Received: 25 February 2019 | Revised: 28 July 2019 | Accepted: 26 August 2019

DOI: 10.1111/ocr.12341

ORIGINAL ARTICLE

Orthodontics & Craniofacial Research WILEY

Cyclic tensile strain promotes the ECM synthesis of cranial base synchondrosis chondrocytes by upregulating miR-140-5p

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 81400536

Abstract

Objective: This study aimed to explore the role of miR-140-5p in cranial base synchondrosis chondrocytes (CBSCs) under cyclic tensile strain (CTS).

Setting and Sample Population: A total of 25 1-week-old Sprague Dawley rats from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, were used.

Material and Methods: The second passage of CBSCs was applied with CTS at 10% elongation (1 Hz) for 24 hours. MiR-140-5p levels in CBSCs were detected by qRT-PCR. The role of miR-140-5p in CBSCs was evaluated by transfection of mimics and inhibitor. RNA sequencing and online search of miRNA databases (TargetScan, miRDB and miRanda) were used in prediction of miR-140-5p targets. A luciferase reporter assay was applied to identify the target gene of miR-140-5p.

Results: Compared with the control, the expression of Col2a1 and Sox9 was significantly higher after CTS ($P < .05$). Also, CBSCs demonstrated higher expression of miR-140-5p after CTS loading for 24 hours ($P < .05$). Overexpression of miR-140-5p promoted ECM synthesis under CTS loading environment, while suppression of miR-140-5p inhibited the effect. Bloc1s2 was a putative target gene of miR-140-5p.

Conclusions: The expression of ECM in CBSCs could be promoted by CTS and miR-140-5p might play a role in this process through targeting Bloc1s2.

KEYWORDS

Bloc1s, cranial base synchondrosis, cyclic tensile strain, MiR-140-5p

1 | INTRODUCTION

Cranial base synchondroses (CBS) are important growth centres of the craniofacial skeleton and play a crucial role in upper face and cranial vault growth.[1] Disturbance of CBS development can lead to a range of craniofacial abnormalities, and CBS may be a treatment target for youngsters with craniofacial abnormalities.[2,3] Mechanical force, cyclic tensile strain (CTS) in particular, plays a fundamental role in the regulation of cartilage morphogenesis, metabolism and maintenance. CTS is a potent antagonist of IL-1β actions and acts as both an anti-inflammatory and a reparative signal in chondrocytes.[4,5] Moderate CTS altered the assembly of the extracellular collagen network and led to a higher amount of cartilage oligomeric matrix protein (COMP) in the matrix.[6] Short-term CTS induced PTHrP expression in postnatal growth plate chondrocytes, which could subsequently affect growth plate development.[7] However, the biochemical and molecular mechanism by which mechanical stimuli influences CBSCs remains unclear.

MicroRNAs (miRNAs) are single-stranded noncoding RNAs (approximately 19-25 nucleotides) that regulate multiple biological processes.[8] Recent studies have underlined the key role of miRNAs in regulating chondrocyte metabolism and functions.[9,10] In particular, miR-140, steadily expressed in healthy human articular chondrocytes, is cartilage-specific and related to embryonic bone development, chondrogenesis, adult cartilage homoeostasis, osteoarthritis (OA) and differentiation of MSCs into chondrocytes.[11]

During chondrogenesis, increased miR-140 correlates with increased SOX9 and COL2A1 expression in human articular chondrocytes.[11] However, the role of miR-140 in CBSCs has never been reported.

The purpose of this study was to investigate the possible effects of CTS on CBSCs. We investigated the effects of miR-140-5p in CBSCs on cartilage extracellular matrix (ECM) and the relationship between miR-140-5p and Bloc1s2 (biogenesis of lysosome-related organelles complex-1 subunit 2).

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of primary CBSCs

Cranial base synchondroses cartilage was obtained from 1-week-old Sprague Dawley rats provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences (SLACCAS). Dissected cartilage samples were sliced into pieces and digested with 0.25% trypsin at 37°C for 30 minutes followed by 0.1% type II collagenase (Vetec) overnight. The isolated chondrocytes were seeded at a density of 1×10^5 cells/cm^2 and cultured at 37°C in DMEM (Gibco BRL) with 10% foetal bovine serum (FBS, Gibco BRL) in a thermal incubator under 5% CO_2. The medium was changed every 2 days. CBSCs were used at passage 2.

