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LncRNA HCG18 regulates the progression of spinal tuberculosis by modulating the hsa-miR-146a-5p/TGF-β1/SMADs pathway

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Abstract

Spinal tuberculosis is the most common extrapulmonary tuberculosis, characterized by intervertebral disc destruction, which seriously affects people's quality of life. Recent studies have suggested that the TGF-β1/SMADs signaling pathway plays an important regulatory role in the process of intervertebral disc destruction caused by spinal tuberculosis. However, the abnormal TGF-β1/SMADs signaling pathway in spinal tuberculosis is not fully understood. Herein, we found for the first time that HCG18 was significantly upregulated in spinal tuberculosis nucleus pulposus clinical samples and confirmed that HCG18 negatively regulates the proliferation and migration ability of nucleus pulposus cells (NPCs). In vitro experiments further suggest that overexpression of HCG18 can significantly promote TGF-β1/SMADs pathway activity and inhibit proliferation, migration, and apoptosis of NPCs, an effect which can be reversed by overexpressing hsa-miR-146a-5p. On the contrary, knocking down HCG18 yields the opposite result. In vivo experiments suggest that knocking down HCG18 can significantly alleviate the destruction of the nucleus pulposus in rats with spinal tuberculosis by inhibiting the activity of the TGF-β1/SMADs pathway. In summary, our research suggests that HCG18 can promote the progression of spinal tuberculosis by alleviating the inhibitory effect of hsa-miR-146a-5p on the TGF-β1/SMADs pathway. This study provides new insights into the occurrence and development of spinal tuberculosis, as well as new strategies for the prevention and treatment of spinal tuberculosis.

Keywords Spinal tuberculosis, Intervertebral disc destruction, HCG18, SMADs signaling pathway, hsa-miR-146a-5p

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Introduction

Tuberculosis is one of the top ten causes of death worldwide, with an annual increase of about 10 million people [1–3]. Spinal tuberculosis (ST) is the most common type of extrapulmonary tuberculosis, which can damage the patient's vertebral bodies and intervertebral discs, leading to the formation of fluid abscesses [4–6]. ST is often accompanied by spinal deformities, paraplegia, and even death, which seriously decrease the quality of life and longevity of people [7, 8]. The most significant characteristic change of ST is intervertebral disc destruction, but its mechanism of destruction is still unclear [8].

Transforming growth factor-β1 (TGF-β1) is an important factor that regulates intervertebral disc metabolism [9, 10]. It can promote collagen degradation by upregulating the expression of matrix metalloproteinases, leading to intervertebral disc destruction [11]. In addition, it plays an important regulatory role in the synthesis of intervertebral disc cell matrix [11]. TGF-β1 mainly activates SMAD homolog 2 (SMAD2) and SMAD homolog 3 (SMAD3) proteins into the nucleus by binding to its specific receptors and exerts biological effects by activating or inhibiting the transcription of target genes through the regulation of SMAD4 and SMAD7 [12, 13]. SMAD4 occupies a central position in the TGF-β1/SMAD signaling pathway and is a common mediator required in the signal transduction process [14]. Studies have shown that TGF-β1 can promote the proliferation of mycobacterium tuberculosis in cells, reduce the phagocytic ability of mononuclear macrophages, and lead to extracellular matrix and bone destruction, cavities, and fibrosis when it accumulates in large quantities [12, 15, 16]. Even that TGF-β1 plays an important role in the pathological process of intervertebral disc destruction caused by ST, the mechanism leading to the imbalance of TGF-β1 expression in ST remains further exploration.

Long non-coding RNAs (lncRNA) is a type of non-coding RNAs (ncRNA) that can act as an endogenous "sponge" of small RNA, such as microRNAs (miRNAs), playing an important biological regulatory role by competing with disease-related miRNAs to regulate their targets [17]. Micro RNAs (miRNAs) may inhibit translation or enhance mRNA degradation in the absence of competitive transcripts [18–21]. The human leukocyte antigen complex group 18 (HCG18) has been reported as an immune-related lncRNA that can be expressed as an immune marker in gliomas [22, 23]. Previous studies have shown that HCG18 could promote intervertebral disc degeneration by regulating hsa-miR-146a-5p [24]. However, the molecular regulatory mechanism of HCG18 in intervertebral disc destruction caused by ST remains largely unknown.

In this study, we detected a significant upregulation of HCG18 expression in clinical samples of ST, while its expression level was significantly negatively correlated with hsa-miR-146a-5p. By utilizing a strategy that combines in vivo and in vitro assays, we further confirmed that HCG18 can sponge hsa-miR-146a-5p and up-regulate the activity of the TGF-β1/SMADs pathway, thereby activating the intervertebral disc degeneration and promoting the progression of ST. Our study provides new insights into the occurrence and development of ST, as well as new strategies for the prevention and treatment of intervertebral disc degeneration caused by ST.

Table 1 The patients information

	Spinal tuberculosis	Spinal trauma
Gender	15 males, 15 females	15 males,15 females
Age	42.33 ± 12.30	40.93 ± 11.44

Materials and methods

Specimen collection

The patients who underwent surgery due to ST or spinal trauma in Weifang People's Hospital were involved in this study. A total of 30 ST patients were involved in the test group and a total of 30 spinal trauma patients were involved in the control group (Table 1). To avoid confounders, we recruited 15 males and 15 females in each group. There was no statistically significant age difference between the two groups of patients ($p = 0.46$). The experimental group was diagnosed based on preoperative imaging and clinical symptoms (Fig. 1), as well as postoperative histopathological examination to exclude other diseases. Patients were excluded from other infectious diseases, tumors, and immunologic diseases of the spine. All clinical specimens in this experiment were collected with the consent of patients following the Helsinki Declaration. The research protocol was approved by the Ethics Committee of Weifang People's Hospital (ethical batch number: KYLL20190412-1).

Cell separation and culture

The isolation method of intervertebral disc nucleus pulposus cells (NPCs) from ST patients and control group patients was based on previous literature [25]. In brief, the nucleus pulposus tissue was placed in sterile PBS and washed to remove blood and necrotic tissue. The nucleus pulposus tissue was cut into small pieces of 1–2 mm^3 and transferred to a solution containing collagenase (0.2 -0.4%) and hyaluronidase (0.1%) for digestion at 37 ℃ for 2–4 h. Adding an equal volume of culture medium containing serum to terminate digestion, and then a cell sieve was used to remove undigested tissue. Finally, the NPCs were collected and cultured in the complete human intervertebral disc nucleus pulposus cell culture medium (Wuhan Ponosi Life Technology Co., Ltd.) and placed in a cell culture box (5% CO_2, 37 ℃). The culture medium

