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IL-1β/NF-kb signaling promotes colorectal cancer cell growth through miR-181a/PTEN axis

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ABSTRACT

To date, the role of miRNA in tumorigenesis has been largely reported. It was found that miR-181a may be involved in the tumorigenesis of colon cancer. The purpose of this study was to investigate the mechanism of miR-181a in colon cancer carcinogenesis. The expression levels of IL-1β, NF-κB (RelA), and miR-181a in colon cancer tissue were higher than that in normal control tissue when assessed by real-timePCR. In addition, it was found that IL-1β induced the expression of miR-181a. The expression of PTEN was regulated by IL-1β-stimulated miR-181a expression. In a PTEN reporter plasmid, miR-181a binding site mutations were introduced. By using a luciferase reporter assay, it was found that wild type reported activity was lower than that of the mutant registration system activity. Furthermore, a siRNA strategy was used to find that IL-1B regulates miR-181a expression via NF-κB and then regulates PTEN expression. Consequently, repression of PTEN by miR-181a promotes colon cancer cell proliferation. Taken together, our data support a critical role for NF-κB-dependent upregulation of miR-181a; this represents a new pathway for the repression of PTEN and the promotion of cell proliferation upon IL-1β induction.

1. Introduction

Colorectal cancer (CRC) is a worldwide disease that is the third most common cause of cancer in both women and men and is also the third most common cause of cancer death [1]. Inflammation plays a major role in CRC development, including tumor initiation, progression, and invasion [2]. Numerous factors have been regarded as potential diagnostic and prognostic markers in CRC [3]. Continuous exposure to inflammatory cytokines, includingIL-1, IL-6, and TNF-α, is known to initiate CRC [4,5].

The nuclear factor kappa B (NF-κB) family members share structural homology with the retroviral onco-protein v-Rel resulting in their classification as NF-κB/Rel proteins [6]. There are five proteins in the mammalian NF-κB family: NF-κB1, NF-κB2, RelA, RelB, and c-Rel. The NF-κB pathway has long been considered a major pro-inflammatory signaling pathway largely based on the activation of NF-κB by pro-inflammatory cytokines and the role of NF-κBin the transcriptional activation of responsive genes including cytokines and chemokines [7,8]. The “canonical” pathway for NF-κB activation is triggered by pro-inflammatory cytokines, such as IL-1β. NF-κB has been reported to be activated by IL-1βin intestinal epithelia cells and colorectal adenomas in azoxymethane- or dextran sulfate sodium-induced mice as well as in colon cancer cell lines [9,10]. Thus far, however, the role of the IL-1β/NF-κB signal pathway in colon carcinogenesis has not been characterized.

NF-κB induces a variety of biological processes through transcriptional gene control in various signaling pathways including several miRNAs [11]. Xu et al. reported that DNA damage-induced NF-κB activation in human glioblastoma cells promotes miR-181b expression and cell proliferation [12]. NF-κB signaling pathway regulates the miR-223/FBXW7 axis in T-cell acute lymphoblastic leukemia (T-ALL) and regulates T-ALL resistance to gamma-secretase inhibitor (GSI) treatment [13]. In addition, NF-κB modulates miR-124, miR-148a, and miR-130a to influence tumor growth and chemo-resistance [11,13–15].

miRNAs are small, non-coding RNA molecules that are highly conserved across species and play key roles as regulators of gene expression [16,17]. miRNAs regulate as much as 60% of human protein coding genes and modulate the level of proteins involved in most biological processes, including cancer development, progression, invasion, and chemo-resistance [7,18]. In cancers,

