

# CRT Silencing Inhibits ECM Synthesis in HGFs Cultured on Three-Dimensional PLGA Scaffolds Via Inhibiting The CaN/NFAT3 Signalling Pathway

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## Abstract

The stability of orthodontic treatment is believed to be greatly affected by the compression and contraction of gum tissue, but the underlying molecular mechanism remains unclear. The aim of the current study was to explore the effects of mechanical forces on CRT, CaN, NFAT3, p-NFAT3 and COL-I expression in human gingival fibroblasts (HGFs) cultured on three-dimensional (3D) poly(lactic-co-glycolic acid) (PLGA) scaffolds. A mechanical force of 25 g/cm<sup>2</sup> was applied to HGFs for 0, 6, 24, 48, and 72h. The expression of CRT, CaN, NFAT3, p-NFAT3, and COL-I were examined by reverse transcription-quantitative polymerase chain reaction and western blotting analysis. The application of mechanical force on HGFs cultured on the 3D PLGA scaffolds led to a significant increase in CRT, CaN, and COL-I expression, as well as a reduction in p-NFAT3 expression. The mechanical force effects were reversed by silencing CRT. By lowering the CRT expression, p-NFAT3 was upregulated, while CaN and COL-I were downregulated in HGFs. These findings suggested that downregulation of CRT inhibited extracellular matrix synthesis, potentially via CaN/NFAT3 signalling pathway. Therefore, CRT may serve as a potential therapeutic target against gingival fibrosis.

## Introduction

During orthodontic treatment, we apply mechanical forces to orthodontic appliances, which are transmitted to the periodontal tissues such as the alveolar bone, gingiva, periodontal membrane to move the teeth<sup>1,2</sup>. In contrast to alveolar bone and periodontal ligament, which can easily be assimilated, gingival tissue generally tends to accumulate after correction of twisted teeth rotation, buccal and labial tooth movement, and closing of the extraction space. The main reason for the accumulation of gum tissue is contraction and compression. This kind of gum tissue, like compressive rubber, has a tendency to relapse to the pretreatment position, which may cause the gaps to reappear at the extraction site or the teeth to return to their original position<sup>3</sup>. It has been reported that orthodontic force can disrupt the balance between collagen synthesis and degradation of gingival tissue, which is very important for maintaining homeostasis, and also increases the multiplication of human gingival fibroblasts (HGFs) and the synthesis of type I collagen (COL-I), which is the main structural component in the extracellular matrix (ECM) of the gingival tissue<sup>4,5</sup>. Such persistent ECM deposition in the gum tissue is the main driver of recurrence after orthodontic treatment<sup>6</sup>. However, the mechanisms by which HGFs perceive and respond to orthodontic forces, that result in ECM deposition are still unclear.

Calreticulin (CRT), located in the endoplasmic reticulum (ER) lumen is a 46-kDa protein that regulates cellular reactions to stress through its roles in its chaperone activation and the unfolded protein reaction<sup>7,8</sup>. Recent literature suggests that CRT adjusts the transcription of multifarious ECM proteins in calcium-dependent manners and plays a posttranscriptional role in collagen transport and matrix assembling<sup>9,10</sup>. Interestingly, CRT expression was shown to be overexpressed in many diverse fibrosis models, including bleomycin-induced pulmonary fibrosis, unilateral ureteral obstruction renal fibrosis model, and chronic fibrosis proliferative diseases<sup>11-13</sup>. Although the mechanisms was not yet clear, heart-specific upregulation of CRT during upgrowth led to interstitial fibrosis<sup>14</sup>. The expression level of CRT was enhanced by factors which were well-known to cause fibrotic diseases and ER stress, including oxidative stress, pneumatoresis, and hyperglycaemia<sup>11,13,15,16</sup>. Therefore, we speculated that CRT might play a role in gingival fibrosis under mechanical force, although the role and mechanisms of CRT in the occurrence and development of gingival fibrosis still remain unknown.

