The H19/let-7 feedback loop contributes to developmental dysplasia and dislocation of the hip

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Short title: H19/let-7 Feedback Loop in DDH

Abbreviations: DDH, developmental dysplasia and dislocation of hip;

KD, knockdown;

lncRNA, long non-coding RNA;

miRNA, microRNA;

PBS, phosphate buffer saline;

FBS, fetal bovine serum;

shRNA, short hairpin RNA;

qRT-PCR, quantitative real time polymerase chain reaction;

CCK-8, Cell Counting Kit-8.

Summary

Developmental dysplasia and dislocation of the hip (DDH) is the most common type of lower limb deformity in pediatric orthopedics. The mechanism of the signaling pathway has been studied in depth. However, the role of epigenetic regulation, such as lncRNA, is still far from clear. In this study, we successfully established a rat model of DDH and demonstrated that H19 was down-regulated in the development of DDH. Further, we constructed H19 knockdown (KD) and overexpression chondrocytes. H19 KD suppressed the proliferation of normal chondrocytes, while overexpression of H19 promoted cell proliferation of DDH chondrocytes. Finally, we revealed that H19 bound to let-7 and inhibited its function, acting as a competing endogenous RNA. Down-regulation of H19 is closely associated with DDH progression and H19 is an important epigenetic factor that regulates the proliferation of chondrocytes. H19 may thus be a potential clinical marker for DDH diagnosis and treatment.

Introduction:

Developmental dysplasia and dislocation of the hip is the most common type of lower limb deformity in pediatric orthopedics (Senaran *et al*. 2007). The vast majority of patients can be diagnosed early, and with application of a brace or surgical treatment can be completely cured. However, there are still a few patients who suffer re-dislocation, residual hip dysplasia, residual acetabular dysplasia or osteoarthritis (Carney *et al*. 2005, Thomas *et al*. 2007). Most of these patients have to undergo total hip arthroplasty before the age of 50, causing the patients themselves and their family great psychological and economic burden (Ashraf *et al*. 2014). In-depth analysis of the molecular mechanism underlying development of this disease and study of the proteins or genes that play key roles in this process should help with screening and identification of these patients.

Hip cartilage is mainly composed of chondrocytes and their extracellular matrix. Chondrocytes differentiate from mesenchymal stem cells. Extracellular matrix, which is secreted by chondrocytes, mainly consists of type II collagen and polysaccharide. These components together fight against joint stress and maintain normal joint shape and function (Bo *et al*. 2012). It has been shown that dysplasia of cells and changes in extracellular matrix components occur in the articular cartilage in DDH. These changes are closely related to cell proliferation, apoptosis and metabolism in chondrocytes (Zhang *et al*. 2015, Wei *et al*. 2016). The Wnt/β-catenin pathway plays a critical role in the development of osteoarthritis (Krase *et al*. 2014). Previous studies also show that overexpression of β-catenin induces chondrocyte dedifferentiation, while significantly inhibiting chondrocyte proliferation and promoting apoptosis (Pei *et al*. 2014). Although the mechanism of DDH and the signaling pathways involved has been studied in depth, research into the epigenetic regulation, such as lncRNA, is still lacking.

Long non-coding RNAs (lncRNAs), which act as epigenetic regulators, are widely involved in the regulation of gene expression at the levels of chromosome modification, transcription, post-transcriptional processing and translation (Li *et al*. 2014). Normal expression of lncRNA is necessary for normal cell function and individual development, while its abnormal expression is often associated with disease (Kazemzadeh *et al*. 2015). For example, tissue-specific expression of lncRNA PCAT-1, which regulates expression of a series of tumor-related genes such as CENPF, BRCA2 and CENPE, plays a key role in the oncogenesis of prostate cancer (Prensner *et al*. 2014). Recently, there have been increasing reports of the effects of lncRNA in osteoblast and chondrocyte proliferation and differentiation. For example, lncRNA

UFC1 has been shown to promote the proliferation of chondrocytes by inhibiting the expression of miR-34a (Zhang *et al*. 2016). High expression of lncRNA HOTAIR promotes the expression of MMP family proteins such as MMP2 / MMP7 / MMP9 and further induces chondrocyte apoptosis (Zhang *et al*. 2016). Although much research on lncRNA and chondrocytes has been reported, the relationship between lncRNA and DDH has not been studied in sufficient detail. Due to the difficulty of obtaining clinical cartilage, we therefore studied DDH using an animal model. In this study, we successfully constructed a rat DDH model and used this to analyze lncRNA expression. We found that H19 is down-regulated in our DDH model and we used this to explore the function and mechanism of H19 in DDH.

