (cat. no. ab185966,1:1000 Abcam); COLLAGEN2(cat.no.ab34712;1:1000 Abcam); AGGRECAN(cat.no.ab3778;1:500; Abcam); ADAR2(cat.no.22248-1-AP;1:1000;Proteintech); YY1(cat.no.66281-1-lg) The image acquisition was performed using the Fluor Chem E imaging system (Protein simple co., Ltd., America). Image processing software (ImageJ; Version14.8; National Institutes of Health)

Toluidine blue stain

Toluidine blue is one of the commonly used synthetic dyes that belongs to quinone imine dyes and demonstrates the proteoglycan constituents of chondromucin aggregates. These dyes generally contain two chromophores, an amine group and a quinone-type benzene ring, which constitutes the chromogen. There are two chromophores and auxiliary chromophores in Toluidine blue, which is a basic dye. The cations in toluidine blue have a dyeing effect. The acidic substances of tissue or cells

would be dyed meeting the cation. Frozen sections were stained with toluidine blue (to visualize matrix proteoglycans). Samples were examined by light microscopy (Olympus microscope, Tokyo, Japan)

The main compositions of Atractylodes lancea volatile oils docked with ADAR2 enzyme

Energy minimized three-dimensional structures were used throughout the docking process. ADAR2 enzyme was processed using a protein preparation wizard to ensure chemical correctness and to optimize the protein's structure for docking. A receptor grid has been generated around the ligand-binding site of the ADAR2 enzyme followed up for the docking process. The Glide docking output contains multiple docking combinations ranked according to Glide score, docking score, binding energy, and other properties.

Statistical analysis.

The experimental data are presented as the mean ± SEM. SPSS 23.0 software (IBM corp., Armonk, NY, USA) was used for statistical analysis. The figures were all produced by GraphPad Prism (Version 6.0; GraphPad Software, Inc., La Jolla, cA, USA). Student's t-test was used to analyze the difference of two groups. The comparison between multiple groups was analyzed using one-way analysis of variance followed by Tukey's multiple comparisons test as the post hoc test. $P<0.05$ was considered to indicate the statistical difference.

Result:

Atractylodes lancea volatile oils analysis.

The chemical compositions of the Atractylodes lancea volatile oils were analyzed by GC-MS, which shows the compound names, retention time (tR) and the percentage content of the individual components (Table.1). The 25 principal components of the oils were preliminarily identified by those stored in the spectrometer database of libraries, which represented about 94.57 % of the total detected constituents. Fig.1 was the mass spectrum of Atractylodes lancea volatile oils.

The function of Atractylodes lancea volatile oils on MSCs.

To explore the potential upregulated effects of Atractylodes lancea volatile oils on MSCs, it was treated with Atractylodes lancea volatile oils at various concentrations for 24 h respectively. A CCK8 assay was used to determine its proliferation, which showed that Atractylodes lancea volatile oils had the effects on MSCs in a dose-dependent manner. The result showed that the concentration of Atractylodes lancea volatile oils greater than 15μg/ml would cause the death of cells (Fig.2A). To determine the best concentration of Atractylodes lancea volatile oils for MSCs cultivation, the researches were tested by q-PCR and western blot. By comparing the expression of chondrocytes genes between the concentration of 0.3μg/ml and 3μg/ml, it was found that the best concentration of Atractylodes lancea volatile oils for MSCs cultivation is 3μg/ml (Fig.2 B, C). The expression of SOX9, COLLAGEN2 and AGGRECAN were all upregulated in the 3μg/ml group. Induced by induction with Atractylodes lancea volatile oils, the MSCs aggregates were stained with toluidine blue. Compared with the control group

and 0.3μg/ml treated group, the 3μg/ml treated group had significantly increased toluidine blue staining (Fig.2D).

The Atractylodes lancea volatile oils impact the expression of ADAR enzyme.

There are three subtypes of ADAR enzymes existing, ADAR1, ADAR2 and ADAR3**.** The expressions of ADAR2 and ADAR3 enzyme significantly increased in MSCs with Atractylodes lancea volatile oils (Atr) cultivation (Fig.3A). Compared with the control group, the expression of ADAR2 increased in MSCs cultivation with Atractylodes lancea volatile oils. To confirm the ADAR enzyme function with Atractylodes lancea volatile oils on MSCs chondrogenic differentiation, the expression of the ADAR enzyme was examined by western blot and q-PCR. And the results showed that the expressions of ADAR2 and ADAR3 all increased in Atr group comparing with the control group (Fig.3B). To better understand the molecular mechanisms of components of Atractylodes lancea volatile oils mediated MSCs chondrogenic differentiation, an in silico molecular docking study was performed with corresponding compositions on the crystal structure of ADAR2 domain to determine if any monomer can directly combine. The dockings of the components of Atractylodes lancea volatile oils with ADAR2 enzyme showed that Atractylodes lancea volatile oils bind to the Kelch domain of ADAR2 enzyme efficiently with a glide score from -2.27 to -7.33. (Tab.2)

