Life Sciences 225 (2019) 72–78

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Lycium barbarum polysaccharides promote osteoblasts viability by regulating microRNA-17/PTEN

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ARTICLE INFO

Keywords:
Lycium barbarum polysaccharides
microRNA-17
PTEN/PI3K/AKT
Viability

ABSTRACT

Aims: *Lycium barbarum* polysaccharides (LBPs) have been reported to promote the proliferation of osteoblasts. Further, the proliferation of osteoblasts has been proved to be associated with microRNA (miR)-17 which targets phosphatase and tensin homolog (PTEN) 3′-untranslated region (UTR) for regulating phosphoinositide 3 kinase/protein kinase B (PI3K/AKT) signaling pathway. Consequently, we aimed to explore a miR-17-relative mechanism by which LBPs mediates proliferation of osteoblasts.

Materials and methods: LBPs were used to stimulate MC3T3-E1 cells, and SF1670 was applied to repress PTEN. miR-17 inhibitor was transfected into cells for down-regulating miR-17. The cell viability and migration were examined by cell counting kit-8 (CCK-8) and 24-well Transwell migration assay, respectively. The expression of Cyclin D1, matrix metallopeptidase (MMP)-2 and MMP-9 was analyzed using Western blot. Quantitative reverse transcription PCR (qRT-PCR) was conducted to detect miR-17. The expression of PTEN and phosphorylation of PI3K and AKT were analyzed by Western blot.

Key findings: We demonstrated that LBPs promoted osteoblasts viability, facilitated migration and positively regulated the expression of miR-17. Additionally, miR-17 inhibitor abolished the positive effects of LBPs on the osteoblasts. Molecularly, miR-17 inhibited the expression of PTEN by targeting its 3′-untranslated region (3′-UTR). Mechanically, LBPs diminished PTEN expression and facilitated the phosphorylation of PI3K and AKT, while the decreased expression of PTEN and enhanced phosphorylation of PI3K and AKT were reversed by miR-17 inhibitor.

Significance: LBPs might fortify osteoblasts viability by up-regulating miR-17 to target PTEN and consequently trigger PI3K/AKT pathway.

1. Introduction

Fracture healing is an ontological process for restoration or regeneration of damaged skeletal organ to its pre-injury cellular composition, structure, and bio-mechanical function. Particularly, osteoblasts, together with chondrocytes and osteoclasts are three major cell types contributing the skeleton [1]. The activity of osteoblasts during the coupled remodeling has been reported to be associated with the bone's original cortical structure [2]. In addition, osteoblasts have been proved to participate in the lamellar bone deposition progress in order to maintain a balance of hard callus resorption by osteoclasts in the remodeling progress [3]. Recently, several studies have been conducted to induce the proliferation and differentiation for facilitating fracture healing [4,5].

Lycium barbarum polysaccharides (LBPs) are a portion of polysaccharides, extracted from *Lycium barbarum* which is eatable and officinal as a type of food supplement and a traditional medicinal herb in China [6]. The multiple biological functionalities of LBPs have been determinate, such as anti-aging, anti-cancers, anti-fatigue, anti-viral, cardio-protective effect, etc. The potential mechanisms by which LBPs play a positive role might contain anti-oxidation, immune-enhancement, as well as modulation of apoptosis and proliferation [7–13]. Remarkably, it has been reported that LBPs protect bone marrow mononuclear cells against injury induced by ionizing radiation [14]. Besides, LBPs mitigate palmitate-induced apoptosis and promote proliferation of osteoblasts [15]. Moreover, its immune-modulatory activities [16] contribute to a therapeutic value since unresolved inflammation normally results in healing failure [17]. In the molecular mechanisms, the regulatory effects of LBPs on the expression of microRNAs (miRs) have been indicated in several studies, while its regulatory effect on microRNA-17 (miR-17) has been seldom studied [18,19].

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https://doi.org/10.1016/j.lfs.2019.04.001
Received 24 December 2018; Received in revised form 20 March 2019; Accepted 1 April 2019
Available online 02 April 2019
0024-3205/

The modulatory function of miR-17 has been verified in the differentiated and proliferative progresses of osteoblast cells [20]. While it is still unclear whether there is a regulatory relationship between miR-17 and LBPs since both of them mediate the proliferative progress of osteoblasts. Furthermore, an antecedent research has discovered and validated that there is a targeting correlation between miR-17 and phosphatase and tensin homolog (PTEN) [9], which has been proved to modulate the phosphorylated expression of protein kinase B (AKT) and phosphoinositide 3 kinase (PI3K) [21]. Apparently, PTEN had been found to be correlated with the cell cycle progression and cell survival via regulating PI3K/AKT signaling pathway [22].

Consequently, we postulated that there might be a miR-17-relative mechanism by which LBPs regulate the proliferation and migration of osteoblasts via PTEN/PI3K/AKT signaling pathway. Accordingly, the present study was conducted to explore whether LBPs modulate the proliferation of osteoblasts by triggering PI3K/AKT signaling pathway via miR-17. Besides, we further verified whether miR-17 triggers PI3K/AKT signaling pathway by targeting PTEN.