2.2 | Application of CTS

Cranial base synchondrosis chondrocytes were plated at a density of 1×10^5 cells/cm^2 on type I collagen–coated BioFlex 6-well culture plates (Flexcell International Corporation). When cells grew to reach 80% confluence in humidified 5% CO_2 at 37°C, FBS-free DMEM was used to synchronize cell growth; 24 hours later, the growth medium was replaced by DMEM with 10% FBS. Cyclic tensile strain (CTS) experiments were performed using FLEXCELL® FX-5000™ Tension system (Flexcell International Corporation). Cells were subjected to a cyclic mechanical biaxial tensile strain (CTS) at 10% elongation (1 Hz) for 24 hours, and CBSCs kept static at the same time were used as negative control.

2.3 | Quantitative real-time PCR

Total RNA was isolated by TRIzol reagent (Invitrogen). According to manufacturer's instructions, the extracted RNA was reverse-transcribed with HiScript II Q RT SuperMix Kit (Vazyme) for mRNA and miScript II Reverse Transcription Kit (Qiagen) for miRNA, respectively. qRT-PCR was carried out using QuantiFast® SYBR® Green PCR Kit (Qiagen) to determine the expression level of gene transcripts for miR-140-5p, Sox9, and Col2a1. U6 and GAPDH were used as internal references. mRNA primers were purchased from Generay Biotech (Generay). The primers used in the present study were as follows:

- Col2a1 forward, 5′-TAGGGCCTGTCTGTTTCTTG-3′; reverse, 5′-CCTAGAGTGACTGCGGTTA –3′;
- Sox9 forward, 5′-CAGAGAACGCACATCAAGAC-3′; reverse, 5′-TTGTAGTGCGGAAGGTTGAA –3′;
- GAPDH forward, 5′-GCAAGTTCAACGGCACAGTC-3′; reverse, 5′- ATACTCAGCACCAGCATCACC-3′.

The microRNA-specific primer sequences were designed and synthesized by Generay Biotech (Generay) based on the miRNA sequences obtained from the miRBase database (Release 20.0) as follows:

- miR-140-5p, CAGTGGTTTTACCCTATGGTAG;
- U6,CAAGGATGACACGCAAATTCG

Universal primer was purchased from Qiagen (Qiagen). The expression of mRNAs and miRNA was determined using the $2^{-\Delta\Delta CT}$ method.

2.4 | Western blot analysis

Cranial base synchondrosis chondrocytes were lysed in cold RIPA buffer (Solarbio) containing 1% PMSF, and protein concentration was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were then blocked with Tris-buffered saline and Tween (TBST) containing 5% skim milk. The membrane was subsequently incubated with primary antibodies against Bloc1s2 (1:2000, Abclonal) antibodies at 4°C overnight. The membranes were washed three times with TBST and then incubated with HRP-conjugated secondary antibodies (1:5000, Jackson) for 1 hour at room temperature. Antibody against β-actin diluted 1:5000 (Santa Cruz Biotechnology) served as an endogenous control. All experiments were performed in triplicate.

2.5 | Immunofluorescence analysis

Cranial base synchondrosis chondrocytes were seeded on BioFlex culture plates and treated with 1Hz 10% CTS for 24 hours. Subsequently, cells were rinsed in PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.2% Triton X-100 and blocked in PBS containing 5% goat serum (Solarbio). The cells were washed with PBS and incubated overnight at 4˚C with antibodies specific to COL2A1 (1:200 dilution, Abcam). Following this, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Abclonal) for 60 minutes at room temperature in a darkroom. Finally, DAPI was added to each sample for nuclear counterstaining. Fluorescent images were examined using an inverted fluorescence microscope (ZEISS).

2.6 | Cell transfection

RFect^{PM} (Biodai) was used to perform transfection, in accordance with the manufacturer's instruction. miR-140-5p mimics, miR-140-5p inhibitor and their negative controls (Qiagen) were diluted with serum-free DMEM, respectively. The diluted RFect^{PM} was then added to the diluted miR-140-5p mimics, miR-140-5p

inhibitor or their negative controls. After incubated at room temperature for 20 minutes, the above mixture was added into the cell suspension, which was then incubated at 37°C, 5% CO_2 for 24 hours. After that, the transfection mixture was replaced by DMEM with 10% FBS. Cells were cultured for 48 hours before following assays.

For cell transfection experiments, CBSCs were divided into the following groups: (a) miR-140-5p mimics group; (b) control group for miR-140-5p mimics; (c) miR-140-5p inhibitor group; (d) control group for miR-140-5p inhibitor.