We assumed that the chondrogenic differentiation of MSCs depended on ADAR2 enzyme. ADAR2 silencing significantly attenuates the effect of MSCs chondrogenic differentiation. To investigate the association between the MSCs differentiation and ADAR2, transfection of siRNA-ADAR2 was performed in the primary medium and

induction with Atractylodes lancea volatile oils. MSCs were transfected with siRNA-ADAR2-1, siRNA-ADAR2-2 and siRNA-ADAR2-3(100nM) for 24h. As presented in Fig.3C, western blotting results showed that siRNA-ADAR2-3 was the most effective segment to silence ADAR2 expression. Compared with the control group, the expressions of chondrocytes genes downregulated in siADAR2 group and upregulated obviously in Atr group. After silencing the ADAR2, MSCs were cultured with Atractylodes lancea volatile oils. It was found that the expressions of chondrocytes genes decreased comparing with the Atr group (Fig.3D). These western blotting results indicated that the capacity of chondrogenic differentiation of MSCs was restricted by ADAR2 enzyme. Comparing with the control, siADAR2 and siADAR2+Atr group, MSCs which were treated with Atr staining deeper. (Fig3E).

The expression of miR−181a-5p decreased in the MSCs chondrogenic differentiation.

To determine whether miRNAs exist in the MSCs chondrogenic differentiation with Atractylodes lancea volatile oils, the authors used a miRNA microarray assay to detect the association of mRNAs with chondrogenic differentiation. A miRNA microarray assay was performed to determine the miRNAs with specific expression in the MSCs induced by Atractylenolide. (Fig.4A) RT-qPCR indicated that, the expressions of miR-181a-5p, miR-181b-5p, miR-181c-5p, miR-181d-5p were all downregulated in Atr group. Especially, the expression of miR-181a-5p decreased significantly in contrast to the control group (Fig.4B) Taken together, the authors took miR-181a-5p containing

Atractylodes lancea volatile oils into the following study.

miR-181a-5p is an obstacle to the process of MSCs chondrogenic differentiation.

The fold change of miR-181a-5p mimic was measured by q-PCR. The expression of miR-181a-5p inhibitor was upregulated comparing with the control group (Fig.5A). The relative expression of miR-181-5p was measured by western blotting. The expression of miR-181a-5p mimic decreased in MSCs chondrocytes differentiation comparing the miR-181a-5p inhibitor group, which confirmed that miR-181a-5p was the negative regulator for MSCs differentiation (Fig.5B). The MSCs were treated with mimic and inhibitor of miR-181a-5p staining with Toluidine blue. The results exhibited that the dyeing of control and inhibitor groups were all deeper than mimic group (Fig.5C), which determined that reducing the expression of miR-181a-5p was beneficial for MSCs chondrogenic differentiation.

To verify the different expression of miR-181a-5p in Atr and siADAR2, the fold change showed that miR-181a-5p increased significantly in the group of siADAR2, comparing with the control group and Atr group. Furthermore, according to the sequences results, the conversions of adenosine to inosine were regarded as adenosine to guanosine. (Fig.5D) These analyses showed that ADAR2 enzyme edited the precursor of miR-181a-5p in MSCs chondrocytes differentiation. The base-pairs of pre-miR-181a-5p were replaced, so as the function of miR-181a-5p might be changed. This suggests an active regulatory role for miR-181a-5p in the editing of miRNAs in MSCs chondrocytes differentiation, mediated by the negative regulation of ADAR2 transcripts.

These results assumed that miR-181a-5p positively regulated MSCs chondrogenic differentiation, which may be depended on the editing of ADAR2 enzyme.

miR-181a-5p directly targets the 3'UTR of YY1 mRNA in MSCs. Using bioinformatics software(TargetScan,www.targetscan.org;miRBase,www.mirbase.org), the present study demonstrated that there were predicted binding sites of miR‑181a-5p in the 3'-UTR of YY1 mRNA. Western blotting exhibited that the expression of YY1 decreased in miR-181a-5p mimic group comparing with the control group and miR-181a-5p inhibitor group (Fig.6A). To verify this result, a dual-luciferase reporter system assay was performed in MSCs transfected with miR-181a5p mimic, miR-181a5p inhibitor, YY1 WT and YY1 MUT. Compared with the NC group, the relative luciferase activity of MSCs transfected with wt-mimic significantly decreased, and mut-mimic showed no significant change (Fig.6B). These results confirmed that the 3'‑UTR of YY1 mRNA might be the target of miR-181a-5p in MSCs. The plasmid profile was presented in Fig. 6C.

To analyze the effect of YY1 in vitro, the present study performed RNA interference (RNAi) to silence the expression of YY1 in MSCs which were transfected with siRNA-YY1 (100nM) for 24 h in 6-well plates ($1x10^5$ cells per well) to select the most effective segment. Western blotting results demonstrated that siRNA-YY1-3, which transfected for 24h was the most effective segment to silence the expression of YY1(Fig.6D).