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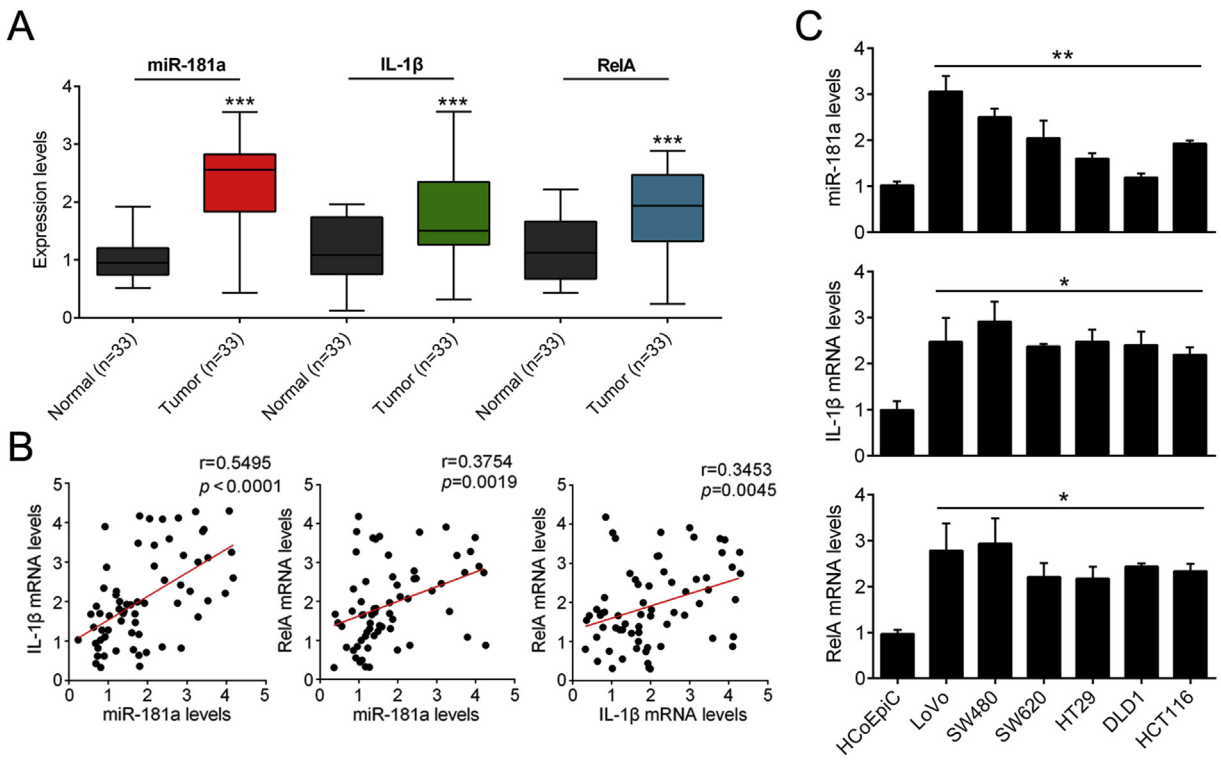

Fig. 1. The expression levels of miR-181a, IL-1β, and NF-κB (RelA) are up-regulated in colon cancer and are positively correlated. By using real-time PCR, the expression levels of miR-181a, IL-1β, and NF-κB (RelA) in 33 paired samples were analyzed. (A) Significantly higher levels of miR-181a, IL-1β, and RelA expression in the tumor samples were found relative to the levels in the adjacent normal tissues ($P < 0.0001$).(B) A positive correlation existed between the miR-181a level and the IL-1βmRNA level, the miR-181a level and the RelA mRNA level, and the IL-1β mRNA level and the RelA mRNA level. (C) Compared with the immortalized colon cancer cell line, HCoEpiC, miR-181a, IL-1β, and NF-κB expression in all six colon cancer cell lines were higher. The data are presented as mean ± SD of three independent experiments.

including CRC, miR-181a attracts the most research attention on account of its correlation with inflammation and cancer chemoresistance [19,20]. Liu et al. suggested that the LPS-TLR4-miR-181a signaling pathway plays a significant role in pancreatic cancer invasion and progression [21]. miR-181a confers cervical cancer resistance to radiation therapy through targeting the pro-apoptotic *PRKCD* gene [22]. Moreover, stable transfection of CRC cell lines with miR-181a promoted cell motility and invasion as well as tumor growth and liver metastasis, while silencing its expression resulted in reduced migration and invasion [23]. However, whether the expression level of miR-181a can be regulated by IL-1β/NF-κB during CRC growth has not been determined.

In this work, we found that miR-181a induction upon IL-1βstimulation was dependent on the activation of NF-κB, which enhanced miR-181a gene transcription. Moreover, the upregulated miR-181a directly targeted phosphatase and tensin homolog (PTEN) and negatively regulated its expression, which resulted in promotion of cell survival upon IL-1β induction.

2. Materials and methods

2.1. Tissue samples, cell lines, and plasmids transfection

A total of 33 pairs of primary colon cancer and their matched adjacent normal tissues were collected. All samples were obtained from patients who underwent surgical resections at Xiangya Hospital of Central South University (Changsha, China), snap-frozen in liquid nitrogen, and then stored at −80 °C for further use. This project was approved by the Ethics Committee of Xiangya Hospital of Central South University.

Seven human cell lines, including five colon cancer cell lines (LoVo, SW480, SW620, SW116, and HCT116) and two human colon immortalized cell lines(HCoEpiC and U6), were purchased from American Type Culture Collection (ATCC). Cells were grown routinely in RPMI-1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) and cultured in a 37 °C humidified atmosphere of 5% CO_2. Ectopic expression of miR-181a in cells was achieved by transfection with miR-181a mimics (Genepharma, Shanghai, China) using Lipofectamine2000 (Invitrogen, CA, USA). Overexpression of PTEN was performed using a PTENORF expression clone (GeneCopoecia, Guangzhou, China). Cells were transfected for 24 h or 48 h. Transfected cells were used for further assays or RNA/protein extraction.