2.7 | RNA sequencing

RNA samples of transfected cells were collected after 24 hours post-CTS, and mRNA sequencing was performed by a service provider (Generay) using Illumina Hiseq System. Differential detection of mRNA signals was defined as a foldchange (log_2 ratio) <−1.00 (down-regulated) or >1.00 (upregulated) with $P < .05$.

2.8 | Prediction of miR-140-5p targets

MiR-140-5p target genes were predicted through online search of miRNA databases (TargetScan, miRDB and miRanda). In addition, the results of RNA sequencing were also used for predicting the miR-140-5p targets. For this study, genes that were predicted by the two methods were defined as potential miR-140-5p targets.

2.9 | Dual-luciferase reporter assay

For dual-luciferase reporter assays, miR-140-5p mimics/controls and Bloc1s2 wild/mutant 3′-untranslated region (3′UTR) plasmids (Genechem) were co-transfected in 293T cells by Lipofectamine 2000 (Life Technologies), and then, luciferase assays were performed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. All experiments were performed in triplicate. Results were expressed as the firefly luciferase activity normalized to Renilla luciferase activity.

2.10 | Statistical analysis

Each experiment was repeated at least three times. All the data were presented as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) or two-tailed Student's *t* test using SPSS 17.0 (SPSS Inc). $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Effects of CTS on CBSCs

To explore the effect of CTS on CBSCs, a 10% strain load at 1 Hz was applied for 24 hours. Types II collagen mRNA expression and protein secretion increased after CTS loading. The qRT-PCR results showed that the expression of Col2a1 and Sox9 in CTS group was significantly higher than that of the control ($P < .05$; Figure 1A). MiR-140-5p was significantly upregulated in CBSCs after CTS loading for 24 hours ($P < .05$; Figure 1B). Furthermore, Alcian blue results showed that CBSCs stimulated with CTS included more ECM components than unstimulated cells (Figure 1C). Immunofluorescence staining showed that the ECM-related protein (Col-II) increased in CBSCs after CTS (Figure 1D).

