Original Article

# Enhanced reparatory effect of EI1 on dental pulp via extracellular matrix remodeling by miR-181b-2-3p inhibitor

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**Abstract** *Background/purpose:* Extracellular matrix (ECM) is crucial for dental pulp repair. The aim of this paper is to investigate the ECM remodeling effect of miR-181b-2-3p (a micro-RNA) and to verify the reparatory effect of EI1 (an epigenetic drug) and miR-181b-2-3p inhibitor on dental pulp.
*Materials and methods:* Levels of ECM-related factors in EI1-treated human dental pulp cells (hDPCs) were measured by qRT-PCR and Western blot. The anti-inflammation effect of EI1 was examined in Lipopolysaccharide-stimulated hDPCs. miR-181b-2-3p mimics or inhibitors were transfected into hDPCs and then the cells' functions were detected. A dual luciferase reporter assay was used to identify the targets of miR-181b-2-3p. Pulpotomy using miR-181b-2-3p antagomirs and EI1 as pulp capping materials was performed in male six-week-old Sprague-Dawley rats.
*Results:* EI1 upregulated ECM-related genes expression in hDPCs, but failed to upregulate the collagen1A1 (COL1A1) protein level. Pro-inflammatory factors were downregulated by EI1 in Lipopolysaccharide-stimulated hDPCs. Overexpression of miR-181b-2-3p downregulated the expression of transforming growth factor-$\beta$2 (TGF-$\beta$2) and fibronectin type III domain-containing protein 5 precursor (FNDC5), while the inhibition had the opposite effect. Dual luciferase reporter assays demonstrated that miR-181b-2-3p targets TGF-$\beta$2, FNDC5 and integrin alpha 4 protein (ITGA4). Compared to EI1 was used alone, EI1 combined with the inhibitor upregulated the protein levels of COL1A1, fibronectin (FN1) and TGF-$\beta$2 in hDPCs, promoted hDPCs migration, and exhibited reparatory effects on inflamed rat pulp tissue.
*Conclusion:* miR-181b-2-3p inhibitor could enhance the reparatory effect of EI1 via ECM remodeling in dental pulp both *in vitro* and *in vivo*.

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## Introduction

With the increasing knowledge about the reparatory potential of dental pulp, vital pulp therapy in teeth with irreversible pulpitis has become more and more popular.[1] Recent studies have proved that the epigenetic regulation (including DNA methylation, histone modifications, and non-coding RNA) participates in the progression of dental pulpitis.[2–4] The enhancer of zeste homolog 2 (EZH2) is a key epigenetic regulator in dental pulpitis which can upregulate pro-inflammatory factors, enchance macrophage chemotaxis, and impair the human dental pulp cells (hDPCs) mineralization.[5,6] Moreover, EZH2 acclerates the extracellular matrix (ECM) degradation and promotes the dental pulp inflammation.[7] The pulpal healing responses require active interactions between hDPCs and ECM, which plays an important role in regulating hDPCs proliferation, migration and differentiation.[8,9] Epigenetic regulation of ECM remodeling, such as the EZH2 inhibition, maybe effective in dental pulp repair.

We recently proved that EI1 (a selective EZH2 inhibitor) has the reparatory ability on hDPCs through regulating levels of several matrix metalloproteinases and collagen1A1 (COL1A1), but had limited in vivo therapeutic effect for rat pulpitis.[7] MicroRNAs (miRNAs, miRs) have been identified as key regulators in diverse biological processes including inflammation and immune response. They are small non-coding RNA molecules that bind to target mRNAs and repress gene expression at posttranscriptional levels in the vast majority of cases.[10] Differential miRNA expression has been detected in healthy and diseased human dental pulps.[11] We focused on the miRNA-mediated regulation in EI1-treated hDPCs and found that miR-181b was upregulated in a preliminary study.

The miR-181 family members are evolutionally conserved across all vertebrate species. They play important roles in preimplantation, embryonic development, neurogenesis, and immune modulation.[12,13] miR-181b regulates ECM remodeling in vascular smooth muscle cells by inhibting transforming growth factor-β (TGF-β) signaling.[14] In chondroblasts and articular chondrocytes, inhibition of miR-181b can reduce the expression of matrix metalloproteinase 13 while induce type II collagen expression.[15] The imbalance of miR-181b level in dental pulp may cause ECM remodeling and affect hDPCs' functions. To the best of our knowledge, no study has been conducted in this context.

In this study, we aimed to investigate the potential involvement of miR-181b inhibitor and EI1 in regulating ECM remodeling of dental pulp. We hypothesized that miR-181b might impair the reparatory effect of EI1 in regulating dental pulp repair.

## Materials and methods

### Cell culture, LPS treatment, and odontogenic differentiation

HDPCs were kindly provided by Oral Stem Cell Bank operated by Beijing Tason Biotech Co. Ltd. (Beijing, China) (http://www.kqgxb.com). hDPCs were treated with 2 μM EI1 (APExBIO, Houston, TX, USA). Lipopolysaccharide (LPS)-stimulated (Sigma-Aldrich, St Louis, MO, USA) hDPCs were treated with 1 μg/mL LPS. To induce odontogenic differentiation, hDPCs were exposed to osteogenic media (OM) comprised of 0.01 mM dexamethasone disodium phosphate (Sigma-Aldrich), 0.1 mM L-ascorbic acid phosphate (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma-Aldrich). The OM was changed every 2–3 days.