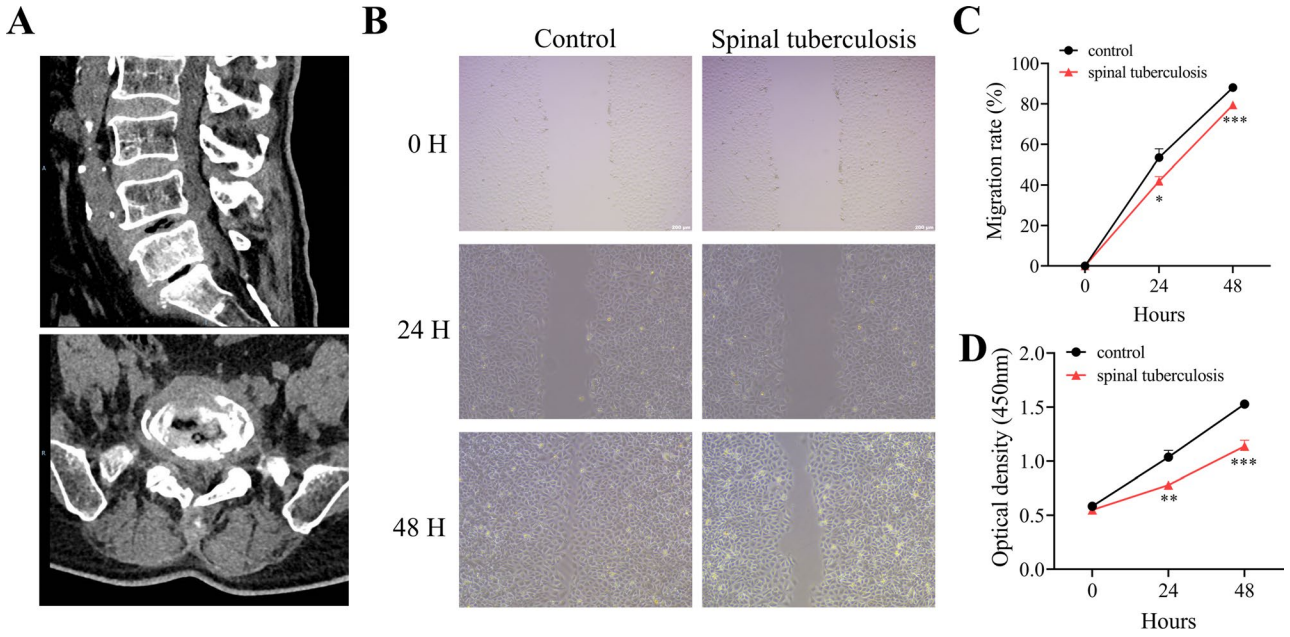

Fig. 1 Extraction of nucleus pulposus cells from tuberculosis infected intervertebral discs. (**A**) The CT scan shows the nucleus pulposus destruction in sagittal and horizontal planes in ST patient. (**B**, **C**) The wound healing assays indicated that tuberculosis infection significantly reduced the migration ability of NPCs ($n=3$, * $p<0.05$, *** $p<0.001$). (**D**) The CCK-8 assays indicated that tuberculosis infection significantly reduced the proliferation ability of NPCs ($n=3$, ** $p<0.01$, *** $p<0.001$)

was changed every 3 days until the adherent cells were expanded to 80% and then digested with 0.25% trypsin.

Cell transfection

Small interfering RNAs have been widely used for knocking down lncRNAs [26–28]. The small interfering RNA of HCG18 (5'-TTGGCTTCAGTCCTGTTCATCAG-3'), overexpression plasmids of HCG18, hsa-miR-146a-5p inhibitor (sequence: 5'- ACCCAUGGAAUUCAGUCUCA-3') and mimics (sequences: 5'- UGGAACUGAAUUCCAUGGUU-3', 5'- CCCAUGGAAUUCAGUCUCUU-3') were constructed by Hanbio Biotechnology (Shanghai, China). In summary, the NPCs were seeded on 12 well or 96 well plates with serum-free culture medium. When the cell density reached 50%, the above nucleic acid molecules were transfected into NPCs using liposome 2000, and each experiment was repeated three times.

CCK-8 assays

The NPCs of the ST group and control group were seeded on 96-well plates (100 μ L/well) and then placed in the incubator for pre-culture. After 24 h of transfection, a volume of 10μ L CCK-8 solution was added to each well, and incubated the plate in the incubator for 2 h. The supernatant was transferred to a new 96-well plate and the absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader.

Wound healing assays

The NPCs were seeded on 12-well plates. After the cells adhered to the wall, the 20 μ L pipette tips were used to draw a horizontal line evenly with a width of about 500 μ m. Then the cells were placed in a 37 ℃, 2% CO_2 cell incubator. After 24 and 48 h of incubation, the displacement of cells at the scratch site was recorded using an optical microscope (TS2-S-SM, Nikon, Japan). The cell migration ability was calculated based on cell migration rate.

Nuclear and cytoplasmic separation

The NPCs were seeded on 12-well plates. After the density reached 80%, the NPCs were collected. A total of 1 ml Lysis buffer was added to each well. The cytoplasmic and nuclear components were separated and collected according to the official guidelines.

Fluorescence in situ hybridization (FISH) assay

The intracellular localization of HCG18 in NPCs was determined by fluorescence in situ hybridization (FISH) assay. First, the CY3-labeled probes for HCG18 (sequence:5′-CY3-TCCCACCACACATCTTGCTGCTCCCTAAC-3′) were designed and synthesized by Geneph-Pharma (Shanghai, China). The NPCs were seeded on 24-well plates. The NPCs were fixed with 4% polyformaldehyde when the cell density reached 80%. The intracellular localization of HCG18 was detected using the RiboTM fluorescent in situ hybridization kit (Ribo

Biotechnology, Guangzhou, China), according to the official guidelines. The nuclear was stained by DAPI under ultraviolet excitation. U6 and 18 S are used as internal references for the nucleus and cytoplasm, respectively.

Apoptosis

The NPCs were seeded on 12-well plates. The cell culture medium was removed and the cells were washed three times with PBS. Adding 195 μl of Annexin V-FITC binding buffer and 5 μl of Annexin V-FITC into each well. Then, 10 μl of propidium iodide staining solution was added to each well. The cells were incubated in an incubator for 15 min, and then immediately observed under a fluorescence microscope. Green fluorescence indicates Annexin V-FITC positive cells, while red fluorescence indicates propidium iodide positive cells. NPCs stained only with green fluorescence present as apoptotic cells, those stained with both green and red fluorescence present as necrotic cells, while those not stained with any fluorescence present as normal cells.

Quantitative reverse transcription polymerase reaction (qRT-PCR)

The total RNAs were extracted by TRIZOL method and then reverse transcribed into cDNA by reverse transcription reagent. Then cDNA was amplified by fluorescence quantitative PCR using the StepOnePlus quantitative PCR system (Applied Biosystems, US). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are used as internal reference genes for miRNAs and mRNAs/lncRNAs, respectively. All the primers (Table 2) used in this study were synthesized by GenephPharma (Shanghai, China). The relative expression of miRNAs, mRNAs, and lncRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blots

The protein extraction kit was used to extract the tissues or cells. The proteins were separated on SDS-PAGE gel and then transferred to the PVDF membrane. Next, the PVDF membrane was mixed with GAPDH (Abcam, US; ab9485; 1:5000) and phosphor-SMAD2 (Abcam, US; ab280888; 1: 1000), phosphor-SMAD3 (Abcam, US; ab52903; 1: 1000), SMAD4 (Abcam, US; ab40759; 1:1000), SMAD7 (Abcam, US; ab216428; 1:1000). After incubating the antibody at 4 ° C overnight, the PVDF membrane was incubated with the corresponding second antibody (A32731) at 25 ° C for 1 h. Finally, the ECL colorimetric solution was added to the PVDF membrane to display protein bands.

Luciferase reporter gene experiment

The 293T cells were seeded into 96 well plates. The firefly luciferase reporter gene plasmid with wild-type and mutant (hsa-miR-146a-5p binding site mutation) HCG18 and SMAD4 sequences were constructed by Hanbio Biotechnology and then transfected into into 293T cells with or without hsa-miR-146a-5p mimics. In addition, an equal amount of sea cucumber luciferase reporter gene plasmids was added to each group as a reference. After 48 h of transfection, two fluorescence activities were detected using an enzyme-linked immunosorbent assay (ELISA) reader, and the relative fluorescence activity was further calculated.