2. Materials and methods

2.1. LBPs preparation and purification

The LBPs were extracted and purified from *Lycium barbarum*. The *Lycium barbarum* was obtained from Ning Xia (China). We prepared LBPs with reference to the method of Yu et al. [23]. Shortly, the dried fruit of *Lycium barbarum* was grounded into small pieces with a blender. The small pieces of *Lycium barbarum* (400 g) was extracted with hot water (6 L) for 3 h. After concentration, the crude extraction was deproteinated with Sevag method and the resulting aqueous fractions were extensively dialyzed against running distilled water for 2 days with Spectra/Por 1 dialysis sacks (molecular weight cut off 6–8 kD, Spectrum Laboratories Inc., Shanghai, China). The retentate was concentrated to 400 mL under reduced pressure and precipitated by three volumes of 95% ethanol. Subsequently, the precipitate was washed with acetone before centrifugation. The resulting precipitate was vacuum-dried at -40 °C to generate brown powder. The polysaccharide content of the extract was examined by phenolsulfuric method. The polysaccharide content of the extract reached to 95%. The freeze-dried powder of LBPs was freshly diluted with Dulbecco's modified eagle medium (DMEM) (Sigma, St. Louis, MO, USA) for the following experimental use.

2.2. Cell culture and treatment

The mouse preosteoblast cell line MC3T3-E1 was purchased from American type culture collection (ATCC; Rockville, MD, USA). The MC3T3-E1 cells were incubated in α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies Corporation), 100 units/mL penicillin (Sigma), and 100 mg/mL streptomycin (Sigma) at an incubator-containing 5% CO_2 and 95% humidified air at 37 °C. MC3T3-E1 cells were pre-incubated with LBPs (10–800 μg/mL) for 24 h. PTEN inhibitor (SF1670) (Sigma) was applied to repress PTEN at a concentration of 5 μM for 30 min.

2.3. Transfection

For silencing the expression of miR-17, we constructed a virus vector (miR-17 inhibitor) and its relative negative control (NC) synthesized by GenePharma (Shanghai, China). The transfection was conducted by using Lipofectamine 3000 reagent (Invitrogen) according its instructions.

2.4. Cell counting kit-8 assay

The cell viability was assessed by a cell counting kit-8 method (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, the CCK-8 solution was added into the cell culture medium after pre-incubation of LBPs. The culture was continuously incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C for 1 h. Finally, the absorbance was measured with a Microplate Reader at 450 nm (Bio-Rad, Hercules, CA).

2.5. Migration analysis

Cell migration was examined by a modified two-chamber migration assay with a polycarbonate Transwell membrane filter (8 μm pore size; Corning, NY, USA). MC3T3-E1 was suspended in 200 μL serum-free medium and deposited on the upper compartment of 24-well Transwell culture chamber with a concentration of 1×10^5 cells/mL. The complete medium (600 μL) was added into the lower compartment. After incubation for 6 h at 37 °C, MC3T3-E1 cells were fixed with methanol (Sigma). Non-traversed cells were removed from the upper surface of the filter with a cotton swab. Traversed cells on the lower side of the filter were stained with crystal violet and counted with a microscope. The data were presented as the percentage of migrated cells in three differently random areas.

2.6. DNA constructs and luciferase reporter assay

The 3′-untranslated region (3′-UTR) of PTEN was predicted to interact with miR-17. A mutated sequence of PTEN (various nucleotides within the binding sites were mutated) within the predicted target site was inserted into the *XbaI/FseI* sites of the pGL3 control vector (Promega, Madison, WI, USA). The mutant (mut) 3′-UTR of PTEN was amplified using the 3′-UTR of PTEN as a template. For analyzing the luciferase activity, MC3T3-E1 cells were co-transfected with the PTEN wild (wt) 3′-UTR or the PTEN mutant 3′-UTR and miR-17 mimic or its negative control mimic (NC mimic). The transfection was performed with the DharmaFECT Duo transfection reagent (Cambridge, UK) for 48 h. The luciferase assays were conducted with the Dual-Glo Luciferase assay (Promega, Beijing, China) referring to its instruction.

2.7. Western blotting determination

The expression of proteins was analyzed by Western blot assay. The total protein was extracted from MC3T3-E1 cells with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified with a BCA protein assay kit (Pierce, Appleton, WI, USA). The separated protein was transferred onto a polyvinylidene difluoride (PVDF) (Millipore, Billerica, MA, USA) membrane. The protein blots were incubated with antibodies against Cyclin D1 (ab16663, 1:100), MMP-2 (ab37150, 1 μg/mL), MMP-9 (ab38898, 1:1000) (all obtained from Abcam, Cambridge, UK), PTEN (9188, 1:1000), t-PI3K (4255, 1:1000), p-PI3K (13857, 1:1000), t-AKT (9272, 1:1000), p-AKT (9614, 1:1000), and β-actin (4967, 1:1000) (all from Cell signaling technology, Beverly, MA). Subsequently, the secondary antibody conjugated-horseradish peroxidase (7074; 1:5000; all from Cell signaling technology) was applied to probe the primary antibodies. The signals of target protein were visualized by a Bio-Rad ChemiDoc XRS with an Image Lab Software (Bio-Rad, Shanghai, China). The relative expression of protein was evaluated by normalizing the expression of individual β-actin.