The calcineurin (CaN)/nuclear factor of activated T cells 3 (NFAT3) signalling is an important intracellular signalling pathway that can participate in the process of cell fibrosis by mediating a variety of cytokines<sup>17-19</sup>. Calreticulin is an essential protein serine/threonine phosphatase that is regulated by calcium ion/calmodulin (CaM)<sup>20</sup>. NFAT3 is the main substrate of CaN and is an important nuclear transcription factor that mediates intracellular signal transduction<sup>21,22,23</sup>. Additionally, CRT plays an important role in the regulation of downstream Ca<sup>2+</sup>-dependent CaN/NFAT signalling pathway<sup>8</sup>. In the absence of calreticulin, the release of ER Ca<sup>2+</sup> is compromised, results in downstream Ca<sup>2+</sup>/CaN signal transduction defects, as well as decreasing NFAT and myocyte enhancer factor 2C (MEF2C) nuclear translocation<sup>24,25</sup>. Calreticulin-deficient mouse could be saved from embryonic death via constitutive active CaN, which causes nuclear translocation of MEF2C and NFAT offering proof that CRT is an upriver regulator of CaN signalling. Therefore, we speculated that CRT might play a role in ECM synthesis via regulating the activation of CaN/NFAT3 signalling under mechanical force.

In comparison with two-dimensional (2D) culture, three-dimensional (3D) culture can more accurately reflect the morphological characteristics of cells in tissues. For example, HGFs cultured on 3D nanoporous fiber scaffolds exhibit a spindle shape instead of being a clustered plane on 2D model<sup>26</sup>. The poly lactide-co-glycolide (PLGA) biological scaffold, which can synchronously imitate the influences of mechanical force and hypoxic environment, can afford a 3D HGFs culture model that is more analogous to the tissue-like environment of natural ECM<sup>4</sup>. In the current study, we aimed to establish a 3D co-culture model of HGFs and PLGA scaffolds to explore the role of CRT, CaN, NFAT3, and phosphorylated NFAT3 (p-NFAT3) in HGFs mechanotransduction and to investigate the role of CRT in ECM deposition in HGFs. Our research offers novel insights into the mechanism of gingival reconstruction during orthodontic tooth movement.

## Methods

### 3D culture of HGFs

Gingival tissue specimens were obtained from healthy premolar teeth of patients (age 12-25 years) who had planned to undergo orthodontic treatment at the affiliated stomatology hospital of Guangxi Medical University. The patients had no systemic diseases, gum inflammation, or gum hyperplasia. This research was reviewed and approved by the Medical Ethics Committee of Guangxi Medical University (approval number: 20150304-22) and patients and their families were fully informed and offered written consent. The research was performed according to the principles of the revisory Declaration of Helsinki (World Medical Association General Assembly, 2008).

The gingival tissue was scraped from the root surface and cut into small tissue blocks (1 mm<sup>3</sup>). These tissue pieces were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., MA, USA), supplemented with 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., MA, USA) and 1% (v/v) penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc., MA, USA) and maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. The PLGA scaffold (size, 1 cm × 1 cm × 300 μm; molar ratio of lactic acid to glycolic acid, 75:25; pore size, 100–120 μm; porosity, 85%) was synthesised by solvent casting/particle leaching technology. The 4th generation (1 × 10<sup>5</sup>) HGFs in good growth conditions in the logarithmic growth phase with a single cell suspension were inoculated on a single sheet of PLGA scaffold to establish a co-culture model of HGFs and PLGA scaffolds.

### Application of mechanical force

The above HGFs-PLGA co-culture model was placed into a sterile well plate and incubated for 24 h and then moved to a new well plate. The cells in the PLGA co-culture model were subjected to a compressive force of 25 g/cm<sup>2</sup> by placing a sterilized cylindrical bottle containing lead granules for 6, 24, 48, and 72 h. The stress value was determined based on previous studies, under a force of 25g/cm<sup>2</sup>, the proliferation index of HGFs was at the maximum<sup>4</sup>. In addition, the stress value was also in line with the level reported by Schwarz<sup>46</sup>, who suggested that the proper force for moving teeth should be within the scope of capillary pressure, which is 7–26 g/cm<sup>2</sup>. The cells in the control group were cultured under the same conditions without applying a mechanical force. In another experiment, the 3D-cultured cells were infected with CRT-siRNA lentivirus and NC lentivirus (GeneChem, Shanghai, China). After 3 d of infection, green fluorescent protein (GFP) expression was observed by fluorescent microscopy, and a mechanical force of 25 g/cm<sup>2</sup> was applied to the cells for 24 h. Quantitative real-time PCR and western blot analysis were performed to determine the knock-down efficiency, and a dose-response assay using the Cell Counting Kit-8 (CCK8; Dojindo, Japan) was performed to assess cytotoxicity.