Animal modeling

The *Mycobacterium tuberculosis* H37Rv was purchased from the Shanghai Institute of Biological Products. We conducted power calculations to determine the appropriate sample size for our study. The sample size was calculated using G*Power software. The results showed the minimum sample was 4.3. Thus, we determined that 6 samples per group would be required to detect a statistically significant difference between the experimental and control groups. A total of 18 eight-week-old female SD rats were purchased, of which 12 rats were injected with 0.1 ml of H37Rv standard strain suspension at a concentration of 1×10^7 CFU/ml into the spine, and 6 rats were injected with 0.1 ml of physiological saline [29]. After 3 days of modeling, rats were divided into three groups: spinal tuberculosis group injected with physiological saline, spinal tuberculosis injection group with si-HCG18 loaded transfection reagent, and blank control group injected with physiological saline. After 12 weeks of feeding, spinal specimens were taken for hematoxylin-eosin (H&E) and Safranin O staining. The expression levels of HCG18, hsa-miR-146a-5p, and SMADs pathways were detected by qPCR or WB.

Statistical analysis

The data is described as mean ± standard deviation and analyzed using GraphPad Prism 7.5 (GraphPad Software, USA). Using a student t-test to compare the differences

Table 2 The primers used in this study

	Forward Primer	Reverse Primer
HCG-18	GCTAGGTCCTCTACTTTCTG	CAGAAAGTAGAGGACCTAGC
miR-146a	AGCCATCTTCACAGCAGGTT	CAAGGGAAGGCCATGTCTAT
SMAD4	CTCATGTGATCTATGCCCGTC	AGGTGATACAACTCGTTCGTAGT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCATC

between the two groups, $P < 0.05$ indicates statistical significance. Pearson correlation was used to analyze the correlation between molecular expressions.

Results

Changes in proliferation and migration ability of NPCs infected with tuberculosis

We first detected the migration capability of NPCs from patients with ST or fracture. The results showed that the migration rates of NPCs in ST patients significantly decreased, compared to those in the control group (Fig. 1B, C). In addition, CCK-8 experiments showed that the proliferation activity of NPCs in ST patients was significantly declined, compared to the control group (Fig. 1D). The above results suggest that tuberculosis infection can significantly reduce the migration and proliferation activity of NPCs.

HCG18 expression level significantly increases in spinal tuberculosis patients

We detected HCG18 expression in the nucleus pulposus tissue of ST patients using qPCR. The results showed that the expression of HCG18 in the nucleus pulposus tissue of ST patients was significantly higher than that in the control group, suggesting that the expression of HCG18 was positively correlated with the progress of ST and could be used as an important molecular marker (Fig. 2A). The nuclear-cytoplasmic separation experiments showed that the expression level of HCG18 in the cytoplasm was significantly higher than that in the nucleus (Fig. 2B), which was further confirmed by FISH experiments (Fig. 2C).

We also validated the effects of overexpression or knockdown of HCG18 on the proliferation, apoptosis, and migration of ST NPCs. The results showed that compared with the control group, overexpression of HCG18 significantly inhibited the proliferation and migration activity of NPCs and reduced their apoptosis activity, while knockdown of HCG18 significantly enhanced the proliferation and migration activity of nucleus pulposus cells and promoted their apoptosis activity (Fig. 2D-I). The above results suggest that HCG18 is a key factor regulating the proliferation and migration activity of nucleus pulposus cells.

HCG18 can bind to and inhibit hsa-miR-146a-5p

Previous studies suggest that hsa-miR-146a-5p is a key downstream target of HCG18 [30, 31]. In this study, we also predicted that hsa-miR-146a-5p is a downstream target of HCG18 (Fig. 3A). The results of the luciferase reporter gene experiment showed that the activity of luciferase was significantly reduced when hsa-miR-146a-5p mimics were co-transfected with a luciferase reporter gene plasmid containing HCG18 in 293T cells (Fig. 3B, C). We next detected the expression of hsa-miR-146a-5p in the nucleus pulposus tissue of ST patients. Overexpression of HCG18 could significantly decrease the expression of hsa-miR-146a-5p, but knocking down HCG18 could significantly increase the expression of hsa-miR-146a-5p (Fig. 3D). We also validated that the expression of hsa-miR-146a-5p in the nucleus pulposus tissue of ST patients with spinal tuberculosis was significantly reduced than that of the control group, and was significantly negatively correlated with the trend of HCG18 (Fig. 3E, F).

The expression of SMAD4 is significantly correlated with hsa-miR-146a-5p expression

Previous studies have suggested that hsa-miR-146a-5p can bind to the SMAD4, thereby inhibiting its expression [32, 33]. In our study, we also predicted that SMAD4 is a downstream target of hsa-miR-146a-5p (Fig. 4A). We constructed luciferase reporter gene plasmids containing wild-type or mutant sequences of the SMAD4 3' - UTR end and co-transfected it with hsa-miR-146a-5p mimics into 293T cells. The results showed that compared with the control group, the fluorescence activity was significantly reduced when co-transfected with the wild-type luciferase reporter gene plasmid of the SMAD4 3 '- UTR sequences and hsa-miR-146a-5p mimics, while the wild-type luciferase reporter gene plasmid of the SMAD4 3' - UTR sequences and the mimics control group, as well as the mutant luciferase reporter gene plasmid of the SMAD4 3 '- UTR sequences and hsa-miR-146a-5p mimics or mimics, showed no significant changes in fluorescence activity (Fig. 4B, C). These results indicate that hsa-miR-146a-5p mimics can target and recognize the SMAD4 3' - UTR sequences.

We further detected the expression of SMAD4 in the nucleus pulposus of patients in the ST group and control group. The qPCR results showed that the expression of SMAD4 in the nucleus pulposus of ST patients significantly increased (Fig. 4D), which was significantly negatively correlated with hsa-miR-146a-5p but positively correlated with HCG18 (Fig. 4E, F).

HCG18 promotes proliferation and differentiation of ST NPCs by regulating the hsa-miR-146a-5p/SMAD/TGF-β1 pathway

To further clarify the regulatory effect of HCG18 on the SMADs pathway in ST NPCs, we overexpressed or knocked down HCG18 in ST NPCs via in vitro experiments, and detected the expression changes of the hsa-miR-146a-5p/SMADs/TGF-β1 pathway. The results showed that overexpression of HCG18 can significantly inhibit the expression of hsa-miR-146a-5p (Fig. 5A), leading to increased mRNA expression of SMAD4 (Fig. 5B) and protein expression levels of phospho-SMAD2,