### Chromogenic in situ hybridization (CISH)

This experiment was granted consent by the Ethics Committee of the Peking University School and Hospital of Stomatology, Beijing, China (Approval Number: PKUSSIRB-202171200). Normal human pulp tissue and the inflamed pulp tissue collected from the teeth diagnosed with pulpitis clinically (n = 6) were fixed with an in situ hybridization fixative (Servicebio, Wuhan, China), embedded, and sliced. CISH experiments were performed using standard methods. The probe concentrations were 60 nM for miR-181b-2-3p, and 2.0 nM for the positive control probe 18S rRNA. Yellow or brownish-yellow were considered as a positive staining.

### Transient transfection

HDPCs were transfected with 25 nM miR-181b-2-3p mimics or 50 nM miR-181b-2-3p inhibitors (Genepharma, Shanghai, China) with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), following the instructions.

### RNA isolation and reverse transcription and quantitative real-time PCR (qRT-PCR)

RNA isolation and reverse transcription were performed as the previous study described.[7] Stem-loop method (RiboBio, Guangzhou, China) was used to synthesize cDNA from miRNA, followed by real-time PCR using SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). Conditions were applied as follows: 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, and 60 °C for 1 min using the Applied Biosystems (Foster City, CA, USA). The primer sequences were shown in Supplementary Table S2.

## Dual-luciferase reporter assay

The wild-type (WT) TGF-β2-3′ untranslated region (UTR), FNDC5-3′UTR, and ITGA4-3′UTR containing one of the miR-181b-2-3p targeting sites and their corresponding mutated 3′UTR sequences were synthesized (Tsingke Biotechnology Co., Ltd, Beijing, China) and cloned into pmirGLO plasmid (Promega, Madison, WI, USA). 293T cells were seeded into 12-well plates at a density of 20,000. Co-transfection was performed the following day using 600 ng of constructed plasmid and 40 pmol of each miRNA using Lipofectamine 3000 (Invitrogen). Cell lysates were collected after 48 h of transfection. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System kit (Promega).

## *In vivo* experimental pulp infection model

All procedures were approved by the Ethics Committee for Animal Research, Peking University Health Science Center, Beijing, China (approval number LA2022045, 2022), and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and the ARRIVE guidelines 2.0.

Thirty male six-week-old Sprague-Dawley rats were purchased from the Laboratory Animal Service Center of the Peking University Health Science Center and were randomly divided into six groups of five rats each. Cavities on the occlusal face were prepared and the coronal pulpal tissue was wiped off. After the LPS (1 μg/mL) treatment, a gelatin sponge soaked in different materials was used to cover the pulp exposure: normal saline (NS) (Solarbio, Beijing, China); rno-miR-181b-2-3p agomir (25 nM, Genepharma), rno-miR-181b-2-3p antagomir (50 nM, Genepharma), DMSO (2 μM, Sigma-aldrich) and EI1 + rno-miR-181b-2-3p antagomir/negative control. The cavities were sealed with MTA (DENSPLY, Konstantz, Germany) subsequently and then Fuji IX glass-ionomer cement (GC, Tokyo, Japan). The animals were sacrificed seven days after surgery. The specimens were divided, stained with hematoxylin-eosin (H&E) for routine histology, Masson's trichrome staining, and immunohistochemical staining. The

**Table 1** Evaluation criteria for histologic examination of pulpal inflammation.

| Inflammatory infiltrate | Characterization of inflammatory infiltrate |
| --- | --- |
| Grade 0 | No inflammatory signs underneath the capping |
| Grade 1 | Light inflammatory infiltrate with the presence of cells, such as polymorphonuclear leukocytes and mononuclear leukocytes underneath the capping |
| Grade 2 | Localized moderate cellular inflammatory infiltrate underneath the capping |
| Grade 3 | Severe cellular inflammatory infiltrate or with abscess characteristics underneath the capping |

sections were observed to examine the pulpal inflammation. The grading criteria of Kiba et al. with some modifications were used for histologic evaluation (Table 1).[16]

## Statistical analysis

All statistical data were processed using GraphPad Prism 9.0 and presented as mean ± standard deviation. Significance was determined via either Student's *t*-test or one-way analysis of variance tests. The histologic evaluation results were analyzed statistically via Kruskal-Wallis test. Differences were considered statistically significant at the $P < 0.05$ level.

## Results

### EI1 inhibited the expression of pro-inflammatory factors in hDPCs

The expression of interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α) were significantly upregulated at 24 h and 48 h in LPS-treated hDPCs (Fig. 1A and B). EI1 decreased expression of *IL-1β, IL-6, IL-8, IL-10,* and *TNF-α* in LPS-treated hDPCs (Fig. 2C). These outcomes suggested that EI1 had the potential to suppress inflammatory response during dental pulpitis.