Methods:

Rat model of developmental dysplasia of the hip and hip joint collection

All the animals used in experiments were maintained according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and protocols were approved by the Institutional Ethics Committee of the Children's Hospital of Fudan University (Shanghai, China). Twenty pregnant Wistar rats (8 weeks, 200–250 g) were purchased from Shanghai Laboratory Animal Co., Ltd., Shanghai, China. Forty male neonates and 1-day old pups were used in this study and were randomly divided into two groups. The DDH model was created by fixation of the hind limbs in an extension and adduction position with medical tape from day 1 to day 12, and normal rats of the same age constituted the control group. During the period of fixation, the model rats were fed by their mothers along with the control group. At different days, sacrifice was performed by means of overdose of general anesthetic. Total hips were excised immediately for subsequent procedures.

Safranin O staining

For histochemical staining, the isolated hips were fixed in 4% paraformaldehyde for 24 h. The hips were then decalcified in 10% ethylene diamine tetraacetic acid until completely demineralized and embedded in paraffin. After sectioning, the samples were deparaffinized with xylene and the safranin O staining process was performed according to a standard protocol. All the chemical reagents used in this experiment were bought from Sinopharm Chemical Reagent Company, Shanghai, China.

Isolation of primary chondrocytes

Primary rat chondrocytes were isolated from rat hips. The acetabulum cartilage from normal rats was isolated and rinsed twice with PBS. The isolated cartilage pieces were incubated in a 10 cm dish with 10 mL of collagenase solution at 0.5 mg/mL overnight at 37□ under 5% CO_2. Then the suspension of isolated cells was filtered through a sterile cell strainer (BD Falcon™, San Jose, CA, USA) by setting over a 50 mL tube and centrifuging for 5 min. Finally, the rat chondrocytes were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) in a cell incubator at 37°C with 5% CO_2 (Thermo Fisher Scientific, Waltham, NJ, USA).

Immunofluorescence staining

The chondrocytes were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100. The cells were blocked with 10% FBS in PBS and incubated with BrdU antibody overnight at 4□ (1:1000, ab8152, Abcam, Cambridge, MA, USA). Next day the cells were rinsed, then incubated with anti-rabbit secondary antibody (1:1000, ab150116, Abcam) for 1 h at room temperature in the dark. After washing, the cells were stained with DAPI for 5 min and then images were captured using a fluorescence microscope (TE2000, Nikon, Tokyo, Japan).

Plasmids, lentivirus supernatants and infection of chondrocytes

The shH19 (H19 short hairpin RNA (shRNA) knockdown) sequences were as follows: shscramble-F, gatccCAGCGCTGACAACAGTTTCATCTCGAGATGAAACTGTTG TCAGCGCTGTTTTTg; shscramble-R, aattcAAAAACAGCGCTGACAACAGTTT CATATATCTCATGAAACTGTTGTCAGCGCTGg; shH19 -1F, gatccGACTGAGG GGCTAGCTCGGTTCAAGAGACCGAGCTAGCCCCTCAGTCTTTTTg; shH19 -1R, aattcAAAAAGACTGAGGGGCTAGCTCGGTCTCTTGAACCGAGCTAGCC CCTCAGTCg; shH19 -2F, gatccGAGAAGAAGCAGCTGACCTTTCAAGAGAA GGTCAGCTGCTTCTTCTCTTTTTg; shH19 -2R, aattcAAAAAGAGAAGAAGCA GCTGACCTTCTCTTGAAAGGTCAGCTGCTTCTTCTCg. The shH19 sequences were ligated into the PLVX-shRNA2 vector which expressed a classic scrambled shRNA and green fluorescent protein (ZsGreen). The rat H19 sequences were