2.8. qRT-PCR

The TRIzol reagent kit (Life Technologies Corporation, Carlsbad, CA, USA) was applied to isolate the total RNA in the MC3T3-E1 cells according to the manufacturer's instructions. The expression of miR-17 at RNA level was analyzed using a Taqman MicroRNA Reverse

Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-17 and U6 (Applied Biosystems, Foster City, CA, USA) with quantitative reverse transcription PCR (qRT-PCR). Thereinto, U6 was an internal control for relative quantification of miR-17.

2.9. Statistical analyses

Statistical manipulation was performed by using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA). The values were averaged and expressed as mean ± standard error of the mean (SEM). The error bars presented SEM of the individual values. Two-tailed Student's *t*-tests were conducted to evaluate the differences between two groups. One-way analysis of variance (ANOVA) was employed for multiple comparisons of variables. The statistical significance was considered with *P*-value at a level of 0.05.

3. Results

3.1. LBPs promoted osteoblast MC3T3-E1 cells viability, migration, and MMPs accumulation

To explore the functions of LBPs from *Lycium barbarum* on the viability and migration, osteoblast MC3T3-E1 cells were exposed to 10–800 μg/mL LBPs for 24 h, followed by cell viability and migration assays. Our results showed that LBPs significantly enhanced the cell viability in a concentration-dependent manner while the cell viability leveled off gradually with the increase of concentration > 500 μg/mL ($P < 0.05$) (Fig. 1A). Afterwards, MC3T3-E1 cells were pre-incubated with LBPs at concentration of 500 μg/mL. The obviously elevated expression of Cyclin D1 implied that LBPs promoted osteoblast cells proliferation ($P < 0.05$) (Fig. 1B) since Cyclin D1 has been proposed to play a central role in the regulation of continued cell cycle progression in actively proliferating cells [24]. Besides, the relative migration of MC3T3-E1 cells was markedly enhanced by LBPs ($P < 0.05$) (Fig. 1C). It has been proved that MMP-2 and MMP-9 perform a crucial role in bone formation in the progression of fracture healing [25–27]. We found that the protein expression of MMP-2 and MMP-9 was enhanced in MC3T3-E1 cells after pretreatment with LBPs ($P < 0.05$ or $P < 0.01$) (Fig. 1D). Conclusively, our results indicated that LBPs effectively promoted MC3T3-E1 cells proliferation, and migration, and the accumulation of MMPs (MMP-2 and MMP-9).

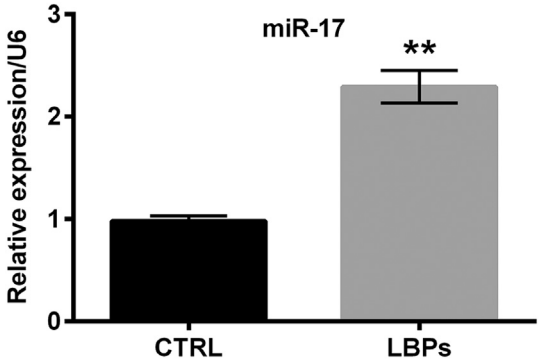

Fig. 2. Expression of miR-17 was up-regulated by LBPs.

3.2. The expression of miR-17 was up-regulated by LBPs

It has been proven that miR-17 participates in the mediation of proliferation progress [28–30]. For validating whether miR-17 was modulated by LBPs, we detected the expression of miR-17 at RNA level by qRT-PCR assay. Our results indicated that the expression of miR-17 was remarkably enhanced in osteoblast MC3T3-E1 cells with an

