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DNA methylation regulates miR-140-5p and miR-146a expression in osteoarthritis

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ABSTRACT

Aims: Previous studies have demonstrated that transcriptional silencing of miRNAs due to DNA hypermethylation is associated with different pathologies. It has also been reported that abnormal expression of miR-140-5p and miR-146a is linked to osteoarthritis (OA) progression. In this study, we investigated the role of DNA methylation on miR-140-5p and miR-146a expression in OA.
Main methods: miR-140-5p and miR-146a expression was investigated by qRT-PCR. The methylation status of miR-140 and miR-146a regulatory regions was analyzed using qMSP and bisulfite sequencing analysis. SMAD-3 and NF-kB binding to miR-140 and miR-146a regulatory regions was assessed by ChIP assay and knockdown experiments. OA-related genes' expression was evaluated in 5-AzadC, miRNAs inhibitor and 5-AzadC/miRNAs inhibitor-treated cells.
Key findings: Hypermethylation of specific CpG sites in miR-140 and miR-146a regulatory regions was associated with downregulation of miR-140-5p and miR-146a in OA chondrocytes and synoviocytes, respectively. 5-AzadC-induced miR-140-5p and miR-146a upregulation was observed in OA chondrocytes and synoviocytes. Moreover, we found decreased binding affinity of SMAD-3 and NF-kB transcription factors on the hypermethylated miR-140-5p and miR-146a regulatory regions, respectively. Downregulation of MMP-13 and ADAMTS-5 in 5-AzadC-treated OA chondrocytes was prevented by miR-140-5p inhibitor transfection. Similarly, 5-AzadC-treated OA synoviocytes showed decreased expression of IRAK-1, IL1B and IL-6, which was reversed following 5-AzadC-/miR-146a inhibitor treatment.
Significance: Our results strongly suggest the impact of DNA methylation on miR-140-5p and miR-146a suppression in OA chondrocytes and synoviocytes, contributing to OA pathogenesis.

1. Introduction

Epigenetic mechanisms can modify gene expression without changes in DNA sequence and depend on environmental stimuli and developmental stage [1]. Epigenetic modifications have been associated with many pathological conditions including osteoarthritis (OA) [2,3]. OA is an age-related disease characterized by progressive degradation of cartilage extracellular matrix (ECM [4]). It affects approximately 40% of individuals over the age of 70 and is the leading cause of pain and loss of joint function [5]. Its prevalence is steadily increasing due to population ageing and increased life expectancy, imposing thus a major threat on population health [6]. However, its aetiopathogenesis is not yet fully understood.

DNA methylation at CpG sites comprises a major epigenetic mechanism. Genome-wide DNA methylation studies in cartilage tissues have revealed differentially methylated CpG sites near or within OA-related genes [2,3]. It has been also shown that the methylation status of regulatory regions can modulate OA-related gene expression by impacting on the DNA binding ability of transcription factors [7–10]. microRNAs (miRNAs) are also epigenetic regulators of gene expression that have been implicated in OA pathogenesis [2,3]. Several previous studies have demonstrated differentiated expression of miRNAs that regulate processes involved in OA development [11–13]. MiRNAs expression is regulated by genetic factors (miR-SNPs), as well as

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Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; qMSP, quantitative methylation specific PCR; SMAD-3, SMAD family number 3; NF-kB, nuclear Factor Kappa-B; ChIP, chromatin immunoprecipitation; 5-AzadC, 5-Aza-2′-Deoxycytidine; MMP-13, matrix metalloproteinase 13; ADAMTS-5, disintegrin and metalloproteinase with thrombospondin motifs 5; IRAK-1, IL-1 Receptor Associated Kinase 1; TRAF-6, TNF Receptor-Associated Factor 6; TNFA, Tumor Necrosis Factor-Alpha; IL1B, Interleukin-1 Beta; IL-6, Interleukin-6; U6 snRNA, U6 small nuclear RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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epigenetic mechanisms [14–16]. Transcriptional silencing of miRNAs due to DNA hypermethylation has been demonstrated in different types of cancer, suggesting it comprises mechanism that contributes to aberrant miRNAs expression [14,17].

MiR-140-5p is specifically expressed in articular cartilage and is upregulated during chondrogenesis [18]. In addition, its expression has been found to be reduced in knee OA synovial fluid and articular chondrocytes [11,19,20]. Target genes of miR-140-5p have been associated among other with inflammation, autophagy and chondrocyte metabolism [matrix metalloproteinase 13 (*MMP-13*), disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS-5*)] [21–26]. Moreover, increased expression of MMP-13 and ADAMTS-5 in OA chondrocytes has been associated with miR-140-5p downregulation [21,23]. Although, little is known about the mechanisms controlling miR-140-5p expression in OA, previous studies have demonstrated the function of SOX-9, C/EBPα and SMAD-3 on the transcriptional activity of regulatory regions of miR-140-5p [27–29].

In addition, miR-146a is also deregulated in OA and its expression pattern is correlated with inflammatory responses in OA articular cartilage and synovium tissues [30,31]. It was shown that miR-146a acts as a negative inflammatory mediator by targeting TNF Receptor-Associated Factor 6 (*TRAF-6*) and IL-1 Receptor Associated Kinase 1 (*IRAK-1*) genes and subsequently impairing the expression of inflammatory factors, as Interleukin-1 Beta (IL1B), Tumor Necrosis Factor-Alpha (TNFA) and Interleukin-6 (IL-6) [32,33]. In addition, miR-146a expression was shown to be induced in response to lipopolysaccharide (LPS) and proinflammatory mediators [33]. Wang et al. [34] reported that histone deacetylase (HDAC) inhibitors enhanced miR-146a transcription by increasing the DNA-binding ability of NF-kB in osteoarthritis fibroblast-like synoviocytes. On the contrary, hypermethylation of NF-kB binding site in *miR-146a* promoter has been associated with decreased miR-146a expression in prostate cancer [35].

In the present study, we aimed at investigating whether DNA methylation contributes to transcriptional regulation of miR-140 and miR-146a in OA chondrocytes and synoviocytes and subsequently to OA pathology.

2. Material and methods

2.1. Sequence of miR-140 and miR-146a regulatory region

500 base pairs of the 5′-flanking region (miR-140 regulatory sequence) directly upstream of *miR-140* gene and 500 base pairs of *miR-146a* promoter upstream of the transcriptional start site (TSS) of primary transcript of miR-146a (pri-miR-146a) were obtained from Ensembl genome browser. The above regions were scanned for predicted transcription factor binding sites with the TFBIND software (http://tfbind.hgc.jp/). SMAD-3 and NF-kB binding sites were selected to be investigated based on previous functional studies [29,33].

2.2. Patients and tissue samples

Human articular cartilage and synovium were obtained from 20 patients (16F/4M; mean age 69.5 ± 7.9 years) with primary OA undergoing knee replacement surgery at the Orthopaedics Department of University Hospital of Larissa. All osteoarthritic cartilage specimens were taken from the main defective area with visible severe cartilage lesions (advanced OA) and focal or diffuse exposure of subchondral bone. Radiographs were obtained before surgery and the Kellgren-Lawrence system was used for the assessment of OA severity. All patients had a Kellgren-Lawrence grade > 2. The assessment of the radiographs by two independent expert observers was blinded. Patients with rheumatoid arthritis or other autoimmune disease, as well as chondrodysplasias, infection induced OA or post-traumatic OA were excluded from the study. Normal articular cartilage and synovium were obtained from 15 individuals (10F/5M; mean age 56.9 ± 10.8 years), undergoing knee fracture repair surgery or amputation surgery, with no history of joint disease and who did not show clinical manifestations compatible with OA when specifically explored by radiography. All samples were collected from the Orthopaedics Department of University Hospital of Larissa. Informed consent was obtained from all individuals of the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Local Ethical Committee of the University Hospital of Larissa.