obtained from NCBI. H19 PF:CCGGAATTCGGGGTGGGGGGTAATGGGGAAAC; H19 PR: CCGTCTAGATTTTTTGACTGTAACTGTATTTATTGGT. The H19 sequences were ligated into the pLVX-IRES-ZsGreen1 vector which expressed the green fluorescent protein (ZsGreen).Thelentivirus was packaged with 293T cells and then infection the chondrocytes. Next, the cells were selected through Flow cytometry using FACS Calibur (BD Biosciences). Then, ZsGreen positive cells expressing ZsGreen protein were observed using fluorescence microscopy (TE2000, Nikon, Japan).

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8) reagent detects the number of living cells and is widely used for cell proliferation assays. For the CCK-8 assay, the chondrocytes were seeded into 96-well plates at a density of 1×10^3 cells/well. After 12, 24, 36, 60 or 72 h, 10 μL of the reagent (Dojindo, Kumamoto, Japan) was added to each well of the 96-well plate and the plate was incubated for 2 h at 37°C . Finally, the absorbance at 450 nm was recorded using a SpectraMax M5.

For cell cycle analysis, cells were fixed in 70% ice-cold ethanol and followed by RNase A treatment and staining with 50 μg/mL of propidium iodide for DNA content analysis in a FACSCalibur BD flow cytometer. The data were collected using the BD FACSuite analysis software. The acquired FACS data were analyzed by ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Western Blotting

Cultured cells were lysed in strong RIPA buffer containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The obtained protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Primary antibodies targeting COL2A (sc-28887, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Caspase-3 (ab90437, Abcam), active Caspase 3 (ab2302, Abcam), or GAPDH (ab70699, Abcam) were incubated with the blots overnight at 4°C , following by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Detection of HRP was performed using the Super Signal West Pico Chemiluminescent Substrate (Pierce).

Reverse Transcription, qRT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. For each sample, 500 ng of RNA was reverse transcribed to cDNA using the Prime-Script RT reagent kit (TaKaRa, Dalian, China). Then the obtained cDNA was amplified with the Takara Ex Taq PCR kit (TaKaRa), and quantitative real time polymerase chain reaction (qRT-PCR) amplification was conducted with the Stratagene Mx3000 QPCR system (Stratagene, La Jolla, CA, USA) and analyzed by the $^{\Delta\Delta}$CT method. The primer sequences used in these assays are as follows:

H19-F:ACGGGGAGCCAGACATTCATC, H19-R:TGAGAAGAAGCAGCTGACCT

C; Gapdh-F:TGGAGTCTACTGGCGTCTT, Gapdh-R:TGGAGTCTACTGGCGTCT.

Statistical Analysis

The error bars represent the SEM of three independent experiments. Data are represented as mean ± SEM; n = 3. *, **, and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (Student's t test).

Results:

LncRNA H19 is down regulated in DDH development.

Safranin O staining showed that cartilage cells in the control group were arranged in an orderly manner and the intercellular matrix was continuous (Fig. 1a). However, cells in the DDH group were disordered and the main cells were spindle-shaped. There were a large number of gaps in the cell matrix in the DDH model group (Fig. 1a). It has been shown that expression of the collagen II component Col2A1 is reduced in the development of DDH (Vonk *et al*. 2014). Herein, we analyzed the expression level of Col2A1 and found that it was significantly down-regulated in DDH (Fig. 1b). We further examined the expression of lncRNA H19 in the DDH model. The results showed that, compared with the control group, the expression of H19 was significantly decreased in DDH group. To investigated the role of H19,.we constructed two shRNAs targeting H19: shH19-1 and shH19-2 (Fig. 1c). Then we packaged the lentivirus and infected normal chondrocytes which were isolated from the control group of rats. Successfully-infected chondrocytes carry the GFP protein

and the infected cells were selected by flow cytometry (Fig. 1d). After confirming successful infection, we further validated the expression of the gene by qRT-PCR. Compared with the control (scrambled shRNA), shH19-1 and shH19-2 both efficiently down-regulated H19 lncRNA (Fig. 1e).