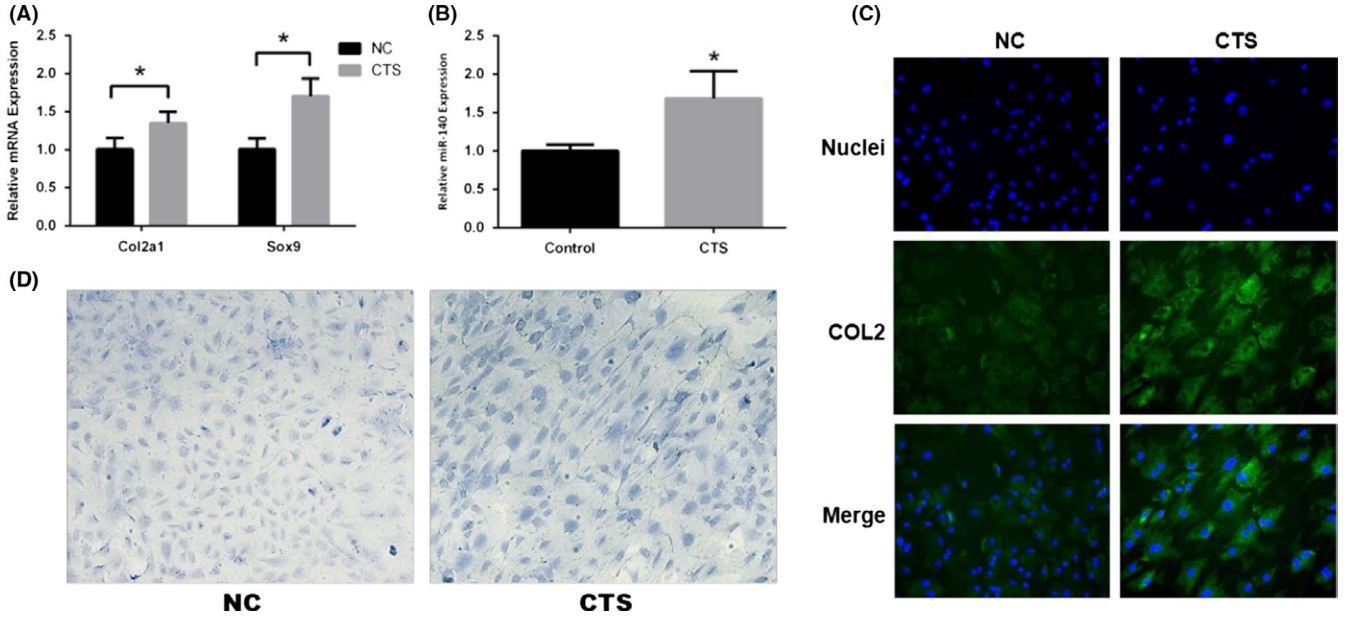

FIGURE 1 Effects of CTS on CBSCs. A, The expression of Col2a1 and Sox9 (*$P < .05$); B, miR-140-5p levels in CBSCs after CTS (*$P < .05$); C, Alcian blue staining for CBSCs; (d) Immunofluorescence staining for Col-II. CBSC, cranial base synchondrosis chondrocytes; CTS, cyclic tensile strain

3.2 | Overexpression of miR-140-5p promoted ECM synthesis of CBSCs under CTS loading

To detect the role of miR-140-5p in CBSCs under CTS, CBSCs were transfected with miR-140-5p mimics, inhibitor and controls before CTS loading. qRT-PCR showed that miR-140-5p was significantly upregulated by mimics ($P < .001$) and downregulated by inhibitor ($P < .05$; Figure 2A). In terms of ECM synthesis, miR-140-5p overexpression remarkably increased Col-II under CTS compared to the control. Meanwhile, these effects were reversed by miR-140-5p suppression (Figure 2B).

3.3 | Bloc1s2 was a putative target of miR-140-5p

The set of highest-ranking predicted miR-140-5p target genes is listed in Table 1. miRDB and miRanda showed that the Bloc1s2 mRNA 3′UTR might be directly targeted by miR-140-5p (Figure 3A). The dual-luciferase reporter assay demonstrated a significant difference in the luciferase activity among the miR-140-5p-NC + Bloc1s2-3′UTR, miR-140-5p-NC + Bloc1s2-3′ UTR mut, miR-140-5p mimics + Bloc1s2-3′ UTR and miR-140-5p mimics + Bloc1s2-3′UTR mut groups ($P < .01$; Figure 3B). Compared with the miR-140-5p-NC + Bloc1s2-3′UTR, miR-140-5p-NC + Bloc1s2-3′UTR mut and miR-140-5p mimics + Bloc1s2-3′UTR mut groups, the luciferase activity was significantly decreased in the miR-140-5p mimics + Bloc1s2-3′UTR group ($P < .01$). To explore the relationship between miR-140-5p and Bloc1s2 under a mechanical environment, CBSCs was transfected with miR-140-5p mimics, miR-214-5p inhibitor and corresponding controls before CTS. The mRNA expression of Bloc1s2 was significantly downregulated by miR-140-5p overexpression ($P < .001$; Figure 3D). Western blot analysis showed a similar trend (Figure 3C).

4 | DISCUSSION

Cranial base synchondroses, composed of mirror-image growth plates, are cartilaginous segments between ossification centres and critical for cranial and upper facial development. Disturbance of CBS development can lead to a number of craniofacial abnormalities.[2,12] CBS shares great similarities with long bone growth plates but is also different in many aspects such as late ossification and reaction to mechanical stimuli. A previous study has shown that appropriate