ADAR2 and YY1 silencing impaired MSCs differentiating into chondrocytes. Compared with the control group, the expressions of SOX9, COLLAGEN2 and were

significantly decreased in the siRNA-ADAR2 and siRNA-YY1 group, and the expression of miR-181a-5p increased significantly in the siRNA-ADAR2 and siRNA-YY1 group (Fig.6E). These results indicated that miR-181a-5p might promote MSCs chondrogenic differentiation with silencing ADAR2 and YY1 expression. Moreover, the stain of toluidine blue showed that the degree dyeing of MSCs with siADAR2, siYY1 and siADAR2+siYY1 were all lighter than negative control groups. (Fig.6F). These results showed that ADAR2 and YY1 were upregulated factors in MSCs chondrogenic differentiation.

Discussion:

Cartilage injury is a causative factor leading to osteoarthritis, and Chinese medicine may serve a protective role in the delay of cartilage injury. Promoting the MSCs chondrogenic differentiation contributes to the treatment of cartilage injury in the clinic. However, few studies have focused on the roles of miRNA in MSCs chondrogenic differentiation with effective components of Chinese medicine. The present study has explored that Atractylodes lancea volatile oils are beneficial for MSCs chondrogenic differentiation through ADAR2 enzyme regulating miR-181a-5p. The significant results of the present study were as follows: i) Atractylodes lancea volatile oils promoted the MSCs chondrogenic differentiation; ii) miR-181a-5p decreased in MSCs chondrogenic differentiation and the mRNA of YY1 was the effective target; iii) ADAR2 enzyme may edit the base pair of miR-181a-5p; iiii) miR-181a-5p negatively regulated the MSCs chondrogenic differentiation without the interference of ADAR2

353 enzyme and YY1. The results also demonstrated that the ADAR2-miR-181a-5p
354 signalling pathway may serve an essential role in MSCs chondrogenic differentiation,
355 and this pathway may be a pivotal target for treatment in cartilage injury.

356 ADAR2 edits the pri-miR-142-3p and impairs cell proliferation and migration[30],
357 which has been reported. An important result of the present study was that ADAR2
358 enzyme edited base pair of pre-miR-181a-5p so that the function of miR-181a-5p would
359 be changed. Previous studies have demonstrated that the ADAR enzyme was critical
360 for human embryonic stem cells differentiation and neural induction by regulating
361 miRNA biogenesis via direct RNA interaction[31]. And the ADAR2, which was
362 identified as a promising target for an innovative anti-tumoral strategy, was treated as a
363 radar enzyme that maintains a degree of editing in the miRNA population and balances
364 miRNA expression[32]. It has been reported about the RNA editing in inhibiting cancer
365 cell proliferation and differentiation[33], which focused on an ADAR2-catalyzed RNA
366 editing site within microRNA seed region[34, 35]. The present study suggested that the
367 expression of miR-181a-5p has upregulated on MSCs chondrogenic differentiation,
368 which was due to the editing by ADAR2 enzyme.

369 Another important finding of the present study was that Atractylodes lancea
370 volatile oils promoted MSCs chondrogenic differentiation. Since ancient time,
371 Atractylenolide’s roles have been discovered in many other diseases, such as
372 expectorant tocolytic effects, and the key components responsible for the usage of
373 Atractylenolide were the volatile oils[36]. Microarray data and q-PCR detection
374 identified that Atractylodes lancea volatile oils specifically inhibited the expression of

miR-181a-5p in the chondrogenic differentiation of MSCs. These results were consistent with the previous study that suggested that Atractylenolide, one of the major sesquiterpenes of Rhizoma atractylodis, induced MSCs to differentiate into chondrocytes, which is promising for bony disease therapy[37]. And the present result of docking showed that Atractylodes may bind to ADAR2 enzyme, then activate the signalling of MSCs chondrogenic differentiation.

It has been reported that miR-181a-5p inhibits chondrocyte proliferation and promotes chondrocyte apoptosis[38, 39]. In the present study, an important mechanism was revealed that miR-181a-5p was observed to regulate MSCs chondrogenic differentiation by targeting the mRNA of YY1 which is a ubiquitously expressed transcription factor that functions in cooperation with various cofactors to regulate gene expression. Bioinformatics analysis predicted that miR-181a-5p targets YY1Mrna and YY1 served an important transcript factor which came from the promoter region of SOX9.In previous study, it has been reported that the deficiency of YY1 reduced not only the severity of arthritis and joint destruction but also related pro-inflammatory cytokine[40], and the YY1 which regulates the expression of the cartilage-specific gene in mesenchymal stem cells[41] was the activator for GDF5 during the process of osteoarthritis[42]. To verify YY1 functions in MSCs chondrogenic differentiation, RNAi experiments were performed with YY1 silencing. The present study results confirmed that YY1 inhibited the expression of miR-181a-5p, which resulted in the increased level of MSCs chondrogenic differentiation. The previous study has investigated that miR-181a-5p was a critical mediator that took part in the destruction

of lumbar facet joint cartilage [43]. And the recent study has confirmed that the miR-181a-5p was a negative factor on MSCs chondrogenic differentiation.