2.3. Primary cultures of normal and OA human articular chondrocytes and synoviocytes

Articular cartilage and synovium tissues were dissected and subjected to sequential digestion with 1 mg/ml pronase and 1 mg/ml collagenase P (Roche Applied Science, Germany). Isolated chondrocytes and synoviocytes from individual specimens were separately cultured with Dulbecco's Modified Eagles Medium/Ham's F-12 (DMEM/F-12) (Thermo Fisher Scientific, USA) plus 5% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) at 37 °C under a humidified 5% CO_2 atmosphere until reaching confluence for 4–6 days.

2.4. Treatment of OA chondrocytes and synoviocytes with 5-AzadC, siRNA against SMAD-3/NF-KB and miRNAs inhibitor

Osteoarthritic articular chondrocytes and synoviocytes were counted and seeded onto six-well plates at a density of 3×10^5 cells/well. One-day post-seeding cells were treated with 5 μM 5-Aza-2′-Deoxycytidine (5-AzadC) (Sigma-Aldrich, USA) or dimethyl sulfoxide (DMSO) (control). Media containing DMSO or 5-AzadC was exchanged every three days and lasted for two weeks. Trypan blue staining was used to determine cell viability after treatment with DMSO or 5-AzadC. For transfection experiments, OA chondrocytes and synoviocytes were treated with 5-AzadC for 12 days, then media was removed and 100 pmol of siRNA against SMAD-3 or miR-140-5p inhibitor (Thermo Fisher Scientific, USA) was added in OA chondrocytes whereas 100 pmol of siRNA against NF-kB or miR-146a inhibitor (Thermo Fisher Scientific, USA) was added in OA synoviocytes for 72 h. miR-140-5p inhibitor (100 pmol) and miR-146a inhibitor (100 pmol) were transfected into OA chondrocytes and synoviocytes for 72 h, respectively. Transfection was performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA). After treatments, cells were harvested and RNA and DNA were extracted for further experimentation.

2.5. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total cellular RNA was extracted from chondrocytes and synoviocytes using TRIzol reagent (Thermo Fisher Scientific, USA). One microgram of RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript III reverse transcriptase (Thermo Fisher Scientific, USA). Quantification of miR-140, miR-146a, COL2A1, COL1A1, MMP-13, ADAMTS-5, TRAF-6, IRAK-1, IL6, IL1B, and TNFA expression was performed with an ABI 7300 real-time PCR instrument (Applied Biosystems, USA) and the results were analyzed using 7300 system SDS software. A 20-μl reaction mixture was prepared, containing 2 μl of cDNA, 10 μl of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA) and 500 nM of each primer. Amplification conditions were: initial step at 95 °C for 10 min, followed by a 2-step PCR program of 95 °C for 15 s and 60 °C for 60 s for 40 cycles. The oligonucleotide primers used for amplification were designed using Primer3 Input (version 0.4.0) and the sequences are shown in Table 1 and Supplementary Table 1. miRNAs and mRNAs expression was normalized to U6 small nuclear RNA (U6 snRNA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal control, respectively, using the $2^{-\Delta\Delta CT}$ method [36].

2.6. DNA methylation analysis by Quantitative methylation specific PCR (qMSP)

qMSP for specific regions of the regulatory elements of *miR-140* and *miR-146a* genes was performed using real-time PCR instrument (ABI 7300, Applied Biosystems, Foster, CA). Two pairs of primers were designed with Methyl Primer Express software for targeting regions; one that specifically recognized methylated DNA and another that recognized unmethylated. 2 μl of bisulfite-treated genomic DNA were amplified with 2× EpiTect Master Mix (Qiagen, USA) and 0,75 μM primers (Table 1 and Suppl Table 1) in qMSP reaction. Amplification conditions were: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 27 s, with a final extension of 72 °C for 10 min. DNA methylation values were calculated by interpolating the difference of the unmethylated primer cycle threshold minus the methylated primer cycle threshold (CtU–CtM) in a standard curve. Mixtures of methylated and unmethylated human control samples with 0%, 10%, 25%, 50%, 75%, 90% and 100% methylated DNA (Qiagen, USA) were used to construct a standard curve for calculation of DNA methylation values [9].

2.7. Bisulfite DNA sequencing analysis

Bisulfite-treated DNA was amplified by PCR using primers for BSP-PCR derived from the Methlyl Primer Express (software v1.0) (Table 1). PCR products were cleaned using QIAquick PCR Purification kit (Qiagen, USA) and then were sequenced using a Bigdye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and analyzed on ABI 3130 Genetic Analyzer (Applied Biosystems, USA). The methylation percentage for each CpG site in the regulatory regions was calculated by measuring the ratio between peak height values of cytosine (C) and thymine (T), yielding the basic equation for the methylation percentage to be $(C/[C + T] * 100)$ [9].

2.8. Chromatin Immunoprecipitation (ChIP) assay

ChIP was performed using a ChIP assay kit (Upstate USA, USA) on cultured chondrocytes and synoviocytes. Briefly, protein-DNA complexes were cross-linked by incubating cells in 1% formaldehyde-containing medium for 10 min at 37 °C. Cell pellets were re-suspended in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 8.1) with protease inhibitors (Roche Applied Science, Germany). Cell lysates were sonicated to shear DNA to fragments between 200 and 1000 bps and were pre-cleaned using Salmon Sperm DNA/Protein A Agarose. Samples were taken at this point as positive controls in the PCR reaction (input chromatin). Next, cell lysates were incubated with monoclonal antibody against SMAD-3 or NF-kB (R&D Systems, USA) overnight at 4 °C. Human purified IgG was used as control antibody (R&D Systems, USA). DNA-protein complexes were collected using Salmon Sperm DNA/Protein A Agarose beads, followed by washing, elution and reverse cross-linking. DNAs were extracted with phenol-chloroform and precipitated with ethanol. Recovered DNAs were re-suspended in TE buffer and were later analyzed by PCR (Primers are shown in Table 1). PCR products were fractionated on 3% agarose gels and were stained with ethidium bromide. The relative signal intensity of each band was quantified using the NIH Scion Image Scion Image and normalized to the Input chromatin of the same sample.

2.9. Statistical analysis

Data was analyzed using the SPPS 24 software. Statistical significance of genes expression was determined using Mann-Whitney *U* test, One-Way ANOVA or *t*-test where appropriate. Spearman's rank correlation coefficient was used to analyze correlative relationships.

Table 1

Primer sequences for (a) qRT-PCR, (b) qMSP, (c) BSP and (d) ChIP assay.