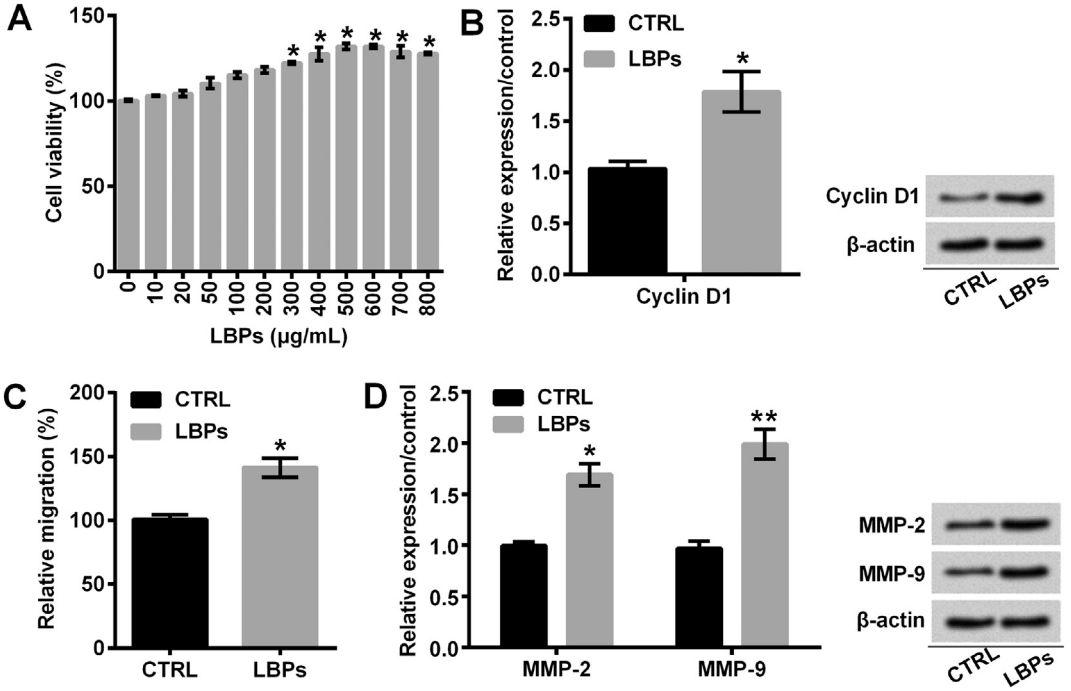

Fig. 1. Viability and migration of osteoblast MC3T3-E1 cells were elevated by LBPs, with the production of MMPs.
(A) The viability was assessed by CCK-8 method. LBPs promoted the viability of MC3T3-E1 cells. MC3T3-E1 cells were pretreated with LBPs (10–800 μg/mL) for 24 h in the LBPs group. (B) Western blot was conducted to assess the expression of Cyclin D1. The protein expression of Cyclin D1 was up-regulated by LBPs. (C) Relative migration of MC3T3-E1 cells was examined with a modified two-chamber migration assay. The relative migration of MC3T3-E1 cells was facilitated by LBPs. (D) The expression of MMP-2 and MMP-9 was determined by Western blot assay. The protein expression of MMP-2 and MMP-9 was increased by LBPs. MC3T3-E1 cells were pretreated with LBPs (500 μg/mL) for 24 h in the LBPs group. MC3T3-E1 cells were not stimulated with LBPs in the CTRL group. CTRL: control; LBPs: *Lycium barbarum* polysaccharides; MMP: matrix metallopeptidase; CCK-8: cell counting kit-8. *, $P < 0.05$; **, $P < 0.01$.