Conclusion

Our finding suggests several important clinical implications of these results. Firstly, there are currently few therapies on effect components of Chinese medicine to protect against cartilage injury progression. The present study has revealed that the Atractylodes lancea volatile oils can promote MSCs chondrogenic differentiation or be used alone to stimulate chondrogenic differentiation. Secondly, miR-181a-5p may be a potential biomarker for the early diagnosis of cartilage injury. miR-181a-5p decreased in the MSCs chondrogenic differentiation, which may remind people to prevent osteoarthritis-related disease. Finally, it is crucial to identify the ADAR2 enzyme as a regulator for miR-181a-5p to protect against cartilage injury effectively. The above study indicates that the Atractylodes lancea volatile oils can be used as an alternative strategy in the induction of chondrogenesis of MSCs for treatment purpose.

Abbreviations

Atr: Atractylodes lancea volatile oils

OA: Osteoarthritis

MSCs: mesenchymal stem cells

ADAR enzyme: Adenosine deaminase acting on RNA (ADAR) enzymes

YY1: yinyang1

miR: microRNA

Acknowledgements

Not applicable.

Authors' contributions

YSY performed the experiments and wrote the manuscript. WWS performed the experiments and analyzed the data. WQ and ZL provided the reagents and materials. YLX and Ziwei Luo performed GC-MS. HP, XRL and ZFH performed the Toluidine blue staining and cells culturing, DFC designed the experiments and gave pratical technical guidance. All authors read and approved the final draft of the manuscript.

Funding

This study was supported by the Natural Science Foundation of Guangdong Province, China (grant no. 2017A030312009)

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal use was approved by the Ethical Committee of the Guangzhou

University of Chinese Medicine.

Competing interests

The authors declare that there is no competing interest.

Consent for publication

Not applicable

Figure legends

Fig.1 GC-MS analysis of Atractylodes lancea volatile oils. The identification of Atractylodes lancea volatile oils was analyzed by GC-MS.

Fig.2 Atractylodes lancea volatile oils enhance MSCs chondrogenic differentiation. (A)The MSCs were seeded at a density of 4×10^4 cells in 96-well plates. Cells were treated as control, DMSO, 0.3μg/ml,3μg/ml,15μg/ml,30μg/ml groups. The CCK8 assay analyzed the cytotoxicity of Atractylodes lancea volatile oils for MSCs chondrogenic differentiation. ****P<0.05 vs. control group (B) The MSCs were seeded at a density of 2.1×10^6 cells in 6mm dishes for 7days and treated with different concentrations of Atractylodes lancea volatile oils. Cells were then harvested, and the mRNA levels of SOX-9, type II collagen and Aggrecan were analyzed by q-PCR. *, **, *** P<0.05, ****P<0.001 vs. control group (C)The protein levels of SOX-9, type II collagen and Aggrecan were analyzed by Western blot. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. *, ** P<0.05 vs. control group (D) The MSCs were seeded at a density of 1.2×10^6 in 6-well plates for 7days.The toluidine blue staining was used for chondrogenic differentiation, see the higher panel.

Fig.3 ADAR2 enzymes express positively in Atractylodes lancea volatile oils(Atr). The MSCs were seeded at a density of 7×106 cells in 10mm dishes and treated with Atractylodes lancea volatile oils. (A) The protein levels of ADAR1, ADAR2 and ADAR3 were analyzed by Western blot. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. * P<0.05 vs. control group (B) The mRNA levels of ADAR1, ADAR2 and ADAR3 were analyzed by q-pcr. **, *** P<0.05 vs. control group (C)Cells were treated with silent segments of ADAR2 and then analyzed by Western blot to identify the most effective segment. * P<0.05 vs. control group (D)Cells were treated with control, siADAR2, Atr and siADAR2+Atr group, and then analyzed by Western blot to identify the effect of siADAR2 in chondrogenic differentiation with Atractylodes lancea volatile oils. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. *, ** P<0.05, ****P<0.001 vs. control group, #### P<0.001vs. Atr group (E) The toluidine blue staining identified the MSCs chondrogenic differentiation in treatment with siADAR2 and Atractylodes lancea volatile oils.

Fig.4 An inverse association between Atractylodes lancea volatile oils and miR-181-5p in MSCs chondrogenic differentiation. Cells were treated with Atractylenolide and then analyzed by miRNA microarray assay. (A) The expression of miR-181a-5p decreased

in the miRNA microarray assay. (B) There are four subtypes of miR-181-5p, and the expression of miR-181a-5p decreased in Atractylenolide group, comparing with the control group. ** P<0.05 vs. control group

Fig.5 miR-181a-5p downregulates the MSCs chondrogenic differentiation. Cells were treated with mimic and inhibitor of miR-181a-5p. (A)The mRNA levels of SOX-9, type II collagen and Aggrecan were analyzed by q-pcr. *, *** P<0.05, ****P<0.001 vs. control group (B)The protein levels of SOX-9, type II collagen and Aggrecan were analyzed by Western blot. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. *, ** P<0.05 vs. control group (C)The toluidine blue identified the MSCs chondrogenic differentiation in treatment with miR-181a-5p. (D) Cells were treated with control, Atr and siADAR2 group, and then analyzed by q-pcr and Sanger sequence. *, *** P<0.05 vs. control group

