Inhibition of miR-205 promotes proliferation, migration and fibrosis of tenocytes through targeting MECP2: Implications for rotator cuff injury

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Abstract

Background. The production of inflammatory mediators is critical for tenocytes proliferation and migration, which play an important role in rotator cuff injury repair and regulation of collagen. MicroRNA (miRNA)-205 (miR-205) promotes the secretion of inflammatory factors. The mechanism of the tenocytes regulation by miR-205 remains unknown. In this paper, we showed that miR-205 can regulate the proliferation, migration and fibrosis of tenocytes.

Objectives. To investigate the function and mechanism of miR-205/MeCP2 pathway on the proliferation, migration and fibrosis of rotator cuff tenocytes, in order to provide a new perspective on the repair of rotator cuff tear injury.

Materials and methods. The tenocytes were collected under sterile conditions from the Achilles tendons of Sprague Dawley (SD) rats (weighing 150–200 g). The cells of passages 2–4 were used for the following experiments. All miRNA and vectors were transfected with Lipofectamine 2000. Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR), Cell Counting Kit-8 (CCK-8) assay, luciferase reporter assay, and migration assay were performed. Then, immunoblotting analysis and statistical analysis were conducted.

Results. The CCK-8 and migration assay revealed that miR-205 inhibition resulted in increased tenocytes proliferation, migration and fibrosis. The miR-205 reduced the mRNA and protein expression levels of MECP2, which is involved in cell proliferation and migration of tenocytes. The miR-205 inhibited luciferase intensity under the control of the 3'UTRs of MECP2.

Conclusions. The inhibition of MECP2 reversed the effect of miR-205 inhibitor on tenocytes, including the proliferation and migration of tenocytes, indicating that miR-205 may be valuable in miRNA-based therapies for rotator cuff injury.

Key words: microRNA, fibrosis, miR-205, cell proliferation and migration, rotator cuff injury

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Background

Rotator cuff injury often leads to pain, limited motion of shoulder and, in general, lowers the quality of life. As a common weighing of shoulder, rotator cuff injury includes several types like injury to tendinopathy, partial tears and complete tears.[1,2] Therefore, novel repair techniques are required. Biologic repair techniques have been considered to be the potential methods of restoring the histological structure. For example, the employment of cytokines and/or certain cells could possibly help in promoting the regeneration of rotator cuff tendons.[3,4]

A tendon is a fibrous cord of connective tissue at the end of a muscle, by which the muscle attaches to a bone or other structure.[5] Tenocytes are the basic functional units of tendons. They synthesize and secrete collagen and other extracellular matrix, and maintain the metabolism of tendon tissue. Tenocytes are essential for the production of the extracellular matrix during the tendon regeneration. Many factors contribute to this progression. For example, bone morphogenetic protein 2 (BMP2) can facilitate tendon repair by helping in the migration and proliferation of tenocytes.[6]

MicroRNAs (miRNAs) have been reported to regulate the expressions of various genes by targeting the 3'UTR of mRNA. This resulted in mRNA degradation or translational repression.[7,8] Increasing evidences demonstrated that miRNAs contribute to various biological processes.[9,10] Among them, miR-205 has been widely used in tumor immune regulation and inflammatory pathway regulation. It has been reported that miR-205 can promote the secretion of inflammatory factors.[11] At the same time, the downregulation of the miR-205 expression can promote the recovery of skin wound,[12] but its research on tendon cell function remains unexplored.

Methyl-CpG-binding protein 2 (MECP2) is a member of the methylated binding protein family and an epigenetic regulator associated with methylated DNA,[13] which was originally considered to repress transcription via its interactions with HDAC-Sin3 complex. Nowadays, with emerging studies, MECP2 has been widely reported to reduce inflammation, pain and injury. Moreover, it may play a role in tendon cell function. The evidence showed that MECP2 can promote myofibroblast differentiation and fibrosis, which are pivotal for wound healing.[14]

Objectives

In this study, we investigated the function and mechanism of miR-205/MECP2 pathway on the proliferation, migration and fibrosis of rotator cuff tenocytes, in order to provide a new perspective on the repair of rotator cuff tear injury.

Materials and methods

Tenocytes culture and transfection

All animal experiments were approved by the ethics committee of Hwa-Mei Hospital (University of Chinese Academy of Sciences, Ningbo, China). The tenocytes were collected under sterile conditions from the Achilles tendons of Sprague Dawley (SD) rats (weighing 150–200 g). The cells of passages 2–4 were used for the following experiments. All miRNA and vectors were transfected with Lipofectamine 2000 (Invitrogen, Waltham, USA).