H19 KD suppresses the proliferation of normal chondrocytes.

H19 is closely involved in the lung cancer cell proliferation, therefore we studied its function in chondrocyte proliferation. We used the CCK-8 assay to evaluate cell numbers of the control group and the H19 knockdown (H19 KD) group at 12, 24, 36, 60 and 84 h. The results showed that H19 KD caused a significant decrease in chondrocyte number (Fig. 2a). To confirm the function of H19 in chondrocyte proliferation, we compared BrdU incorporation in the control and H19 KD groups. Compared with the control group, BrdU incorporation in the H19 KD group was significantly reduced, suggesting that H19 KD decreases the proliferation capacity of chondrocytes (Fig. 2b). Consistently, H19 KD in chondrocytes dramatically increased the cell population at G0/G1 phase, whereas it reduced the cell population at G2/M phase (Fig. 2c and 2d). These data suggest that the proliferative ability of H19 KD chondrocytes is significantly inhibited.

H19 affects chondrocyte proliferation but not apoptosis.

Cell apoptosis is closely related to DDH progression. Therefore we examined apoptosis by flow cytometry. We found no significant difference in the number of apoptotic cells between the H19 KD and control chondrocytes (Fig. 3a). Caspase-3, an important terminal cleavage enzyme, is a crucial mediator of apoptosis, and activated Caspase-3 is a key indicator of apoptosis. H19 KD did not alter the protein levels of Caspase-3 or of activated Caspase-3 (Fig. 3b and 3c), indicating that H19 does not affect chondrocyte apoptosis.

H19 overexpression promotes chondrocyte proliferation.

To further confirm the function of H19 in DDH progression, we over-expressed H19 in chondrocytes isolated from DDH model rats. qRT-PCR analysis showed that H19 was successfully over-expressed (Fig. 4a). Overexpression of H19 promoted the proliferation of chondrocytes from the DDH model rats (Fig. 4b). Similarly, BrdU immunostaining revealed that overexpression of H19 enhanced the proportion of

dividing chondrocytes (Fig. 4c). We then performed flow cytometry to further identify the relationship between H19 and cell cycle regulation. The results showed that overexpression of H19 significantly decreased the cell population at G0/G1 phase and increased the cell population at G2/M phase (Fig. 4d and 4e). These data suggest that overexpression of H19 promotes the proliferation of chondrocytes isolated from our DDH model.

H19 regulates proliferation of chondrocytes through direct binding to let-7.

The molecular mechanism of lncRNA includes RNA–protein interactions as well as acting as a competing endogenous RNA that binds to a miRNA and inhibits its function. It is reported that H19 interacts with miRNA let-7 and functions as a let-7 inhibitor (Kallen *et al*. 2013). We next explored the role of let-7 in the effects of H19 in chondrocytes. Interestingly, the level of let-7 was significantly up-regulated in the development of DDH in our model while H19 was down-regulated in this model (Fig. 4a). Similarly, when H19 was overexpressed, let-7 was down-regulated (Fig. 4b). Thus it appears that the function of H19 is associated with let-7 in this disease. It has been reported that let-7 interacts with H19 directly (Kallen *et al*. 2013). Together, these results suggest that H19 down-regulation impairs proliferation of chondrocytes through regulating the expression of let-7.

Discussion:

DDH is one of the most frequent congenital abnormalities in newborns. It is associated with degenerative lesions of cartilage in the joint, but the molecular mechanism of DDH is still far from clear. We found that the formation and development of DDH may be closely related to H19. These results reveal, for the first time, the role of the epigenetic factor lncRNA in DDH, which helps to deepen the understanding of the pathogenesis of DDH as well as providing a potential molecular marker for the diagnosis of DDH. At present, the treatment of DDH is mainly dependent on surgical methods for infants more than 18 months old, such as hip replacement and associated bony osteotomy (Wenger, *et al*. 2014). Although the vast majority of patients can achieve a satisfactory cure, there are a small number of patients who remain disabled, leaving a heavy burden on the patients and their family (Anuar *et al*. 2015). A deeper understanding of the molecular mechanisms, such as lncRNA, will help with the development of targeted drugs, thereby increasing

treatment options for these patients. In addition to lncRNA, epigenetic regulators include DNA methylation, histone acetylation and deacetylation, as well as miRNA and many other regulators (Wee *et al*. 2014). It may be very interesting to investigate the function and mechanism of these epigenetic regulators in DDH.

