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Cyclic tensile strain promotes chondrogenesis of bone marrow-derived mesenchymal stem cells by increasing miR-365 expression

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ABSTRACT

Aims: The chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) is critical for cartilage regeneration. Tissues constructed from BMSCs through cartilage tissue engineering still exhibit some histological, morphological, and biomechanical differences from normal cartilage tissues. Cyclic tensile strain (CTS) can increase chondrogenic gene expression and reduce hypertrophic gene expression in chondrocytes. miR-365 has been identified as a mechanoresponsive microRNA and is an important regulator of both chondrocyte hypertrophy and differentiation. Therefore, we hypothesized that CTS may promote the chondrogenesis of BMSCs by upregulating the expression of miR-365.

Methods: BMSCs were subjected to CTS to investigate the effects and mechanism on chondrogenesis. An Agilent miRNA microarray was used to profile miRNAs in the CTS-treated BMSCs and 3D-cultured control BMSCs. miR-365 was shown to interact with *HDAC4* mRNA through a luciferase reporter assay. An animal cartilage defect model was constructed and different groups of BMSCs were implanted to investigate their *in vivo* effect.

Key findings: CTS promoted BMSC chondrogenesis. miR-365 was significantly upregulated in CTS-treated cells and played an important role in CTS-mediated chondrogenesis. Luciferase assays showed that *HDAC4* is a direct target of miR-365. An *in vivo* study showed that CTS treatment and miR-365 overexpression could promote cartilage regeneration from BMSCs.

Significance: CTS can promote the expression of miR-365, a crucial mechanosensitive microRNA involved in the chondrogenesis of BMSCs, which directly inhibits the expression of *HDAC4*, in turn, enhancing the chondrogenesis of BMSCs.

1. Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) are capable of self-renewal and have the potential to differentiate into many cell lines, such as osteocytes, adipocytes, and chondrocytes. Therefore, they are treated as powerful primitive cells that, in the future, may be able to cure diseases caused by the inability of the original cells to regenerate [1]. Cartilage diseases in particular are more difficult to manage because they lack this capacity to regenerate [2]. Cartilage tissue engineering provides us new methods for the treating osteoarthritis, and chondrogenesis of BMSCs could be a promising method to repair cartilage defects [3]. The cells produce a cartilage-specific extracellular matrix containing proteins such as aggrecan and collagen II during cartilage formation. Many signaling pathways, including the ROCK, ERK1/2, p38 MAPK, and IGF-1 pathways, are involved in the process. It is believed that TGF-β and a three-dimensional environment are essential for prompting chondrogenesis [4,5]. Cartilage tissues constructed from BMSCs still exhibit some morphological and histological differences compared with normal cartilage tissues [6]. Furthermore, they are prone to osteogenesis and ageing. Therefore, there is still a need to promote chondrogenesis to repair damaged or defective cartilage.

Mechanical loading can promote chondrogenesis of BMSCs without causing chondrocyte hypertrophy [7]. Previously published studies investigating mechanical loading have mostly focused on compression and shear stress [8–10]. However, there are still no studies on the effects of CTS on BMSC chondrogenesis. It has been reported that CTS can reduce hypertrophic gene expression and increase chondrogenic gene expression in chondrocytes [11]. Therefore, we hypothesized that CTS may promote the chondrogenic differentiation of BMSCs.

MicroRNAs (miRNAs) are non-coding RNAs that regulate post-transcriptional gene expression by directly targeting the 3′ untranslated

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region (3′-UTR) of genes [12]. miRNAs are important regulators of a variety of biological processes, including cell differentiation, apoptosis, migration, proliferation, and tumorigenesis [13]. Some miRNAs have also been shown to regulate BMSC chondrogenesis, such as miR-335-5P [14], miR-574-3p [15], miR-23b [16], and miR1-99a [17].

miR-365 has been shown to exert anticancer effects in a variety of human cancer cell lines by modulating cell cycle and inducing apoptosis [18,19]. miR-365 also regulates IL-6 expression in HEK293 and HeLa cells *via* the MAPK/ERK pathway, whereas downregulation of miR-365 is associated with increased expression of IL-6 in macrophages from patients with tuberculosis [20]. miR-365 is considered a mechanoresponsive miRNA. This miRNA is involved in mechanic transduction and stimulates chondrocyte proliferation and differentiation. It is the most significantly up-regulated among 49 miRNAs altered by cyclic loading of chondrocytes [21]. Histone deacetylase 4 (HDAC4) is a major regulator of cartilage development and endochondral ossification as a potent inhibitor of chondrocyte hypertrophy. Decreased HDAC4 levels have been shown to contribute to increased OA-related gene expression in osteoarthritis [22]. Several studies have shown that HDAC4 is a direct target of miR-365 [21,23,24]. In the present study, we demonstrated that CTS could upregulate the expression of miR-365, thereby inhibiting HDAC4 expression, which in turn enhanced chondrogenic differentiation of BMSCs.

2. Materials and methods

2.1. BMSC culture and induction of chondrogenesis

Second-generation Rat BMSCs (Lot Number: 140313J31, Analysis certification can be downloaded from: https://www.cyagen.com/media/uploads/QAPI0800A0_RASMX-01001_CoA_140313J31.pdf) were purchased from Cyagen Biosciences Inc. (Cyagen Biosciences Inc., China) and were subcultured in L-DMEM with 10% FBS. BMSCs at passage 4 were used for chondrogenesis induction. The chondrogenic medium consisted of H-DMEM with 10^{-7} M dexamethasone, 50 μg/ml ascorbate, sodium pyruvate, L-proline, 10% ITS solution, and 10 ng/ml transforming growth factor-β (TGF-β). We divided cells into three groups for chondrogenesis: (1) the CTS-treated group described below, (2) three-dimensional (3D) culture group, and (3) monolayer cells in 6-well plates as a two-dimensional (2D) culture group. The 3D-cultured cells were grown in a Cytodex 3 microcarrier environment, which we have described in our previous studies [25]. Briefly, Cytodex 3 is a collagen-coated microcarrier that enables BMSC adhesion. BMSCs are then suspended in chondrogenic medium to increase the contact area between the cells and the medium to improve cell metabolism. All experiments used 3D-cultured cells as the control unless otherwise indicated.

2.2. Application of cyclic tensile strain

BMSCs were subcultured into Flexcell six-well BioFlex plates (25 mm diameter, Flexcell International Corporation, Hillsborough, NC). Upon reaching near 100% confluency, cells were subjected to CTS with 10% amplitude at 1 Hz for 2 h daily using a computer-controlled Flexcell FX-5000™ Tension System according to the manufacturer's instructions (www.flexcellint.com).

2.3. Agilent miRNA microarray

An Agilent miRNA microarray was used to profile miRNAs in the CTS-treated BMSCs and 3D culture control BMSCs (GEO number: GSE97467). BMSCs were treated with CTS for 7 days. Total RNA was extracted and 100 ng RNA was used per sample on the microarray.