Reverse transcription quantitative real-time polymerase chain reaction

Total RNA was extracted with TRIzol reagent (Qiagen, Hilden, Germany) and phenol/chloroform methods. An miR-205 RT primer was used for the cDNA synthesis of miR-205, and U6 small nuclear RNA was used as an internal control.

CCK-8 assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) assay. Briefly, all transfected cells were incubated with CCK-8 for 2 h at 37°C, and the absorbance was assessed at 450 nm.

Migration assay

Tenocytes were incubated, collected and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal bovine serum (FBS). Subsequently, 10^4 tenocytes were seeded onto the upper chamber. The migrated tenocytes were fixed and stained using crystal violet.

Immunoblotting analysis

Then, 50 μg of protein were loaded in 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Rabbit polyclonal antibodies were used as primary antibodies. The bound antibodies were detected with conventional protocols.

Luciferase reporter assay

The DNA fragment corresponding to 3'UTR of MECP2 was cloned into the pmirGLO Vector (Promega, Beijing, China). The HEK-293T cells were seeded in 24-well plates and co-transfected with miR-205 and the wild-type or mutant 3'UTR, and were harvested for protein extraction. Luciferase intensity was examined with the Dual-Luciferase Reporter Gene Assay kit (Promega).

Statistical analyses

All data were presented as median (range, minimum–maximum). The GraphPad Prism v. 7.0 (GraphPad Software, San Diego, USA) and SPSS v. 18.0 software (SPSS Inc., Chicago, USA) were used for calculation. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used for comparison among 3 groups. All experiments were repeated in triplicate for both duplicate detection and biological duplication. A value of $p < 0.05$ was considered statistically significant.

Results

Inhibition of mir-205 promotes proliferation, migration and fibrosis of tenocytes

To investigate the role of miR-205 in the tendon repair, the inhibition of miR-205 was achieved in tenocytes by transient transfection with miR-205 inhibitor. Cell proliferation was evaluated using CCK-8 assay (Fig. 1A). The results showed that the inhibition of miR-205 increased tenocytes proliferation, compared with control cells. Also, cell migration was enhanced by inhibition of miR-205 by transwell assay (Fig. 1B). In addition,