Fig.6 The 3'-UTR of YY1 was the specific target of miR-181a-5p. (A)The protein level of YY1 was analyzed by Western blot. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. *, *** P<0.05 vs. control group (B) miR-181a-5p reduced the relative luciferase activity of pLUc-YY1. * P<0.05 vs. wt+inhibitor NC group, ** P<0.05 vs. wt+mimic NC group (C) The construction profile of the pLUC-YY1 plasmid. The binding sequence in the 3'-UTR of YY1 was replaced in the pLUc-YY1 MUT plasmid. (D) Western blotting identified the most effective fragment of YY1 in silence. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. * P<0.05 vs. nc group (E)Cells were treated with NC, siADAR2, siYY1, siADAR2 and siADAR2+siYYY1 group. The chondrocytes genes were analyzed by q-pcr to determine the differential expression in siADAR2 and siYY1. *** P<0.05 vs. NC group, **** P<0.01 vs. NC group (F) The toluidine blue identified the MSCs chondrogenic differentiation in treatment with siADAR2 and siYY1.

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Figures

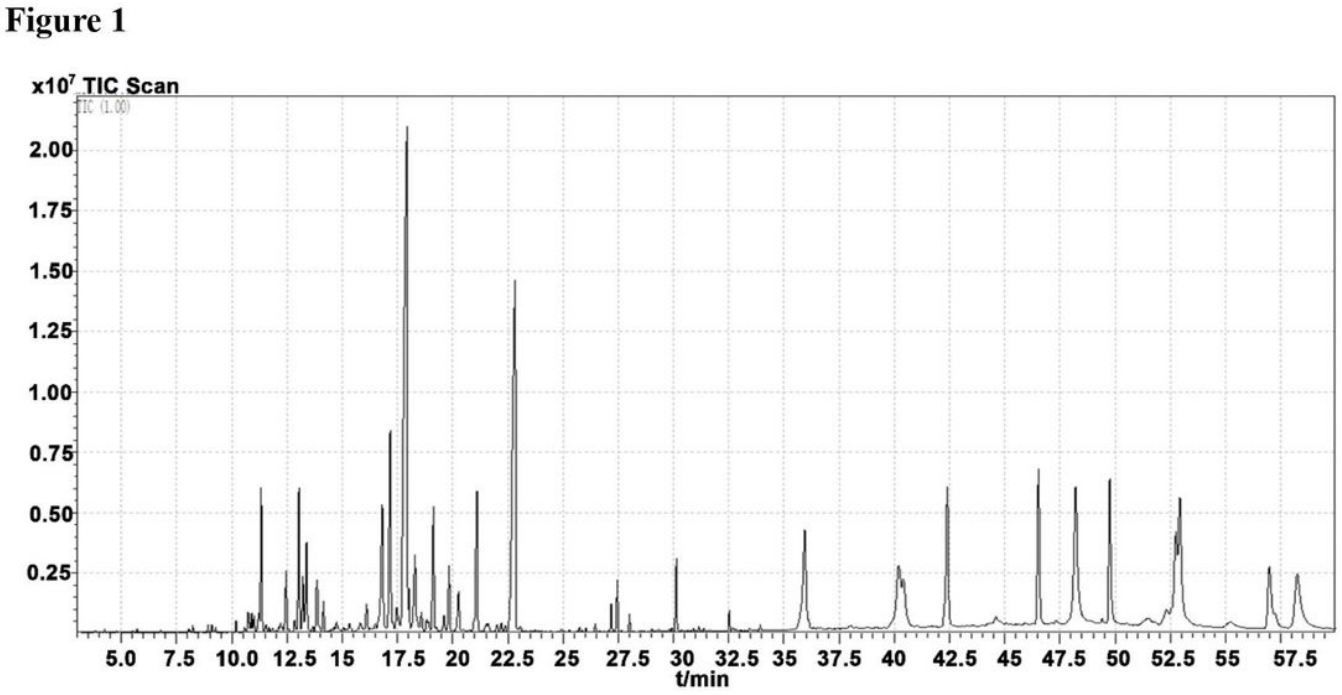

Figure 1

GC-MS analysis of Atractylodes lancea volatile oils. The identification of Atractylodes lancea volatile oils was analyzed by GC-MS.

