

SEMA6D, Negatively Regulated by miR-7, Contributing to Chondrocyte Catabolic and Anabolic Activities via p38 Signaling Pathway

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Research

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Abstract

Background: MiR-7 has been recognized as a promoting factor of osteoarthritis (OA), but the specific down-stream pathway of miR-7 still remains unknown. Further investigation of the molecular regulatory mechanism of miR-7 might help develop a novel therapeutic method for OA.

Results: Here we revealed that Semaphorin 6D (SEMA6D) was a direct target gene of miR-7, of which presented a negatively regulatory relation in vitro and in vivo. Lucubration of SEMA6D suggested that SEMA6D is validated to promote the anabolic metabolism and reduce the catabolism of chondrocytes via inhibiting the activation of p38 pathway.

Conclusions: Present research illustrated that SEMA6D is a negatively regulatory factor of miR-7 and a pivotal mediator of the catabolism and anabolism of chondrocytes. SEMA6D exerts its function via inhibiting the activation of p38 pathway.

Background

Osteoarthritis (OA) has been recognized a degenerated chronic disease with high incidence that mostly occurred in seniors, which was characterized as cartilage degeneration and alteration of secondary subchondral bone, and thereby induced articulus malfunction(1-3). So far, osteoarthritis has become a predominant reason for lower living standard which usually manifested as pain and functional limitation caused by degeneration of articular cartilage(4). According to epidemiological research data, the incidence proportion of OA in the elderly aged over 55-year-old was higher than 50%, much higher incidence (over 60%) occurred at people over 65-year-old(5). The complexity of the etiology and pathogenesis of OA is correlated with many pathological factors including heredity, metabolism and trauma, among which old age is considered to be the most important one(6, 7). As the rapid development of aging population, an urgent for OA remedy has become a crucial way to improve the quality of population. Therefore, searching for effectively therapeutic method for OA has been a research hotspot.

Previous studies revealed that the imbalance of anabolic and catabolic activities in chondrocytes is the predominant reason of OA. Hence, further investigation of the cytoskeleton integrity and factors that influenced anabolic and catabolic activities is being conducted to reveal the molecular mechanism of OA(8). Recently, accumulating evidence proved that microRNAs (miRNAs) participated in regulating the development of osteoarthritis (9). MicroRNA is a kind of non-coding RNA that is capable to mediate cell differentiation, apoptosis and tumorigenesis(10). Previous studies discovered that miRNA also serves as functional role in mediating the metabolism of cartilage matrix and chondrocytes. To date, research report elucidated that miRNA regulatory network, chondrocytes autophagy and alteration of epigenetics would be the targeting therapeutic strategies for osteoarthritis(11). Additionally, our previous research discovered that miR-7 was overexpressed in IL-1 β induced OA chondrocytes via activating PI3K/AKT/mTOR and thereby worsen the course the OA(12). However, the specific molecular mechanism

of miR-7 still remained unknown, which makes it a hindrance in developing novel curing method of osteoarthritis.

Semaphorin 6D (SEMA6D) is a member of semaphorin family, which serves as a crucial factor in mediating the development of nervous system(13, 14). Nowadays SEMA6D has been recognized as regulatory factor in multiple physiological processes and the development of diseases, which include development of visual system, maturation of perinatal cardiomyocyte and breast invasive carcinoma(15, 16). In this study, we adopted multiple databases to produce a bioinformatics prediction of the downstream target gene of miR-7 and found that SEMA6D might be a potential target of miR-7. Further verification of this hypothesis was performed using dual luciferase assay. We proposed an assumption that miR-7 promotes the chondrocyte anabolic activity via mediating SEMA6D.

So far, there is little data about the exact function of SEMA6D in osteoarthritis. Our finding indicated that SEMA6D is negatively regulated by miR-7 and thereby contributing to balance the catabolic and anabolic activities by specific downstream pathway. These discoveries might provide novel therapeutic methods for OA patients.

Results

SEMA6D is a down-stream target gene of miR-7 in chondrocytes.

Our previous studies already proved that miR-7 was down-regulated in normal chondrocytes and thereby exacerbated the course of OA through aggravating cartilage degradation (12). Nonetheless, the downstream regulatory target gene still remains unknown. Further investigation of the regulatory axis of miR-7 could possibly help developing novel therapeutic methods for OA, which therefore encouraged us to continue the lucubration on miR-7. Multiple databases involving TargetScan and starBase were adopted to seek the target gene of miR-7, and SEMA6D was found as a potential regulatory protein of miR-7. Bioinformatics prediction was conducted to discover that there was a direct binding site on SEMA6D sequence of miR-7 (Fig.1A), which suggested the possibility of the existence of interaction between SEMA6D and miR-7. To further confirm this assumption, dual luciferase assay was exerted and found that there was a significant difference between SEMA6D-Mut group and SEMA6D-WT group which were both co-treated with miR-7 mimic (Fig.1B, $p < 0.01$). This result verified that there was a direct interaction between miR-7 and SEMA6D. To identify the exact relation between miR-7 and SEMA6D, miR-7 mimic and miR-7 inhibitor were given to OA chondrocytes, respectively. Then the relative transcription level of SEMA6D was evaluated through qRT-PCR, and the result demonstrated that up-regulation of miR-7 led to transcribed suppression of SEMA6D in OA chondrocytes, while miR-7 inhibitor group presented the opposite phenomenon (Fig.1C, $p < 0.001$), which suggested that SEMA6D was negatively regulated by miR-7 in chondrocytes.

MiR-7 accelerated the development of OA in vivo via down-regulating SEMA6D.