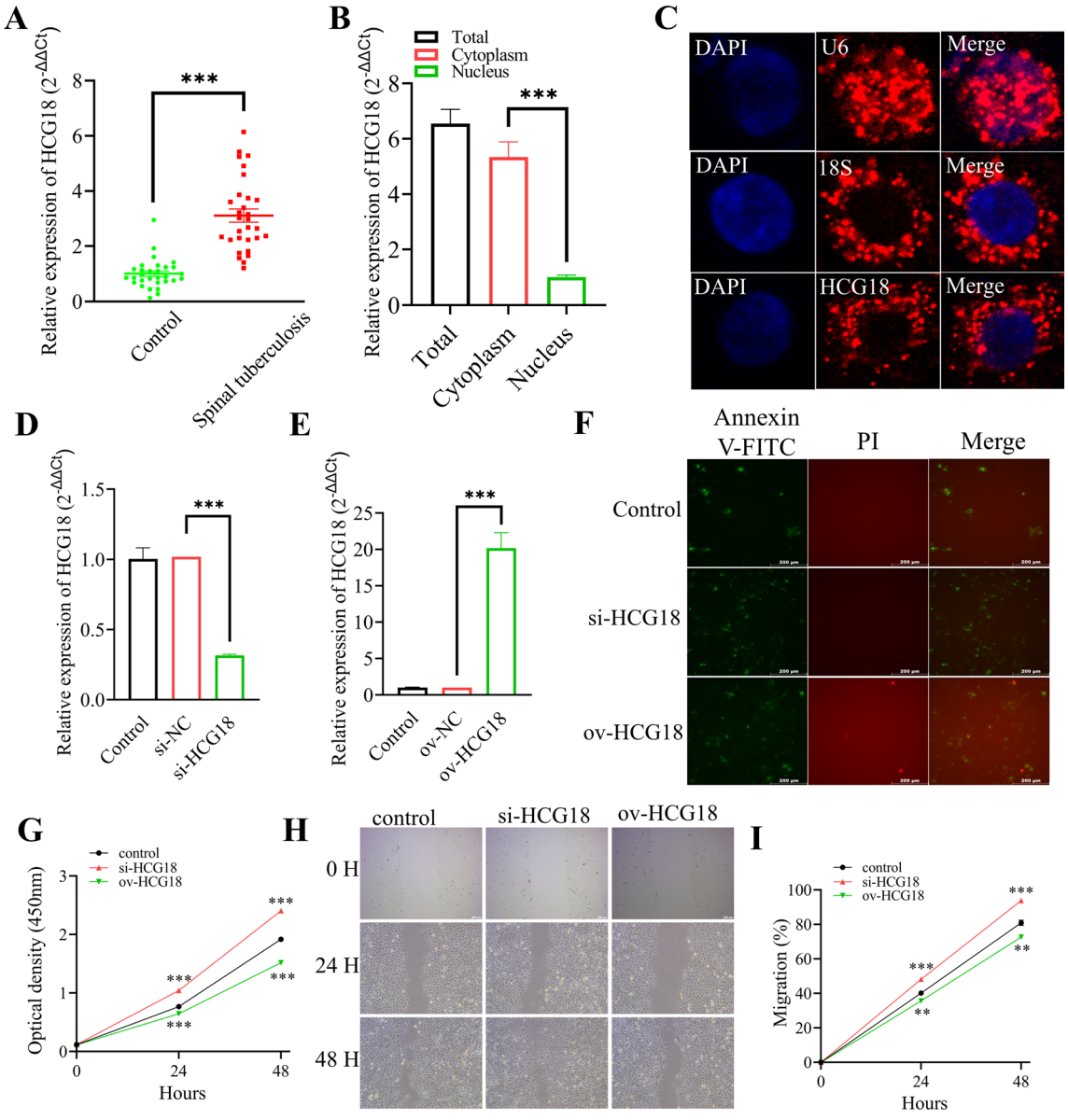

Fig. 2 HCG18 inhibits the proliferation and migration ability but promotes apoptosis of NPCs. (**A**) The expression level of HCG18 significantly increased in clinical nucleus pulposus samples of ST patients ($n = 30$, *** $p < 0.001$). (**B**) The nuclear cytoplasmic separation assays indicated that the expression level of HCG18 in the cytoplasm is significantly higher than that in the nucleus ($n = 3$, *** $p < 0.001$). (**C**) FISH assays indicated that HCG18 is mainly distributed in the cytoplasm of NPCs ($n = 3$). (**D**, **E**) Identification of HCG18 inhibitors and overexpression plasmids ($n = 3$, *** $p < 0.001$). (**F**) Knocking down HCG18 can significantly inhibit apoptosis of NPCs, while overexpression of HCG18 can significantly promote apoptosis of NPCs ($n = 3$). (**G**) Knocking down HCG18 significantly promoted the migration capability of NPCs, while overexpression of HCG18 significantly inhibited the migration capability of NPCs ($n = 3$, *** $p < 0.001$). (**I**) Knocking down HCG18 can significantly promote the proliferation capability of NPCs, while overexpression of HCG18 can significantly inhibit the proliferation capability of n NPCs ($n = 3$, ** $p < 0.01$, *** $p < 0.001$)

phospho-SMAD3, SMAD4, and TGF-β1, and decreased protein expression levels of SMAD7 (Fig. 5C). However, the above results can be remedied by co-transfection of hsa-miR-146a-5p mimics (Fig. 5A-C). Further functional experiments indicated that overexpression of HCG18 can significantly inhibit proliferation and migration, but promote apoptosis of NPCs, an effect which can be reversed by overexpressing hsa-miR-146a-5p (Fig. 5D-G).

On the contrary, knocking down HCG18 can significantly promote the expression of hsa-miR-146a-5p (Fig. 6A), leading to decreased mRNA expression of SMAD4 (Fig. 5B) and protein expression levels of phospho-SMAD2, phospho-SMAD3, SMAD4, TGF-β1, and increased protein expression levels of SMAD7 (Fig. 6C). However, the above results can be remedied by co-transfection of hsa-miR-146a-5p inhibitor (Fig. 6A-C). Further functional experiments indicated that knocking