2.2. RNA extraction and SYBR green quantitative PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA). Mature miR-181a expressions in cells were detected using a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China). Expression of U6 was used as an endogenous control. PTEN expression was measured by a SYBR green qPCR assay (Takara, Dalian, China). Data were processed using the $2^{-\Delta\Delta CT}$ method.

2.3. CCK-8 cell proliferation assay

Cell proliferation rates were measured using Cell Counting Kit-8 (CCK-8) (Beyotime, Hangzhou, China). In each 96-well plate, 0.5×10^4 cells were seeded for 24 h, transfected with the indicated miRNA or siRNA, and further incubated for 24 h, 48 h, 72 h and 96 h, respectively. Ten microliters of CCK-8 reagent were added to each well for 1 h before the endpoint of incubation. An OD_{450nm} value in each well was determined using a microplate reader.

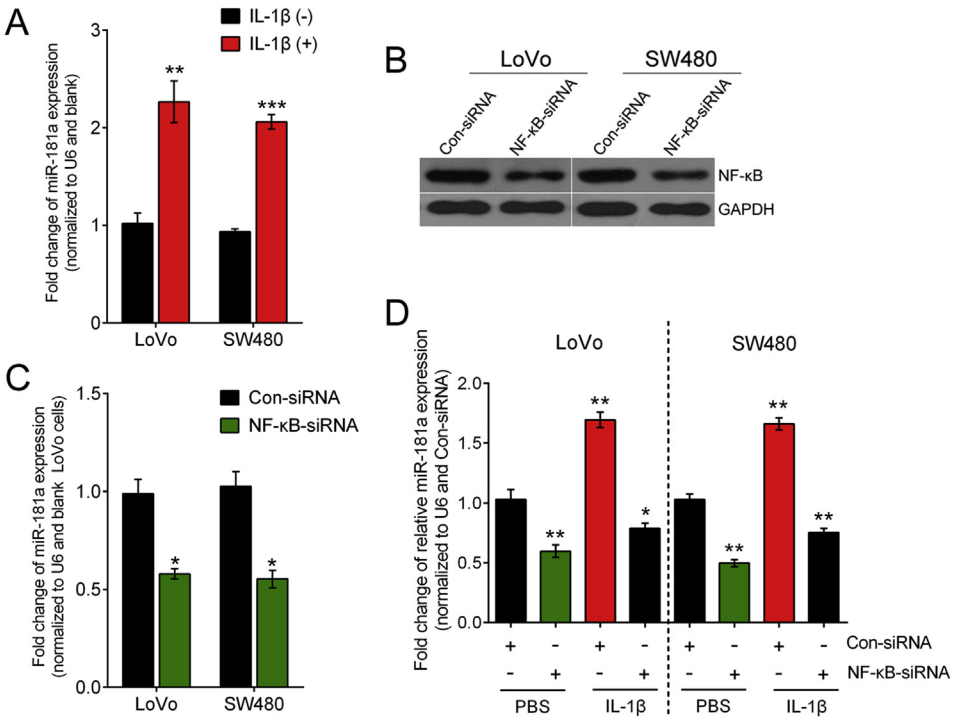

Fig. 2. IL-1β-induced NF-κB activation is required for miR-181a induction. (A) Real-time PCR results showed that IL-1β could induce miR-181a expression. (B) Western blot showed the level of NF-κB was decreased when transfected with NF-κB-siRNA. (C) Knocking down NF-κB by NF-κB-siRNA significantly decreased miR-181a expression. (D) IL-1β-induced miR-181a upregulation was attenuated in the presence of NF-κB-siRNA. The data are presented as mean ± SD of three independent experiments.

2.4. BrdU incorporation assay

BrdU assays were performed to determine DNA synthesis at 24 h and 48 h after transfecting Lovo cells and SW480 cells with miR-181a mimics or mimics negative control (NC). The infected cells were cultured for 24 h or 48 h and incubated with a final concentration of 10 μM BrdU (BD Pharmingen, San Diego, CA, USA) for 2 h–24 h. At the end of the incubation period, the medium was removed, and the cells were fixed for 30 min at RT, incubated with peroxidase-coupled anti-BrdU-antibody (Sigma-Aldrich) for 60 min at RT, washed with PBS, and incubated with peroxidase substrate (tetramethyldbenzidine) for 30 min. Then, the absorbance values were measured at 490 nm.

2.5. Western blot analysis

Immunoblotting was performed to detect the expression of NF-κB and PTEN in colon cell lines. Cultured or transfected cells were lysed in RIPA buffer with 1% PMSF. Protein was loaded onto a SDS-PAGE minigel and transferred onto PVDF membrane. After being probed with 1:1000 diluted rabbit polyclonal p65, NF-κB or PTEN antibody (Abcam, MA, USA) at 4 °C overnight, the blots were subsequently incubated with HRP-conjugated secondary antibody (1:5000). Signals were visualized using ECL substrates (Millipore, MA, USA). β-actin was used as an endogenous protein for normalization.