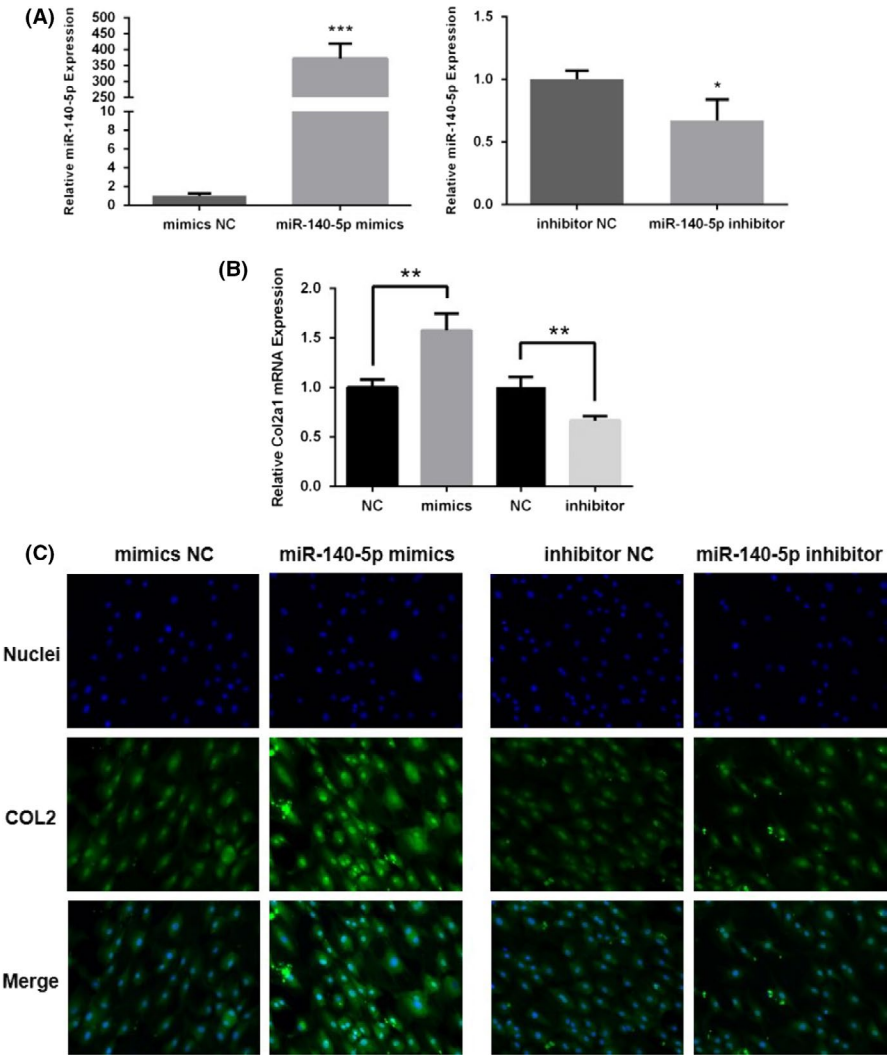

FIGURE 2 Overexpression of miR-140-5p promoted Col-II expression in CBSCs. CBSCs were transfected with miR-140-5p mimics, inhibitor and controls. A, miR-140-5p expression was upregulated in mimics transfected cells and downregulated in inhibitor transfected cells (*$P < .05$, ***$P < .001$); B, overexpression of miR-140-5p promoted Col2a1 in CBSCs under CTS, while suppression inhibited Col2a1 (**$P < .01$); C, immunofluorescence staining for Col-II. CBSC, cranial base synchondrosis chondrocytes; CTS, cyclic tensile strain

TABLE 1 MiR-140-5p targets identified by bioinformatics prediction and microarray analysis

	Transcripts regulated by miR-140-5p treatment (RNA sequencing)		MiR-140-5p targets predicted by bioinformatic tools		
Gene name	Foldchange (log_2 Ratio of miR-140-5p/control)	*P*-value	TargetScan	miRDB (score)	miRanda
Bloc1s2	−1.193575028	4.31E-09		1 (94)	1
Pdgfra	−1.1929853	8.81E-28	1	1 (91)	
Adam10	−1.132123315	1.01E-27		1 (84)	
Slc6a4	−1.041696703	1.33E-10			1
Nrm	−1.284103386	0.011825629			1
Fblim1	−1.344686619	7.51E-06			1

Note: Only the top six highest-ranking predicted miR-138 target genes are listed.