(a)		
Stem loop U6	CACGGAAGCCCTCACACCGTGTCGTTC	
Stem loop miR-146a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCCATG	
Stem loop miR-140	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCA	
U6	F: GCTTCGGCAGCACATATACTAAAAT	R: CTCACACCGTGTCGTTCCA
miR-146a	F: GTGCAGGGTCCGAGGT	R: CGGCGGTGAGAACTGAATTCC
miR-140-5p	F: GCCTCAGTGGTTTTACCC	R: GTGCAGGGTCCGAGGT
GAPDH	F: GAGTCAACGGATTTGGTCGT	R: GACAAGCTTCCCGTTCTCAG
ADAMTS-5	F: CAGCAGTGCAACCTGACATT	R: CAGATTCTCCCCTTTCCACA
MMP-13	F: TGGCATTGCTGACATCATGA	R: GCCAGAGGGCCCATCAA
IL1B	F: GGGCCTCAAGGAAAAGAATC	R: TTCTGCTTGAGAGGTGCTGA
IL-6	F: ATGCAATAACCACCCCTGAC	R: GAGGTGCCCATGCTACATTT
TNFA	F: CCCTGAAAACAACCCTCAGA	R: AAGAGGCTGAGGAACAAGCA
IRAK-1	F: AGACCCTGTCTCTGCCAAAA	R: CAGGCTGGAGTGCAGTCATA
TRAF-6	F: TGTTGCAGCAGCTATTTTGC	R: GCACAAGGCGGTAGTGATTT
(b)		
M-miR-146a	F: TGGGTAGTCGATAAAGTTTTC	R: AATACTTTAACCTACGCGCT
U-miR-146a	F: ATTGGGTAGTTGATAAAGTTTTT	R: TAATACTTTAACCTACACACTC
M-miR-140	F: TGTTTGGGGTTAGTAAAATTGTAAC	R: AAAATCTCGAACGAAAAAATCG
U-miR-140	F: TTTGGGGTTAGTAAAATTGTAATGG	R: AAAAAATCTCAAACAAAAAAATCACT
(c)		
BSP miR-146a	F: ATGAAATAAGGGAGTTTTTTGT	R: CTACTCCCCTACAACCTCC
BSP miR-140	F: AAAGTTAATAGGTTTTTTGGGAAA	R: ACACAAAAAAAAACACACACAA
(d)		
miR-146a pr	F: AAAAGCCAACAGGCTCATTG	R: ATCCTGCACAGAACGCTCTAG
rsmiR-140	F: AGAGAGAGAGAGCGCTGTGG	R: TGGCAGGACACAGAGAGAGA

F: forward; R: reverse; M: methylated; U: unmethylated, pr: promoter; rsmiR-140: miR-140 regulatory region.

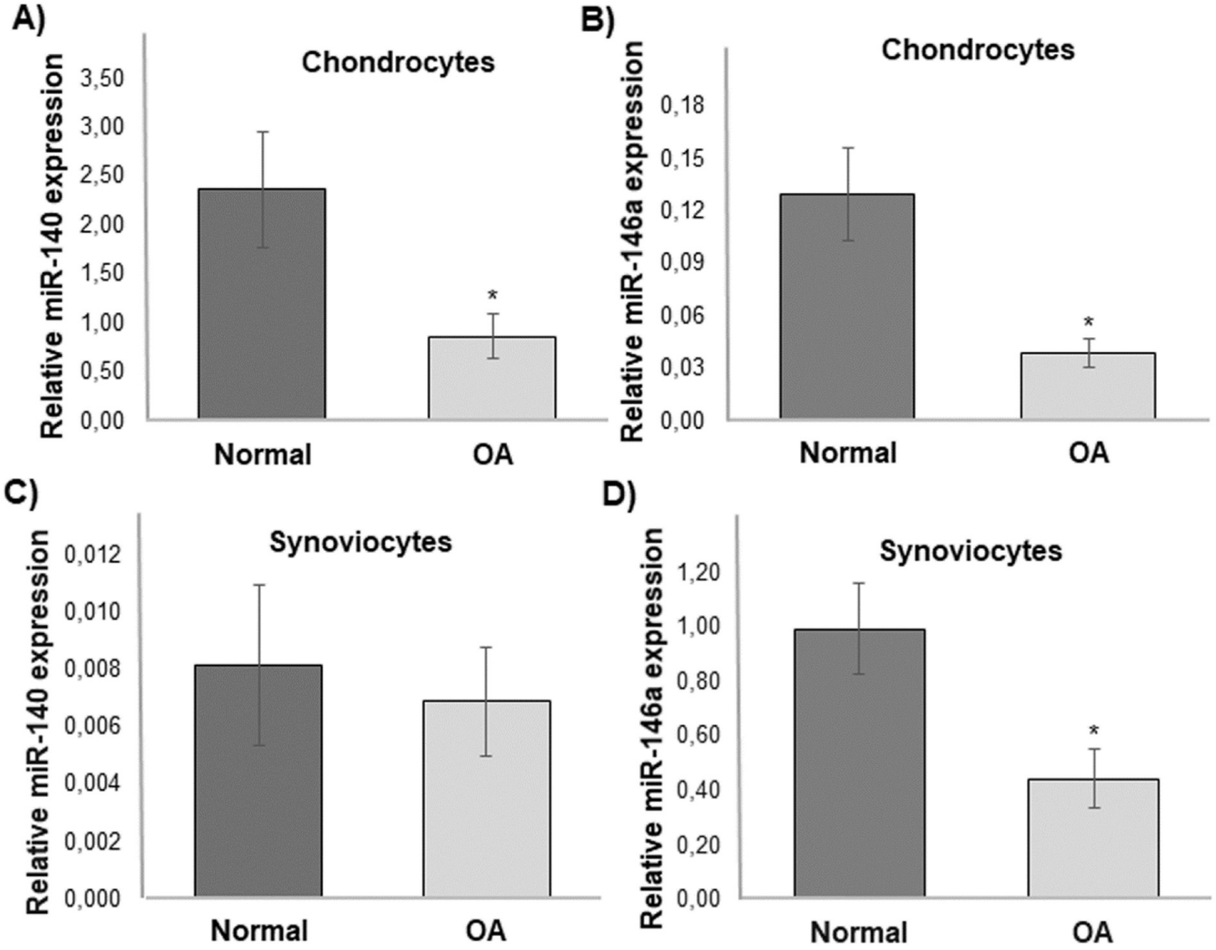

Fig. 1. Differential expression of miR-140-5p and miR-146a in OA chondrocytes and synoviocytes. Relative miR-140-5p (A) and miR-146a (B) expression was analyzed in cultured normal and OA chondrocytes by qRT-PCR and normalized against U6 expression. Similarly, the relative miR-140-5p (C) and miR-146a (D) expression was analyzed in synoviocytes of normal individuals and OA patients. Values are the mean ± SEM. $*p < 0.05$.

$p < 0.05$ was considered statistically significant.

3. Results

3.1. Deregulation of miR-140-5p and miR-146a expression levels in OA chondrocytes/synoviocytes

Since, as mentioned, miR-140-5p and miR-146a were found in previous studies to be among the differentially expressed miRNAs in OA tissues, we firstly aimed at assessing their expression levels in chondrocytes and synoviocytes of normal individuals ($n = 15$) as well as OA patients ($n = 20$) with qRT-PCR analysis. Our results showed that miR-140-5p and miR-146a expression levels were significantly reduced in OA chondrocytes as compared to normal chondrocytes ($p < 0.05$) (Fig. 1A and B). Moreover, we found that miR-146a expression was also downregulated in OA synoviocytes but no significant difference was observed in miR-140-5p expression in the same cell type (Fig. 1C and D).

3.2. Differential DNA methylation status of miR-140 regulatory region between normal and OA chondrocytes

MiR-140 regulatory sequence is located upstream of precursor sequence of *miR-140* gene and has several potential binding sites for transcription factors regulating its expression [29]. At position −120 bp in miR-140 regulatory sequence a SMAD-3 binding site has been previously descripted by Tardif et al. [29]. We scanned this region for the presence of CpG sites and found that eleven CpG sites are located around this area (Fig. 2A). Using qMSP technology, we evaluated DNA methylation levels of the above region of miR-140 regulatory sequence in normal and OA chondrocytes and observed that it was significantly hypermethylated in OA chondrocytes ($p < 0.05$) (Fig. 2B). Moreover, we found significant negative correlation between miR-140-5p expression and methylation levels of miR-140 regulatory region in chondrocytes ($p < 0.05$) (Fig. 2C). To identify which of the CpG sites at position −120 bp in miR-140 regulatory sequence are differentially methylated between OA and normal chondrocytes and affect its expression level in OA chondrocytes, we performed bisulfite DNA sequencing. From the eleven CpG sites that were analyzed, eight CpG sites were found to be highly methylated in OA compared to normal chondrocytes ($p < 0.05$) (Fig. 2D). There was no difference in the methylation status of the regulatory region of miR-140 in synoviocytes between OA and normal samples (data not shown), which correlated with similar expression levels of miR-140-5p observed in OA and normal cells. Moreover, analysis of the methylation status of CpG islands located on *miR-140* promoter did not reveal differences between normal and OA chondrocytes (Suppl Fig. 1A).