Figure 2

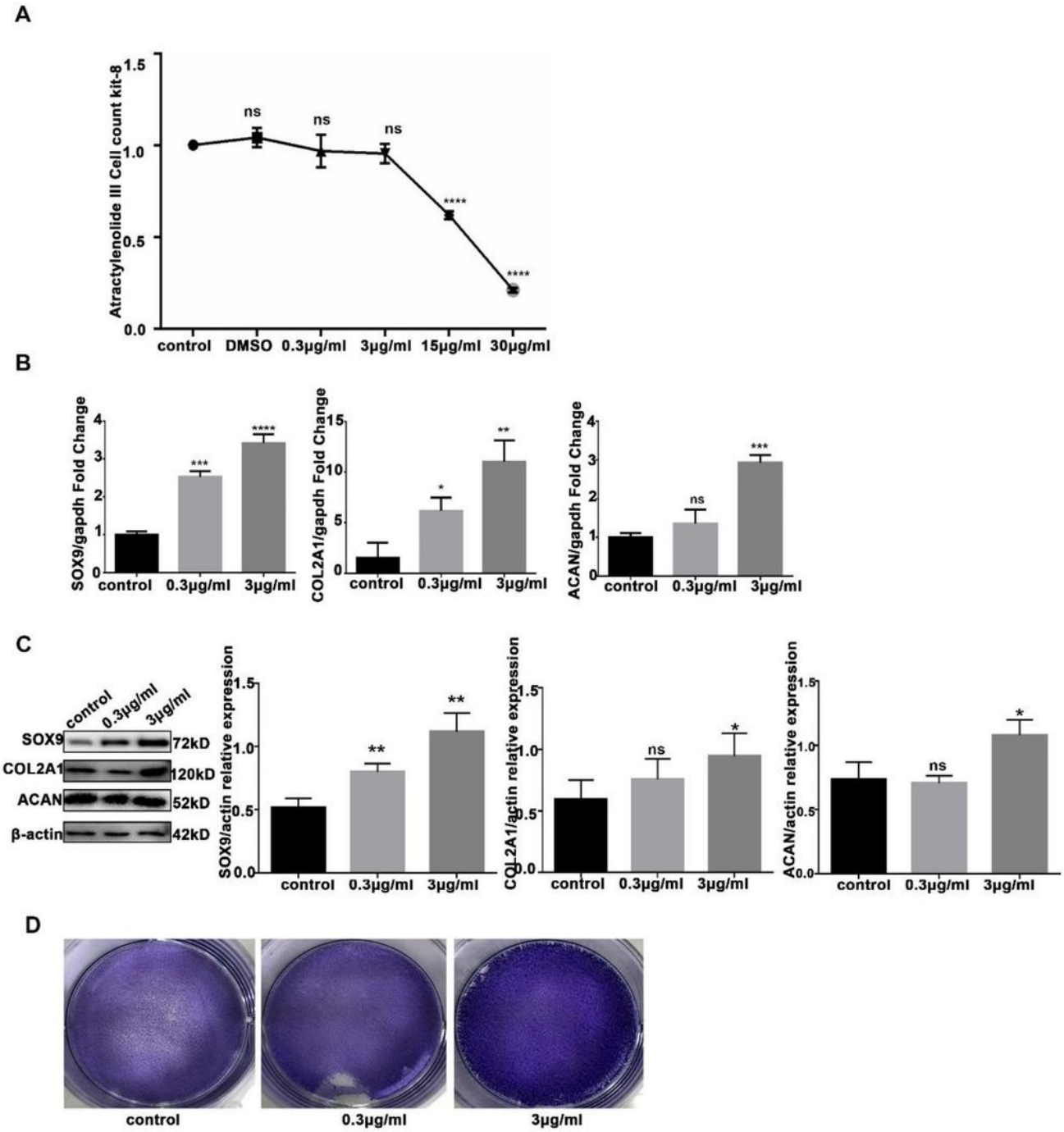

Figure 2

Atractylodes lancea volatile oils enhance MSCs chondrogenic differentiation. (A)The MSCs were seeded at a density of 4×104 cells in 96-well plates. Cells were treated as control, DMSO, 0.3μg/ml,3μg/ml,15μg/ml,30μg/ml groups. The CCK8 assay analyzed the cytotoxicity of Atractylodes lancea volatile oils for MSCs chondrogenic differentiation. ****P<0.05 vs. control group (B) The MSCs were seeded at a density of 2.1×106 cells in 6mm dishes for 7days and treated with different