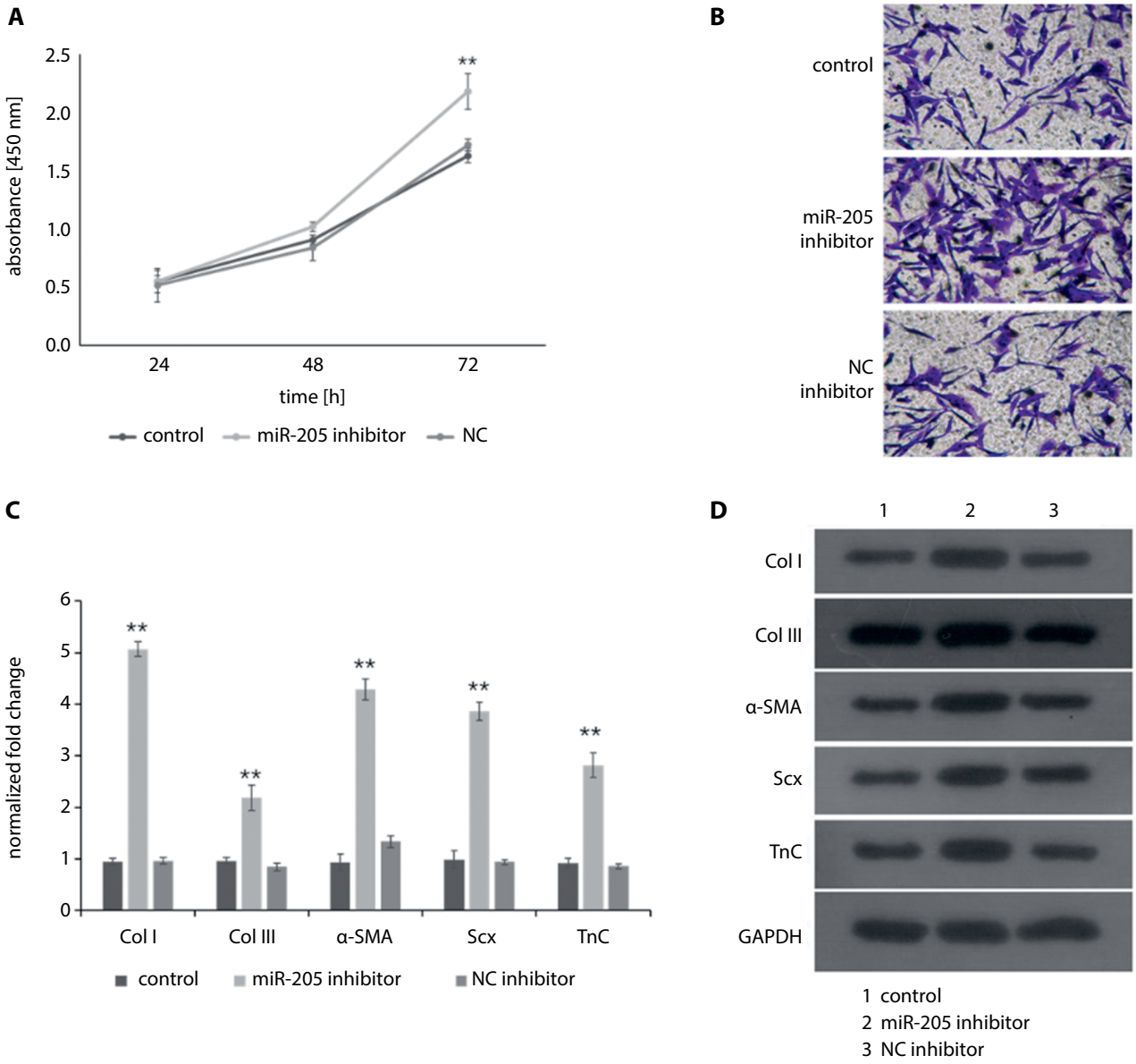

Fig. 1. Inhibition of miR-205 promotes proliferation, migration and fibrosis of tenocytes. Cells were transiently transfected with miR-205 inhibitor or control. A. Cell proliferation was measured with Cell Counting Kit-8 (CCK-8) assay at various timepoints following the transfection; B. Transfected cells were subjected to migration assay. The image displays the cells stained with crystal violet; C. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for the mRNA expression levels of the fibrosis markers; D. Protein levels of the fibrosis markers. Data are from experiments repeated in triplicate for both duplicate detection and biological duplication; values are presented as the median (range, minimum–maximum). Boxplots show the minimum value, 1st quartile, median, 3rd quartile, and maximum value. *$p < 0.05$; **$p < 0.01$ compared to negative control (NC)