2.6. Luciferase reporter assay

LoVo and SW480 cells were transfected with miR-181a and pGL3 luciferase reporter constructs harboring the miR-181a target sequence. After 24 h, the activities of firefly luciferase and renilla luciferase in the cell lysates were measured with the Dual-Luciferase Assay System (Promega, Madison, WI, USA). For the luciferase transcription reporter assay, miR-181a gene promoter sequences (WT or site deletion) were cloned into the promoter region of the pGL3-Basic vector, and luciferase activity was measured as described above.

2.7. Statistical analysis

All data from 3 independent experiments were expressed as mean ± SD and processed using SPSS17.0statistical software. The expression of miR-181a in colon tissues and their matched adjacent normal bone and myeloid tissues were compared by Wilcoxon's paired test. The difference among the groups in migration and invasion assay was estimated by Student's t-test or one-way ANOVA. A P value of<0.05 was considered to be statistically significant.

3. Results

3.1. The expression levels of miR-181a, IL-1β, and NF-κB (RelA) are up-regulated in colon cancer and are positively correlated

By using real-time PCR, we analyzed the expression levels of miR-181a, IL-1β, and NF-κB (RelA) in 33 paired samples. Significantly higher levels of miR-181a, IL-1β, and RelA expression in the tumor samples were found relative to the levels in the adjacent normal tissues ($p < 0.0001$) (Fig. 1A). We observed a positive correlation between themiR-181a level and IL-1βmRNA level, the miR-181a level and RelA mRNA level, and the IL-1β mRNA level and RelA mRNA level (Fig. 1B). Compared with the immortalized colon cancer cell line, HCoEpiC, miR-181a, IL-1β, and NF-κB expressions in all six colon cancer cell lines were higher (Fig. 1C).

3.2. IL-1β-induced NF-κB activation is required for miR-181a induction

Recently, emerging evidence has confirmed that IL-1β activates NF-κB [24]. To determine the mechanism involved in miR-181a