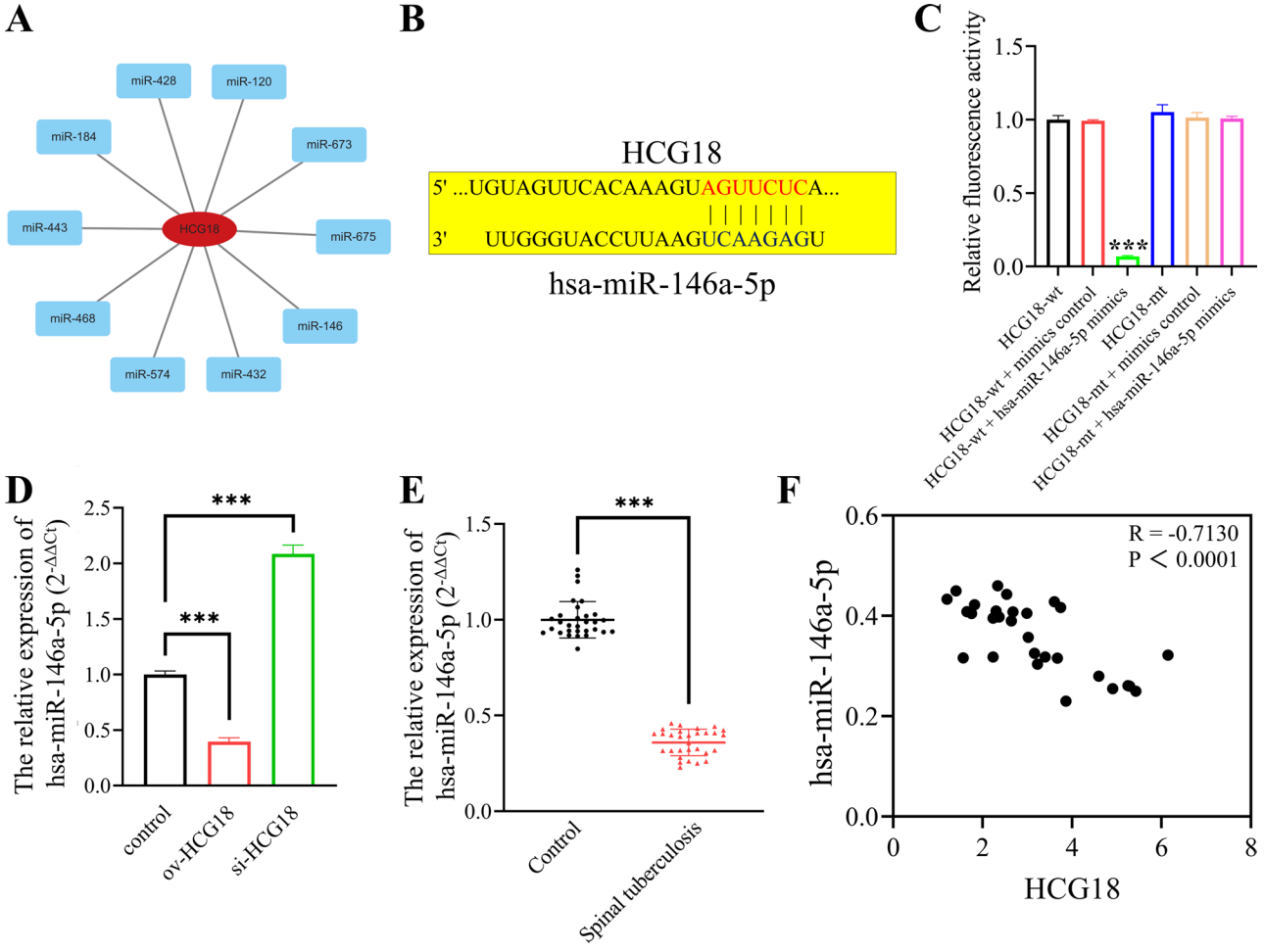

Fig. 3 hsa-miR-146a-5p is a potential downstream target of HCG18. (**A**) Venn diagram displays the potential downstream targets of HCG18. (**B**) The binding sites between HCG18 and hsa-miR-146a-5p. (**C**) The luciferase reporter gene assays showed that the luciferase activity of 293T cells was significantly reduced when the luciferase reporter gene plasmid containing the wild-type HCG18 sequence was co-transferred with hsa-miR-146a-5p ($n=3$, *** $p<0.001$). (**D**) Knocking down HCG18 significantly promoted hsa-miR-146a-5p expression, while overexpression of HCG18 significantly inhibited hsa-miR-146a-5p expression ($n=3$, *** $p<0.001$). (**E**) The expression level of hsa-miR-146a-5p was significantly reduced in clinical nucleus pulposus samples of spinal tuberculosis ($n=30$, *** $p<0.001$). (**F**) The expression level of hsa-miR-146a-5p was negatively correlated with HCG18 in spinal tuberculosis nucleus pulposus tissue ($n=30$, $p<0.0001$, $R=-0.7130$)

down HCG18 can significantly promote the proliferation and migration, but inhibit the apoptosis of NPCs, an effect which can be reversed by knocking down hsa-miR-146a-5p (Fig. 6D-G).

Knocking down HCG18 in vivo can significantly alleviate nucleus pulposus destruction in rats with spinal tuberculosis

We further performed in vivo experiments to validate the mechanism of HCG18 regulating the progression of ST. As expected, qPCR showed a significant decrease in HCG18 and SMAD4, but a significant increase in hsa-miR-146a-5p expression in the nucleus pulposus tissue of ST rats treated with si-HCG18 (Fig. 7A). The results of WB experiments indicate a decrease in protein expression of phospho-SMAD2, phospho-SMAD3, SMAD4, and TGF-β1, as well as an increase in SMAD7 expression level after si-HCG18 treatment (Fig. 7B). The HE and Safranin O staining results suggest that compared with the control group, nucleus pulposus destruction is significant in rats with ST (Fig. 7C). However, injection of si-HCG18 significantly alleviated bone destruction in ST rats (Fig. 7C). The above results further suggest that knocking down HCG18 in vivo can significantly alleviate nucleus pulposus destruction in rats with ST by regulating the hsa-miR-146a-5p /SMADs/TGF-β1 pathway (Fig. 8).

Discussion

The spine is the most susceptible area for tuberculosis of the entire body, with vertebral tuberculosis accounting for the majority [4]. Tuberculosis infection can cause damage to the nucleus pulposus of intervertebral