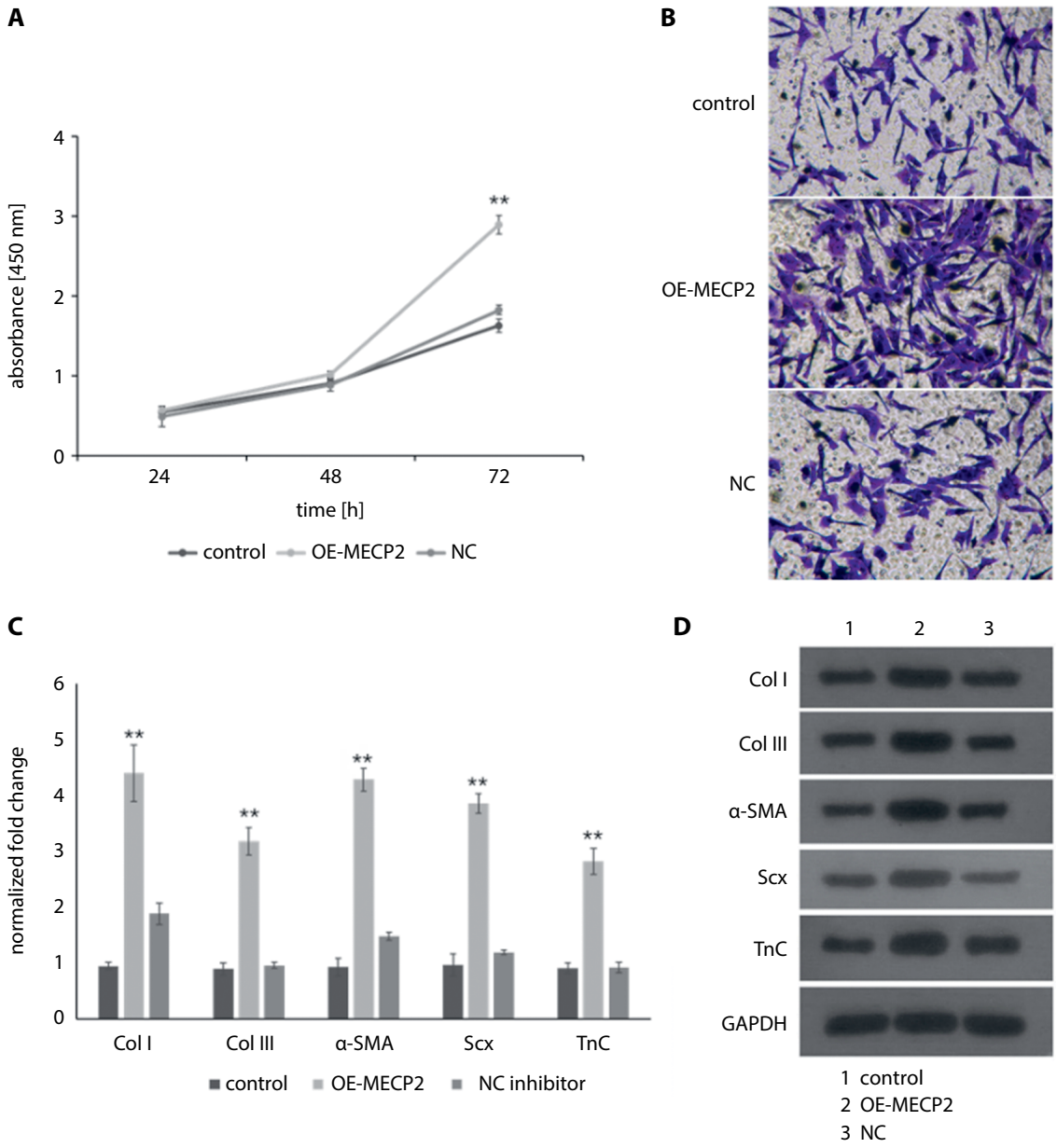

Fig. 2. The MECP2 promotes proliferation, migration and fibrosis of tenocytes. Cells were transiently transfected with MECP2 or control. A. Cell proliferation was measured with Cell Counting Kit-8 (CCK-8) assay at various timepoints following the transfection; B. Transfected cells were subjected to migration assay. The image displays the cells stained with crystal violet; C. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for the mRNA expression levels of the fibrosis markers; D. Protein levels of the fibrosis markers. Data from experiments repeated in triplicate for both duplicate detection and biological duplication; values are presented as the median (range, minimum–maximum). Boxplots show the minimum value, 1st quartile, median, 3rd quartile, and maximum value. *p < 0.05; **p < 0.01 compared to negative control (NC)

the mRNA and protein levels of fibrosis maker of tenocytes were detected to be enhanced by inhibition of miR-205, including Col I, Col III, α-SMA, Scleraxis (Scx), and tenascin C (TnC) (Fig. 1C,D). Altogether, these findings demonstrated that the inhibition of miR-205 can promote proliferation, migration and fibrosis of tenocytes.

MECP2 promotes proliferation, migration and fibrosis of tenocytes

To gain more insight into the mechanisms underlying the biological function of MECP2 in the tendon repair progress, the ectopic expression of MECP2 was achieved in tenocytes by transient transfection. The CCK-8 assay was applied to determine cell proliferation, which showed that ectopic expression of MECP2 increased tenocytes proliferation compared with the control cells (Fig. 2A). Cell migration was enhanced by ectopic expression of MECP2 (Fig. 2B) and the levels of fibrosis makers of tenocytes, including Col I, Col III, α-SMA, Scx, and TnC, were also found to be enhanced (Fig. 2C,D). These results demonstrated that the overexpression of MECP2 can promote proliferation, migration and fibrosis of tenocytes.

miR-205 targets and negatively regulates MECP2

To confirm the direct regulation of miR-205 on MECP2, we cloned the 3'UTR-containing miR-205 binding sites (Fig. 3A) and this fragment was inserted into the 3' flank of the reporter gene. Dual luciferase reporter assay (Promega) was performed, and the result demonstrated that miR-205 significantly downregulated luciferase intensity of MECP2 (Fig. 3). To further validate this result, site mutations were introduced into the binding sites. As expected, miR-205 could not change the fluorescence intensity and mRNA level of MECP2 containing the mutant 3'UTR (Fig. 3B,C). Taken together, our studies proved that *MECP2* can be a direct target gene of miR-205.