2.4. Cell transfection

miR-365 mimics/inhibitor or negative control were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The miR-365 mimics/inhibitor and negative control were synthesized by Shanghai Gene Pharma Co., and were transfected into BMSCs at a final concentration of 100 nM. The medium was replaced 6 h later. Cell transfection was repeated after 2 weeks during chondrogenesis. For the *in vivo* study, the BMSCs were transfected with a lentiviral vector expressing pre-miR-365 (Hanbio, Shanghai, China) at a multiplicity of infection (MOI) of 30 virus particles per cell. The medium was changed after 12 h and EGFP expression was observed after 72 h.

2.5. RNA extraction and RT-PCR

Three samples from each group were randomly selected for RNA extraction and RT-PCR analysis. Samples from each group were mixed with 1 ml of Trizol reagent (Invitrogen, USA). RNA (200 ng) was reverse transcribed using a cDNA synthesis kit (Thermo Scientific). The mRNA expression levels were quantified by real-time PCR using a SYBR Green/ROX qPCR Mix Kit. The mRNA levels were normalized to levels of *GAPDH*, and for chondrogenic mRNA, *B2M* was chosen for normalization. miR-365 levels were normalized to levels of U6. The $2-\Delta\Delta Ct$ method was used to assess relative expression. Primer sequences are shown in Table 1.

2.6. Western blot analysis

The cells were washed three times with cold PBS, then lysed on ice for 30 min in ice-cold RIPA (Thermo Scientific) containing 10 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 12,000 ×*g* for 10 min before collection. Protein concentration was determined by a BCA protein assay kit. Protein (30 mg) was added to each well. After SDS-polyacrylamide gel electrophoresis, proteins were transferred to PVDF membranes. Transmembrane blocking was performed and incubated overnight at 4 °C in the appropriate primary antibody. The membrane was washed three times with PBST and then incubated with the appropriate secondary antibody for 2 h. After adding the ECL luminescent agent, the film was developed in a dark room. Images were semi-quantitatively analyzed using Image J.

Table 1
Primers used for real-time PCR.

Gene	Primer sequences (5′ to 3′)
1. MIR-365	Forward: CGCGTAATGCCCCTAAAAAT Reverse: AGTGCAGGGTCCGAGGTATT
2. Col l II	Forward: GCTCCCAGAACATCACCTACCA Reverse: ATTCCTGCTCAGGCCCTCC
3. ANCN	Forward: ATGGCTTCCACCAGTGCG Reverse: CGGATGCCGTAGGTTCTCA
4. SOX9	Forward: CTCGAAACCGACTGGCAACT Reverse: AACAAGCGGTCCAAAGGAAA
5. GAPDH	Forward: CCACTTTGTGAAGCTCATTTCCT Reverse: TCGTCCTCCTCTGGTGCTCT
6. IHH	Forward: GCTTGTAGGCAAGAGGCACGAG Reverse: CTCCTGCTGCTGCTGCTTCTG
7. HDAC4	Forward: GGCTTCCTTGTGGTGGTGTTGG Reverse: TGTACTCTCCTCGGCATGGTGTC
8. ALP	Forward: CCTCTTGGGTCTCTTTGAGC Reverse: CAATCCTGCCTCCTTCCA
9. COLL X	Forward: CCCTTCTGCTGCTAGTGTC Reverse: GTCTTGGTGTTGGGTTGTG
10. B2M	Forward: ACTCCCCAAATTCAAGTGTACT Reverse: TCCTTCAGAGTGACGTGTTTAA
11. Annexin V	Forward: GCAGAACTAACAGCCATAA Reverse: AGAACCAACCAACATCCTC

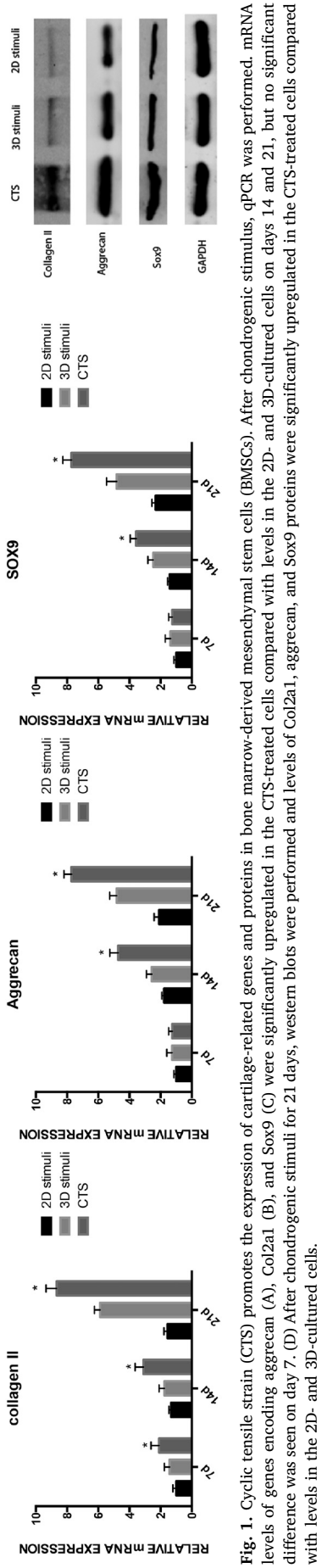

Fig. 1. Cyclic tensile strain (CTS) promotes the expression of cartilage-related genes and proteins in bone marrow-derived mesenchymal stem cells (BMSCs). After chondrogenic stimulus, qPCR was performed. mRNA levels of genes encoding aggrecan (A), Col2a1 (B), and Sox9 (C) were significantly upregulated in the CTS-treated cells compared with levels in the 2D- and 3D-cultured cells on days 14 and 21, but no significant difference was seen on day 7. (D) After chondrogenic stimuli for 21 days, western blots were performed and levels of Col2a1, aggrecan, and Sox9 proteins were significantly upregulated in the CTS-treated cells compared with levels in the 2D- and 3D-cultured cells.

2.7. Alcian blue staining

Treated chondrogenic cells or samples were fixed in 4% paraformaldehyde for 15 min, and then stained with Alcian blue for 20 min. The excess stain was then washed with water. Images were captured using a confocal microscope (LSM 710; Zeiss).

2.8. Immunohistochemical analysis

Induced cells or *in vivo* samples were harvested for immunohistochemical analysis of collagen II. Samples were washed twice with PBS and then fixed with 4% formaldehyde for 10 min at room temperature. Cells or sections were treated with H_2O_2 for 10 min to inhibit endogenous peroxidase. Next, the cells or sections were blocked in PBS containing 0.5% BSA and incubated over night with primary antibodies against collagen II. The cells were then washed three times with PBS for 10 min each and then incubated with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, England) for 1 h as a secondary antibody. The images were visualized by confocal microscopy (LSM 710; Zeiss).