### Laser scanning using confocal microscope

The 3D-cultured cells that were treated with compressive force were washed twice with phosphate-buffered saline (PBS). Then, 4% paraformaldehyde was applied for cell fixation for 30 min and washed with PBS three times for 5 minutes each time. The complex was treated with DAPI (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 10 min at 37°C. Immunofluorescence micrographs (Olympus Corporation, Japan) were obtained using a laser confocal microscope (light wavelength of 405nm).

### Quantitative real-time PCR

Total RNA was extracted from the HGFs-PLGA Complex using the Trizol kit (Aidlab Biotechnology Co., Ltd., Beijing, China) in each group. The obtained RNA was reverse-transcribed to cDNA using the ExScript RT kit (Takara Bio, Inc., Otsu, Japan), RT-qPCR was performed based on the (SYBR) manufacturer's instruction. The 2<sup>-ΔΔCt</sup> was considered to be the relevant expression of the target gene in each group. Each experiment was repeated three times, and the average value was recorded.

The primers used had the following sequences:

*GAPDH* forward: 5'-TGTGTCCGTCGTGGATCTGA-3'

*GAPDH* reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3'

*CRT* forward: 5'-GCACTTGGATCCACCCAGAA-3'

*CRT* reverse: 5'-GAAGTTGTCAAAGATGGTGCCAGA-3'

*RCaN1* forward: 5'-CCAGACAAGCAGTTTCTGATCTCC-3'

*RCaN1* reverse: 5'-AGTCGCTGCGTGCAATTCATA-3'

*COL-I* forward: 5'-CTACTCTCTCAACTCACTCTTCACTTA-3'

*COL-I* reverse: 5'-TCACTCACCACCCTATCTTCCA-3'

### Western blotting analysis for protein expression

To separate the HGFs from the HGFs-PLGA 3D culture model, 0.25% trypsin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used. The cells were harvested in ice-cold RIPA buffer (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitor cocktails. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were immersed in 5% skimmed milk at 25 °C for 2 h. The primary antibodies were then incubated at 4 °C overnight, including the anti-CRT (ab92516, Abcam), anti-CaN (ab140131, Abcam), anti-NFAT3 (CST, USA), anti-p-NFAT3 (Santa, USA), and anti-COL-I (ab138492, Abcam). The membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signalling Technology, Boston, USA) at 25 °C for 2h. Enhanced chemiluminescence (ECL) was used for development. ChemicDoc MP all-round imaging system (Bio-Rad, Cal., USA) was used for photography, while the Image Lab v3.0 software (Bio-Rad, Cal., USA) was used for the gray-value analysis of the protein bands.

### Statistical analysis

Statistical analyses were performed using SPSS 20.0 (IBM, Chicago, IL, USA) statistical software. The results were expressed as mean ± standard deviation (SD), while t-test methods were used for comparison between two groups, with one-way analysis of variance used for comparison among multiple groups.

The inspection level of  $\alpha=0.05$ ,  $P<0.05$  were considered to be statistically significant.

## Results

### Laser scanning using confocal microscope

As shown in Figure 1, HGFs grew densely, crisscrossing and interconnecting with each other in all directions on the 3D HGFs-PLGA scaffold model under a confocal laser microscope (Olympus Corporation, Tokyo Metropolis, Japan). The samples were scanned in layers, and it could be seen that there were cells at different levels.

### Improvement in the expression levels of CRT, COL-I, and activation of the CaN /NFAT3 in HGFs

As shown in Figure 2a and 2b, quantitative real-time polymerase chain reaction (RT-qPCR) showed a significant increase in the levels of CRT and CaN under mechanical stimulation compared with those of the control group, with the peak mRNA expression levels in the 24-h group. Calreticulin and CaN showed the highest protein expression at 24h (Figure 2c, 2d). In addition, with respect to the levels of NFAT3 phosphorylation, the p-NFAT3/NFAT3 ratio was significantly decreased within the first 24h and then increased over time (Figure 2e). These results illustrated that mechanical force was a sufficient stimulus to induce CRT and CaN/NFAT3 activation in HGFs.