3.3. Differential DNA methylation status of miR-146a promoter between normal and OA synoviocytes

Two NF-kB binding sites have been previously identified at position −440 bp and −127 bp in *miR-146a* promoter [33]. To investigate the presence of CpG sites near the NF-kB binding sites, we scanned *miR-*

146a promoter and found that it contains ten CpG sites around the NF-kB binding site at position −440 bp (Fig. 3A). To explore the contribution of DNA methylation to the decreased miR-146a expression that was observed in OA chondrocytes and synoviocytes, we evaluated DNA methylation levels of *miR-146a* promoter near the NF-kB binding site at position −440 bp, in both cell types. Analysis by qMSP demonstrated increased methylation levels of *miR-146a* promoter in OA synoviocytes compared to normal ($p < 0.05$) (Fig. 3B). We also observed that miR-146a expression was inversely correlated with DNA methylation of *miR-146a* promoter in synoviocytes ($p < 0.05$) (Fig. 3C). CpG sites were then analyzed by bisulfite DNA sequencing, which revealed that three of the ten CpG sites were significantly hypermethylated in OA synoviocytes compared to the normal cells ($p < 0.05$) (Fig. 3D). In addition, there was no difference in the methylation status of miR-146a promoter near the NF-kB binding site at position −440 bp between OA and normal chondrocytes (Fig. 3E), suggesting another route of regulation for this microRNA expression in this cell type. Scanning the region around the NF-kB binding site at

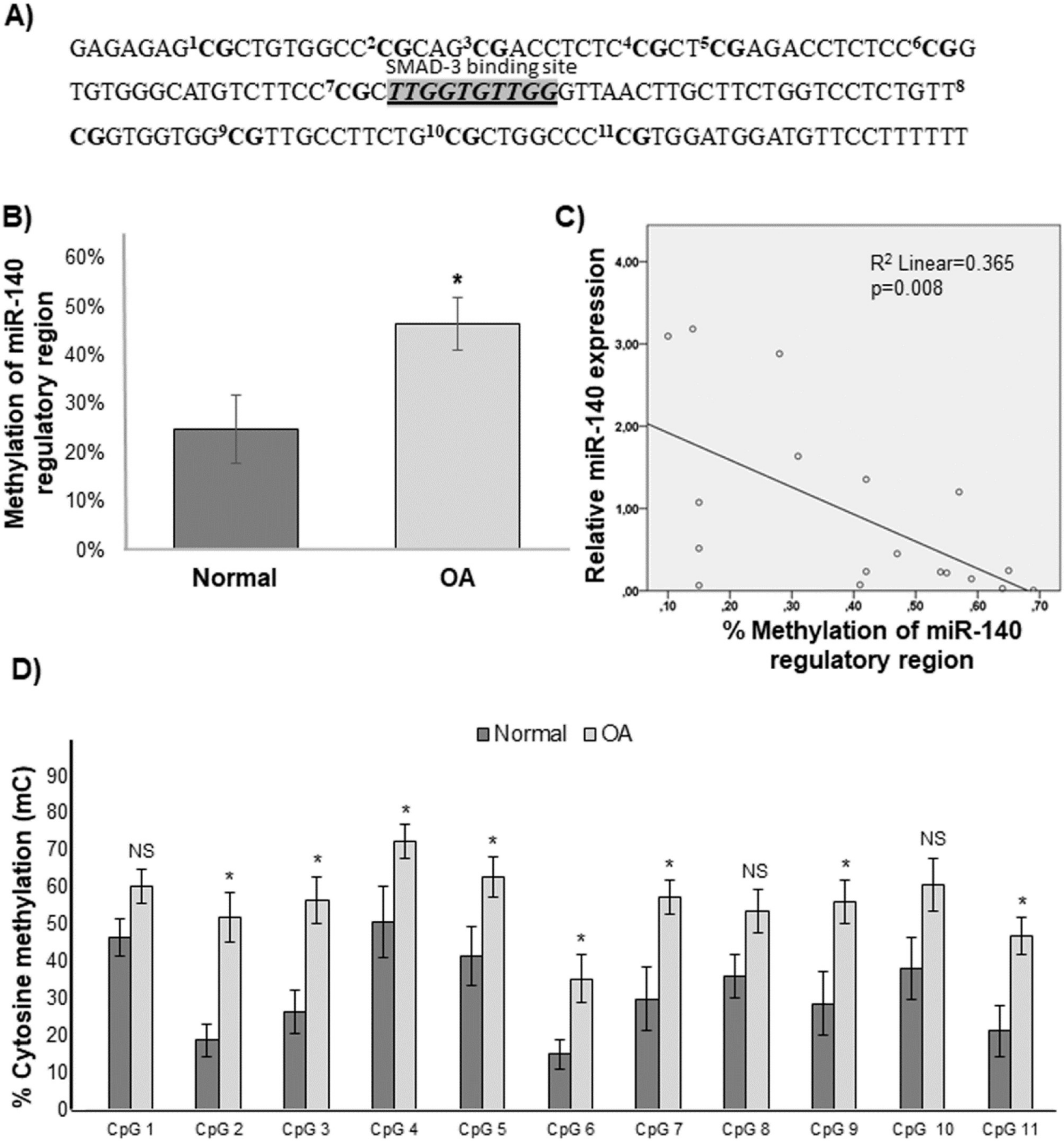

Fig. 2. Hypermethylation of specific CpG sites in miR-140 regulatory region was associated with decreased miR-140-5p expression in OA chondrocytes.
(A) Schematic representation of miR-140 regulatory region showing the SMAD-3 binding site and eleven CpG sites. (B) Methylation status of miR-140 regulatory region near the SMAD-3 binding site in normal and OA chondrocytes estimated by qMSP. (C) Spearman's rank correlation analysis was used to determine the association between methylation levels of miR-140 regulatory region and miR-140-5p expression in chondrocytes. R^2 Linear: coefficient of determination (goodness-of-fit measure of the model). (D) Cytosine methylation of each CpG site located near the SMAD-3 binding sites of miR-140 regulatory region in normal and OA chondrocytes, after bisulfite DNA sequencing analysis. Values are the mean ± SEM. *$p < 0.05$; NS: no significant.