2.9. Luciferase assay

HDAC4 reporter constructs were determined by co-transfection of PGL3HDAC4 or its mutant (0.5 g) with 50 nmol miR-365 mimic or control miRNA in BMSCs. The transfection control was an equal amount of empty vector DNA. Transfection was performed in 6-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cultures were harvested 48 h after incubation and luciferase activity was determined using a DLR-ready luminometer using a Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA). All transfection efficiency firefly luciferase values were normalized using the PRL-TK, Renilla luciferase value.

2.10. Animal model construction

The Animal Experimentation Ethics Committee of Shanghai Tenth Peoples Hospital approved all animal experiments. Eight-week-old male Sprague Dawley rats were used for the *in vivo* experiment. During surgery, rats were anesthetized with 25 mg/kg of 2.5% sodium pentobarbital. We made a lateral parapatellar longitudinal incision to expose the knee joint. A defect (2.5-mm diameter and 2-mm depth) was created in the weight-bearing area of each medial femoral condyle using a drill. All BMSCs were cultured in chondrogenic medium under a Cytodex or CTS environment for 2 weeks prior to implantation. Next, we randomly allocated the 24 animals into four groups, with each group containing 6 animals, as follows: (1) untreated defect, (2) BMSCs, (3) miR-365 infected BMSCs and (4) CTS-treated BMSCs. Fibrin gel with 1×10^5 BMSCs was immediately implanted into the defect cavity. The patella was physically relocated and the joint capsule and subcutaneous tissue were closed. After 8 weeks, the rats were sacrificed and the knee joints were harvested for evaluation.

2.11. Statistical methods

All statistical analyses were performed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA, USA). Data are expressed as mean ± standard deviation (SD) of at least three independent replicates. Inter-group differences were assessed using a Student's *t*-test or one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. CTS promote BMSC chondrogenesis

Aggrecan and Collagen II are two specific proteins in matrix of chondrocytes. The expression of SOX9 is associated with cartilage synthesis. Therefore, genes encoding SOX9, collagen II, and aggrecan are viewed as cartilage synthesis-related genes, and cartilage synthesis can be indicated through their mRNA expression levels. The effect of CTS on BMSC chondrogenic differentiation was examined by applying exogenous CTS. BMSCs were exposed to chondrogenic stimuli for 21 days. On days 7, 14, and 21, levels of the chondrogenic marker genes were examined, and on day 21, the chondrogenic proteins Col2a1, Sox9, and aggrecan, were significantly up-regulated in the CTS-treated cells compared with levels in the 3D and 2D control cells (Fig. 1).

3.2. CTS promotes the expression of miR-365 in BMSCs

Several miRNAs are expressed in response to mechanical stimulation and have been shown to regulate BMSC chondrogenesis. Therefore, we used an Agilent miRNA microarray to examine miRNA expression in CTS-treated and 3D Cytodex culture control BMSCs. Compared with miRNAs in control BMSCs, 18 miRNAs were upregulated and 22 miRNAs were down-regulated in CTS-treated BMSCs (Fig. 2A), and miR-365 was the most up-regulated miR (Fig. 2B). To determine the effect of CTS on miR-365 expression in BMSCs, miR-365 levels in cells on days 3, 14, and 21 were quantified through real-time PCR. miR-365 was significantly upregulated in CTS-treated and miR-365 mimic-treated cells compared with the control 3D-cultured cells on days 3, 14, and 21. The level of miR-365 expression in CTS-treated cells increased over time, increasing to 80-fold on day 21 (Fig. 2C).

3.3. miR-365 plays an important role in enhancing chondrogenesis of BMSCs by CTS

We found that CTS could promote chondrogenesis and miR-365 expression in BMSCs. Therefore, we wondered whether miR-365 plays a role in enhancing CTS-mediated BMSC chondrogenesis. The role of miR-365 in chondrogenic differentiation of BMSCs was examined by transfecting BMSCs with a miR-365 mimic or inhibitor and then applying exogenous CTS; a miR-365 mimic control was used as the negative control. There were six groups: control (3D culture), miR-365 mimics (3D culture), miR-365 inhibitor (3D culture), CTS, CTS + miR-365 mimics, and CTS + miR-365 inhibitor. At 72 h post-infection, BMSCs were exposed to chondrogenic stimuli for 21 days. On day 21, all three chondrogenic marker genes and proteins measured were significantly upregulated in the miR-365 mimics, CTS, and CTS + miR-365 mimics groups. Suppressing miR-365 expression inhibited the level of chondrogenic marker genes and proteins, and this effect could be abolished by CTS. The results indicated that miR-365 could promote chondrogenesis and play an important role in enhancing chondrogenesis of BMSCs by CTS. To confirm the effect of miR-365 on chondrogenesis, then we quantified chondrogenesis by Alcian blue staining and immunocytochemistry for Col2a1 after 21 days of chondrogenic stimuli. The results also showed that miR-365-overexpressing and CTS-treated cells were highly stained compared to cells in the 3D or 2D culture control groups, confirming that miR-365 and CTS promoted chondrogenesis. Collectively, we can conclude that miR-365 plays an important role in enhancing CTS-mediated chondrogenesis in BMSCs.

3.4. CTS does not promote cartilage hypertrophy and ageing during chondrogenesis

During endochondral ossification, ALP expression is associated with the gradual maturation, hypertrophy, and ageing of chondrocytes. Collagen X is a characteristic protein of the osteoblast matrix. Annexin

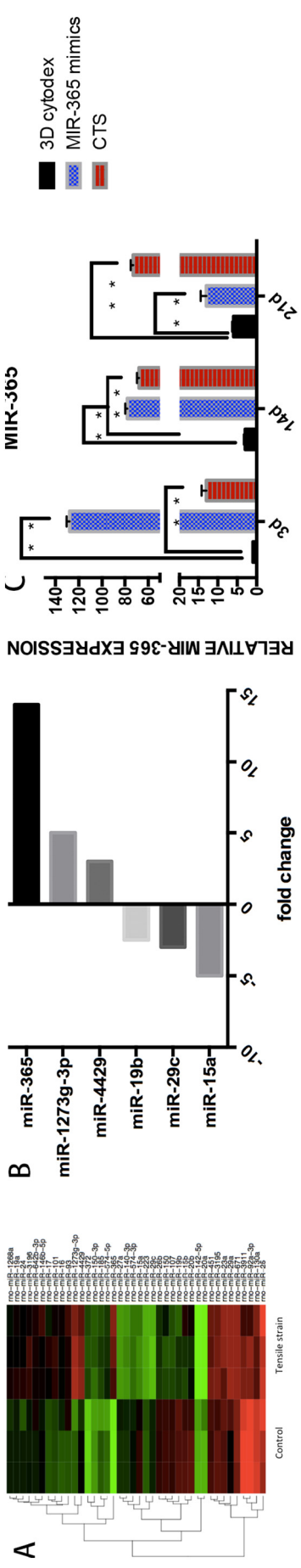

Fig. 2. A) Heat map of miRNAs expressed in cyclic tensile strain (CTS)-treated and control bone marrow-derived mesenchymal stem cells (BMSCs). B) The expression of three top upregulated and downregulated miRNAs in CTS-treated BMSCs compared to expression in control BMSCs. miR-365 was the top upregulated miR. C) Expression of miR-365 was increased in BMSCs after transfection and CTS. In CTS-treated cells, the level of miR-365 expression increased over time and on day 21 the expression in CTS-treated cells had increased about 80-fold.