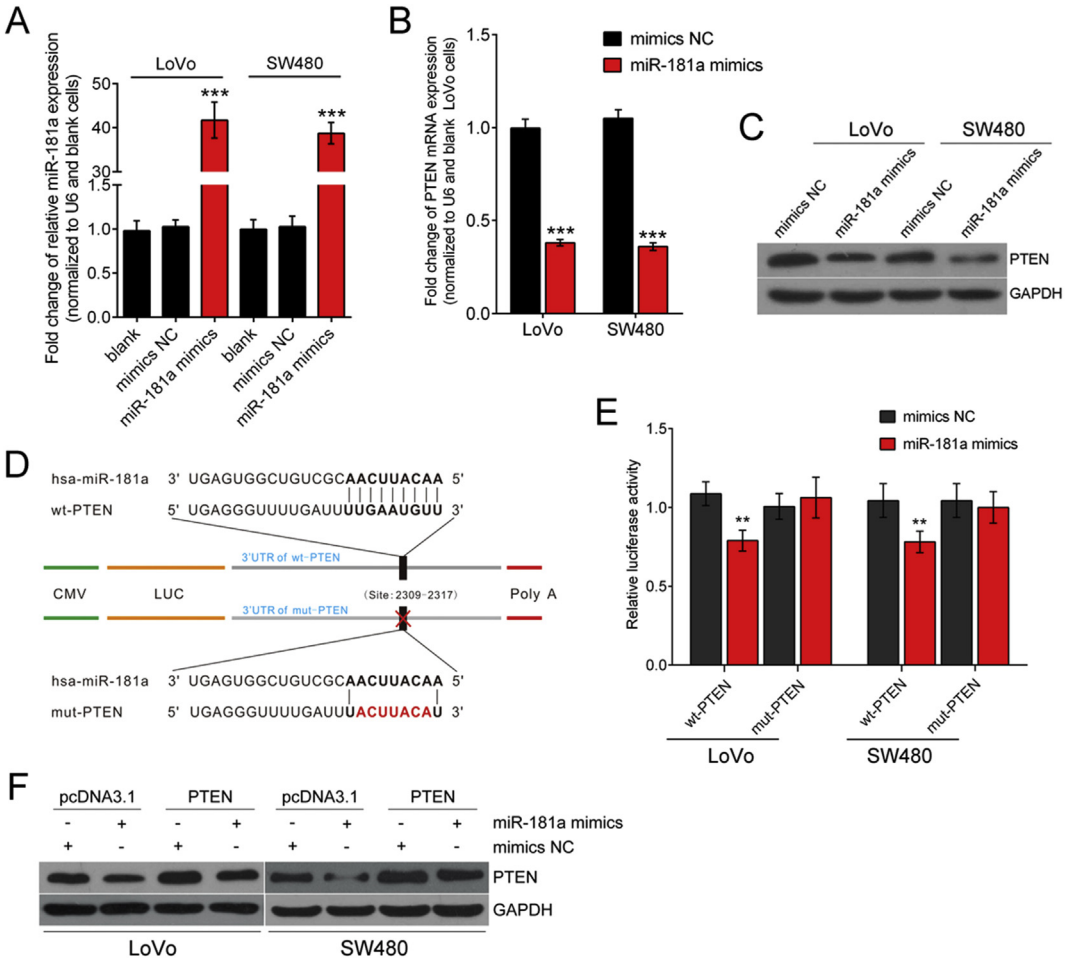

Fig. 3. Expression of PTEN is negatively regulated by miR-181a. (A) The transfection efficiency of miR-181a mimics on miR-181 expression was verified by real-time PCR and the expression level of miR-181a was up-regulated by miR-181a mimics by 40 times compared with U6 and blank cells. (B) Enhanced miR-181a expression in LoVo and SW480 cells significantly repressed PTEN mRNA expression compared with the mimics NC group. (C) Enhanced miR-181a expression in LoVo and SW480 cells significantly repressed PTEN protein expression compared with NC. (D) A point mutation in the miR-181a binding site of the PTEN reporter luciferase was constructed, and then mimic NC and miR-181amimics were co-transfected into LoVo cells and SW480 cells. (E) Compared with wild-type reporter plasmid, the luciferase mut-PTEN reporter was found to be 1.5 times more active, and similar results were obtained by transfection of reporter plasmid into the SW480 cell line. (F) Forced expression of PTEN partially restored the PTEN protein expression inhibited by miR-181a. The data are presented as mean ± SD of three independent experiments.

trans-activation upon IL-1β induction, NF-κB activity was assessed in the present study. It was observed that IL-1β could induce miR-181a expression (Fig. 2A). To explore whether NF-κB activity was present in LoVo and SW480 cells treated with IL-1β, we used a Western blot to determine the level of NF-κB when transfected with NF-κB-siRNA (Fig. 2B). Results showed that NF-κB expression was significantly down-regulated by NF-κB-siRNA transfection compared to Con-siRNA (Fig. 2B, Fig. S1). As expected, we observed that knocking down NF-κB significantly decreased miR-181a expression (Fig. 2C) and that IL-1β-induced miR-181a upregulation was attenuated in the presence of NF-κB-siRNA (Fig. 2D). These results suggested that NF-κB dependent IL-1β treatment was required for regulation of miR-181a.

3.3. *PTEN expression is negatively regulated by miR-181a*

To identify the targets of miR-181a, genes that were predicted by at least five of eleven databases (Diana, microinspector, miranda, mirtarget2, mitarget, nbmirtar, pictar, pita, rna22, rnahybrid, and targetscan) were selected as putative targets. To confirm whether PTEN is the predicted direct target of miR-181a, we transfected LoVo and SW480 cells with miR-181a mimics or mimics NC, and the transfection efficiency was verified by real-time PCR (Fig. 3A). Moreover, enhanced miR-181a expression in LoVo and SW480 cells significantly repressed PTEN mRNA expression compared with NC (Fig. 3B). Consistently, PTEN protein expression was reduced in response to miR-181a overexpression (Fig. 3C, Fig. S2A). To demonstrate the specific effect of miR-181a, we constructed a mutation point in the miR-181a binding site of the PTEN reporter luciferase and co-transfected mimic NC and miR-181amimics into LoVo cells and SW480 cells (Fig. 3D). Results showed that compared with the wild-type reporter plasmid, the luciferase mut-PTEN reporter was found to be 1.5 times more active, and similar results were obtained by transfecting reporter plasmid into the SW480 cell line (Fig. 3E). Moreover, forced expression of PTEN partially restored the PTEN protein expression inhibited by miR-181a (Fig. 3F, Fig. S2B). Taken together, these results indicate that miR-181a plays a critical role in repressing PTEN expression by targeting its 3′ UTR.