H19, an imprinted oncofetal gene, has a central role in carcinogenesis. However，its function in DDH has not been explored. Here, we found that H19 was closely related to the formation of DDH in rats. Its expression was significantly down-regulated as the hip condition deteriorated; further we found that the effect of H19 in the DDH process may be associated with its role in regulating cell proliferation. In the process of hip development, the proliferative ability of chondrocytes is very important, so lack of H19 should lead to limitation of the cell proliferation capacity, leading to hip articular cartilage dysfunction and hip dysplasia. In one previous study, it was reported that H19 is linked to diseases such as cancer and cardiovascular disease (Liu *et al*. 2016, Zhou *et al*. 2015). Our findings reveal the role of H19 in DDH. These results suggest that H19 may be useful as an important disease indicator, and further can be used as a target for developing new drugs or treatment in DDH.

At the molecular level, one of the main functions of lncRNAs is its action as a ceRNA, which can bind to miRNAs and inhibit their function (Zhou *et al*. 2016). Previous studies have found that let-7, which is closely related to cell proliferation in human cells, is an important regulatory factor of H19 (Yan *et al*. 2015). We found that let-7 is a downstream target of H19, through which H19 regulates cell proliferation in rat chondrocytes. Further, we confirmed this result by luciferase assay. This study demonstrated that a similar let-7/H19 regulatory feedback loop is present not only in the human but also in the rat. Therefore, this loop is conserved in these two species. For ethical reasons, it is difficult to obtain human chondrocytes, but because of this conserved pathway, we estimate that the let-7/H19 regulatory pathway may also play an important role in the process of human DDH.

In summary, our study has shown that expression of H19 is significantly down-regulated during DDH development and that H19 may be an important epigenetic regulatory factor in DDH formation (Fig. 5c). We also identified let-7 as an H19 target gene. Our study is the first to reveal that H19 is an epigenetic regulator of DDH and the challenge for future studies will be to better understand this regulation in other epigenetic regulators, such as miRNA and chromatin modifiers.

Acknowledgments

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Figure legends:

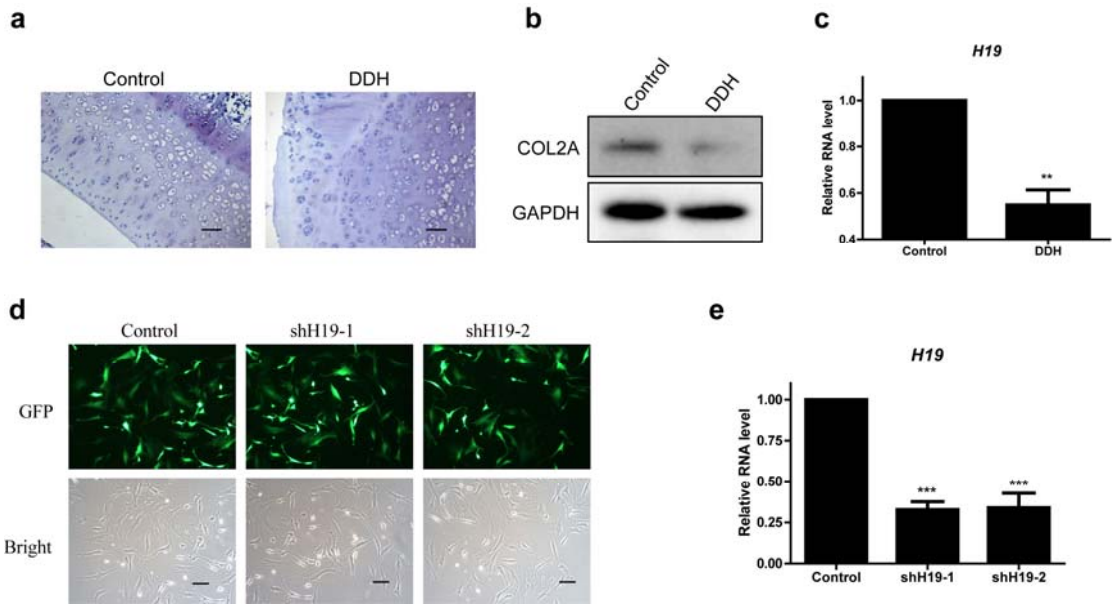


Fig. 1: H19 is down-regulated in a rat DDH model.

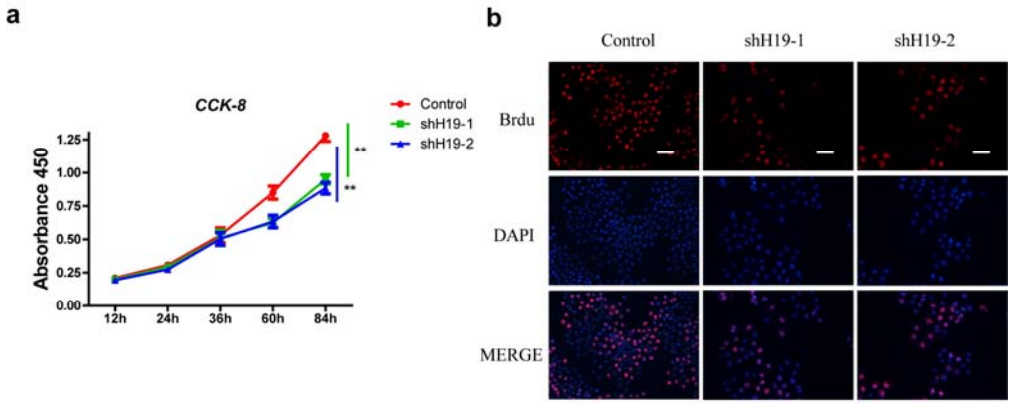
a. Safranin O staining of the Control group and DDH group. Scale bars = 60 μm.

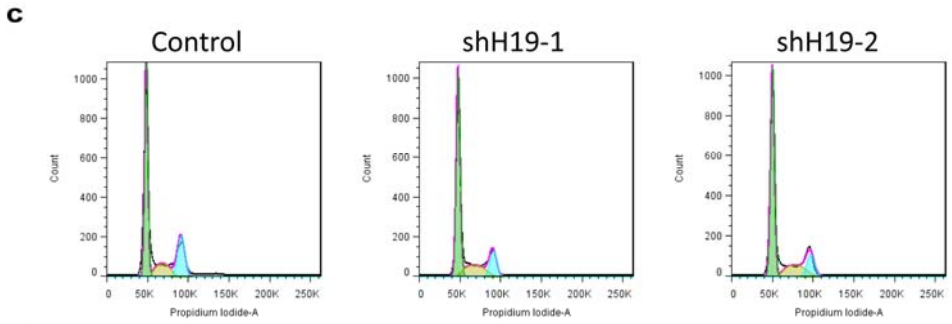
b. Western blot analysis of COL2A in Control and DDH groups.

c. qRT-PCR analysis of the lncRNA H19 in the Control and DDH groups.

d. Morphology of GFP-positive lentiviral-infected chondrocytes. Scale bars = 60 μm.

e. qRT-PCR analysis of the RNA level of H19 in Control and H19 KD chondrocytes. The data are displayed relative to the results of the Control and represented as mean ± SEM; n = 3.





d

	G0/G1phase(%)	Sphase(%)	G2/Mphase(%)
Control	72.06±2.48	7.4±0.78	17.02±1.27
shH19-1	83.23±3.52	7.86±1.02	6.7±1.54
shH19-2	81.51±2.61	7.07±1.37	7.91±1.78

Fig. 2: H19 KD suppresses cell proliferation.

a. CCK-8 assay analysis of the cell proliferation of Control and H19 KD chondrocytes isolated from our rat model.** indicates $P < 0.01$.

b. BrdU immunostaining analysis of the cell proliferation of Control and H19 KD chondrocytes. Scale bars = 60 μm.

c and **d**. Flow cytometric analysis of the cell cycle of Control and H19 KD chondrocytes.