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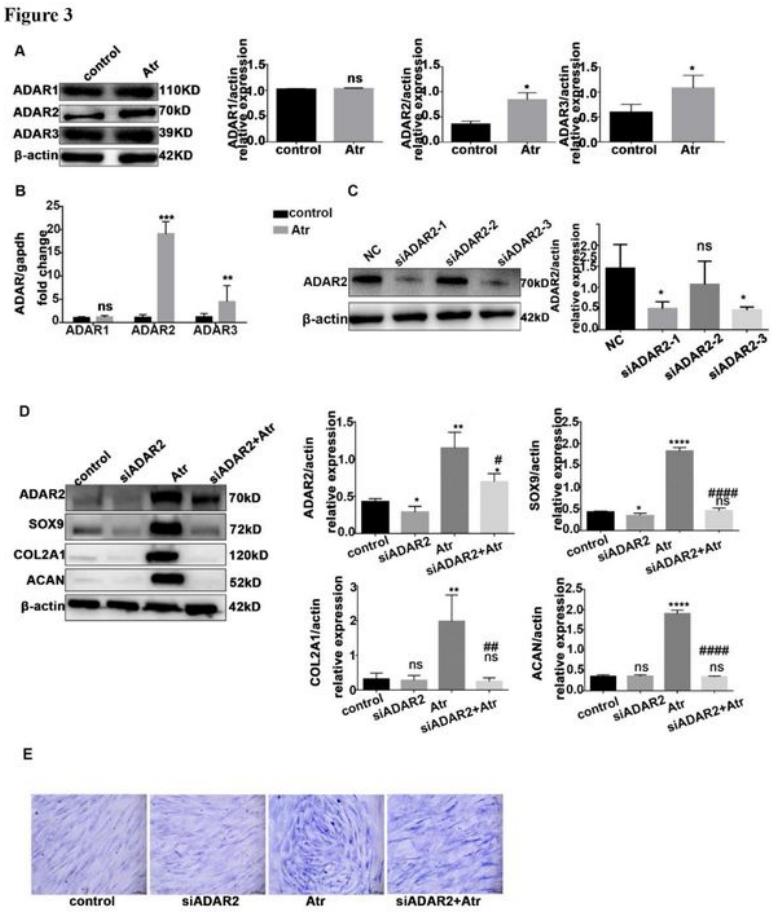


Figure 3

ADAR2 enzymes express positively in Atractylodes lancea volatile oils(Atr). The MSCs were seeded at a density of 7×106 cells in 10mm dishes and treated with Atractylodes lancea volatile oils. (A) The protein levels of ADAR1, ADAR2 and ADAR3 were analyzed by Western blot. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. * $P<0.05$ vs. control group (B) The mRNA levels of ADAR1, ADAR2 and ADAR3 were analyzed by q-pcr. **, *** $P<0.05$ vs. control group (C)Cells were treated with silent segments of ADAR2 and then analyzed by Western blot to identify the most effective segment. * $P<0.05$ vs. control group (D)Cells were treated with control, siADAR2, Atr and siADAR2+Atr group, and then analyzed by Western blot to identify the effect of siADAR2 in chondrogenic differentiation with Atractylodes lancea volatile oils. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. *, ** $P<0.05$, ****$P<0.001$ vs. control group, #### $P<0.001$vs. Atr group (E) The toluidine blue staining identified the MSCs chondrogenic differentiation in treatment with siADAR2 and Atractylodes lancea volatile oils.

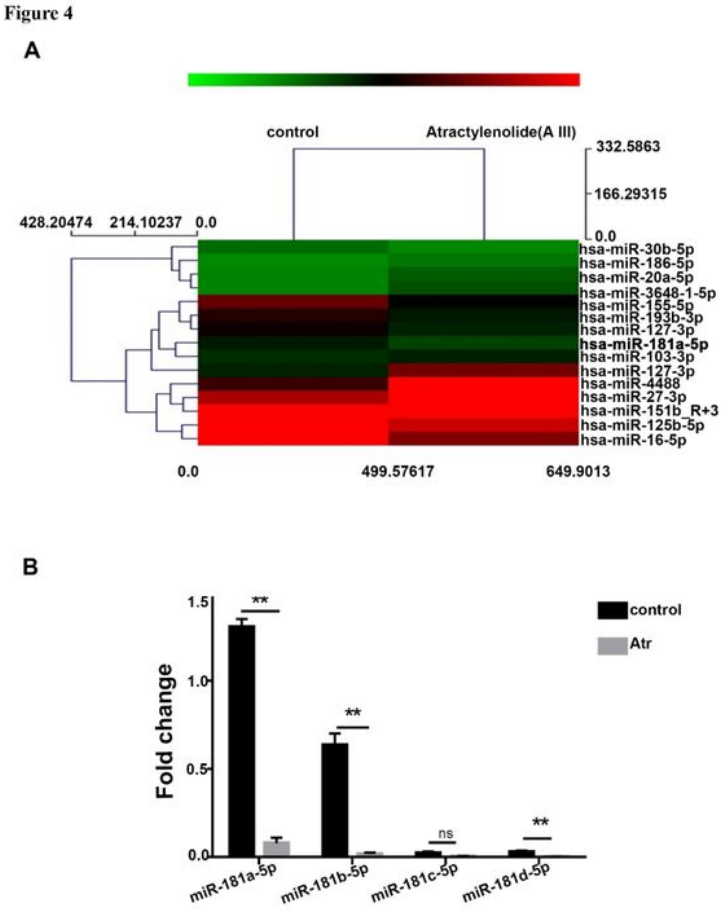
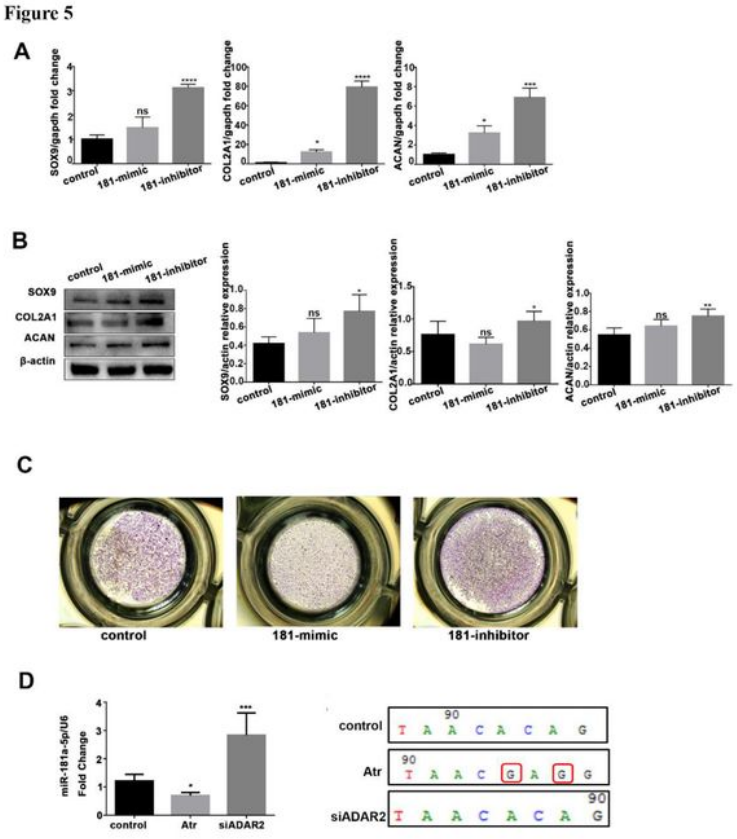