3.4. *Induction of miR-181a promotes cell proliferation via targeting PTEN*

In a previous study, PTEN was reported to be one of the most frequently inactivated tumor suppressor genes. Overexpression of PTEN in different mammalian tissue culture cells affects various

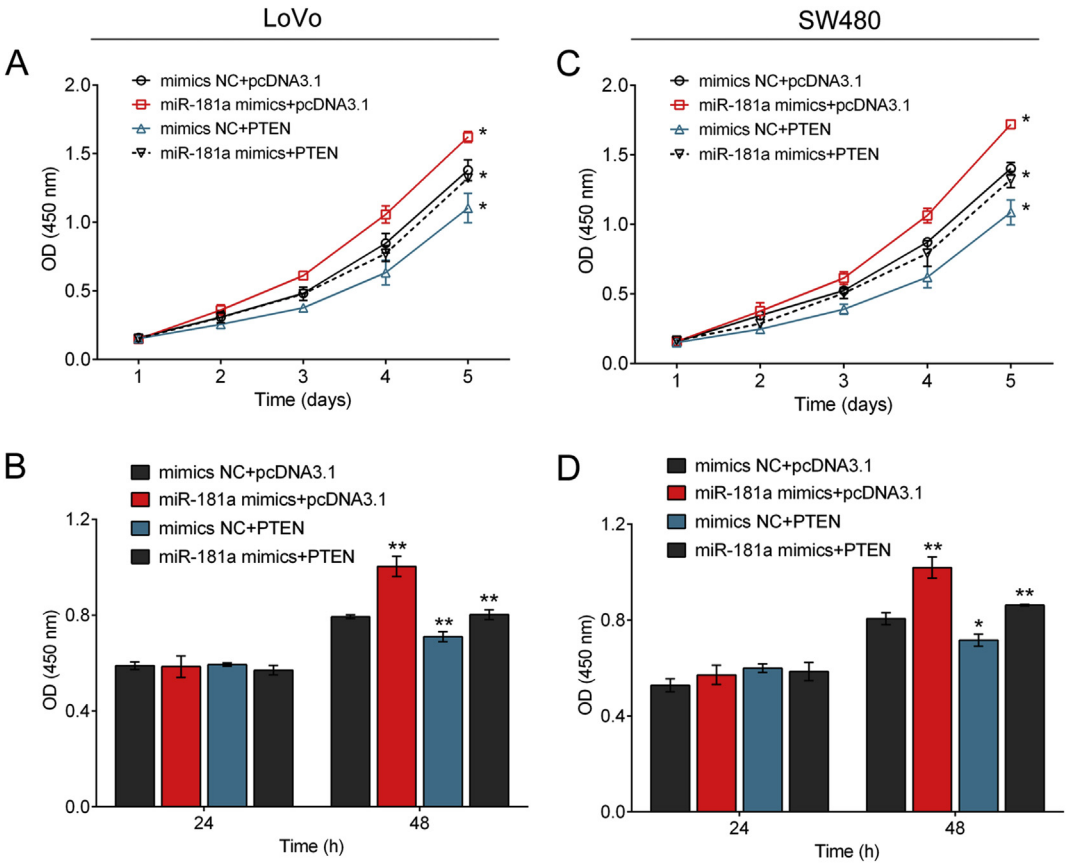

Fig. 4. Induction of miR-181a promotes cell proliferation via targeting PTEN. (A and C) CCK-8 and BrdU assays were used to determine the proliferation of LoVo and SW480 cells. In both LoVo and SW480 cells, the proliferation of miR-181a mimics transfected cells was significantly enhanced compared to that of the NC mimics control group. When the miR-181a mimics and pcDNA3.1-PTEN plasmids were co-transfected into cells, the cell proliferation rate was decreased. (B and D) 48 h after transfection of LoVo and SW480 cells, the transfection efficiency of BrdU in miR-181a transfected cells was increased by 25% compared with the control group of mimic NC transfected cells. The incorporation rate of BrdU was decreased by 12.5% in pcDNA3.1-PTEN transfected cells, whereas the incorporation rate of BrdU was partly recued in cells co-transfected with miR-181a mimics and pcDNA3.1-PTEN. The data are presented as mean ± SD of three independent experiments.

processes, including cell proliferation [25]. In the present study, the CCK-8 and BrdU assays were used to determine the proliferation of LoVo and SW480 cells. In both LoVo and SW480 cells, the proliferation of miR-181a mimics transfected cells was significantly enhanced compared to that of the NC mimics control group. When the miR-181a mimics and pcDNA3.1-PTEN plasmids were co-transfected into cells, the cell proliferation rate was decreased (Fig. 4A and C). Forty-eight hours after transfection of LoVo and SW480 cells, the transfection efficiency of BrdU in miR-181a transfected cells was increased by 25% compared with the control group of mimic NC transfected cells. The incorporation rate of BrdU was decreased by 12.5% in pcDNA3.1-PTEN transfected cells, whereas the incorporation rate of BrdU was partly rescued in cells co-transfected with miR-181a mimics and pcDNA3.1-PTEN (Fig. 4B and D). Taken together, proliferation was enhanced in both LoVo and SW480 cells transfected with miR-181a mimics, whereas proliferation was decreased with overexpression of PTEN. Meanwhile, the pro-survival effect of miR-181a was partly blocked by co-transfection with exogenous PTEN, suggesting that PTEN repression may play a major role in miR-181a-dependentprotection in cells.