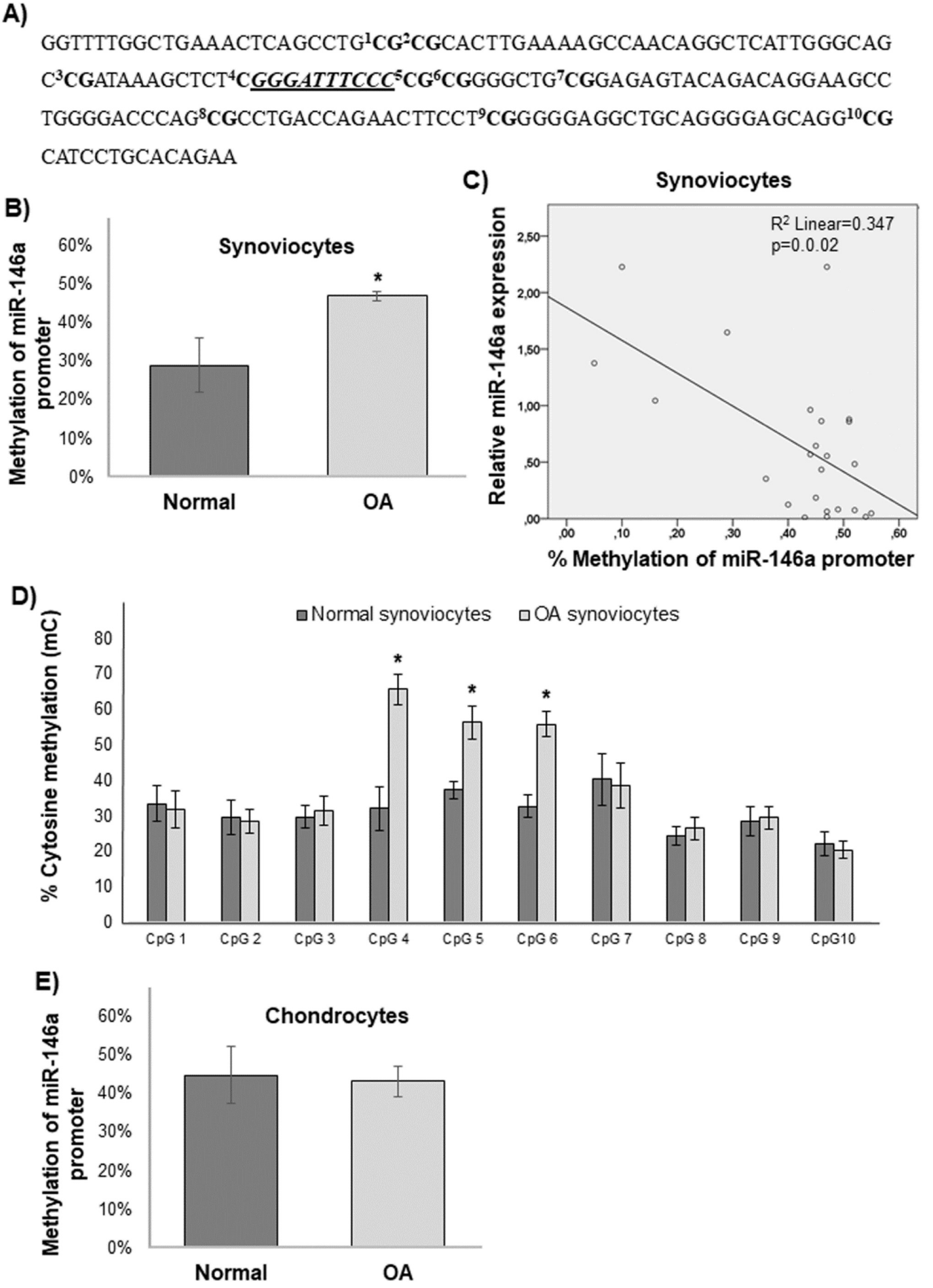

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Fig. 3. Decreased miR-146a expression in OA synoviocytes was associated with the methylation status of specific CpG sites in *miR-146a* promoter. (A) Schematic representation of *miR-146* promoter showing the NF-kB binding site at position −440 bp and ten CpG sites. (B) Methylation status of *miR-146a* promoter near the NF-kB binding site at position −440 bp in normal and OA synoviocytes estimated by qMSP. (C) Comparison of miR-146a expression and methylation status of specific CpG sites in *miR-146a* promoter in synoviocytes using Spearman's rank correlation analysis. R^2 Linear: coefficient of determination (goodness-of-fit measure of the model). (D) Bisulfite DNA sequencing analysis was used to evaluate the cytosine methylation of ten CpG sites located near the NF-kB binding site at position −440 bp of *miR-146a* promoter in normal and OA synoviocytes. (E) Methylation of *miR-146a* promoter near the NF-kB binding site at position −440 bp in normal and OA chondrocytes evaluated by qMSP. Values are the mean ± SEM. *$p < 0.05$.

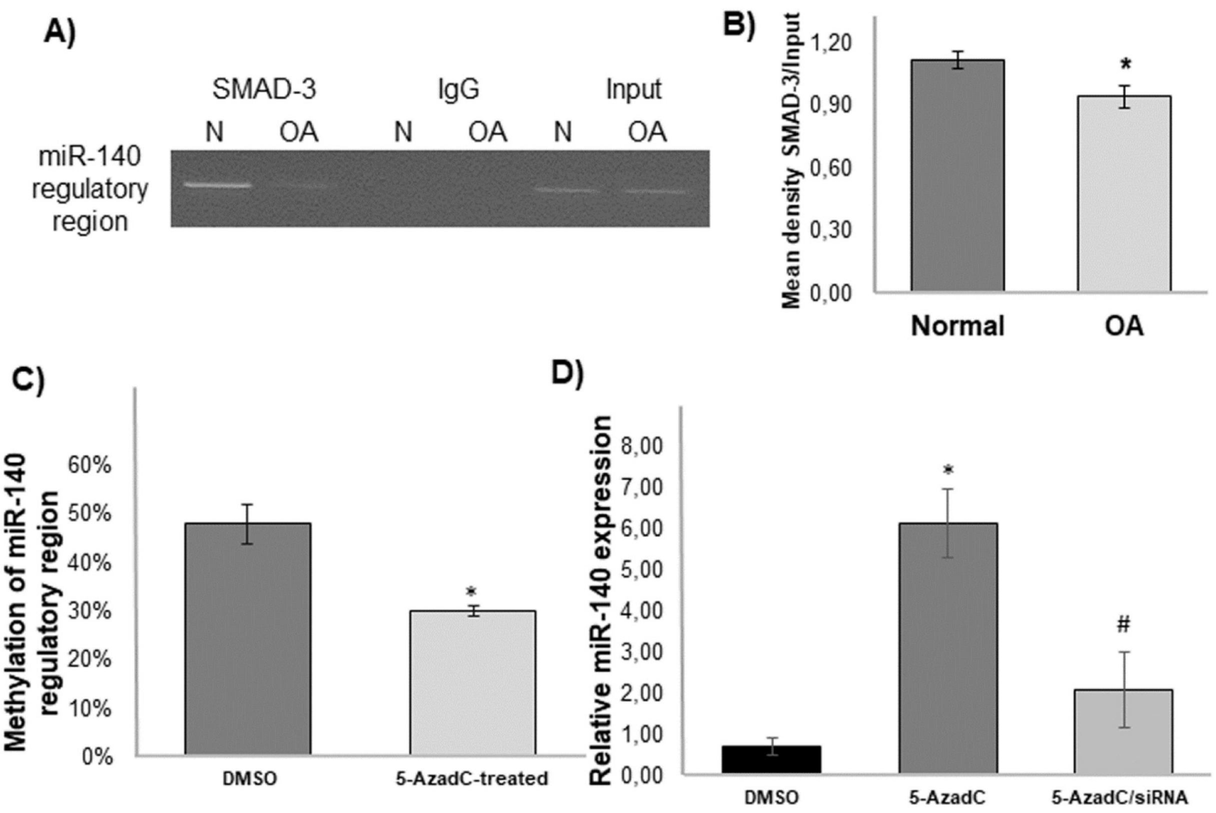

Fig. 4. DNA methylation-mediated repression of miR-140-5p expression in OA chondrocytes was due to impairment of SMAD-3 binding affinity. (A) Representative gel of SMAD-3 binding on miR-140 regulatory region in normal and OA chondrocytes after ChiP assay with the specific antibody against SMAD-3 (B) Densitometric analysis of the band intensity in normal and OA chondrocytes. *$p < 0.05$ OA vs normal. (C) Methylation of miR-140 regulatory region in untreated and 5-AzadC-treated OA chondrocytes by qMSP. *$p < 0.05$ 5-AzadC-treated vs DMSO. (D) Detection of miR-140-5p expression levels by qRT-PCR in DMSO, 5-AzadC-treated and 5-AzadC/siRNA against SMAD-3-treated OA chondrocytes. U6 expression was used for normalization of the qRT-PCR data. *$p < 0.05$ 5-AzadC-treated vs DMSO (control); $^{\#}p < 0.05$ 5-AzadC/siRNA-treated vs 5-AzadC-treated. Values are the mean ± SEM.

position −127 bp, we found eight CpG sites; however, no difference was observed in their methylation status between OA and normal synoviocytes (Suppl Fig. 1B).