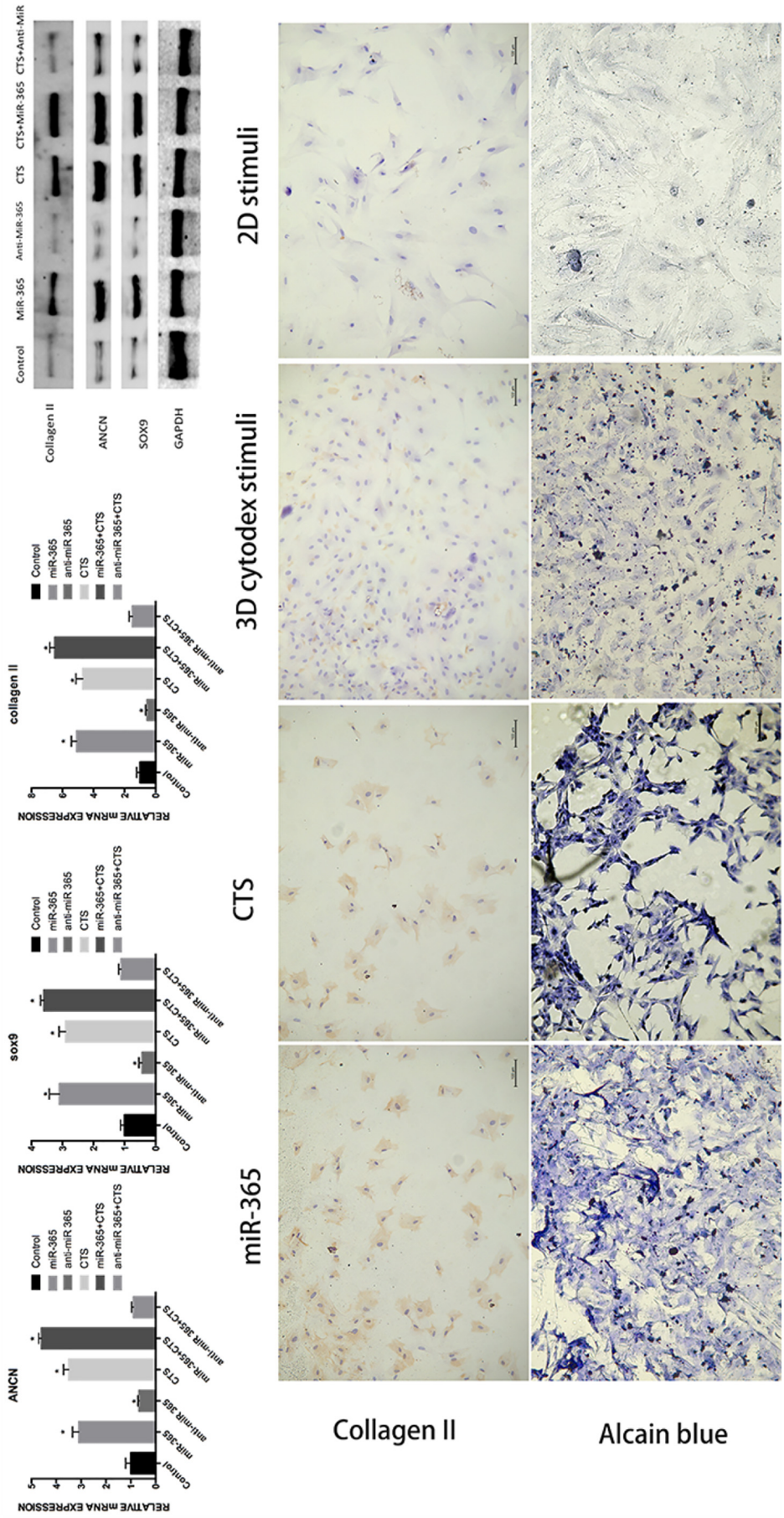

Fig. 3. miR-365 plays an important role in enhancing cyclic tensile strain (CTS)-mediated chondrogenesis of bone marrow-derived mesenchymal stem cells (BMSCs). On day 21, the chondrogenic marker genes (A) and proteins (B), Col2a1 and Aggrecan, as well as Sox9, were significantly upregulated in group miR-365 mimics, CTS, and CTS + miR-365 mimics. Suppressing miR-365 expression could inhibit the level of chondrogenic marker genes and proteins, and this effect could be abolished by CTS. (C) After exposure to chondrogenic stimuli for 21 days, the results of Alcian blue staining and immunocytochemistry for Col2a1 confirmed that miR-365 and CTS promoted chondrogenesis.

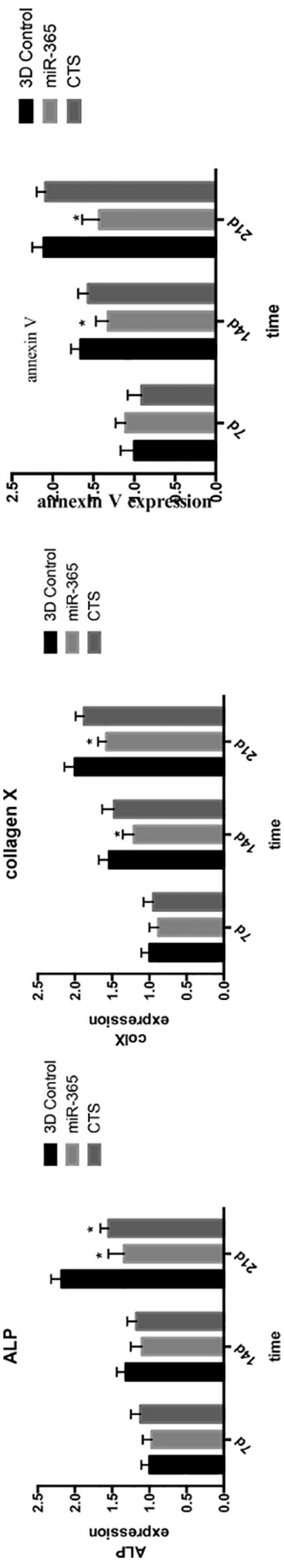

Fig. 4. Cyclic tensile strain (CTS) does not promote cartilage hypertrophy or ageing during chondrogenesis. The mRNA expression levels of genes encoding cartilage hypertrophy-related proteins ALP, collagen X, and annexin V gradually increased and then stabilized at the middle stage of differentiation induction. At the late stage of chondrogenic differentiation, miR-365 expression reduced the levels of ALP, collagen II, and annexin V. CTS reduced the expression of ALP, but not that of collagen X or annexin V.