### EI1 promoted the expression of ECM-related factors in hDPCs

At the mRNA level, the expression of COL1A1, fibronectin (FN1), fibronectin type III domain-containing protein 5 precursor (FNDC5), integrin alpha 4 (ITGA4), ITGA5, integrin beta 1 (ITGB1), transforming growth factor-β1 (TGF-β1), and TGF-β2 were significantly upregulated at 24 h with EI1 treatment, and the expression of COL1A1 and collagen3A1 (COL3A1) expression was dramatically increased at 48 h as compared with the control (Fig. 1D and E). At the protein level, FN1 was highly upregulated in EI1-treated hDPCs while COL1A1, TGF-β1, FNDC5, and TGF-β2 protein levels demonstrated no differences compared to the control (Fig. 1F). The protein levels of ECM-related genes were not completely consistent with the mRNA levels, indicating that there might be some post-transcriptional regulatory mechanisms in the ECM-remodeling process of EI1.

### miR-181b-2-3p was downregulated in the inflamed pulp tissue and during the osteogenic differentiation of hDPCs

The mRNA levels of miR-181b-2-3p displayed more than 15-fold upregulation in EI1-treated hDPCs at 24 h (Fig. 2A). The CISH results revealed that miR-181b-2-3p was widely expressed in normal dental pulp tissue and apparently reduced in the inflamed tissue (Fig. 2B). The mRNA levels of miR-181b-2-3p decreased gradually in the OM group (Fig. 2C).
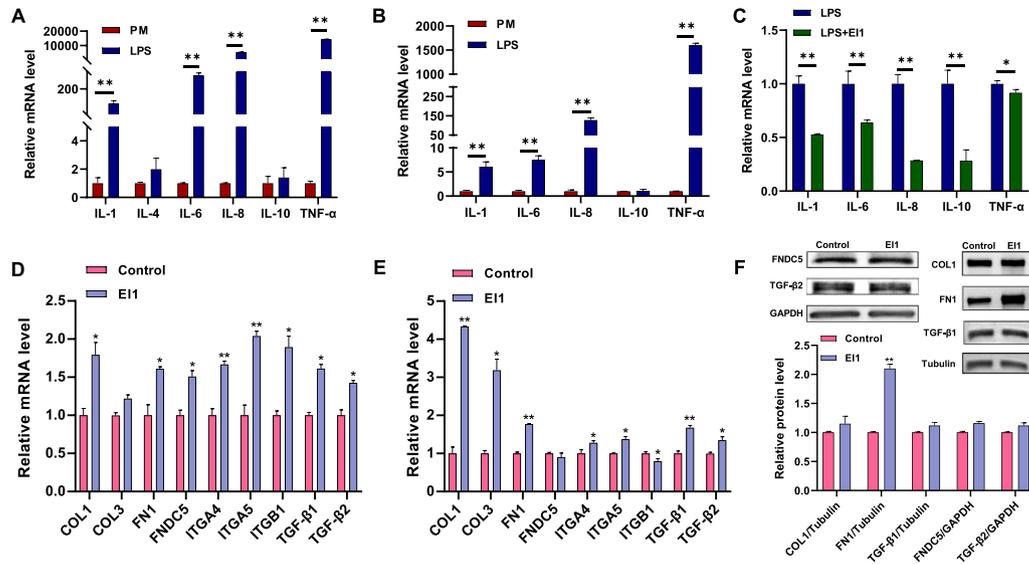
**Figure 1** EI1 has an anti-inflammatory effect in LPS-stimulated hDPCs. EI1 promotes ECM-related factors production in hDPCs. (A, B) qRT-PCR was conducted to determine the expression of inflammatory factors in LPS-stimulated hDPCs at 24 and 48 h. All mRNA values were normalized to GAPDH. (C) qRT-PCR was conducted to determine the expression of inflammatory factors in hDPCs at 48 h after LPS and EI1 treatment. (D, E) The qRT-PCR analysis of ECM-related genes expressions in hDPCs at 24 and 48 h after EI1 treatment. (F) Western blot analysis of ECM-related proteins in hDPCs at 48 h after EI1 treatment. COL1/3: collagen type I/III; Control: 2 μM DMSO (the solvent of EI1) in proliferation medium; EI1: 2 μM EI1 in proliferation medium; FN1: fibronectin; FNDC5: fibronectin type III domain-containing protein 5 precursor; IL-1, -4, -6, -8, and -10: interleukin-1(β), -4, -6, -8, and -10; ITGA4/A5/B1: integrin alpha 4/alpha 5/beta 1 protein; LPS: 1 μg/mL LPS in proliferation medium; PM: proliferation medium; TGF-β1/2: transforming growth factor-β1/2; TNF-α: tumor necrosis factor-α. *P < 0.05, **P < 0.01.
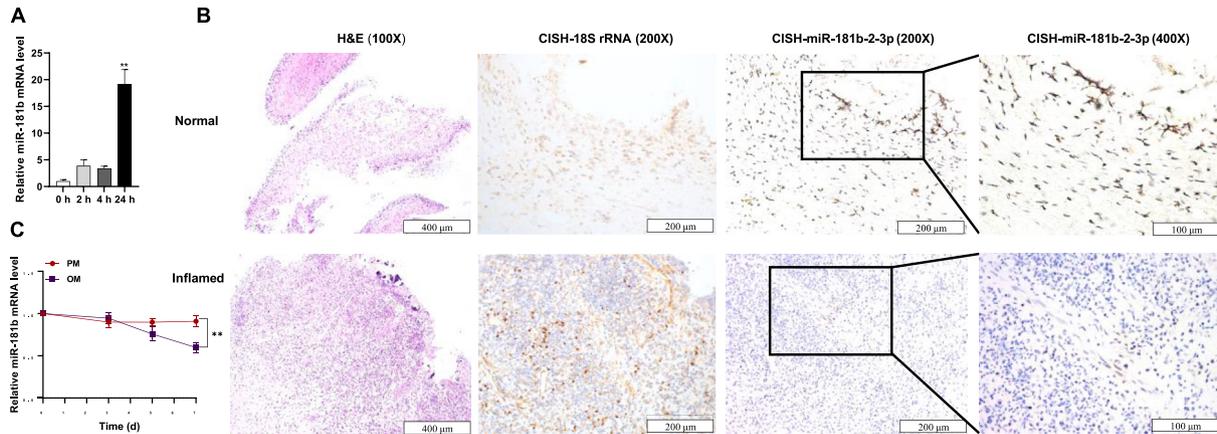