3.4. DNA methylation affects miR-140-5p expression in OA chondrocytes through changes in the binding affinity of SMAD-3 to miR-140 regulatory sequence

As reported above (Fig. 2D) the bisulfite DNA sequencing results suggest possible changes in the binding affinity of SMAD-3 in OA chondrocytes, due to hypermethylation of CpG sites located near the SMAD-3 binding sites. In order to prove this, we performed ChIP assay in normal and OA chondrocytes using an antibody against SMAD-3. As expected, we observed a weaker binding affinity of SMAD-3 in OA chondrocytes compared to normal ($p < 0.05$) (Fig. 4A and B). In order to further validate this finding, we treated OA chondrocytes with the inhibitor of DNA methylation, 5-AzadC with or without siRNA against SMAD-3 and subsequently evaluated miR-140-5p expression by qRT-PCR. 5-AzadC did not cause significant increase in cell death, as revealed by trypan blue testing (data not shown), nor chondrocyte dedifferentiation, as evidenced by lack of reduction of COL2A1/COL1A ratio between treated and untreated OA chondrocytes. (Suppl. Fig. 1C) [37]. 5-AzadC treatment in OA chondrocytes resulted in decreased methylation levels of miR-140 regulatory region, as expected according to qMSP (Fig. 4C) and significant upregulation of miR-140-5p expression levels compared to control. Furthermore, we observed that miR-140-5p overexpression in 5-AzadC-treated OA chondrocytes was reversed by SMAD-3 knockout, which proves that DNA methylation impairs SMAD-3 binding affinity on miR-140 regulatory sequence (Fig. 4D).

3.5. DNA methylation affects miR-146a expression in OA synoviocytes through changes in the binding affinity of NF-kB to miR-146a promoter

As above, in order to validate the bisulfite DNA sequencing results (Fig. 3D) on the binding affinity of NF-kB to the regulatory region of miR-146a, OA and normal synoviocytes were subjected to ChIP assay. We observed that NF-kB binding on *miR-146a* promoter in OA synoviocytes was reduced compared to normal cells ($p < 0.05$) (Fig. 5A and B). In order to further confirm that DNA methylation impacts on the binding affinity of NF-kB to *miR-146a* promoter, we assessed miR-146a expression after treatment of OA synoviocytes with 5-AzadC or with 5-

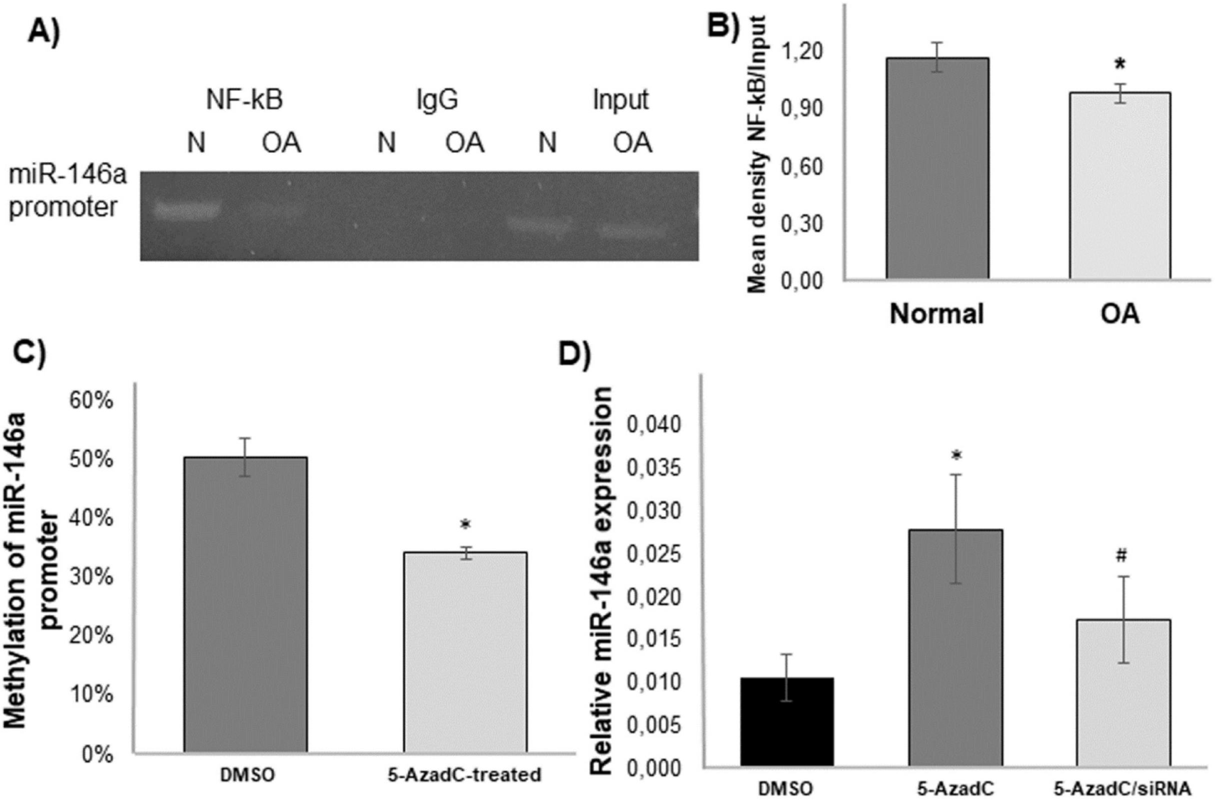

Fig. 5. DNA methylation-mediated repression of miR-146a expression in OA synoviocytes was due to impairment of NF-kB binding affinity. (A) Representative gel of NF-kB binding on *miR-146a* promoter in normal and OA chondrocytes after ChiP assay with the specific antibody against NF-kB (B) Densitometric analysis of the band intensity of NF-kB binding on *miR-146a* promoter in normal and OA synoviocytes after ChIP assay. $^{*}p < 0.05$ OA vs normal. (C) Evaluation of *miR-146a* promoter methylation levels in DMSO and 5-AzadC-treated OA synoviocytes was performed using qMSP. $^{*}p < 0.05$ 5-AzadC-treated vs DMSO. (D) Relative miR-146a expression in DMSO, 5-AzadC-treated and 5-AzadC/siRNA against NF-kB-treated OA synoviocytes were analyzed by qRT-PCR and normalized against U6 expression. Values are the mean ± SEM. $^{*}p < 0.05$ 5-AzadC-treated vs DMSO (control); $^{\#}p < 0.05$ 5-AzadC/siRNA-treated vs 5-AzadC-treated.