V expression also increases during cartilage hypertrophy and ageing. We used ALP, collagen X, and annexin V genes as chondrocyte ageing-related and hypertrophy genes in this experiment. According to the RT-PCR results, the mRNA expression levels of genes encoding ALP, collagen X, and annexin V gradually increased and then stabilized at the middle stage of differentiation induction. However, at the late stage of chondrogenic differentiation, miR-365 reduced the expression of genes encoding ALP, collagen II, and annexin V. CTS reduced the expression of ALP, but not that of collagen X or annexin V (Fig. 3). This result indicated that CTS did not promote cartilage hypertrophy and ageing when promoting chondrogenesis in our experiment (Fig.4).

3.5. HDAC4 is a target of miR-365

We conducted a bioinformatics search to identify possible target genes for miR-365. Two different algorithms, miRanda and Target Scan, consistently predicted HDAC4, a known negative regulator of chondrocyte differentiation, as an assumed target for miR-365. The predicted seeding site was in the 3′-UTR of HDAC4 (Fig. 5A). BMSCs were therefore transfected with a miR-365 mimic or control at 100 nmol. At 48 h post-transfection, RNA and protein were extracted. To determine whether miR-365 inhibits *HDAC4* gene expression, we quantified the expression of *HDAC4* and its downstream gene *IHH* by qPCR. The miR-365 mimic significantly inhibited *HDAC4* expression and increased *IHH* gene levels in BMSCs (Fig. 5C). Western blot analysis also verified these results at the protein level (Fig. 5D, E). To determine the miR-381 target region in *HDAC4* mRNA, we mutated three nucleotides of the putative seed region in the HDAC4 3′-UTR. While transfection with the miR-365 mimic inhibited the luciferase activity of the wild-type HDAC4 3′-UTR reporter, the inhibition was abolished in the mutated HDAC4 3′-UTR reporter gene (Fig. 5B).

3.6. CTS-treated and miR-365-overexpressing BMSCs promote cartilage regeneration in vivo

At last, in order to determine the effect of CTS-treated and miR-365 overexpressing BMSCs on cartilage regeneration *in vivo*, fibrin hydrogels containing 1×10^5 cells from the different groups of treated BMSCs were immediately implanted into the defect cavity. After 8 weeks, the defects were filled with newly formed cartilage-like tissue in the CTS and miR-365-overexpressing BMSCs group, while the control BMSC group still showed a moderate defect, and the untreated group showed significant defect. Immunohistochemical staining for collagen II and Alcian blue staining of sections are shown in Fig. 6. In specimens from the CTS and miR-365 BMSC groups, the regenerated tissue stained positive. The control BMSCs group showed weaker positive staining. The defect group showed less staining than all other groups. These results indicated that CTS-treated and miR-365-overexpressing BMSCs could promote cartilage regeneration *in vivo*.

4. Discussion

Articular cartilage is comprised of water, collagen, and proteoglycans [26,27]. This tissue has little capacity for repair and regeneration it lacks neural and vascular components [28]. Effective treatments for articular cartilage diseases are still lacking [29]. Therefore, it is important to develop new strategies for treating this disease. Mechanical loading is known to be a key factor in cartilage tissue engineering [30]. Published papers investigating mechanical loading have mostly focused on compression and shear stress. However, there are no published studies on the effects of CTS on BMSC chondrogenesis. In this study, we investigated how CTS could regulate chondrogenic differentiation.

First, we found that CTS could promote BMSCs chondrogenesis. Next, we found miR-365 was the top up-regulated microRNA in CTS-treated BMSCs. miR-365 has been identified as a mechanoresponsive microRNA. Additionally, it can affect *RUNX1* and *ACVR1* expression,