Figure 4

An inverse association between Atractylodes lancea volatile oils and miR-181-5p in MSCs chondrogenic differentiation. Cells were treated with Atractylenolide and then analyzed by miRNA microarray assay. (A) The expression of miR-181a-5p decreased in the miRNA microarray assay. (B) There are four subtypes of miR-181-5p, and the expression of miR-181a-5p decreased in Atractylenolide group, comparing with the control group. ** P<0.05 vs. control group



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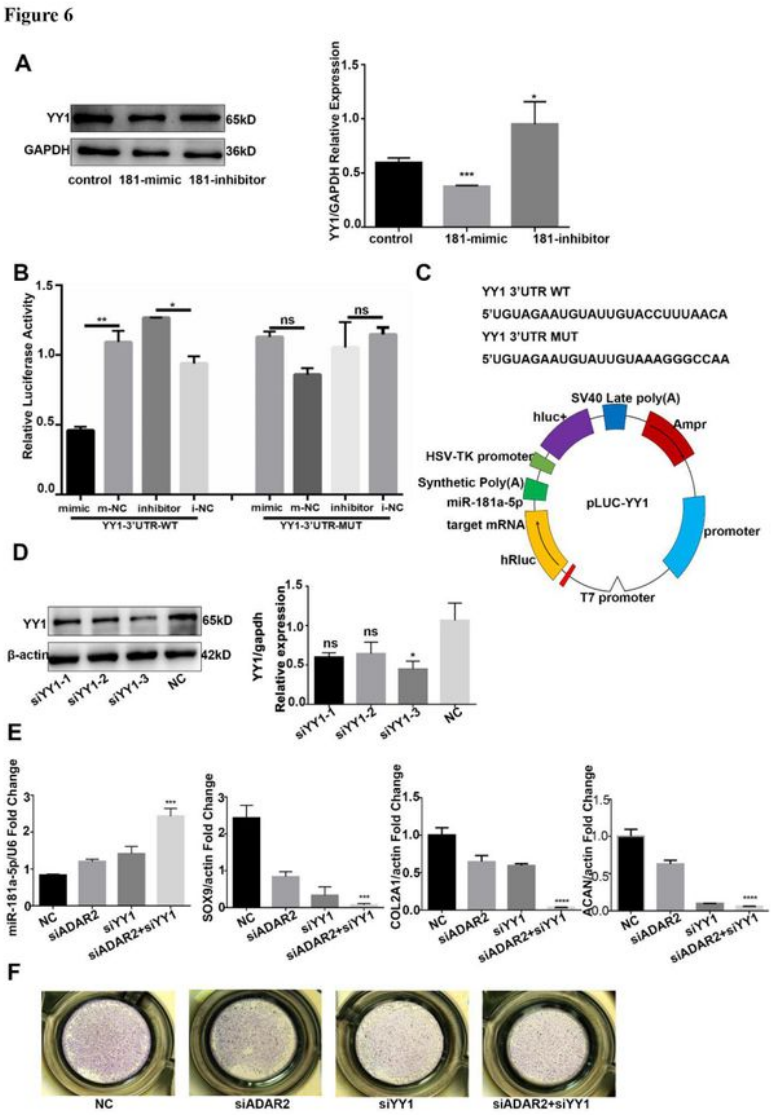

Figure 6

The 3'UTR of YY1 was the specific target of miR181a-5p. (A)The protein level of YY1 was analyzed by Western blot. The grouping of gels cropped from different parts of the same gel with target gene and β-actin. *, *** P<0.05 vs. control group (B) miR-181a-5p reduced the relative luciferase activity of pLUc-YY1.

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