Inhibition of MECP2 reverses the effect of miR-205 inhibitor on tenocytes

To further explore whether MECP2 participates in the miR-205-mediated proliferation, migration and fibrosis of tenocytes, cells were transfected with control, miR-205 inhibitor, inhibitor plus small interfering RNA against MECP2 (si-MECP2), and the inhibitor plus the negative control siRNA (si-NC). The inhibition of MECP2 reverses the effect of miR-205 inhibitor on tenocytes, including the proliferation and migration of tenocytes caused by miR-205 inhibitor (Fig. 4A,B). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and western blot analysis showed that miR-205 inhibitor transfection promoted the protein expression of Col I, Col III, α-SMA, Scx, and TnC, which could be alleviated by downregulated MECP2 in tenocytes (Fig. 4). Together, these results suggested that the inhibition of miR-205 promotes proliferation, migration and fibrosis of tenocytes through targeting MECP2.

Discussion

With the aging of the population and the improvement in the general fitness of the population in China, the number of patients with rotator cuff tears caused by degeneration and sports injury has increased dramatically, and irreparable rotator cuff tear (IRCT) caused by various reasons has become more and more common.[15] Irreparable rotator cuff tear is different from huge rotator cuff tear, since not all huge rotator cuff tears are irreparable.[16] As the name suggests, the torn rotator cuff cannot be repaired in the original footprint area after conventional loosening. In addition, irreparable rotator cuff can affect the quality of tendon due to atrophy and steatosis. Even if its structure is directly repaired, it will fail to function due to its quality defects. The irreparability of rotator cuff is caused by many factors, and its incidence can be as high as 30%.

During the healing process of the tendon injury, double healing mechanisms are observed, which include the division and proliferation of tenocytes, the endogenous healing of repairing tendon rupture and defect, and the exogenous healing participated by the proliferation of tendon adventitia cells. The external healing process will cause