At the same time, we detected COL-I expression in the HGFs in response to compressive force. In comparison to the control group, the mRNA and protein expression levels of COL-I were significantly increased, and the highest expression level was detected at 24 h (Figure 2f, 2g). Therefore, the mechanical force stimulus caused higher collagen expression in the gingival tissue. In addition, when the compressive force was applied for 24 hours, the expression levels of CRT, CaN, and p-NFAT3 all had significant changes, and thus was selected for subsequent experiments.

### Reversal of the mechanical force effects by blocking CRT

As shown in Figure 3a, b, c, d, after infection, the efficiencies were greater than 80% for both CRT-siRNA lentivirus and NC lentivirus, indicating that the CRT-siRNA lentivirus and NC lentivirus were highly infectious to HGFs. Regarding the function of CRT on cell growth, knockdown of CRT expression had no significant effect on the proliferation of HGFs (Figure 3e). The cultures infected with the CRT-siRNA lentivirus had significantly lower levels of CRT mRNA and protein compared to levels in cultures infected with the NC lentivirus (all  $P\leq 0.05$ , Figure 3f, 3g).

According to our analysis, the expression level of CaN was significantly decreased (all  $P\leq 0.05$ , Figure 4a, 4b), and p-NFAT3 expression was increased when HGFs were transfected with si-CRT (all  $P\leq 0.05$ , Figure 4c). It is noteworthy that CRT blockade decreased the expression of COL-I in HGF (all  $P\leq 0.05$ , Figure 4d, 4e). These results suggested that CRT is an effective mediator in increasing CaN/NFAT3 activation and the production of COL-I in HGFs under mechanical force.

## Discussion

Increasing evidence has showed that, compared with 2D culture systems, 3D cell culture systems could more accurately response the microenvironment of cells in the tissue, and thereby the behaviour of the cells cultured on 3-dimension system was more consistent with the actual in vivo cellular reaction<sup>27</sup>. The PLGA is polymerized by polyglycolic acid (PGA) and polylactic acid (PLA), which has not only the advantages of PGA in the aspect of cell stickiness, but also that of PLA with respect to physical stage<sup>28,29</sup>. In addition, it is more in accordance with the periodontal membrane tissue, and can more accurately simulate the anoxic environment<sup>30</sup>. When mechanical forces are applied to teeth, the periodontal cells on the compression side are simultaneously stimulated by mechanical and hypoxic factors due to vascular atresia, which act upon each other and accelerate the formation of osteoclasts, thus promoting the absorption of alveolar bone on the compression side. The combination of these stimuli has been shown to be beneficial for orthodontic tooth movement<sup>31,32</sup>. In this research, due to the limited diffusion after the application of mechanical force, the cells in the central part of the complex were in a state of hypoxia and nutrient deficiency. So, the PLGA 3D model imitated the influences of mechanical forces and low-oxygen environments at the same time, furnishing a firm base for the follow-up experiments.

Converting the mechanical energy of a stimulus signal into a chemical signal is called mechanical transduction<sup>33</sup>. When mechanical force acts on the cell surface and the mechanical signal is transmitted to the cytoskeleton, ER, and nucleus, the stimulation of mechanical force can cause the ER stress response and upregulate the expression of CRT<sup>34</sup>. Calreticulin is an important ER Ca<sup>2+</sup>-buffering protein participating in adjusting ER Ca<sup>2+</sup> reserve and discharge<sup>35,36</sup>, acts as a molecular chaperone, which can help proteins fold correctly<sup>37-39</sup>. So far, the majority of studies have concentrated upon the expression and function of CRT in pulmonary and renal fibrosis. In fibrotic disorders studies, researchers found that CRT was unusually expressed in bleomycin-induced pulmonary fibrosis and unilateral ureteral obstruction (UUO) renal fibrosis<sup>11,12</sup>. However, the expression and function of CRT in gingival fibrosis had not been studied. Therefore, we first detected the expression level of CRT in HGFs under mechanical forces. CRT was more highly expressed in HGFs than that in the group without mechanical force, indicating the application of mechanical force resulted in an increase in CRT expression, suggesting that CRT might be involved in the process of gingival fibrosis. To some extent, we also found that CRT expression increased with a trend, that is, with the extension of the application time of mechanical force, the CRT expression continued to rise to the highest value, and then gradually decreased. This may be due to the fact that with the extension of the application time, the sensitivity of the cells to the stimulus gradually decreases. Additionally, CRT plays an important role in regulating downstream cellular Ca<sup>2+</sup>-dependent signalling through CaN and NFAT<sup>8,40</sup>. Calreticulin-lacking mice die during embryogenesis because of damaged heart development; thus, CRT-lacking mice could be saved from embryonic lethality if CaN was constitutively activated during development<sup>24</sup>, suggesting the importance of the role of CRT in adjusting the ER Ca<sup>2+</sup> discharge and downstream CaN/NFAT signalling.