**Figure 2** The expression of miR-181b-2-3p changes during the osteogenic differentiation of hDPCs and in the inflammed human pulp tissue. (A) The qRT-PCR analysis of miR-181b-2-3p expression in hDPCs at 2 h, 4 h, and 24 h after EI1 treatment. miRNA values were normalized to U6. (B) H&E (original magnification, 100×) stain showing increased inflammatory cells in the inflamed group compared with the normal control. Chromogenic in situ hybridization (CISH) (original magnification, 200× and 400×) showed that miR-181b-2-3p expression was apparently reduced in the inflamed group compared with the normal control. 18S rRNA was used as a positive control. (C) qRT-PCR showed that miR-181b-2-3p expression was dramatically decreased in hDPCs after osteogenic differentiation for 7 days. 18S rRNA: 18S ribosomal RNA; Inflamed: inflamed pulp tissue extirpated from human teeth; Normal: pulp tissue from healthy human teeth that were extracted for orthodontic reasons; OM: osteogenic induction medium; PM: proliferation medium. **P < 0.01.

## miR-181b-2-3p had no effect on biological behaviors of hDPCs

The transfection efficiencies of miR-181b-2-3p mimics and inhibitors in hDPCs were verified by qRT-PCR (Fig. 3A). CCK8 assays demonstrated that miR-181b-2-3p mimic or inhibitor transfection had no significant effect on cell viability in hDPCs (Fig. 3B). Scratch-wound assays demonstrated miR-181b-2-3p did not affect hDPCs migratory abilities (Fig. 3C).
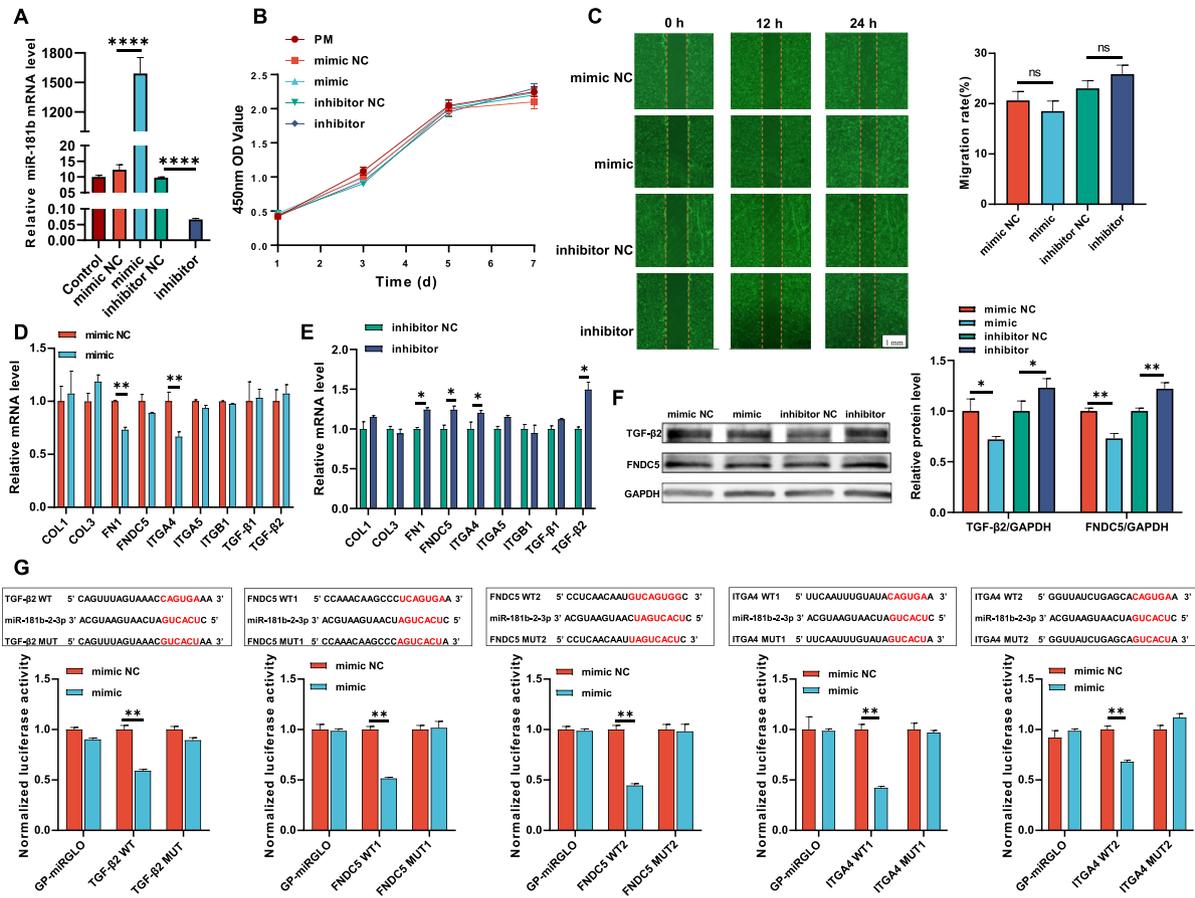
**Figure 3** miR-181b-2-3p inhibits several ECM-related factors expression, while its inhibitor has the opposite effect. (A) The expression of miR-181b-2-3p was examined by qRT-PCR analysis to confirm transfection efficiency. (B, C) CCK8 and scratch assays were used to investigate the effect of miR-181b-2-3p mimic or inhibitor on proliferation and