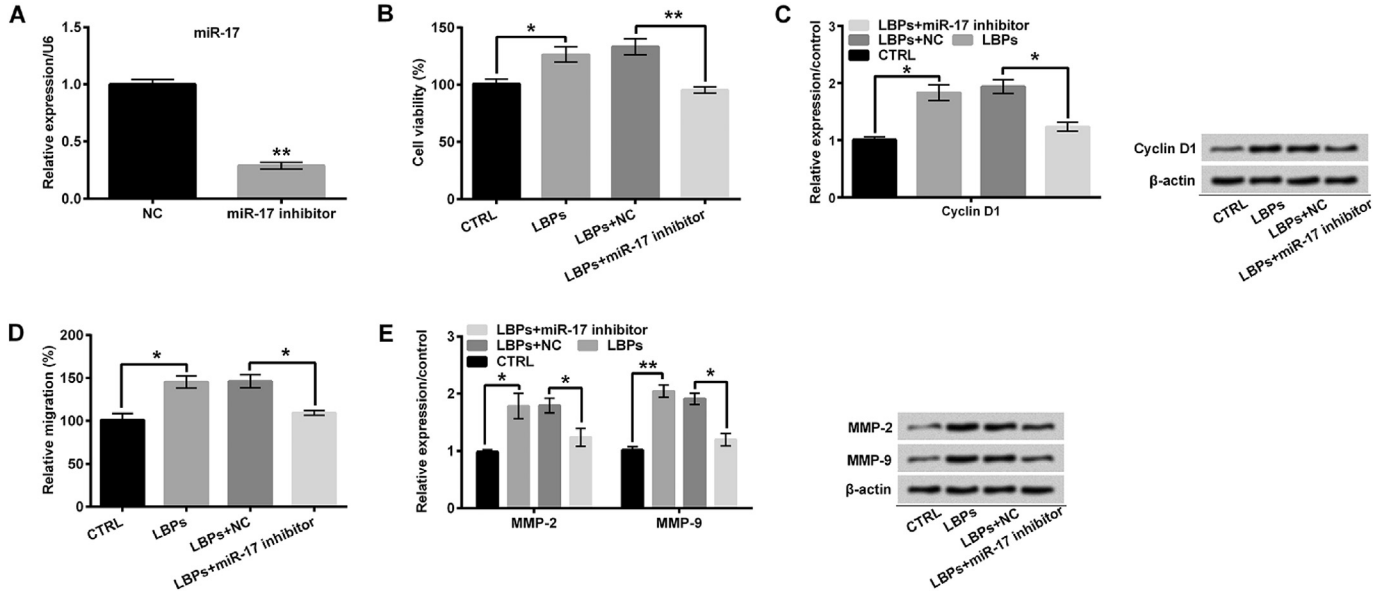

Fig. 3. The LBPs-accelerated cell viability, migration, and MMPs expression were inhibited by miR-17 inhibitor.
(A) The expression of miR-17 was quantified by qRT-PCR. The relative expression of miR-17 was down-regulated by miR-17 inhibitor. (B) The cell viability was assessed by CCK-8 assay. miR-17 inhibitor decreased the viability of MC3T3-E1 cells pretreated with LBPs. (C) The relative expression of Cyclin D1 was analyzed by Western blot. The LBPs-induced expression of Cyclin D1 at protein level was repressed by miR-17 inhibitor. (D) The relative migration of MC3T3-E1 cells were examined with a modified two-chamber migration assay. The relative migration of MC3T3-E1 cells were reduced by miR-17 inhibitor in MC3T3-E1 cells pretreated with LBPs. (E) The expression of MMP-2 and MMP-9 was quantified with Western blot method. The LBPs-induced protein expression of MMP-2 and MMP-9 was abolished by miR-17 inhibitor in the MC3T3-E1 cells. MC3T3-E1 cells were pretreated with LBPs (500 μg/mL) for 24 h in the LBPs group. MC3T3-E1 cells were not stimulated with LBPs in the CTRL group. NC: negative control; miR-17: microRNA-17; CTRL: control; LBPs: *Lycium barbarum* polysaccharides; MMP: matrix metallopeptidase; CCK-8: cell counting kit-8; qRT-PCR: quantitative reverse transcription PCR. *, $P < 0.05$; * $P < 0.01$.

exposure to LBPs ($P < 0.01$) (Fig. 2). This result suggested that LBPs might promote cells proliferation via regulating the expression of miR-17.

The expression of miR-17 was determined by qRT-PCR. MC3T3-E1 cells were pretreated with LBPs (500 μg/mL) for 24 h in the LBPs group. MC3T3-E1 cells were not stimulated with LBPs in the CTRL group. CTRL: control; LBPs: *Lycium barbarum* polysaccharides; miR-17: microRNA-17; qRT-PCR: quantitative reverse transcription PCR. **, $P < 0.01$.

3.3. miR-17 inhibitor abolished the acceleratory effects of LBPs on the proliferation, migration and MMPs expression

In order to verify whether miR-17 was associated with the proliferation and migration progresses mediated by LBPs, we silenced the expression of miR-17 with miR-17 inhibitor. Obviously, the expression of miR-17 was sufficiently down-regulated by miR-17 inhibitor as shown in Fig. 3A ($P < 0.01$). In addition, targeted inhibition of miR-17 using miR-17 inhibitor in MC3T3-E1 cells pretreated with LBPs resulted in the reduced cell viability and expression of Cyclin D1, suggesting that LBPs might augment the proliferation of osteoblast MC3T3-E1 cells by expediting the miR-17 expression ($P < 0.05$ or $P < 0.01$) (Fig. 3B and C). Further, we found that miR-17 inhibitor distinctly abolished the positive effects of LBPs on the migration ($P < 0.05$) (Fig. 3D). Moreover, the relative expression of MMP-2 and MMP-9 at protein level ($P < 0.05$ or $P < 0.01$) was notably suppressed in miR-17-deficient cells in despite of LBPs administration (Fig. 3E). Taken together, these results implied that aggrandizing the expression of miR-17, LBPs strengthened osteoblast cells viability, migration, and MMPs accumulation.

3.4. miR-17 down-regulated the expression of PTEN by targeting its 3′-UTR

PTEN has been identified in the modulation of osteoclast cells migration and differentiation [31]. Accordingly, in order to explore the regulatory effects of LBPs on the expression of PTEN via miR-17, we firstly explored whether the expression of PTEN was regulated by miR-17 inhibitor. Remarkably, the results showed that the protein expression of PTEN was notably aggrandized by miR-17 inhibitor ($P < 0.05$) (Fig. 4A). Therefore, we hypothesized that there might exist a targeting connection between PTEN and miR-17. Although an antecedent study has discovered a targeting relationship between miR-17 and PTEN in spinal cord injuries [32], we further consolidated this conclusion in osteoblast MC3T3-E1 cells with a relative luciferase activity assay. After co-transfecting PTEN 3′-UTR or its mutant 3′-UTR and miR-17 mimic and its NC mimic into MC3T3-E1 cells, our results showed that the relative luciferase activity was obviously decreased by miR-17 mimic in the PTEN-wt osteoblast cells, while this variation was not significant in the PTEN-mut cells. Hence, we considered that the expression of PTEN was facilitated by silencing miR-17 which was expected to mitigate the expression of PTEN by targeting its 3′-UTR.