Under the stimulation of mechanical force, collagen synthesis in the HGFs increased, which was consistent with the results of our previous study and other research<sup>4</sup>. Recent literature has reported that fibroblasts were sensitive to the mechanics of their microenvironment, and abnormal fibroblast mechanosensing can drive the fibrotic program<sup>42</sup>. In recent years, numerous studies have reported the key role of the CaN/NFAT3 signalling pathway in ECM accumulation in the process of tissues fibrosis, including liver fibrosis and cardiomyocyte fibroblasts<sup>19,43</sup>. The deposition of ECM was obviously inhibited when an CaN or NFAT inhibitor was used in these fibrotic tissues<sup>17,18,44</sup>. However, the expression and function of CaN/NFAT3 signalling pathway in gingival fibrosis had not been studied. Thus, we first explored the expression levels of CaN, NFAT3, p-NFAT3 in HGFs under mechanical forces and found that a significant increase in CaN expression, as well as a decrease in p-NFAT3 expression, indicating the application of mechanical force significantly increased the activation of the CaN/NFAT3 signalling pathway. To some extent, we also found that the activity of the CaN/NFAT3 signalling pathway and the expression level of CRT showed the same trend of change, suggesting that the expression of the CaN/NFAT3 signalling pathway and CRT may have rhythmic characteristics. In order to assess CRT and CaN/NFAT3 signalling function in ECM synthesis in HGFs, we infected HGFs with CRT-siRNA lentivirus or control lentivirus. Compared to controls, CRT-siRNA treated cells showed decreased CaN and COL-I expression, increased p-NFAT3 expression. These findings suggested that downregulation of CRT inhibited gingival collagen synthesis, potentially via CaN/NFAT3 signalling pathway. Additionally, Owusu proposed that factors such as high glucose levels, nutrient deprivation, and oxidative stress trigger ER stress. Endoplasmic reticulum stress increases CRT levels; in turn, CRT stimulates Ca<sup>2+</sup> release from the ER, leading to increased cytosolic Ca<sup>2+</sup>, that results in activation of CaN, which dephosphorylates cytoplasmic NFAT, leading to NFAT activation and nuclear translocation, inducing the transcription of related target genes, promoting collagen secretion and procollagen processing into the ECM<sup>45</sup>. Nevertheless, the effects of CRT on HGFs under mechanical forces should be further demonstrated using a more rigorous approach that includes genetic knockouts and overexpression vectors. This would be performed in future studies.

## Conclusion

Our findings demonstrated activation of the CaN /NFAT3 signalling pathway, a significant increase in CRT expression and COL-I synthesis in the HGFs cultured on 3D PLGA scaffolds under the mechanical force. Specifically, blocking CRT could inhibit CaN/NFAT3 activation and collagen synthesis. These findings ultimately offer novel insights into the mechanisms of mechanical conduction and ECM synthesis in HGFs, and may be conducive to the progress of targeted therapeutics by accelerating gingival tissue remodeling.

## Declarations

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## Author contributions

H.W. conceived the study and participated in its design. H.W. drafted the manuscript. H.W., X.Z.C., and H.Y.M. performed the cell experiments. H.W., T.Y.Y., and Q.Q.M., performed sample collection and virus transfection. All authors read and approved the final manuscript.

## Competing interests

The authors declare no competing interests.