migration of hDPCs respectively. (D, E) The qRT-PCR analysis of ECM-related genes expressions in hDPCs after miR-181b-2-3p mimic or inhibitor transfection. (F) Western blot analysis of TGF-β2 and FNDC5 expressions in hDPCs after miR-181b-2-3p mimic or inhibitor transfection. (G) TGF-β2, FNDC5 and ITGA4 were verified as miR-181b-2-3p target genes by dual-luciferase reporter assays. COL1/3: collagen type I/III; Control: proliferation medium containing transfection reagent; FN1: fibronectin; FNDC5: fibronectin type III domain-containing protein 5 precursor; ITGA4/A5/B1: integrin alpha 4/alpha 5/beta 1 protein; MUT: mutant type; PM: proliferation medium; TGF-β1/2: transforming growth factor-β1/2; WT: wild type. ns: not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

## miR-181b-2-3p affected the expression of ECM-related factors in hDPCs

Then, we explored the relationship of miR-181b-2-3p to ECM in hDPCs. miR-181b-2-3p mimic (25 nM) downregulated the mRNA levels of *ITGA4* and *FN1* in hDPCs (Fig. 3D), while miR-181b-2-3p inhibitor (50 nM) upregulated the mRNA levels of *FN1*, *FNDC5*, *ITGA4*, and *TGF-β2* in hDPCs (Fig. 3E). The protein level of TGF-β2 and FNDC5 were significantly downregulated by miR-181b-2-3p mimic, while the inhibition had the opposite effect (Fig. 3F).

## miR-181b-2-3p directly targeted TGF-β2, FNDC5, and ITGA4

To further confirm the connection between miR-181b-2-3p and ECM-related factors, the dual-luciferase reporter assay

was performed. The results showed that miR-181b-2-3p only reduced the luciferase activities of the pmirGLO-TGF-β2-WT, two pmirGLO-FNDC2-WTs, and two pmirGLO-ITGA4-WTs but not those of the mutant reporters (Fig. 3G). These results depicted that miR-181b-2-3p could inhibit the expression of TGF-β2, FNDC5, and ITGA4 by complementarily binding to the 3′ UTR of their mRNAs.