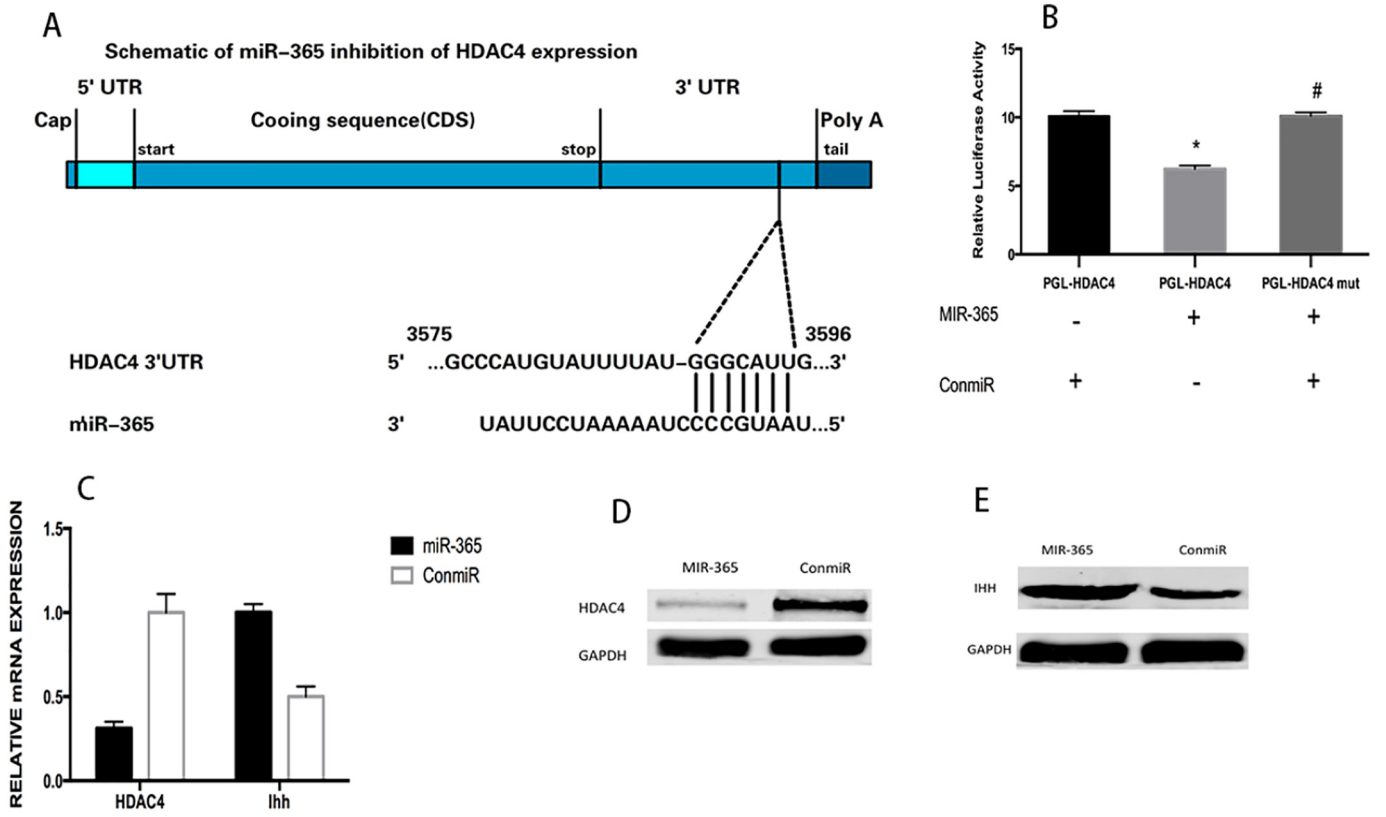

Fig. 5. HDAC4 is a target of miR-365. A) Diagram of predicted miR-365 sequence pairing with a putative target region (miR-365 binding site) in the 3'-UTR of *HDAC4* mRNA. The 5′ end of miR-365 contains a base pair sequence complementary to the putative seed site in *HDAC4* mRNA. B) Inhibition of pGL-HDAC4 reporter gene activity requires the miR-365 seed site. Rat bone marrow-derived mesenchymal stem cells (BMSCs) were transfected with 0.5 μg pGL-HDAC4 of the reporter or pGL-HDAC4 mutant gene (pGL-HDAC4mut) with firefly luciferase and of the PRL-TK gene with Renilla luciferase as an internal control. At 48 h after transfection, cells were harvested for quantification of dual luciferase activities. The Ratio of firefly *vs.* Renilla luciferase represents the relative value of pGL-HDAC4 or pGL-HDAC4mut reporter gene activities. Values are means ± SD (n = 3). *P < 0.05. (C, D, E) BMSCs were transfected with a miR-365 mimic (miR-365) or with a control miRNA (conmiR) at 100 nmol. At 48 h post-transfection, total RNA and protein was extracted from cell lysates. qPCR shows that miR-365 expression decreased the mRNA level of *HDAC4* and increased levels of *IHH* mRNA in BMSCs (C). Western blot analysis was performed with antibodies against HDAC4 and IHH. Inhibition of miR-365 enhanced the protein levels of IHH and inhibited the protein levels of HDAC4 (D, E).

which are known as cartilage matrix regulation genes [31,32]. miR-365 was recently shown to participate in chondrocyte differentiation by directly targeting *HDAC4* [21]. Furthermore, miR-365 expression was found to be elevated in chondrocytes from patients with osteoarthritis, while its levels were decreased by hydrostatic pressure [33]. Therefore, we investigated whether miR-365 plays an important role in promoting BMSC chondrogenesis. The results showed that miR-365 overexpression promoted BMSC chondrogenesis and plays an important role in promoting CTS-mediated chondrogenesis. Additionally, mechanical loading can promote chondrogenesis but can also exacerbate cartilage hypertrophy and ageing [34–36]. In our study, CTS was not found to exacerbate cartilage hypertrophy and ageing during chondrogenesis. The loading regimen parameters such as loading frequency, compressive loading amplitude, and loading duration time are important determining factors for hypertrophy [7]. In our study, the regimen for CTS was 10% amplitude at 1 Hz for 2 h daily, which did not exacerbate cartilage hypertrophy and ageing. It is still unclear if this is the optimal regimen for chondrogenesis, which should be confirmed in more experiments, and other regimens may be able to reduce hypertrophy significantly. Our animal model study confirmed that CTS-treated cells could promote cartilage regeneration *in vivo*. Therefore, collectively we concluded that CTS promotes BMSC chondrogenesis, possibly through promoting the expression of miR-365.

In addition, luciferase reporter assays suggested that miR-365 directly binds *HDAC4*. HDAC4 inhibits endochondral bone formation and chondrocyte hypertrophy [22,37]. Previous studies suggest that miR-365 stimulates chondrocyte differentiation by targeting *HDAC4* and its downstream molecules, including *IHH* and *RUNX2* mRNA [22]. Consistent with this hypothesis, the mRNA levels of *RUNX2* and *IHH* are significantly increased in HDAC4-deficient mice [37]. IHH in particular can promote the chondrogenesis of BMSCs and inhibit cartilage ageing [25]. Consistent with this hypothesis, we also found that the expression of *IHH* was greatly increased in miR-365(+) cells. Therefore, miR-365 is upregulated in osteoarthritis chondrocytes and promotes chondrogenic differentiation. It is possible that a moderate amount of arthritis may stimulate cartilage repair and regeneration as a self-regulation mechanism, and miR-365 is a key intermediate transmission signal for this feedback. In summary, our data suggests a model in which miR-365, activated by CTS, promotes chondrogenesis of BMSCs by inhibiting HDAC4 and inducing IHH expression (Fig. 7).

There are still some limitations in our study. First, we did not apply CTS *in vivo* directly due to the lack of mechanical devices at present. Second, the best regimen of CTS for chondrogenesis still needs to be investigated to balance the effect of chondrogenesis and cartilage hypertrophy. At last, miR-365 may not be the only mechanism underlying the promotion of chondrogenesis by CTS, and more in-depth research needs to be implemented.

5. Conclusion

In summary, we demonstrated for the first time that CTS could promote the expression of miR-365, a crucial mechanosensitive