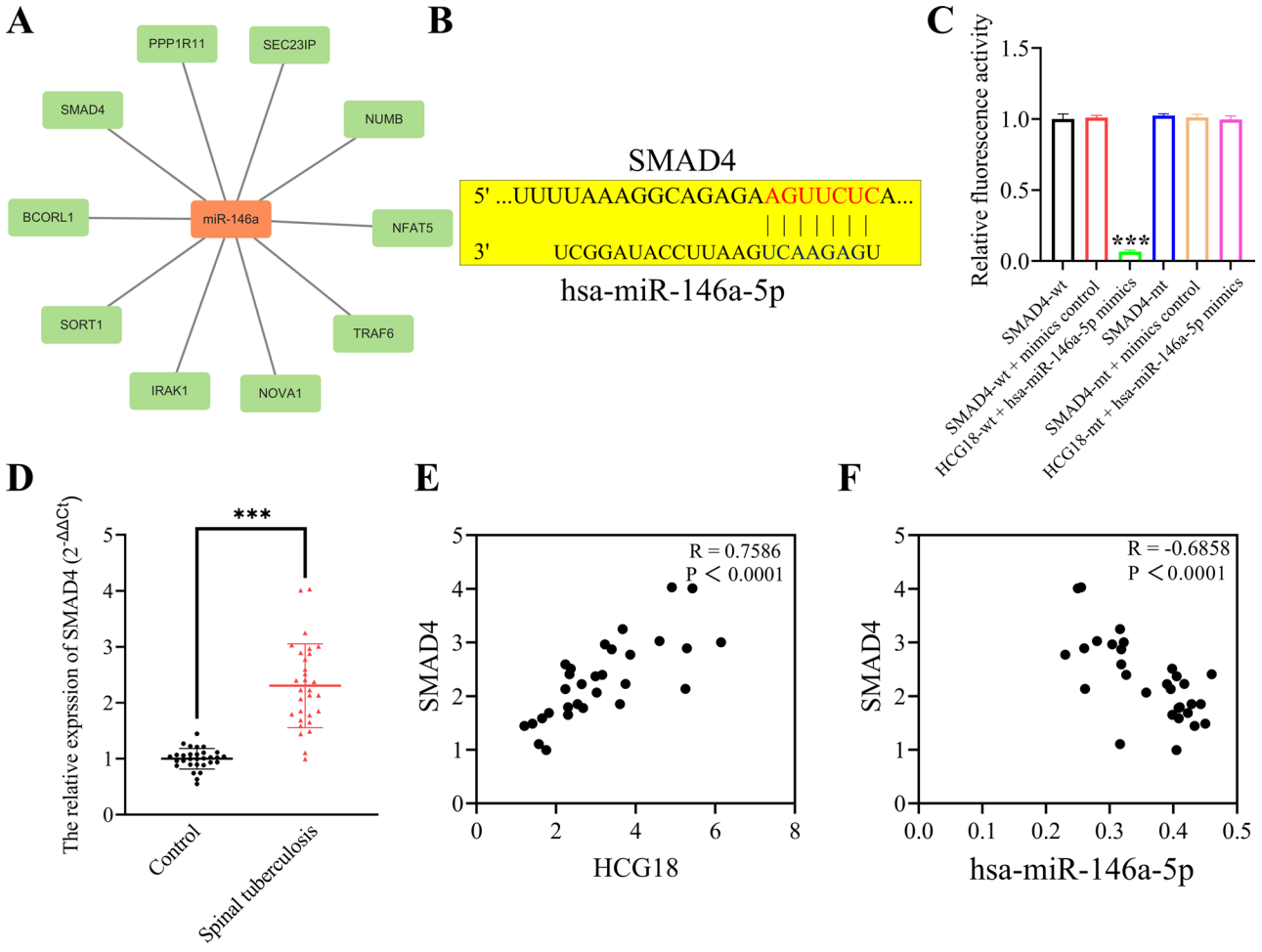

Fig. 4 SMAD4 is a potential downstream target of hsa-miR-146a-5p. (**A**) Venn diagram displays potential downstream targets of hsa-miR-146a-5p. (**B**) The binding sites between SMAD4 and hsa-miR-146a-5p. (**C**) The luciferase reporter gene assays showed that the luciferase activity of 293T cells was significantly reduced when the luciferase reporter gene plasmid containing the wild-type SMAD4 3′-UTR sequence was co-transferred with hsa-miR-146a-5p ($n = 3$, *** $p < 0.001$). (**D**) The expression level of SMAD4 significantly increased in clinical nucleus pulposus samples of spinal tuberculosis ($n = 30$, *** $p < 0.001$). (**E**) The expression level of SMAD4 in spinal tuberculosis nucleus pulposus tissue is significantly positively correlated with the expression level of HCG18 ($n = 30$, $p < 0.0001$, $R = 0.7586$). (**F**) The expression level of SMAD4 in spinal tuberculosis nucleus pulposus tissue is significantly negatively correlated with the expression level of hsa-miR-146a-5p ($n = 30$, $p < 0.0001$, $R = -0.6858$)

discs, which is an important factor in causing intervertebral disc damage [8]. LncRNA has been confirmed to play an important regulatory role in tuberculosis infection and progression [34, 35]. Recent studies suggest that HCG18 is involved in the regulation of intervertebral disc degeneration [23]. Luo et al. found that the expression HCG18 was significantly up-regulated in intervertebral disc degeneration thereby promoting the apoptosis and inflammation of nucleus pulposus (NP) cells [36]. Cao et al. found that high levels of HCG18 result in extracellular matrix degradation therapy promoting the progress of intervertebral disc degeneration [37]. Unlike the degenerative diseases of intervertebral discs mentioned above, the degeneration caused by tuberculosis is mainly due to changes in the activity of intervertebral disc cells, such as proliferation, migration, and apoptosis, caused by tuberculosis infection of nucleus pulposus cells. However, the regulatory role and mechanism of HCG18 in intervertebral disc degeneration and necrosis caused by tuberculosis infection are still unclear. In our study, we found for the first time that HCG18 was significantly upregulated in ST nucleus pulposus tissue, suggesting a close relationship with tuberculosis-induced nucleus pulposus degeneration.

Xi et al. found that HCG18 could promote intervertebral disc degeneration by suppressing hsa-miR-146a-5p [24]. hsa-miR-146a-5p is extremely powerful and is the first miRNA with immune system regulatory effects [38, 39]. Its main function is to negatively regulate immune inflammatory responses, including various immune diseases such as rheumatoid arthritis and lupus erythematosus [40]. In our study, we found that the expression level

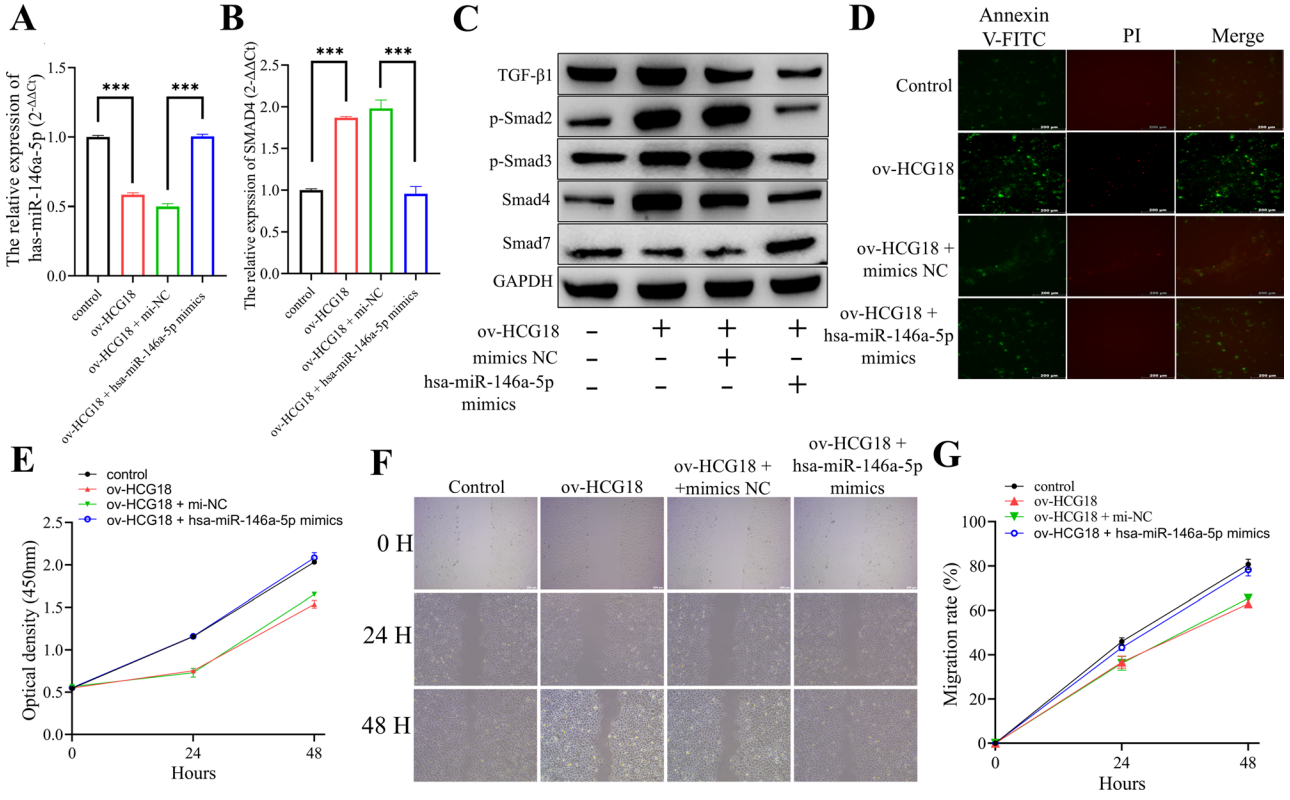

Fig. 5 Overexpression of HCG18 can activate the TGF-β1/SMADs pathway activity by inhibiting hsa-miR-146a-5p expression. (**A**) The expression level of hsa-miR-146a-5p significantly decreased after transfection with HCG18 overexpression plasmid, but could be remedied by co-transfection with hsa-miR-146a-5p mimics ($n=3$, *** $p<0.001$). (**B**) The expression level of SMAD4 mRNA significantly increased after transfection with HCG18 overexpression plasmid, but could be remedied by co-transfection with hsa-miR-146a-5p mimics ($n=3$, *** $p<0.001$). (**C**) Overexpression of HCG18 can significantly increase the protein expression levels of phospho-SMAD2, phospho-SMAD3, SMAD4, and TGF-β1, and decrease the expression levels of SMAD7, an effect which can be remedied by co-transfection of hsa-miR-146a-5p mimics ($n=3$). (**D**) Overexpression of HCG18 significantly increase apoptosis of NPCs, an effect which can be remedied by co-transfection of hsa-miR-146a-5p mimics ($n=3$). Wound healing (**E**, **F**) and CCK-8 (**G**) assays suggest that overexpression of HCG18 can significantly increase the migration and proliferation of NPCs, an effect which can be remedied by co-transfection of hsa-miR-146a-5p mimics ($n=3$, *** $p<0.001$)

of hsa-miR-146a-5p was significantly reduced in ST, suggesting that it may be associated with the progression of ST and negatively correlated with HCG18 expression.