## miR-181b-2-3p inhibitor counteracted the adverse effects of EI1 on cell cycle and migration ability of hDPCs

The cell cycle distribution analysis demonstrated that the EI1 treatment could noticeably induce cell cycle arrest in the G0/G1 phase, and hDPCs depicted significantly decreased G2/M phase proportions (Fig. 4A). In contrast, the miR-181b-2-3p inhibitor plus EI1 increased G2/M phase
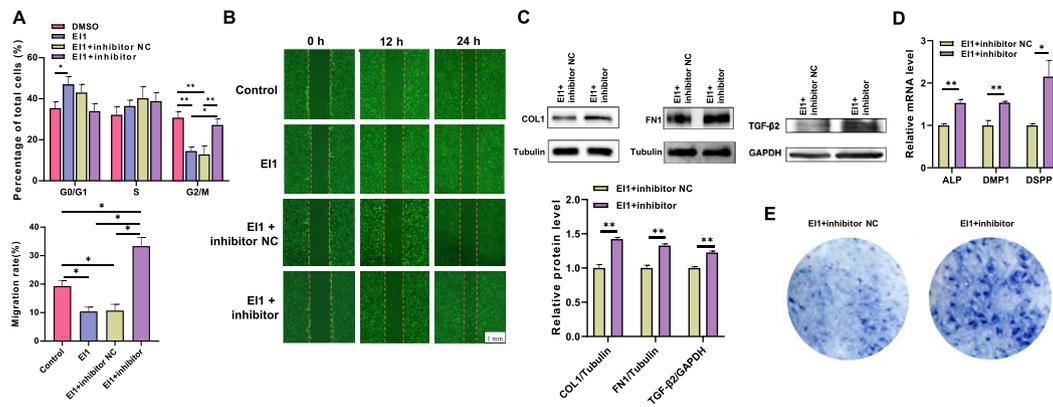
**Figure 4** miR-181b-2-3p inhibitor promotes the reparatory effect of EI1 *in vitro*. (A, B) miR-181b-2-3p inhibitor abolishes the adverse effects of EI1 on migration ability and cell cycle of hDPCs. Statistical quantification of the percentages of hDPCs in different cell cycles from flow cytometry data. Representative images of scratch wound assays. Migration rate = ((T0 h scratch area − T24 h scratch area)/T0 h scratch area) × 100%. (C) Western blot analysis of COL1A1, FN1, and TGF-β2 in hDPCs incubated with EI1 and miR-181b-2-3p inhibitor/NC. (D, E) HDPCs were cultured in the osteogenic induction medium for 7 days. The qRT-PCR analysis of ALP, DMP1, and DSPP expressions and the alkaline phosphate staining in hDPCs incubated with EI1 and miR-181b-2-3p inhibitor/NC. ALP: alkaline phosphatase; COL1/3: collagen type I/III; Control: culture medium (1% FBS); DMP1: dentin matrix protein 1; DSPP: dentin sialophosphoprotein; EI1: 2 μM EI1 in culture medium (1% FBS); FN1: fibronectin; TGF-β2: transforming growth factor-β2. *$P < 0.05$, **$P < 0.01$.

proportion compared with EI1 used alone (Fig. 4A). The scratch-wound healing assay demonstrated that the migration rate of hDPCs was significantly decreased in the EI1 group compared with the control (Fig. 4B). However, the migration rate was strongly enhanced when EI1 was used with miR-181b-2-3p inhibitor, compared to EI1 was used alone (Fig. 4B). The above results showed that the miR-181b-2-3p inhibitor abrogated the adverse effect of EI1 on the cell cycle and migration ability of hDPCs. These results laid a foundation for further detailed researches.

**miR-181b-2-3p inhibitor increased the expression of ECM-related proteins in hDPCs treated with EI1**

Since miR-181b-2-3p inhibitor could increase the protein level of TGF-β2 and FNDC5 in hDPCs, this study concerned the compensatory effect of miR-181b-2-3p inhibitor to EI1 for ECM repair. The protein levels of COL1A1, FN1 and TGF-β2 were upregulated in hDPCs treated with EI1 combined with miR-181b-2-3p inhibitor compared to EI1 used alone (Fig. 4C). We concluded that the synergistic effect of EI1 and miR-181b-2-3p inhibitor promoted the ECM-related proteins production in hDPCs, which is important in the process of dental pulp repair.

**miR-181b-2-3p inhibitor combined with EI1 promoted odontogenesis of hDPCs *in vitro***

After 7 days of culturing in the OM, the results showed that miR-181b-2-3p inhibitor combined with EI1 increased the expression of alkaline phosphatase (ALP), dentin matrix protein 1 (DMP1), and dentin sialophosphoprotein (DSPP) in hDPCs, and enhanced the ALP staining when comparing to the control group (Fig. 4D and E).

**miR-181b-2-3p antagomir promoted the reparatory effect of EI1 on dental pulp**

Finally, we investigated the reparatory effect of EI1 combined with miR-181b-2-3p antagomir *in vivo*. The results of histopathological evaluation of pulpal inflammation are summarized in Table 2. No significant differences in pulpal inflammation were found among all the groups. All teeth in the NS group and agomir group showed moderate inflammation underneath the capping and light disorganization of the odontoblast layer, meanwhile, the rest of the pulp tissues were basically normal (Fig. 5A and B). Among five teeth tested, four teeth exhibited moderate inflammation underneath the capping and basically no disorganization of the odontoblast layer, and one tooth showed light inflammation in the miR-181b-2-3p antagomir group (Fig. 5C). Compared to the NS group and agomir group, immunohistology revealed that cells near the root canal walls were stained positively in the antagomir group (Fig. 5Cd). In the DMSO group, moderate inflammation was observed