3.5. LBPs exerted positive effects on cell viability, migration, and MMPs expression through repressing PTEN by up-regulating miR-17

We have consolidated that PTEN was a target of miR-17 in the anterior study. Additionally, given that PTEN modulated the PI3K and AKT signaling pathways and consequently affects cell cycle progression and cell survival [22], we further designed experiments to prove whether LBPs were involved in triggering PTEN/PI3K/AKT signaling pathway via mediating miR-17. The Western blot results showed that the expression of PTEN was notably repressed by LBPs and subsequently up-regulated by miR-17 inhibitor, revealing that LBPs attenuated the expression of PTEN by augmenting the expression of miR-17 ($P < 0.05$ or $P < 0.01$) (Fig. 5A and 5B). Additionally, LBPs aggrandized the phosphorylation of PI3K and AKT, while miR-17 inhibitor abolished this positively modulatory effect, indicating that LBPs augmented the phosphorylated expression of PI3K and AKT by promoting

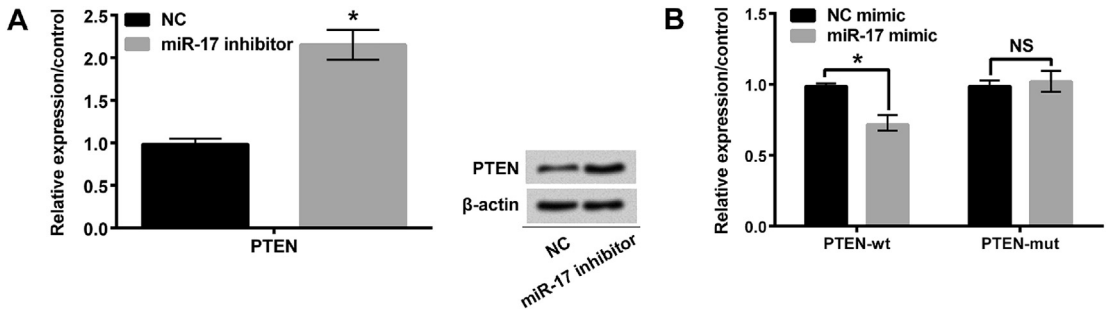

Fig. 4. PTEN was a potential target of miR-17.
(A) The expression of PTEN was evaluated by Western blot assay. The expression of PTEN was promoted by miR-17 inhibitor. (B) The relative luciferase activity assay was performed to probe that miR-17 target PTEN 3′-UTR. The 3′-UTR of PTEN was predicted to interact with miR-17. 3′-untranslated region: 3′-UTR; NC: negative control; miR-17: microRNA-17; PTEN: phosphatase and tensin homolog; NS: not significant; wt: wild type; mut: mutant type. *, $P < 0.05$.

the expression of miR-17 ($P < 0.05$ or $P < 0.01$) (Fig. 5A and 5B). Taken together, these results suggested that LBPs up-regulated miR-17 to suppress the expression of PTEN and consequently triggered PI3K/AKT signaling pathway.

In addition, we stimulated miR-17-deficient MC3T3-E1 cells with PTEN inhibitor (SF1670) after pre-incubated with LBPs. Our results suggested that SF1670 obviously ($P < 0.05$) restored cell viability (Fig. 6A), with an enhanced ($P < 0.01$) accumulation of Cyclin D1 (Fig. 6B). Notably, SF1670 augmented ($P < 0.01$) the migration behaviors (Fig. 6C). The accumulation of MMP-2 and MMP-9 was significantly ($P < 0.05$) promoted by SF1670 (Fig. 6D). These results indicated that LBPs up-regulated miR-17 which post-transcriptionally inhibited PTEN expression, activated PI3K/AKT pathway, and finally facilitated osteoblasts viability, migration and MMPs accumulation.

4. Discussion

It has been proposed that LBPs, isolated from *Lycium barbarum*, promotes osteoblasts differentiation [33], which provides a potential therapy for bone disease treatment. In our study, we primarily consolidated that LBPs promotes the proliferation, migration, and MMPs (MMP-2 and MMP-9) expression of osteoblasts, and subsequently explored the underlying mechanisms by which LBPs exert a positive effect on osteoblast MC3T3-E1 cells.

The proliferation and migration-promoting effects of LBPs on osteoblasts have been presented in our results. An obviously increased expression of proliferative marker (Cyclin D1) induced by LBPs suggested that LBPs mediated the cell cycle. Mounting evidences have suggested that LBPs exert a growth suppressive effect on cancer cells via G0/G1 phase arrest by regulating the expression of Cyclin D1 [34–36], which has been proven as an oncogenic regulator [37]. However, LBPs exert a positive effect on viability in H_2O_2-treated human lens epithelial cells and LPS-induced microglia in an appropriate concentration [38,39] and promote the proliferation of osteoblasts [33]. Additionally, we observed the up-regulation of MMPs in LBPs-treated MC3T3-E1 cells. Given that MMPs are involved in cartilage remodeling and bone formation [26], we proposed that LBPs exhibited a positive function in the wound healing. Taken together, our results implied that LBPs contributed to the proliferation, migration, and MMPs accumulation of osteoblast MC3T3-E1 cells.