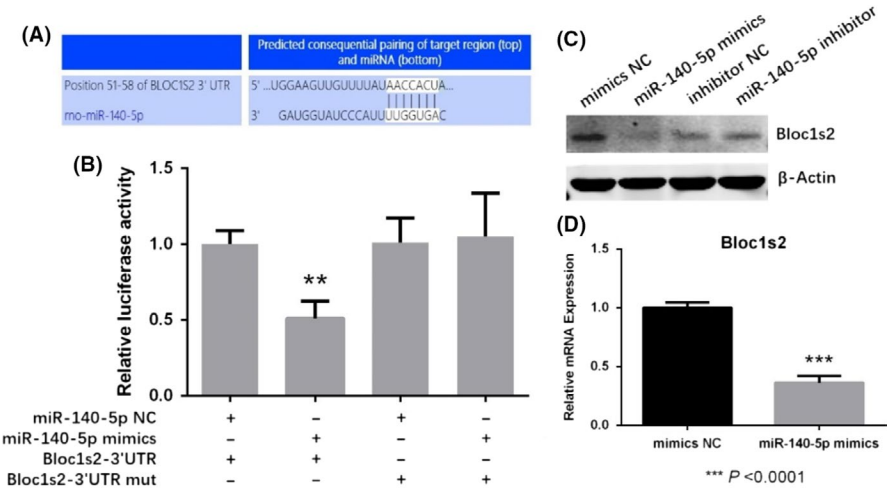

FIGURE 3 Bloc1s2 was a target of miR-140-5p. A, Schematic diagram of putative binding sites of miR-140-5p in the 3'-UTR of Bloc1s2; B, dual-luciferase reporter assay showed Bloc1s2 is the target gene of miR-140-5p. Luciferase activity was much lower following co-transfection of Bloc1s2 wild-type 3′-UTR with miR-140-5p (**P < .01). C, and (d) miR-140-5p negatively regulated the mRNA and protein expression of Bloc1s2 in CBSCs (***P < .0001). CBSC, cranial base synchondrosis chondrocytes

CTS can promote biological changes of CBSCs.[13] However, the effects and mechanism of mechanical tension stress on CBSCs remain unknown.

The present study was undertaken to investigate the effects of mechanical stress on CBSCs. In this in vitro model, we applied CTS via FX-5000™ tension system. Observations were mainly focused on the expression of ECM and the cartilage-specific miR-140-5p. We found that the expression of Col2a1 and Sox9 in CTS group was significantly upregulated. Immunofluorescence staining also suggested that CTS could promote the synthesis of Col-II. These results clearly demonstrate that CTS significantly improves ECM synthesis of CBSCs. Our results were consistent with previous animal studies which showed tensile forces were potent anabolic stimulus for chondral growth in CBS.[14-17] More importantly, miR-140-5p was highly expressed after CTS loading for 24 hours, indicating that miR-140-5p might be involved in ECM synthesis of CBSCs under a mechanical tension environment. Additionally, overexpression of miR-140-5p promoted Col-II expression under CTS, while downregulation had a reversed effect. Although it has been established that miR-140 is involved in regulation of the ECM expression in chondrocytes, this is the first report demonstrating the relationship between miR-140-5p and mechanical stimulation. Our RNA-seq-based study identified several genes regulated by miR-140-5p, including the experimentally confirmed miR-140-5p target gene, Bloc1s2.

Bloc1s2 is a negative regulator of Notch signalling,[18,19] which plays important roles in the development of the neural crest.[20,21] Bloc1s2$^{-/-}$ mice demonstrated craniofacial malformation.[19] The presphenoidal synchondrosis used in this study is entirely composed of neural crest-derived chondrocytes, and the spheno-occipital synchondrosis is also partially neural crest-derived.[22] Notch signalling is also involved in maintaining clonality and proliferation of chondrocytes y.[23-25] Therefore, we hypothesize that Bloc1s2 might be associated with cranial base synchondroses and craniofacial development. However, little is currently known about the function of Bloc1s2 and further investigation is needed to elucidate its function and involvement in CTS-promoted ECM synthesis.

Further work will be required to explore the relationship between miR-140-5p and other targets, including Pdgfra and Adam10, which were evident in the current results. In summary, our study offers strong evidence that CTS can promote the production of ECM in CBSCs under suitable conditions. MiR-140-5p may play an important role in this process. And Bloc1s2 was a putative target gene of miR-140-5p. The cranial base synchondrosis has received increasing attention in orthodontics since its growth can influence the maxillary–mandibular complex. Involvement of mechanical signals in the

CBS growth might have important clinical implications, such as the beneficial effects of rapid maxillary expansion (RME) and reverse-pull headgear (RPHG) in young Class III patients, both of which might induce growth modification not only in the maxilla but also in the anterior cranial base.

5 | CONCLUSIONS

To sum up, the expression of ECM in CBSCs could be promoted by CTS and miR-140-5p might play a role in this process. Bloc1s2 was a putative target gene of miR-140-5p. Further studies are necessary to investigate the function and involvement of Bloc1s2 in CTS-promoted ECM synthesis.

ACKNOWLEDGEMENTS

This work was supported by the grants from National Natural Science Foundation of China (81400536).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Cheng M, Chu F, Feng Q, Shen G. Cyclic tensile strain promotes the ECM synthesis of cranial base synchondrosis chondrocytes by upregulating miR-140-5p. *Orthod Craniofac Res*. 2019;00:1–6. https://doi.org/10.1111/ocr.12341