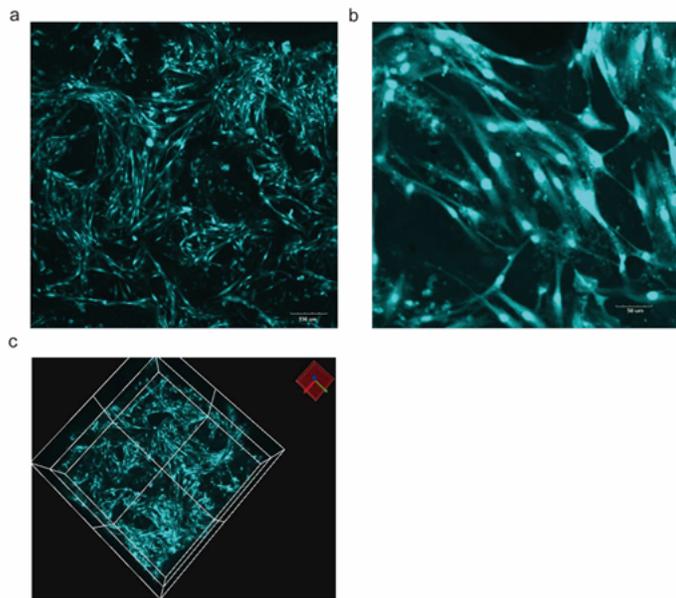
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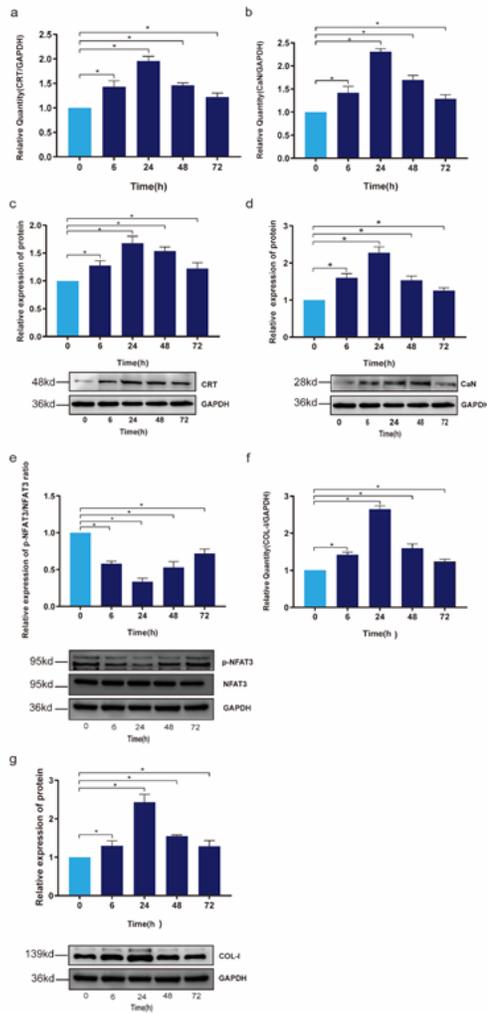
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## Figures