The overexpression of miR-17 has been detected in multiple cancers [28]. Besides, the elevated expression of miR-17 promotes proliferation of the lung epithelial progenitor cells [40]. Uniformly, LBPs regulate the proliferation of osteoblasts. The regulatory effects of LBPs on the expression of miRs have been reported to exert a positive function against H_2O_2-mediated oxidative damage, cardiac contractile dysfunction, and hypoxia-induced injury in recent studies [18,19,41]. Consequently, we proposed a hypothesis that LBPs might play an essential regulatory role in the expression of miR-17. In an attempt to identify this assumption, we conducted qRT-PCR assay to analyze the expression

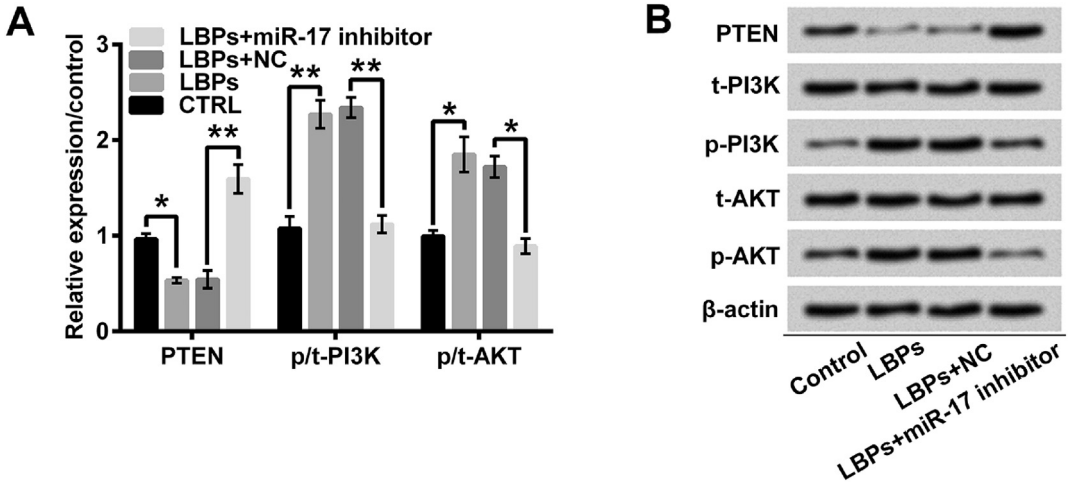

Fig. 5. miR-17 inhibitor reversed the modulatory effects of LBPs on the expression of PTEN and phosphorylated expression of PI3K and AKT.
(A) miR-17 inhibitor elevated the relative expression of PTEN which was decreased by LBPs. The LBPs-induced phosphorylated expression of PI3K and AKT was abolished by miR-17 inhibitor. (B) The signal bands of PTEN, t-/p-PI3K, and t-/p-AKT were visualized after SDS-PAGE. The relative expression of regulators was normalized after Western blot analysis. CTRL: control; LBPs: *Lycium barbarum* polysaccharides; NC: negative control; miR-17: microRNA-17; PTEN: phosphatase and tensin homolog; PI3K: phosphoinositide 3 kinase; AKT: protein kinase B; p-: phosphorylated; t-: total. *, $P < 0.05$; **, $P < 0.01$.