A study has found that all-trans-retinoic acid can induce hsa-miR-146a-5p to regulate the proliferation of acute myeloid leukemia cells by targeting SMAD4 [41]. Liu et al. found that hsa-miR-146a-5p regulates human epithelial fibroblast differentiation by targeting SMAD4 and subsequently modulating TGF-β1 [42]. All the above literature reveals that SMAD4 is a downstream target of hsa-miR-146a-5p. The TGF-β1/SMADs signaling is a key pathway for the occurrence and development of intervertebral disc destruction in ST [42]. Studies have revealed that high-level TGF-β1 can activate SMAD2 and SMAD3 proteins by binding to specific receptors and entering the nucleus, thereby promoting nucleus pulposus degeneration by regulating the metabolism of nucleus pulposus cells [43]. SMAD4 plays a central role in the TGF-β1/SMADs signaling pathway and is a key mediator in promoting the nuclear entry of SMAD2/3 protein, while SMAD7 exerts inhibitory effects in this process [44, 45]. Additionally, current evidence suggested that SMAD4 was closely related to intervertebral disc degeneration [46], with significantly higher expression in intervertebral disc degeneration tissues than in normal intervertebral disc tissues, and hsa-miR-146a-5p is a key inhibitor of SMAD4. In the present study, we confirmed that the expression level of hsa-miR-146a-5p is significantly negatively correlated with the expression levels of SMAD2, SMAD3, SMAD4, and TGF-β1, while positively correlated with SMAD7, suggesting that hsa-miR-146a-5p has an inhibitory effect on the TGF-β1/SMAD pathway.

Based on the above results, we speculate that HCG18 can regulate ST nucleus pulposus degeneration through the hsa-miR-146a-5p/TGF-β1/SMADs pathway. We first further found through gain/loss of function assays and rescue assays that overexpression of HCG18 significantly promotes TGF-β1/SMADs pathway activity and apoptosis, but inhibits proliferation and migration activity of nucleus pulposus cells, which can be reversed by

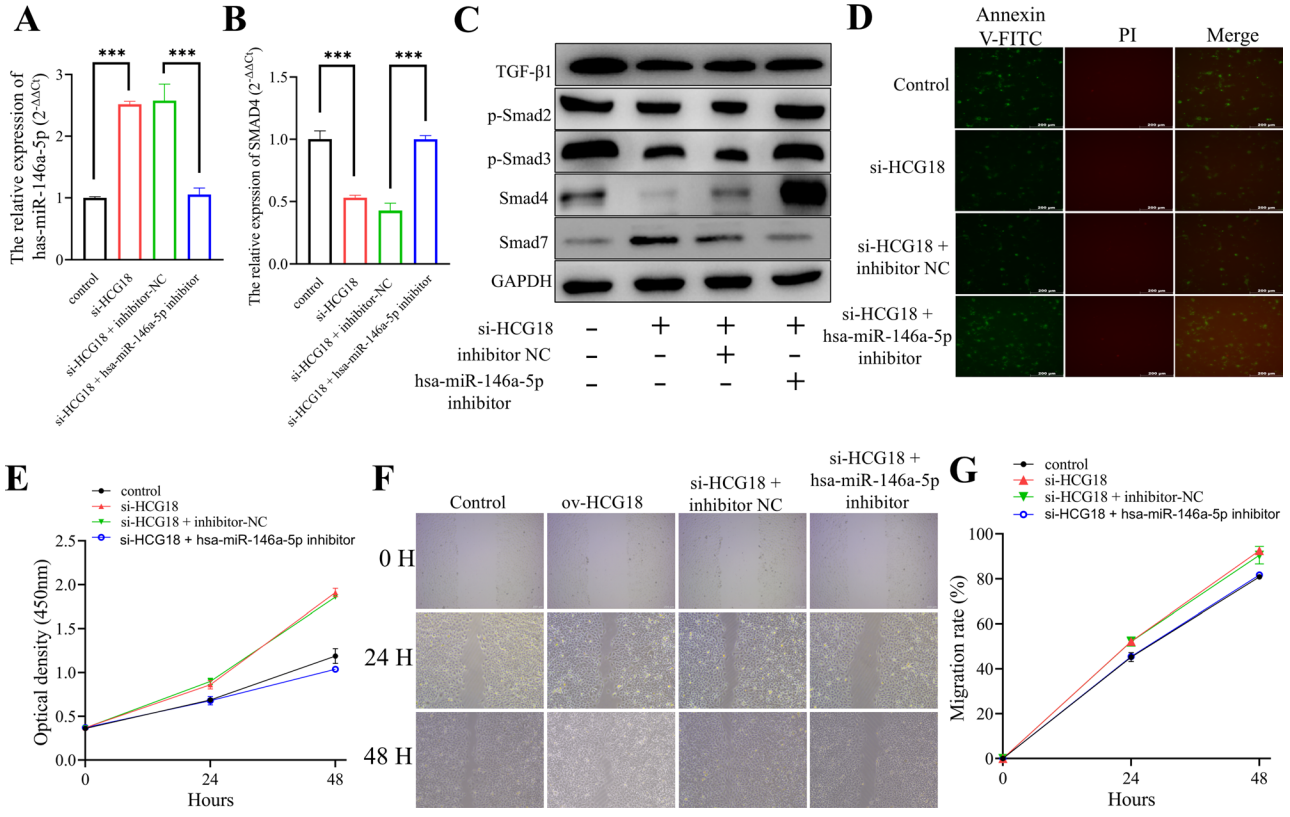

Fig. 6 Inhibition of HCG18 can inhibit TGF-β 1/SMADs pathway activity by promoting hsa-miR-146a-5p expression. (**A**) The expression level of hsa-miR-146a-5p decreased after HCG18 knocking down, but could be reversed by co-transfection with hsa-miR-146a-5p inhibitor ($n=3$, *** $p<0.001$). (**B**) The expression level of SMAD4 mRNA significantly increased after HCG18 knockdown, but can be reversed by co-transfecting hsa-miR-146a-5p inhibitor ($n=3$, *** $p<0.001$). (**C**) Knocking down HCG18 can significantly reduce the protein expression levels of phospho-SMAD2, phospho-SMAD3, SMAD4, and TGF - β 1, and increase expression levels of SMAD7, an effect that can be remedied by co transfection of hsa-miR-146a-5p inhibitor($n=3$). (**D**) Knocking down HCG18 can significantly reduce apoptosis of NPCs, an effect that can be remedied by co transfection of hsa-miR-146a-5p inhibitor ($n=3$). Wound healing (**E**, **F**) and CCK-8 (**G**) assays suggest that knocking down HCG18 can significantly increase migration and proliferation of NPCs, an effect which can be remedied by co-transfection of hsa-miR-146a-5p inhibitor ($n=3$, *** $p<0.001$)