3.5. *Inhibition of miR-181a/NF-κB restores PTEN repression by IL-1β*

To confirm the role of miR-181a and NF-κB in IL-1β induced PTEN repression, miR-181a/NF-κB inhibition was achieved, and the expression level of PTEN was determined. Results showed thatIL-1β induced NF-κB expression but reduced PTEN expression on the protein level (Fig. 5A, Fig. S3A). Inhibition of miR-181a was achieved by miR-181a inhibitor, and the inhibitory efficiency was verified by real-time PCR (Fig. 5B). Moreover, results from Western blot analysis showed that PTEN repression induced by IL-1βcould be restored by either miR-181a or NF-κB inhibition (Fig. 5C and D, Fig. S3B and C). In conclusion, these results suggest that NF-κB dependent IL-1β stimulation is required for CRC cell growth regulation, most likely through regulation of the miR-181a/PTEN axis.

4. Discussion

IL-1 is a major pro-inflammatory cytokine that is produced by malignant or micro environmental cells [26]. IL-1 also functions as a pleiotropic cytokine involved in tumorigenesis and tumor invasiveness; therefore, it represents a feasible candidate for a modulatory cytokine that can tilt the balance between inflammation and immunity toward the induction of antitumor responses [27].

NF-κB is one of the most important intracellular nuclear transcription factors, and it plays a central role in the transcriptional regulation of many genes that are influenced by various stimuli [28,29]. Recently, NF-κB has been shown to regulate miRNA expression, and in turn, some miRNAs modulate NF-κB expression directly or indirectly [28,30–32]. Emerged evidence has confirmed that some inflammation-related cytokines activate NF-κB, including IL-1β [33,34].

miRNA regulation by the inflammatory pathway is important for the regulation of colon cancer cell growth [5]. In the present study, real-time PCR results detected higher levels of IL-1β, NF-κB (RelA),