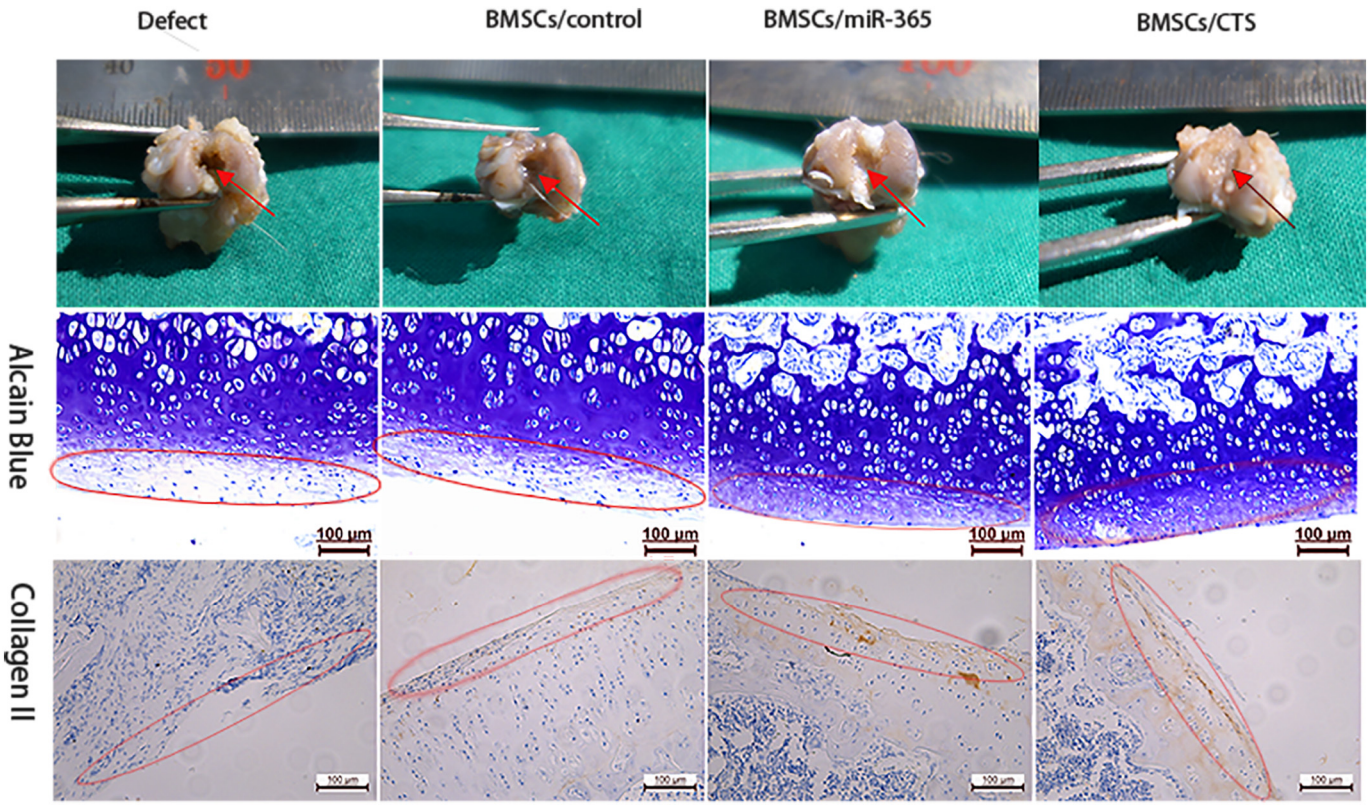

Fig. 6. Cyclic tensile strain (CTS)-treated and miR-365-overexpressing bone marrow-derived mesenchymal stem cells (BMSCs) promote cartilage regeneration *in vivo*. The defects were filled with newly formed cartilage-like tissue in the CTS and miR-365-BMSCs group, while the BMSCs group still showed a moderate defect and the untreated group showed a significant defect. In specimens from the CTS-treated and miR-365-expressing BMSCs, the regenerated tissue stained positive for Alcian blue and collagen II. The control BMSCs group showed weaker positive staining. The untreated defect group showed less staining than all other groups (the rough margins of defects are indicated in the red circle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microRNA involved in the chondrogenesis of BMSCs, which directly targets *HDAC4* and inhibits its expression. CTS, therefore, promotes the expression of IHH, which in turn enhances BMSC chondrogenesis. Therefore, our results indicate that CTS promotes BMSC differentiation into chondrocytes by miR-365 and this may help develop new stem cell or drug therapies for treating cartilage disease.

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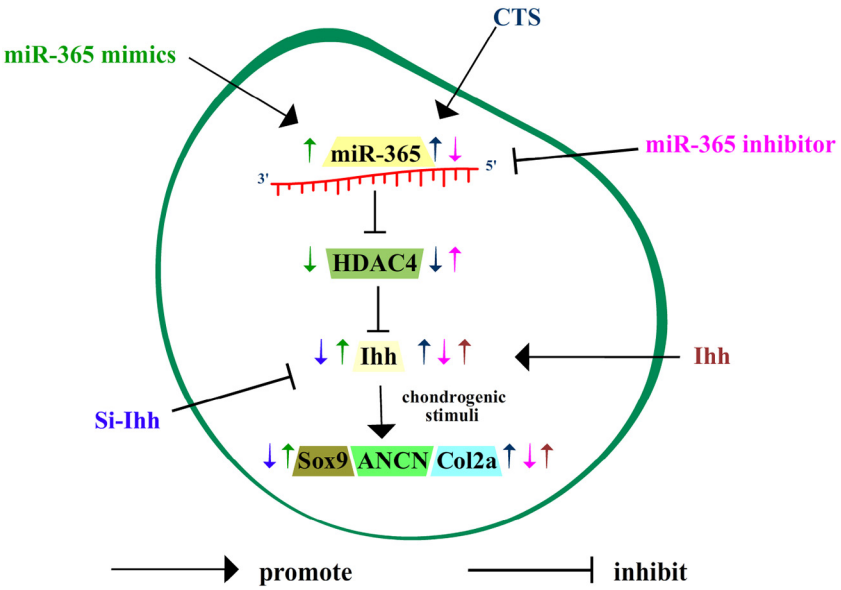

Fig. 7. Model of the possible mechanisms by which cyclic tensile strain promotes chondrogenesis of bone marrow-derived mesenchymal stem cells.

References

[1] V.D. Roobrouck, C. Clavel, S.A. Jacobs, F. Ulloa-Montoya, S. Crippa, A. Sohni, et al., Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions, Stem Cells 29 (2011) 871–882.

[2] N.W. Marion, J.J. Mao, Mesenchymal stem cells and tissue engineering, Methods Enzymol. 420 (2006) 339–361.

[3] K. Gopal, H.A. Amirhamed, T. Kamarul, Advances of human bone marrow-derived mesenchymal stem cells in the treatment of cartilage defects: a systematic review, Exp. Biol. Med. 239 (2014) 663–669.

[4] T.D. Bornes, A.B. Adesida, N.M. Jomha, Mesenchymal stem cells in the treatment of traumatic articular cartilage defects: a comprehensive review, Arthritis Res. Ther. 16 (2014) 432.

[5] S.L. Vega, M.Y. Kwon, J.A. Burdick, Recent advances in hydrogels for cartilage tissue engineering, Eur. Cell. Mater. 33 (2017) 59–75.

[6] T. Zhang, F. Wen, Y. Wu, G.S. Goh, Z. Ge, L.P. Tan, et al., Cross-talk between TGF-beta/SMAD and integrin signaling pathways in regulating hypertrophy of mesenchymal stem cell chondrogenesis under deferral dynamic compression, Biomaterials 38 (2015) 72–85.

[7] J.R. Choi, K.W. Yong, J.Y. Choi, Effects of mechanical loading on human mesenchymal stem cells for cartilage tissue engineering, J. Cell. Physiol. 233 (2018) 1913–1928.

[8] O.F.W. Gardner, G. Musumeci, A.J. Neumann, D. Eglin, C.W. Archer, M. Alini, et al., Asymmetrical seeding of MSCs into fibrin-poly(ester-urethane) scaffolds and its effect on mechanically induced chondrogenesis, J. Tissue Eng. Regen. Med. 11 (2017) 2912–2921.

[9] L.M. Kock, J. Malda, W.J. Dhert, K. Ito, D. Gawlitta, Flow-perfusion interferes with chondrogenic and hypertrophic matrix production by mesenchymal stem cells, J. Biomech. 47 (2014) 2122–2129.

[10] N.S. Remya, P.D. Nair, Mechanoresponsiveness of human umbilical cord mesenchymal stem cells in in vitro chondrogenesis-a comparative study with growth factor induction, J. Biomed. Mater. Res. A 104 (2016) 2554–2566.