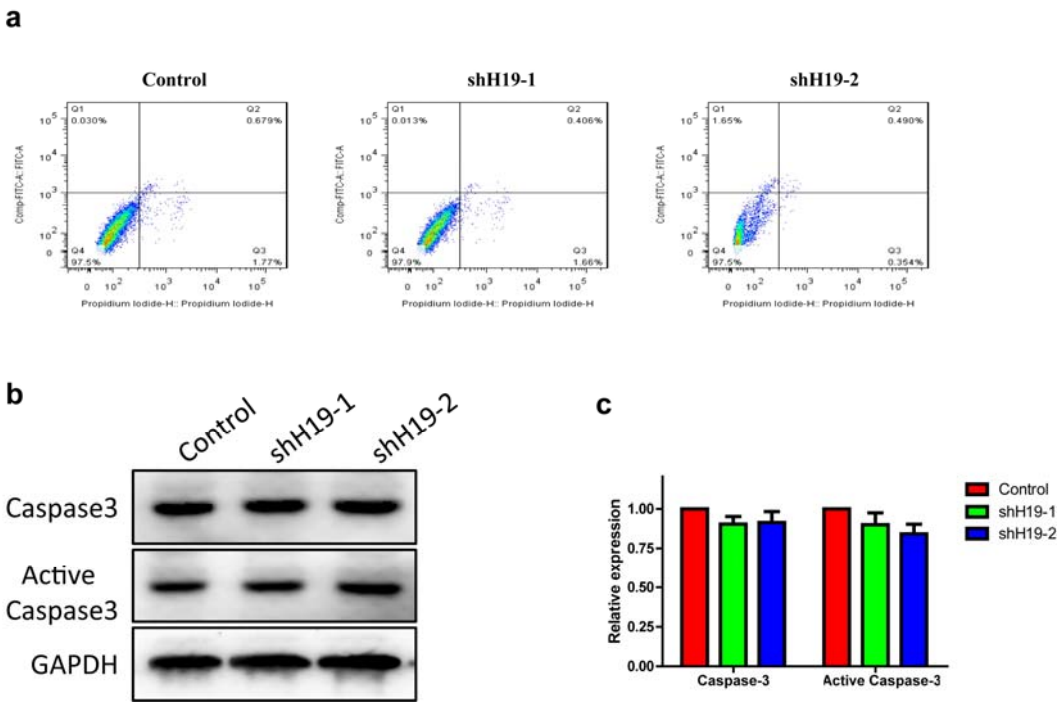
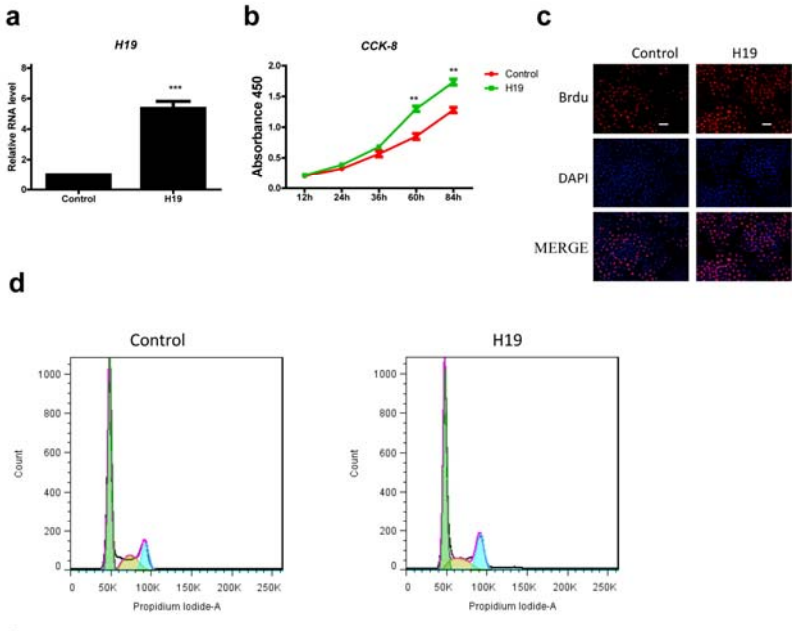