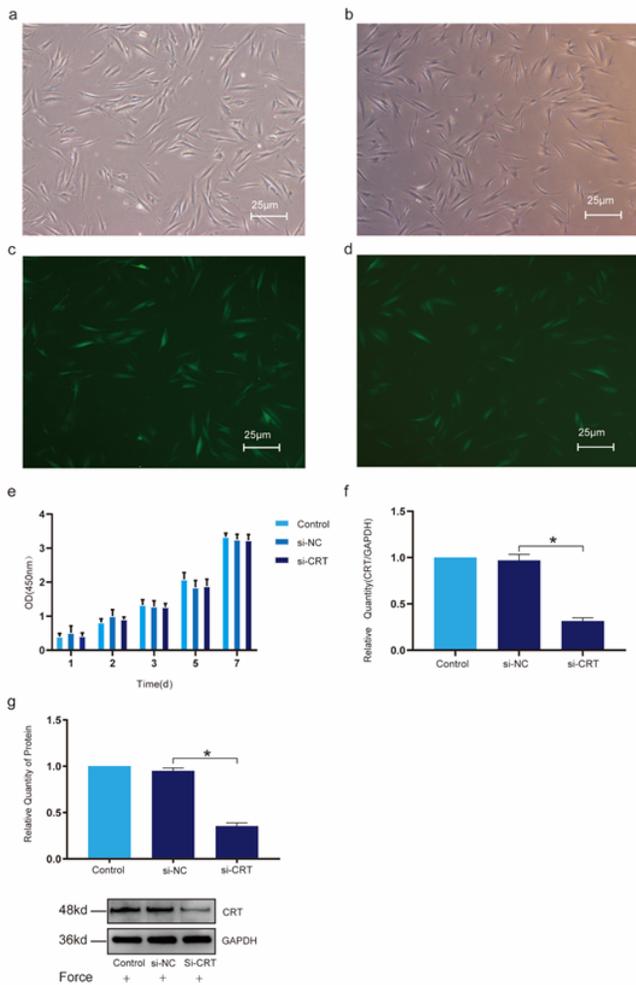
**Figure 1**

The morphology of HGFs cultured on 3D scaffold under confocal laser scanning microscope. (a) 3D compound culture model (100 X) of the HGFs; (b) 3D compound culture model of the HGFs (600X); (c) 3D culture model of the HGFs (layered; scanning). 3D, three-dimensional; HGF, human gingival fibroblast.



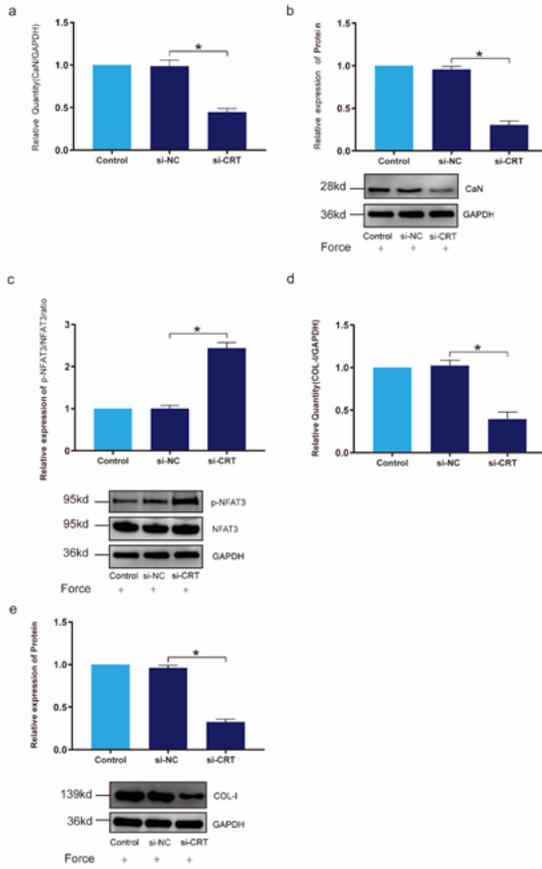
**Figure 2**

Mechanical force increased the expression of CRT, COL-I and activation of the CaN/NFAT3 signaling pathway in HGFs. The mRNA expression: (a) CRT, (b) CaN, (f) COL-I and protein expression: (c) CRT, (d) CaN, (e) the p-NFAT3/NFAT3 ratio, and (g) COL-I were evaluated after being treated with a compressive force of 25 g/cm<sup>2</sup> on the HGFs at different time points. Data are presented in the form of means ± standard deviations and were obtained from three independent experiments. \* p < 0.05 vs. 0 h control.



**Figure 3**

Fluorescence of HGFs infection with the siRNA-CRT lentivirus or NC lentivirus for 72 h (40 ×). (a, b) Images under an ordinary optical microscope; (c, d) Images under the fluorescence microscope; (a, b) siRNA-CRT group; (c, d) siRNA-NC group; (e) Cell growth rate for the 1st, 2nd, 3rd, 4th and 5th day after transfection with siRNA-CRT lentivirus and NC lentivirus; RT-qPCR and Western Blot analysis were used to detect the mRNA and protein expression of CRT (f, g) and data were presented in the form of means ± standard deviations and were obtained from three independent experiments. \*  $p < 0.05$ .



**Figure 4**

The mRNA expression: (a) CaN, (d) COL-I and protein expression: (b) CaN, (c) p-NFAT3/ NFAT3 ratio and (e) COL-I were evaluated after being treated with a compressive force of 25 g/cm<sup>2</sup>, with the siRNA-CRT lentivirus and NC lentivirus on HGFs for 24h. Data were presented as means ± standard deviations and were obtained from three independent experiments. \* p < 0.05.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementary information CRT silencing inhibits ECM synthesis in HGFs cultured on three-dimensional PLGA scaffold via inhibiting the CaN/NFAT3 signalling path](#)