**Table 2** Summary of the histological observation data of pulpal inflammation.

| Grade | Group | | | | | |
|---|---|---|---|---|---|---|
| | NS | Agomir | Antagomir | DMSO | EI1+ antagomir NC | EI1+ antagomir |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 1 | 0 | 2 | 2 |
| 2 | 5 | 5 | 4 | 5 | 3 | 3 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 |

The figures for each group indicate the number of specimens among the five teeth tested. NS: normal saline.
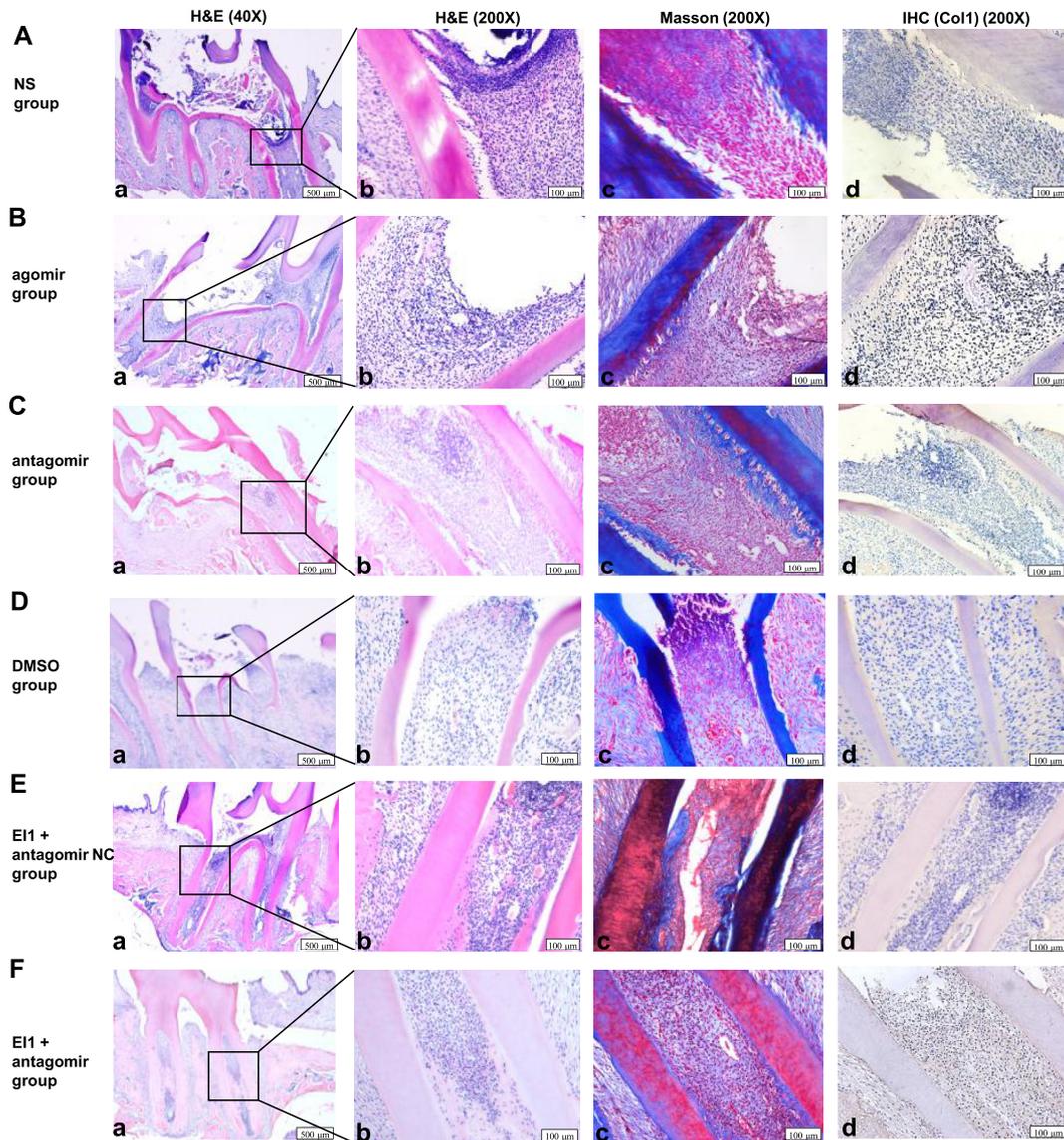
**Figure 5**  miR-181b-2-3p antagomir promotes the reparatory effect of EI1 at day 7 after the surgery *in vivo*. H&E staining (original magnification, 40× and 200×), Masson-Trichrome staining (original magnification, 200×), and immunohistochemistry for Col1 (original magnification, 200×) were shown. (A, C) Moderate inflammation and light disorganization of the odontoblast layer was observed underneath the capping. Immunohistology showed no obvious positive Col1 expression. (B) Moderate inflammation and basically normal odontoblast layer was observed underneath the capping. Immunohistology showed cells near the root canal walls were stained positively. (D, E) Light to moderate inflammation and basically normal odontoblast layer was observed underneath the capping. Masson-Trichrome staining results displayed that newly formed blue-stained collagen fibers were yielded underneath the capping and near the root canal walls in both groups. A strong Col1 immunoreactivity was observed in the cells near the root canal walls in the EI1 and miR-181b-2-3p antagomir NC group. In the EI1 and antagomir group, immunohistology revealed an increase in Col1 underneath the inflammatory infiltration both in the center of root canal and near the root canal walls. Col1: collagen type I; IHC: immunohistochemical staining; NS: normal saline.