AzadC/siRNA against NF-kB using qRT-PCR. Firstly, it was confirmed, as above, that -AzadC treatment decreased the methylation levels of *miR-146a* promoter (Fig. 5C). Subsequently, it was observed that miR-146a was upregulated in 5-AzadC-treated OA synoviocytes compared to control ($p < 0.05$) and this effect was reversed using siRNA against NF-kB ($p < 0.05$) (Fig. 5D). This result proves that DNA methylation on specific CpG sites of *miR-146a* promoter impairs the binding affinity of NF-kB.

3.6. DNA methylation-mediated downregulation of miR-140-5p expression enhances the expression of catabolic genes in OA chondrocytes

Functional studies have previously demonstrated that MMP-13 and ADAMTS-5 are targeted by miR-140-5p. To identify whether DNA methylation-mediated suppression of miR-140-5p in OA chondrocytes enhances the above catabolic genes' expression, we treated these cells with 5-AzadC or 5-AzadC/miR-140-5p inhibitor or miR-140-5p inhibitor and then evaluated MMP-13 and ADAMTS-5 mRNA expression levels. qRT-PCR revealed that in 5-AzadC-treated OA chondrocytes the expression levels of catabolic factors MMP-13 and ADAMTS-5 were downregulated, whereas no significant difference was observed in miR-140-5p inhibitor-treated OA chondrocytes. MMP-13 and ADAMTS-5 expression levels were significantly increased in 5-AzadC/miR-140-5p inhibitor-treated chondrocytes compared to 5-AzadC-treated and miR-140-5p inhibitor-treated OA chondrocyte ($p < 0.05$) (Fig. 6A), suggesting the negative impact of DNA methylation-mediated downregulation of miR-140-5p on MMP-13 and ADAMTS-5 expression levels.

3.7. DNA methylation-mediated downregulation of miR-146a enhances the expression of inflammatory factors in OA synoviocytes

It is known that miR-146a inhibits TRAF-6 and IRAK-1 expression and subsequently influences the expression of inflammatory factors, such as IL1B, IL-6, and TNFA [32,33]. To investigate the role of miR-146a suppression due to its promoter hypermethylation on the inflammatory status of OA synoviocytes, we evaluated TRAF-6, IRAK-1 IL1B, IL-6 and TNFA mRNA levels after treatment with 5-AzadC or 5-AzadC/miR-146a inhibitor or miR-146a inhibitor in OA synoviocytes. We observed that IRAK-1, IL1B and IL-6 expression levels were markedly decreased in 5-AzadC-treated OA synoviocytes and increased in 5-AzadC/miR-146a inhibitor-treated OA synoviocytes. Moreover, significant increased IRAK-1, IL1B and IL-6 expression was observed in 5-AzadC/miR-146a inhibitor-treated OA synoviocytes compared to miR-146a inhibitor-treated ($p < 0.05$) (Fig. 6B), suggesting the impact of DNA methylation-mediated regulation of miR-146a on the expression of the above inflammatory factors known to be involved in OA pathogenesis. No difference in TRAF-6 and TNFA expression was observed between 5-AzadC/miR-146a inhibitor-treated and 5-AzadC-treated OA synoviocytes, implying a different pathway of regulation for these genes (Fig. 6B).

4. Discussion

MiR-140-5p and miR-146a have been identified as regulators of inflammation-related pathways and play a crucial role in the catabolic action of OA chondrocytes through regulation of MMPs and ADAMTSs expression [21–23,31,38–40]. In the present study, we found that both miR-140-5p and miR-146a were significantly downregulated in OA

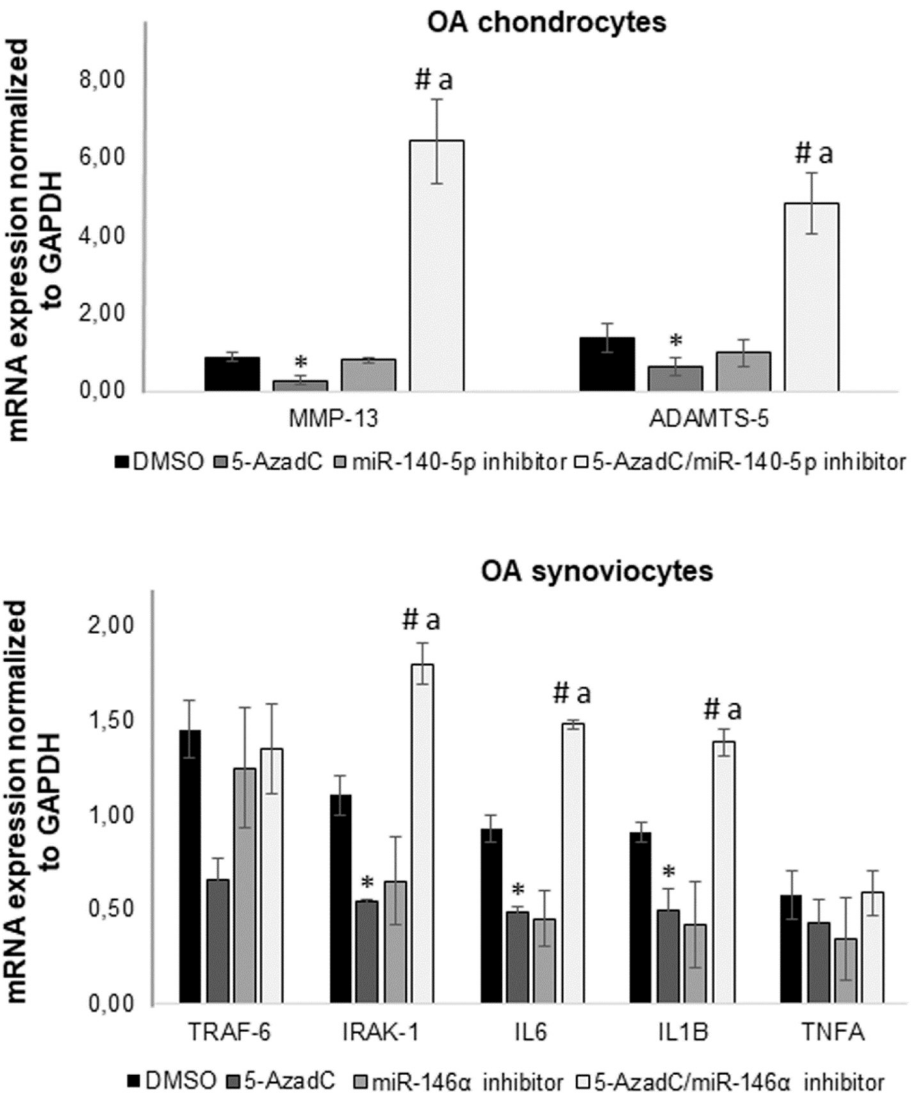

Fig. 6. miR-140-5p and miR-146a downregulation by DNA methylation contributes to OA pathogenesis. (A) Detection of MMP-13 and ADAMTS-5 expression by qRT-PCR in DMSO, 5-AzadC-treated, miR-140-5p inhibitor-treated and 5-AzadC/miR-140-5p inhibitor-treated OA chondrocytes. GAPDH expression was used for normalization of the qRT-PCR data. (B) Relative IRAK-1, TRAF-6, IL1B, IL-6 and TNFA expression in DMSO, 5-AzadC-treated, miR-146a inhibitor treated and 5-AzadC/miR-146a inhibitor OA synoviocytes were analyzed by qRT-PCR and normalized against GAPDH expression. Values are the mean ± SEM. $^{*}p < 0.05$ 5-AzadC-treated vs DMSO (control); $^{\#}p < 0.05$ 5-AzadC/miRNA inhibitor-treated vs 5-AzadC-treated; $^{a}p < 0.05$ 5-AzadC/miRNA inhibitor-treated vs miRNA inhibitor-treated.