Fig. 3: H19 does not affect apoptosis but does promote cell proliferation of chondrocytes.

a. Flow cytometric analysis of apoptosis of Control and H19 KD chondrocytes.

b and c. Western blotting analysis of apoptosis-related genes in Control and H19 KD chondrocytes. The data are displayed relative to the results of Control.



e

	G0/G1phase(%)	Sphase(%)	G2/Mphase(%)
Control	80.06±2.18	7.0±0.96	9.02±1.27
H19	72.23±2.26	8.86±1.42	16.14±1.84

Fig. 4: H19 overexpression promotes chondrocytes proliferation.

a. qRT-PCR analysis of the RNA levels of H19 in the Control and H19 groups. The data are displayed relative to the results of the Control and are represented as mean ± SEM; n = 3. *** indicates $P < 0.001$.

b. CCK-8 assay analysis of the cell proliferation of Control and H19 chondrocytes isolated from Control rats.** indicates $P < 0.01$.

c. BrdU immunostaining analysis of the cell proliferation of Control and H19 chondrocytes. Scale bars = 60 μm.

d and **e**. Flow cytometric analysis of the cell cycle of Control and H19 chondrocytes.

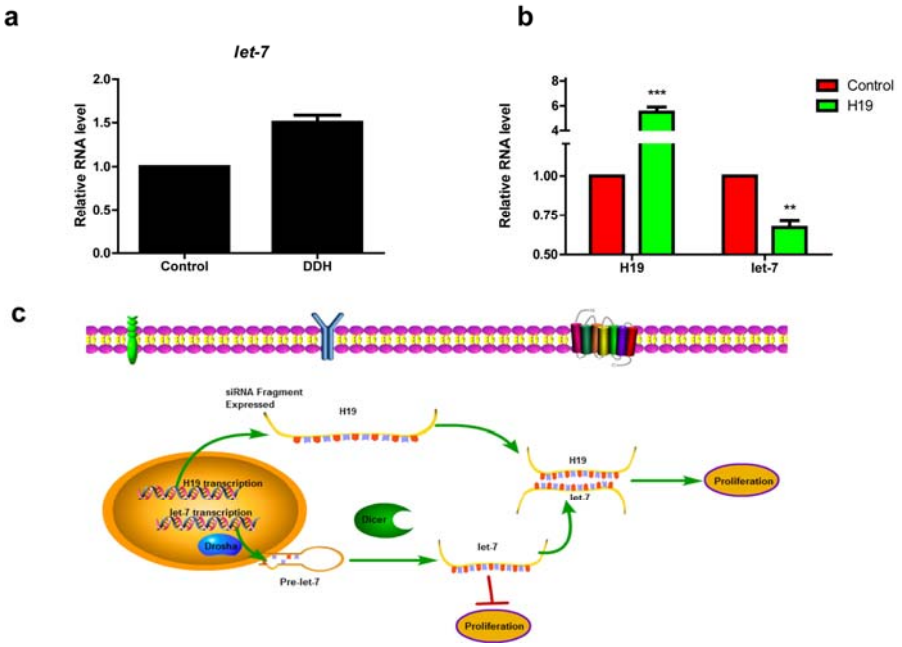


Fig. 5: H19 promotes proliferation of chondrocytes through let-7.

a. qRT-PCR analysis of the RNA level of let-7 in chondrocytes from Control and DDH model rats.

b. qRT-PCR analysis of the RNA level of H19 and let-7 in chondrocytes from Control and H19 chondrocytes.

c. Schematic diagram.