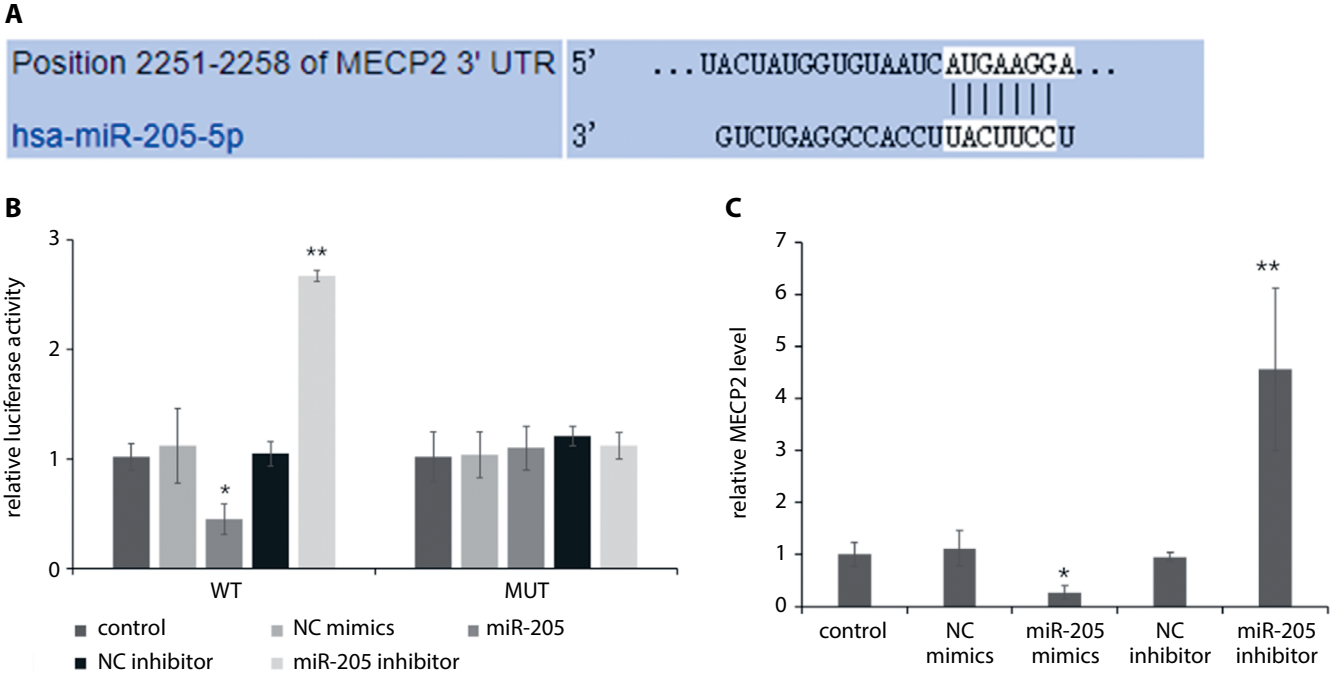

Fig. 3. The miR-205 targeting and negative regulation of MECP2. A. Alignments between miR-96 and the binding sites in the sequences at the 3'UTR of MECP2 mRNA; B. Cells were co-transfected with miR-96 and the WT or mutant 3'UTR of MECP2 mRNA, and subjected to luciferase reporter assay for the analysis of the effect of miR-96 on the intensity controlled by the 3'UTR; C. The mRNA expression level of MECP2 using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The β-actin was used as an internal control. Boxplots show the minimum value, 1st quartile, median, 3rd quartile, and maximum value. **p < 0.01 compared to mimics negative control (NC); ##p < 0.01 compared to inhibitor NC