underneath the capping in all teeth, and light disorganization of the odontoblast layer was observed as well (Fig. 5D). In the EI1 and miR-181b-2-3p antagomir/NC group, among five teeth tested, two teeth exhibited light inflammation and three teeth exhibited moderate inflammation underneath the capping and the rest of the pulp tissues were basically normal (Fig. 5E and F). Masson-Trichrome staining results displayed that newly formed blue-stained collagen fibers were yielded underneath the capping and near the root canal walls in the EI1 and miR-

181b-2-3p antagomir NC group (Fig. 5Ec), and a strong Col1 immunoreactivity was observed in the corresponding region (Fig. 5Ed). In the EI1 and antagomir group, more reparatory dentine were observed and immunohistology revealed an increase in Col1 underneath the inflammatory infiltration both in the center of root canal and near the root canal walls (Fig. 5Ed). These results further illustrated that the synergistic effect of EI1 and a miR-181b-2-3p inhibitor could promote the dental pulp repair *in vivo* by inhibiting ECM degradation.

## Discussion

Vital pulp therapy in inflamed teeth has attracted more and more attention from researchers.[17−20] Epigenetics-based therapeutics have been proven to be effective in tissue repair by regulating ECM remodeling but have not been extensively studied in dental pulp.[21,22] EI1, a selective EZH2 inhibitor, has the potential to promote ECM remodeling in dental pulp.[7] In this study, we first verified the anti-inflammatory and ECM-remodeling effect of EI1 *in vitro* and further demonstrated that miR-181b-2-3p inhibitor could enhance the reparatory effect of EI1 via ECM remodeling in dental pulp both *in vitro* and *in vivo*.

ECM-related factors play important roles in the behaviors of hDPCs.[23−25] We found that EI1 could upregulate the mRNA levels of these ECM-related genes in hDPCs, but not the protein level of COL1A1, TGF-β, and FNDC5. Since miRNA modulation could affect the ECM-remodeling process in EI1-treated dental pulp, we searched for the key miRNA in this process. In this study, miR-181b-2-3p was significantly upregulated in the EI1-treated hDPCs, and was found to participate in the inflammation and repair process of dental pulp, which is in agreement with a previous study.[11]

miR-181b has an anti-fibrotic effect in different tissues, such as blood vessels, cartilage, and liver.[15,26,27] The specific role of miR-181b in dental pulp ECM remodeling has not been clear. To verify the target genes of miR-181b-2-3p, HEK-293T cells were co-transfected with constructed pmirGLO plasmid and miR-181b-2-3p/NC and they were chosen because of their high transfection efficiency.[28] The dual-luciferase assays demonstrated that the miR-181b-2-3p mimic repressed the 3′UTR of TGF-β2, FNDC5, and ITGA4. These ECM-related factors are responsible for migration and collagen synthesis.[29−32] The reason why the mRNA levels of TGF-β2 and FNDC5 were not significantly changed may be that miR-181b-2-3p silences them by translation repression rather than degradation.[10] The follow-up experiments were performed on this basis. The combination of EI1 and a miR-181b-2-3p inhibitor effectively increased COL1A1, FN1 and TGF-β2 production in hDPCs. Among these factors, fibronectin is known to be important for cellular adhesion and migration,[33,34] and TGF-β2 is a vital protein in collagen matrix synthesis and dental tissue repair,[35] which may explain why the migration and odontogenesis abilities of hDPCs were both elevated.

The inhibition of miR-181b-2-3p also promote the reparatory effect of EI1 via ECM remodeling *in vivo*. The construction of rat pulpitis model in maxillary first molars has been widely used for testing the healing of pulp tissue after capping with experimental materials.[36] In our study, both EI1 and miR-181b-2-3p antagomir increased Col1 expression slightly when applied alone, mainly focused on the cells near the root canal walls. In contrast, the combination of EI1 and miR-181b-2-3p antagomir significantly increased the formation of reparatory dentine and Col1 expression in inflamed dental pulp. The unusual phenomenon of Masson-Trichrome staining in some samples that both dentine and the pulpal tissue were colored red may result from the inadequate decalcification degree of root dentine and the dye of large molecular size failed to penetrate the root dentine.[37]

In conclusion, we showed that EI1 has anti-inflammatory ability *in vitro* and miR-181b-2-3p has an ECM-remodeling effect in dental pulp by modulating TGF-β2, FNDC5, and ITGA4. Moreover, miR-181b-2-3p inhibitor could help EI1 create a better condition for inflamed pulp tissue preservation, thus achieving better reparative effects on dental pulp.

## Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2023.05.002.

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