[11] C. Chen, X. Wei, Z. Lv, X. Sun, S. Wang, Y. Zhang, et al., Cyclic equibiaxial tensile strain alters gene expression of chondrocytes via histone deacetylase 4 shuttling, PLoS One 11 (2016) e0154951.

[12] A.M. Mohr, J.L. Mott, Overview of microRNA biology, Semin. Liver Dis. 35 (2015) 3–11.

[13] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.

[14] X. Lin, L. Wu, Z. Zhang, R. Yang, Q. Guan, X. Hou, et al., MiR-335-5p promotes chondrogenesis in mouse mesenchymal stem cells and is regulated through two positive feedback loops, J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res. 29 (2014) 1575–1585.

[15] D. Guerit, D. Philipot, P. Chuchana, K. Toupet, J.M. Brondello, M. Mathieu, et al., Sox9-regulated miRNA-574-3p inhibits chondrogenic differentiation of mesenchymal stem cells, PLoS One 8 (2013) e62582.

[16] O. Ham, B.W. Song, S.Y. Lee, E. Choi, M.J. Cha, C.Y. Lee, et al., The role of microRNA-23b in the differentiation of MSC into chondrocyte by targeting protein kinase A signaling, Biomaterials 33 (2012) 4500–4507.

[17] E.A. Lin, L. Kong, X.H. Bai, Y. Luan, C.J. Liu, miR-199a, a bone morphogenic protein 2-responsive MicroRNA, regulates chondrogenesis via direct targeting to Smad1, J. Biol. Chem. 284 (2009) 11326–11335.

[18] J. Nie, L. Liu, W. Zheng, L. Chen, X. Wu, Y. Xu, et al., MicroRNA-365, down-regulated in colon cancer, inhibits cell cycle progression and promotes apoptosis of colon cancer cells by probably targeting cyclin D1 and Bcl-2, Carcinogenesis 33 (2012) 220–225.

[19] M. Zhou, W. Liu, S. Ma, H. Cao, X. Peng, L. Guo, et al., A novel onco-miR-365 induces cutaneous squamous cell carcinoma, Carcinogenesis 34 (2013) 1653–1659.

[20] Q. Song, H. Li, H. Shao, C. Li, X. Lu, MicroRNA-365 in macrophages regulates Mycobacterium tuberculosis-induced active pulmonary tuberculosis via interleukin-6, Int. J. Clin. Exp. Med. 8 (2015) 15458–15465.

[21] Y.J. Guan, X. Yang, L. Wei, Q. Chen, MiR-365: a mechanosensitive microRNA stimulates chondrocyte differentiation through targeting histone deacetylase 4, FASEB J. 25 (2011) 4457–4466.

[22] K. Cao, L. Wei, Z. Zhang, L. Guo, C. Zhang, Y. Li, et al., Decreased histone deacetylase 4 is associated with human osteoarthritis cartilage degeneration by releasing histone deacetylase 4 inhibition of runt-related transcription factor-2 and increasing osteoarthritis-related genes: a novel mechanism of human osteoarthritis cartilage degeneration, Arthritis Res. Ther. 16 (2014) 491.

[23] D. Xu, Y. Gao, N. Hu, L. Wu, Q. Chen, miR-365 ameliorates dexamethasone-induced suppression of osteogenesis in MC3T3-E1 cells by targeting HDAC4, Int. J. Mol. Sci. 18 (2017).

[24] X. Yang, Y. Guan, S. Tian, Y. Wang, K. Sun, Q. Chen, Mechanical and IL-1beta responsive miR-365 contributes to osteoarthritis development by targeting histone deacetylase 4, Int. J. Mol. Sci. 17 (2016) 436.

[25] P.C. Liu, K. Liu, J.F. Liu, K. Xia, L.Y. Chen, X. Wu, Transfection of the IHH gene into rabbit BMSCs in a simulated microgravity environment promotes chondrogenic differentiation and inhibits cartilage aging, Oncotarget 7 (2016) 62873–62885.

[26] J.C. Bernhard, G. Vunjak-Novakovic, Should we use cells, biomaterials, or tissue engineering for cartilage regeneration? Stem Cell Res Ther 7 (2016) 56.

[27] C. Madeira, A. Santhagunam, J.B. Salgueiro, J.M. Cabral, Advanced cell therapies for articular cartilage regeneration, Trends Biotechnol. 33 (2015) 35–42.

[28] A.R. Tan, C.T. Hung, Concise review: mesenchymal stem cells for functional cartilage tissue engineering: taking cues from chondrocyte-based constructs, Stem Cells Transl. Med. 6 (2017) 1295–1303.

[29] E.A. Makris, A.H. Gomoll, K.N. Malizos, J.C. Hu, K.A. Athanasiou, Repair and tissue engineering techniques for articular cartilage, Nat. Rev. Rheumatol. 11 (2015) 21–34.

[30] A.C. Shieh, K.A. Athanasiou, Principles of cell mechanics for cartilage tissue engineering, Ann. Biomed. Eng. 31 (2003) 1–11.

[31] F.J. Blanco, C. Ruiz-Romero, New targets for disease modifying osteoarthritis drugs: chondrogenesis and Runx1, Ann. Rheum. Dis. 72 (2013) 631–634.

[32] M. Mura, S. Cappato, F. Giacopelli, R. Ravazzolo, R. Bocciardi, The role of the 3′UTR region in the regulation of the ACVR1/Alk-2 gene expression, PLoS One 7 (2012) e50958.

[33] S. Cheleschi, A. De Palma, A. Pecorelli, N.A. Pascarelli, G. Valacchi, G. Belmonte, et al., Hydrostatic pressure regulates microRNA expression levels in osteoarthritic chondrocyte cultures via the Wnt/beta-catenin pathway, Int. J. Mol. Sci. 18 (2017).

[34] A. Cochis, S. Grad, M.J. Stoddart, S. Fare, L. Altomare, B. Azzimonti, et al., Bioreactor mechanically guided 3D mesenchymal stem cell chondrogenesis using a biocompatible novel thermo-reversible methylcellulose-based hydrogel, Sci. Rep. 7 (2017) 45018.

[35] O.F.W. Gardner, N. Fahy, M. Alini, M.J. Stoddart, Joint mimicking mechanical load activates TGFbeta1 in fibrin-poly(ester-urethane) scaffolds seeded with mesenchymal stem cells, J. Tissue Eng. Regen. Med. 11 (2017) 2663–2666.

[36] Z. Li, L. Kupcsik, S.J. Yao, M. Alini, M.J. Stoddart, Mechanical load modulates chondrogenesis of human mesenchymal stem cells through the TGF-beta pathway, J. Cell. Mol. Med. 14 (2010) 1338–1346.

[37] R.B. Vega, K. Matsuda, J. Oh, A.C. Barbosa, X. Yang, E. Meadows, et al., Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis, Cell 119 (2004) 555–566.