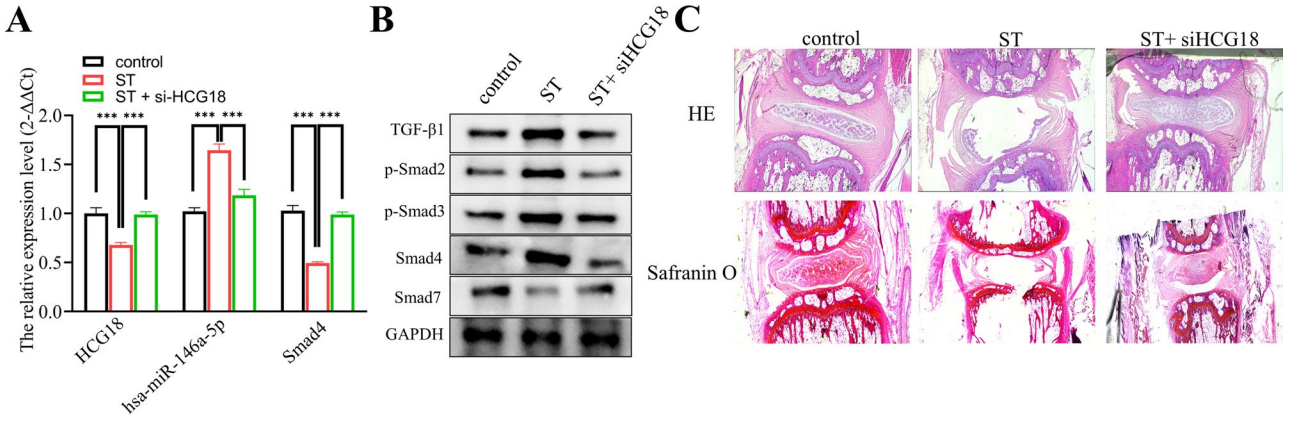

Fig. 7 Knocking down HCG18 in vivo can significantly alleviate nucleus pulposus destruction in rats with spinal tuberculosis. (**A**) Knocking down HCG18 in vivo significant decresed the expression of HCG18 and SMAD4, but increase the expression of hsa-miR-146a-5p expression in the nucleus pulposus tissue of ST rats ($n=6$, *** $p<0.001$). (**B**) Knocking down HCG18 in vivo can significantly decrease the protein expression levels of phospho-SMAD2, phospho-SMAD3, SMAD4, and TGF-β1, and increase expression levels of SMAD7 in nucleus pulposus samples from ST rats ($n=6$). (**C**) The HE and Safranin O staining showed that nucleus pulposus destruction is significant in rats with spinal tuberculosis, but could be improved by injection of si-HCG18 ($n=6$)

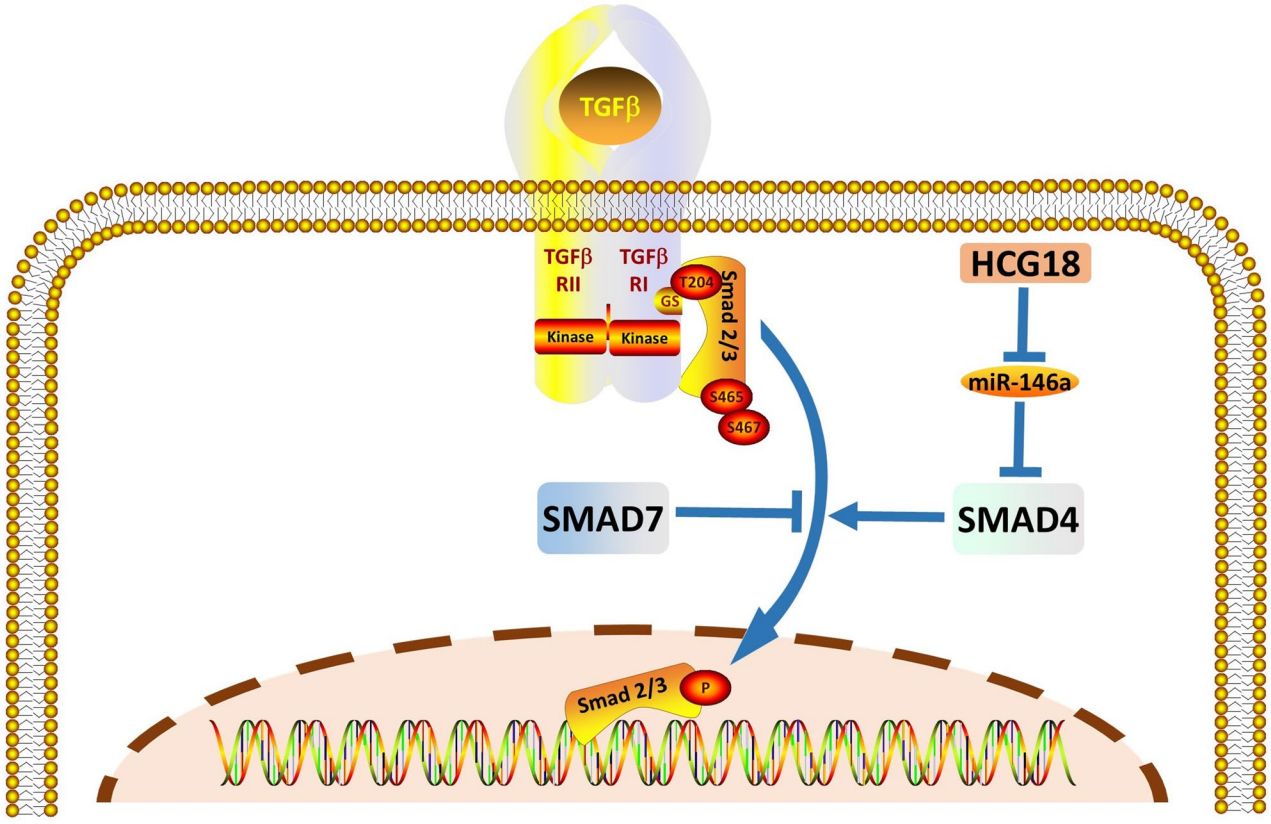

Fig. 8 HCG18 can promote the progression of spinal tuberculosis nucleus pulposus destruction by alleviating the inhibitory effect of hsa-miR-146a-5p on the TGF-β1/SMADs pathway

increasing hsa-miR-146a-5p expression. Knocking down HCG18 yields the opposite result. Further confirmation shows that HCG18 can upregulate the activity of the TGF-β1/SMADs pathway by inhibiting hsa-miR-146a-5p, thereby inhibiting the proliferation and migration ability of nucleus pulposus cells. Meanwhile, we have confirmed that knocking down HCG18 in vivo can significantly inhibit the activity of the TGF-β1/SMAD pathway, thereby alleviating the destruction of the nucleus pulposus in ST rats.

Of course, there are still certain shortcomings in this study. Our study revealed the expression trend of HCG18 in ST tissues based on sample detection and confirmed the regulatory effect of HCG18 on TGF-β1/SMAD pathway activity and proliferation and migration activity of nucleus pulposus cells through in vitro cell experiments. The therapeutic efficacy of HCG18 for ST was carried out using si-HCG18. However, more advanced targeting strategies are needed to improve the targeting and transfection efficiency of HCG18.

In summary, we found that HCG18 is a key regulatory factor in the progression of ST. HCG18 can promote the progression of ST nucleus pulposus destruction by alleviating the inhibitory effect of hsa-miR-146a-5p on the TGF-β1/SMADs pathway. This study provides new insights into the molecular mechanisms underlying the occurrence and development of ST, as well as new ideas for the treatment of ST.

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Author contributions
Feng Li, Xiaopeng Li, Hongdong Tan, and Gaoyang Chen designed this study. Xiao Zhang, Xiaodong Zhao participated in the study conduction.Feng Li, Xiaopeng Li, Hongdong Tan participated in data collection and analysis. Feng Li, Xiaopeng Li, Hongdong Tan, and Gaoyang Chen drafted and revised the manuscript. All authors have approved the publication.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All experiments in the present study were under the ethical standards formulated in the Helsinki Declaration and were approved by the ethics committee of the First Affiliated Hospital of Weifang Medical University. Written informed consent was received from each participant.

Disclosure statement
None.

Competing interests
The authors declare no competing interests.

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