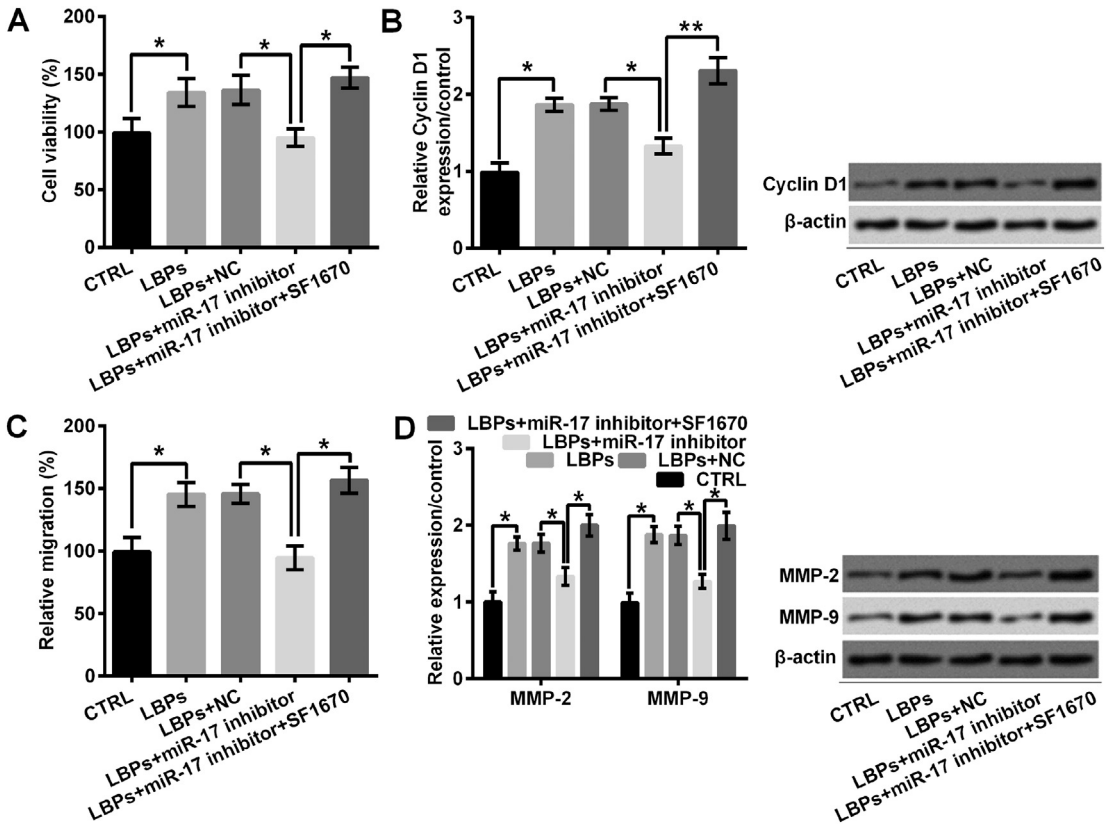

Fig. 6. PTEN down-regulation elicited a protective effect on cell viability and migration, as well as the accumulation of MMPs.
(A) Cell viability was carried out by CCK-8 assay. (B) Protein expression of Cyclin D1 was assessed with Western blot assay. (C) A modified two-chamber migration assay was performed to examine the relative migration of MC3T3-E1 cells. (D) Protein expression of MMP-2 and MMP-9 was determined by Western blot assay. miR-17-deficient MC3T3-E1 cells were pre-incubated with LBPs (500 μg/mL) for 24 h and then stimulated with SF1670 (5 μM). CTRL: control; LBPs: *Lycium barbarum* polysaccharides; MMP: matrix metallopeptidase; CCK-8: cell counting kit-8; NC: negative control; miR-17: microRNA-17; PTEN: phosphatase and tensin homolog. *, $P < 0.05$.

of miR-17. The results indicated that LBPs prominently expedited the expression of miR-17. The preceding argumentation that LBPs facilitated the expression of miR-17 implied that LBPs might mediate the proliferation, migration and MMPs expression of osteoblasts by up-regulating miR-17. Hence, we further validated the plausible mechanism by silencing miR-17 through transfection with miR-17 inhibitor. Evidences from our experiments have consistently shown that the accelerative function of LBPs on osteoblasts proliferation, migration and MMPs expression was abolished by miR-17 inhibitor.

Inhibition of PTEN activity normally contributes to the increased phosphorylated expression of AKT and enhances osteoblasts proliferation [42]. Of particular attention in the current study was the up-regulated expression of PTEN induced by miR-17 inhibitor, which suggested that the expression of PTEN might be negatively modulated by miR-17. As a consequence, we considered that a targeting modulation between miR-17 and PTEN might exist in the proliferative and migratory progresses of osteoblasts. An antecedent study has actually discovered a targeting relationship between miR-17 and PTEN in spinal cord injuries [9]. Consistently, PTEN as a potential target of miR-17 has been discussed in numerous studies [43–45]. In the present study, we further consolidated this conclusion in osteoblast MC3T3-E1 cells with a relative luciferase activity assay. Dramatically, our results suggested that targeting the 3′-UTR of PTEN, miR-17 repressed the expression of PTEN in the osteoblasts.

The modulatory effects of LBPs on signaling pathway, containing Brf2/HO-1, PTEN, PI3K/AKT, JNK, et al., have been explored in multiple cancer cells or normal cells in a severity stimulus circumstance [15,46,47]. In addition, a recent study proved that the intramembranous and late endochondral fracture healings are improved and the phosphorylated expression of AKT is aggrandized in the mice lacking PTEN [21]. Subsequently, we analyzed the expression of PTEN and phosphorylated expression of PI3K and AKT for adequately understanding the potentially pathway-relative mechanisms by which LBPs mediated osteoblasts proliferation, migration, and MMPs production. The results from Western blot assay showed that LBPs mitigated the expression of PTEN, while expedited the phosphorylation of PI3K and AKT. Considering that PTEN is a target of miR-17, we further authenticated whether LBPs suppressed the expression of PTEN via elevating the expression of miR-17 by transfecting miR-17 inhibitor into osteoblasts. The results indicated that LBPs might repress the expression of PTEN by up-regulating miR-17. Sequentially, the LBPs-induced phosphorylation of PI3K and AKT was abolished by miR-17 inhibitor. Given that the phosphorylated expression of AKT is modulated by PTEN [21], it remains incompletely understood whether the phosphorylation of AKT is directly regulated by LBPs or indirectly mediated by LBPs via PTEN. In our further exploration, we found that LBPs virtually repressed the expression of PTEN and promoted the phosphorylation of PI3K and AKT via up-regulating miR-17.

In our present study, we demonstrated that LBPs exerts a proliferation- and migration-promoting function on osteoblasts by up-regulating miR-17, with MMPs accumulation. In addition, we proposed an underlying mechanism that LBPs might promote the expression of miR-17 which represses the expression of PTEN and consequently triggers PI3K/AKT pathway by targeting PTEN.

Conflict of interest

Authors declare that there is no conflict of interests.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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