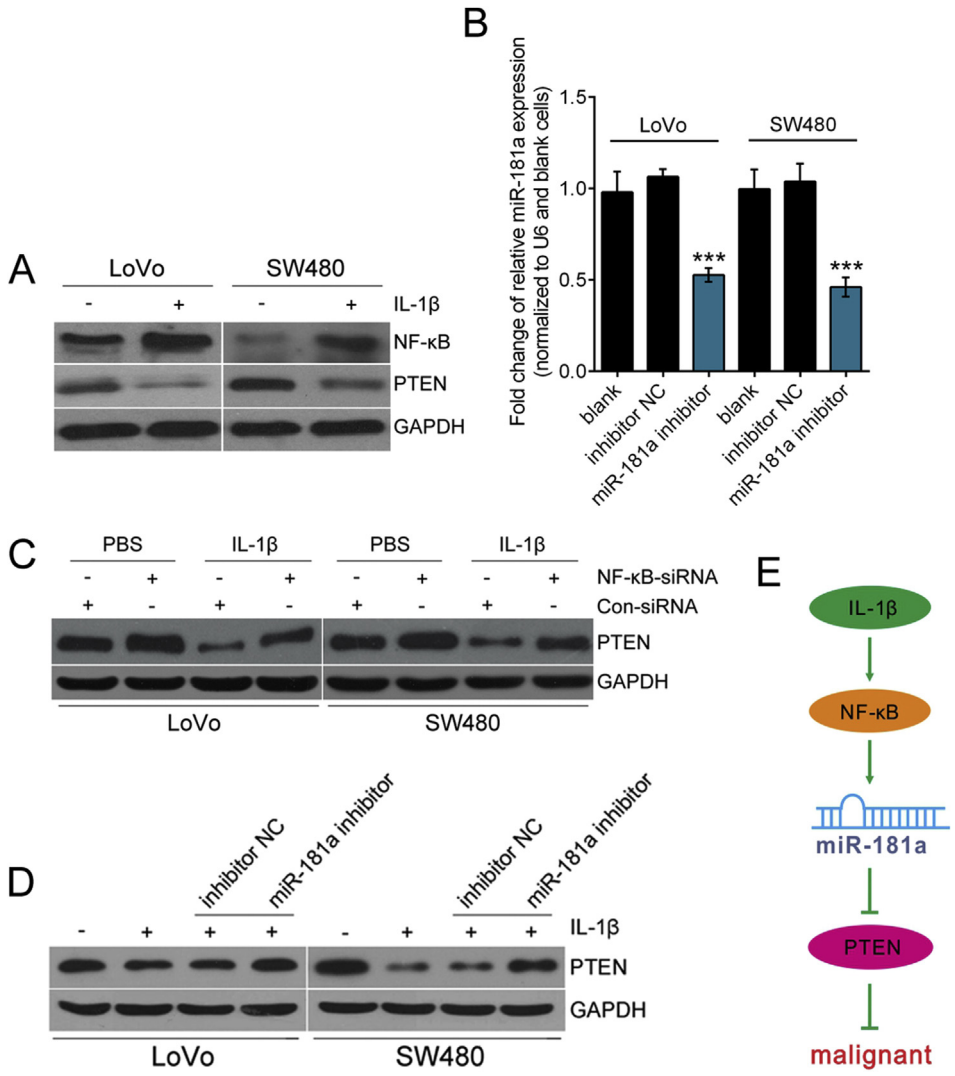

Fig. 5. Inhibition of miR-181a/NF-κB restores PTEN repression by IL-1β. (A) IL-1β induced NF-κB expression but reduced PTEN expression on the protein level. (B) Inhibition of miR-181a was achieved with a miR-181a inhibitor and the inhibitory efficiency was verified by real-time PCR. (C and D) Results from Western blot analysis showed that PTEN repression induced by IL-1β could be restored by either miR-181a or NF-κB inhibition. (E) A flow chart of the IL-1β/NF-κB/miR-181a/PTEN pathway that may contribute to CRC progression. The data are presented as mean ± SD of three independent experiments.

and miR-181a in patients with colon cancer compared to control subjects and immortalized cell lines. In addition, we first observed a positive correlation between the miR-181a level and the IL-1β mRNA level, the miR-181a level and the RelA mRNA level, and the IL-1β mRNA level and the RelA mRNA level.

To verify whether IL-1βregulates miR-181a expression through NF-κB, NF-κB activity was examined in the present study. We found thatmiR-181a expression was promoted by IL-1β but reduced by knocking down NF-κB. In addition, NF-κB inhibition restored promotion of miR-181a expression by IL-1β. These data revealed that IL-1βregulates miR-181a expression through NF-κB. However, the role of this regulation progression in CRC cell growth and the underlying mechanism was still unclear.

PTEN is an omnipresent tumor-suppressor gene, and its dysregulation results in many solid tumors [35,36]. Targeted deletion of PTEN leads to the development of colon cancer [37]. In addition, IL-1βand NF-κB significantly enhance PTEN repression [38]. Accumulating evidence suggests that NF-κB regulates PTEN in cancers, directly or indirectly through miRNAs [39,40]. On the other hand, PTEN has been reported to be regulated by inflammatory factors, such as LPS, TNF-α and IL-6 [21,39,41]. In the present study, it has been revealed that PTEN expression on both the mRNA and protein levels were significantly down-regulated in response to miR-181a overexpression. In addition, bioinformatics studies suggest that a target site of miR-181a is in the 3′UTR of PTEN mRNA. Given that IL-1βregulates miR-181a expression through NF-κB, it was assumed thatIL-1β/NF-κB regulates PTEN by miR-181a directly targeting its 3′UTR.To confirm the assumption, we first cloned the 3′UTR of PTEN into the pMIR-REPORT luciferase reporter and found that miR-181a significantly inhibited PTEN expression by directly binding to the 3′UTRof PTEN, consistent with a previous study by Wei et al. that demonstrates that miR-181a mediates a metabolic shift in colon cancer cells via the PTEN/AKT pathway [37].

To detect the specific role of the miR-181a/PTEN axis in CRC progression, proliferation of LoVo and SW480 cells was assessed. Results showed that proliferation was enhanced in both LoVo and SW480 cells transfected with miR-181a mimics, whereas proliferation decreased with overexpression of PTEN. Meanwhile, the pro-survival effect of miR-181a was partly blocked by co-transfection with exogenous PTEN, suggesting that PTEN repression may play

a major role in miR-181a-dependentprotection in cells.

Moreover, to confirm the role of miR-181a and NF-κB in IL-1β induced PTEN repression, miR-181a/NF-κB inhibition was achieved and the expression levels of PTEN were then determined. Results showed that compared with the control group, PTEN repression induced by IL-1β was restored by either miR-181a or NF-κB inhibition. All the above results suggest that IL-1β/NF-κB regulates the miR-181a/PTEN axis in CRC progression, which plays a major role in CRC growth and progression.

Taken together, our data might provide new insights into the pathophysiological mechanism of CRC and suggest a novel drug target for CRC treatment. Furthermore, overexpression of PTEN in LoVo and SW480 cells could reverse the effect of miR-181a and IL-1βto restore the proliferation of LoVo and SW480 cells. In conclusion, our observations indicated that the NF-κB/miR-181a/PTEN pathway upon IL-1β treatment contributes to malignancy.

5. Conclusion

In the present study, we identified anIL-1β/NF-κB/miR-181a/PTEN pathway that may contribute to CRC progression and represents a novel potential therapeutic target for treating CRC.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2016.06.001.

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