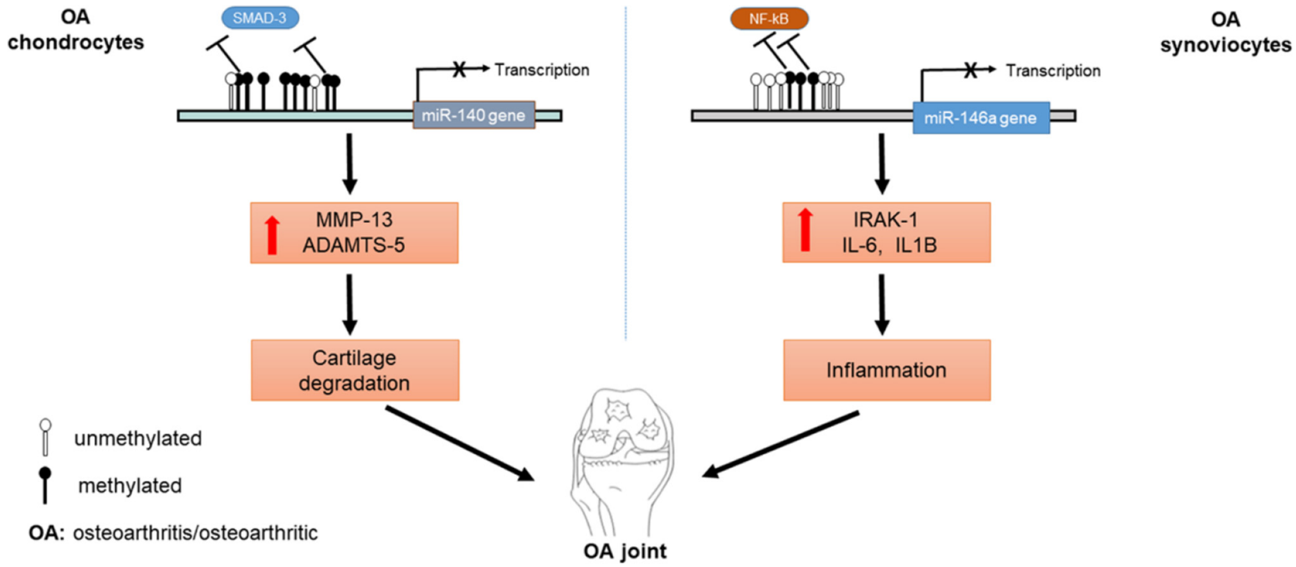

Fig. 7. DNA methylation-mediated regulation of miR-140-5p and miR-146a is implicated in OA pathology. In OA chondrocytes (left), hypermethylation of miR-140 regulatory region results in miR-140 downregulation and increased expression of its target genes, MMP-13 and ADAMTS-5, involved in cartilage degradation. In OA synoviocytes (right) DNA methylation-mediated miR-146a suppression is associated with increased expression of its target gene, IRAK-1, and the inflammatory factors, IL1B and IL-6. Increased catabolic and inflammatory actions of joint cells lead to cartilage degradation and OA progression.

chondrocytes compared to normal, whereas miR-146a expression was also downregulated in OA synoviocytes compared to normal. Decreased expression of these two miRNAs has been previously reported in OA tissues [11,19,20,23,31,40], but the underlying mechanisms contributing to their downregulation remains unclear.

Our analyses revealed hypermethylation of miR-140 regulatory region and *miR-146a* promoter in OA chondrocytes and synoviocytes, respectively. Moreover, the percentage of methylation displayed a strong negative correlation with miR-140-5p and miR-146a expression in OA chondrocytes and synoviocytes, confirming the role of DNA methylation on the transcriptional regulation of these miRNAs. Although, it has been previously reported that DNA methylation changes contribute to abnormal expression of several miRNAs in human cancer [35,41,42], there is no previous data until now, demonstrating the regulatory role of DNA methylation on aberrant expression of miRNAs, and especially miR-140-5p and miR-146a in joint diseases, such as OA.

Furthermore, it has been shown that DNA methylation participates in gene transcription regulation by preventing transcription factors from binding to specific elements in the genes' regulatory region [43]. In our study, we demonstrated that hypermethylation of miR-140 regulatory region led to impairment of SMAD-3 binding affinity in this region. We have previously demonstrated that TGFβ/SMAD-3 signaling is over-activated in OA [44]. We now propose that the previously TGFβ/SMAD-3 over-activation did not increase the transcriptional expression of its target gene, *miR-140*, due to hypermethylation of its regulatory region. Tardif et al. have previously reported negative regulation of miR-140 expression in OA based on TGF-β signaling-mediated control of SMAD-3 function [29]. In the present study, we propose a different mechanism, where hypermethylation in miR-140 regulatory region at a different site than the previously identified SMAD-3 binding site [29], impairs the binding ability of SMAD-3 and as a consequence downregulates miR-140-5p expression in OA chondrocytes.

We also found that DNA methylation contributed to transcriptional silencing of *miR-146a* promoter in OA synoviocytes and that this hypermethylation was responsible for the reduced binding affinity of NF-kB transcription factor. MiR-146a has been reported previously to be a NF-kB-dependent gene, as it was shown that NF-kB can bind on *miR-146a* promoter after LPS, IL-β and TNFa stimulation in a human monocytic cell line [33]. Moreover, an association between methylation levels of NF-kB binding site on *miR-146a* promoter and miR-146a expression levels was demonstrated in prostate carcinoma cells [35]. We demonstrate here, for the first time, that the methylation status of *miR-146a* promoter can also affect the binding ability of NF-kB in OA synoviocytes and therefore control miR-146a expression levels.

It is known that miR-140-5p is an important factor in OA development [21]. MiR-140 inhibits mediators of inflammation and cartilage degradation, whereas upregulates chondrogenic proteins [45]. It has also been reported that miR-140-5p downregulation results in over-activation of inflammatory pathways and increased catabolic genes' expression contributing to progressive cartilage degradation in OA joints [23–25,45]. In our study, we demonstrated that DNA methylation-mediated repression of miR-140-5p influences the expression of specific target genes, as MMP-13 and ADAMTS-5, which are known to contribute to OA progression. MMP-13 and ADAMTS-5 are significantly overexpressed in OA articular cartilage and induce OA development through excessive ECM degradation [46,47]. The deeper understanding of MMP-13 and ADAMTS-5 expression regulation provided here could be useful for OA diagnosis, treatment and/or prevention.

Finally, we confirmed the inflammatory role of miR-146a in OA, as we found decreased IRAK-1, IL-1b and IL-6 expression levels in 5-AzadC-treated OA synoviocytes. The inflammatory component of OA is well-recognized, as chondrocytes and synoviocytes express inflammatory mediators (IL-1b, IL-8, TNFa) that enhance their catabolic action in the joint [48]. Modulation of inflammatory pathways is considered an attractive concept for OA treatment. Our results demonstrate that methylation-based miR-146a regulation could be part of a new therapeutic strategy for patients with cartilage diseases.

5. Conclusion

The present study sheds light on the epigenetic regulation of miR-140-5p and miR-146a expression in OA. We provide novel evidence that DNA methylation modulates miR-140-5p and miR-146a expression through changes in the binding affinity of SMAD-3 and NF-kB to their regulatory regions (Fig. 7). Moreover, we proved that modulation of methylation status of miR-140-5p and miR-146a regulatory regions can control their expression and as a result regulate catabolic and inflammatory factors that are involved in OA pathogenesis and progression. Importantly, as DNA methylation is a reversible process, our study emphasizes the high potential of epigenetic regulation of miRNAs for the development of new OA treatment strategies.

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Declaration of competing interests

The authors declare no competing or financial interests.

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