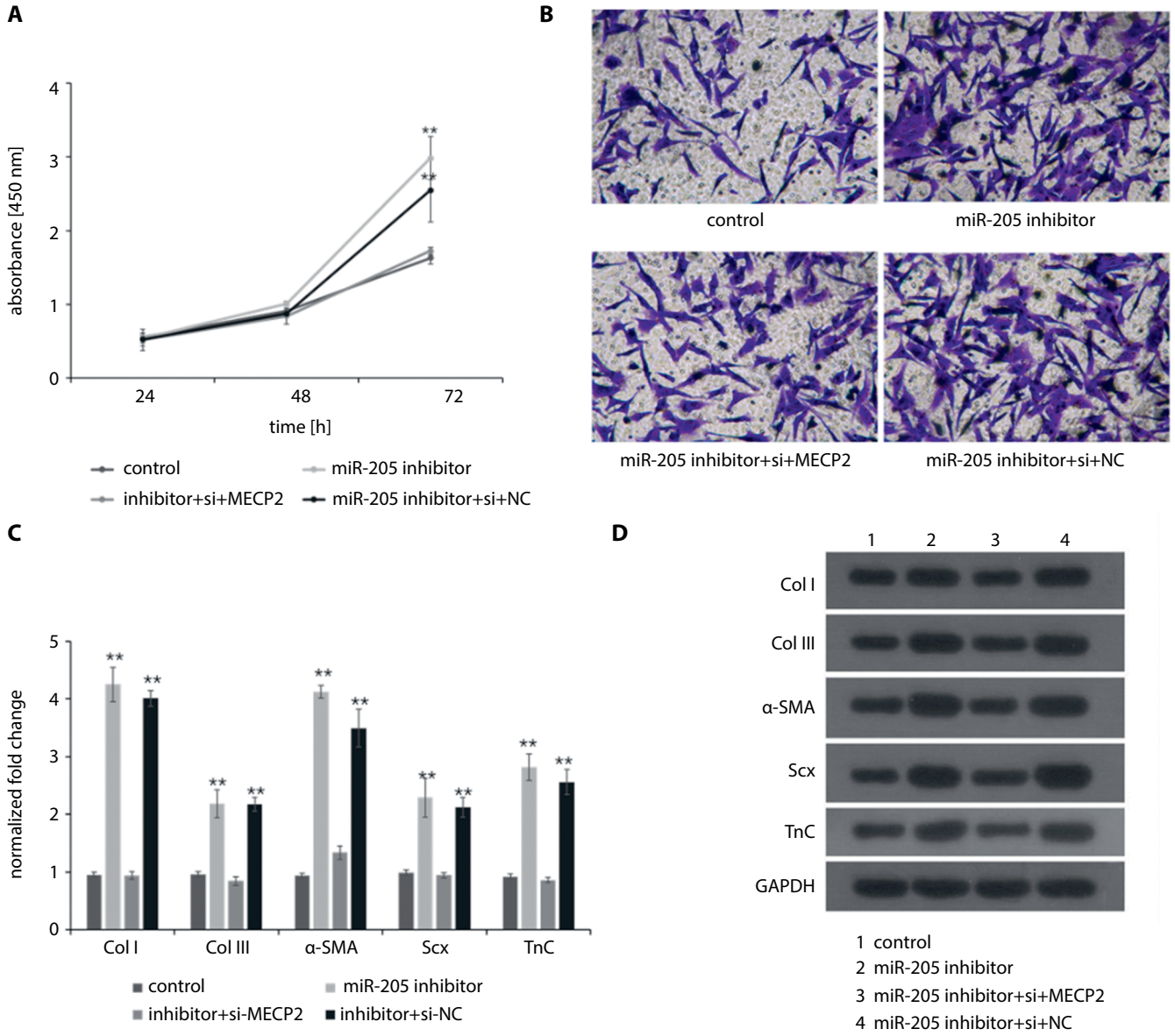

Fig. 4. The inhibition of MECP2 reverses the effect of miR-205 inhibitor on tenocytes. Cells were transiently transfected with miR-205 inhibitor, inhibitor plus small interfering RNA against MECP2 (si-MECP2), and the inhibitor plus the negative control siRNA (si-NC), or control. A. Cell proliferation was measured with Cell Counting Kit-8 (CCK-8) assay at various timepoints following the transfection; B. Transfected cells were subjected to migration assay. The image displays the cells stained with crystal violet; C. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for the mRNA expression levels of the fibrosis markers; D. Protein levels of the fibrosis markers. Data from experiments repeated in triplicate for both duplicate detection and biological duplication; values are presented as the median (range, minimum–maximum). Boxplots show the minimum value, 1st quartile, median, 3rd quartile, and maximum value. **p < 0.01 compared to control, ##p < 0.01 compared to miR-205 inhibitor+si-NC

the adhesion between tendon and surrounding tissue, and restrict tendon sliding. It can regulate the proliferation of tenocytes and the internal healing mechanism of tendon dominant, promoting tendon healing and preventing postoperative adhesion of tendon.[17] Related growth factors play a key role in the repair of tendon and other soft tissue injuries.[18] Distinct molecular mechanism underlying the tendon injury, repair and regeneration will have a great influence on the treatment of tendon injury.

The miRNAs have been reported to function as key regulators of various biological processes. There is evidence that tendon injury repair is related to the differentially expressed miRNAs.[19–21] It has also been reported that exogenous miR-29a downregulated BMP2 and BMP12, while miR-26a and miR-30d did not have a significant effect on tenocyte gene expression, suggesting that miR-29a contributes to tendon homeostasis and can serve as a potential therapeutic target in treating tendinopathy.[22] In our study, we observed that the inhibition of miR-205 promoted tenocyte proliferation, migration and fibrosis, which indicated that miR-205 might negatively regulate the progress of tendon injury repair.

The MECP2 can inhibit the transcription of its downstream target genes and play a role in the transcriptional regulation through the specific binding with methylated DNA, thus, it is an important transcriptional inhibitor.[23]

The MECP2 cannot inhibit the corresponding downstream target gene efficiently when its expression is downregulated, which leads to the occurrence of various diseases.[24] It has been reported that MECP2 plays a pivotal role in Rett syndrome pathology and a novel double mutation that can affect the transcription repression domain of MECP2 and cause a severe phenotype of Rett syndrome.[25–27] The results of our study reported here show that MECP2, the target of miR-205 in tenocytes was identified as an important transcriptional inhibitor, which might be a novel critical factor in the proliferation, migration and fibrosis of tenocytes.

Limitations

Given that miR-205 is a 22-mer small RNA differentially expressed in various tissues, targets other than MECP2 should be present in tenocytes. Therefore, whether and how miR-205 regulates proliferation, migration or fibrosis of tenocytes through targeting other factors is still to be studied.

Conclusions

The inhibition of miR-205 could promote the proliferation, migration and fibrosis of tenocytes. In addition, MECP2 is the direct target of miR-205. The miR-205 acts on tenocytes through targeting MECP2. These data implicate a multifactorial effect of miRNA on tenocyte and gene expression. Our findings may provide important information on miRNAs in tenocytes. The results of this study may be valuable for further experimental investigation on the mechanism of the repair of rotator cuff tear injury.

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