Further understanding the specific role of SEMA6D in vivo was performed in human cartilage tissues and mice cartilage tissues. As depicted in Fig.2A was the immunohistochemistry consequences of SEMA6D in human and mice cartilage tissues, results showed that SEMA6D was in higher expression level both in normal human and mice chondrocytes when compared with OA chondrocytes. Next, to further verified the correlation of miR-7 and SEMA6D in vivo, miR-7 mimics and miR-7 inhibitor were given to normal mice and OA mice to investigate the nuance of SEMA6D expression, and negative controls (mimics-NC, inhibitor-NC) were also given to OA mice. Research consequences manifested that up-regulation of miR-7 would decrease the expression quantities of SEMA6D in vivo when compared with the mimic-NC group, while miR-7 inhibitor in OA mice presented lower existence of SEMA6D, which confirmed the negatively regulatory effect of miR-7 toward SEMA6D. The statistics of SEMA6D expression in normal and OA mice tissues were performed in Fig.2B, which conspicuously indicated the lower-than-usual expression quantities of SEMA6D in OA chondrocytes. These results possibly suggested that SEMA6D might have pathological regulatory function in the course of OA.

SEMA6D was overexpressed in chondrocytes with strong anabolism.

It was reported that the development of OA was closely correlated with the anabolic and catabolic activity of chondrocytes (17, 18). Multiple inflammatory mediators and mechanical stimulation collectively affect the physiological process of chondrocytes and thereby induce excessive catabolic activity and trigger osteoarthritis (19-21). Although previous experiments demonstrated that miR-7 negatively regulated SEMA6D in OA chondrocytes, there is still much confusion about the roles of miR-7 and SEMA6D in mediating the development of OA. We next proposed a hypothesis that SEMA6D might participate in regulating the catabolism and anabolism of chondrocytes. To confirm our assumption, we then constructed chondrocytes with excessive catabolic activity using fibronectin fragment (FN-f)(22), and chondrocytes with excessive anabolic activity by osteogenic protein-1 (OP1)(8). Chondrocytes were given different concentration of FN-f (0 μ M, 0.5 μ M, 1 μ M and 2 μ M) and OP1 (0ng/ml, 50ng/ml, 100ng/ml and 150ng/ml) to induce catabolism and anabolism, respectively. The status of catabolism and anabolism induced by FN-1 and OP1 presented dose dependent. Subsequently, the transcriptional levels of miR-7 and SEMA6D were detected by qRT-PCR, and the results depicted in Figure.3A and 3B demonstrated miR-7 expressed at a highest level in the chondrocytes with highest catabolic activity level induced by FN-f, which indicated that miR-7 possibly partakes in promoting the catabolism of OA chondrocytes. Contrarily, the expression quantities of SEMA6D presented the lowest in chondrocytes with highest catabolic activity. These results suggested that SEMA6D might dedicate in preventing the catabolism in chondrocytes, and the research consequence further illustrated that the regulatory effect of miR-7 and SEMA6D was opposite in chondrocytes. Subsequently, metalloproteinase 2 (MMP-2) was chosen as a biomarker in this study to examine the injury degree in cartilage cells with catabolism stimulation brought by FN-f, of which MMP-2 has proven up-regulated in OA chondrocytes with high level of catabolism(23). The results of Western blot showed that MMP-2 was significantly up-regulated in 2 μ M of FN-f treated group (Fig.3C, $p < 0.001$), and SEMA6D was in the lowest expression level in 2 μ M of FN-f treated group (Fig.3C, $p < 0.001$). These consequences illustrated that SEMA6D was down-regulated in chondrocytes with active catabolism, and SEMA6D might have a pathological correlation with the catabolic activity of

OA chondrocytes. From the above research consequence we could conclude that SEMA6D possibly participated in decreasing the catabolism of OA chondrocytes, however, whether SEMA6D is validated in promoting the anabolism of chondrocytes still remains unknown. Therefore, we utilized OP1 to induce active anabolism in chondrocytes and detected the transcriptional level of miR-7 and SEMA6D through qRT-PCR. Results demonstrated that 150ng/ml of OP1 treated group presented the lowest level of miR-7, while the transcriptional quantities of SEMA6D was the highest (Fig.3D-3E, $p < 0.001$). Similarly, the results of western blot showed that MMP-2 was down-regulated when given OP1 treated and presented dose dependent toward OP1. Contrarily, SEMA6D was significantly up-regulated when given OP1 treated, and the expression level also manifested dose dependent (Fig.3F, $p < 0.001$). These consequences collectively illustrated that SEMA6D was pathologically correlated with the catabolism and anabolism in chondrocytes and might act as a favourable factor in activating anabolism.

SEMA6D reduced the catabolism of OA chondrocytes.

According to literature research, the expression quantities of matrix metalloproteinases (MMPs), including MMP-2 and MMP-13, are increased in the cartilage tissues of OA patients(24, 25). To further investigate the functional role of SEMA6D in OA chondrocytes, we constructed and transfected small interfering RNA SEMA6D (si-SEMA6D) and pcDNA3.1-SEMA6D into chondrocytes for SEMA6D knockdown and overexpression, respectively. FN-f was used as inducer to construct OA chondrocytes. Next, we examined the mRNA and proteins expression level of MMP-2, MMP-13 and SEMA6D using qRT-PCR and western blot. After transfection of si-SEMA6D and pcDNA3.1-SEMA6D, we first scrutinized the expression level of SEMA6D. The quantities of SEMA6D in normal chondrocytes was significantly lower in si-SEMA6D transfected group (Fig.4A, $p < 0.05$), while pcDNA3.1-SEMA6D transfected chondrocytes presented significantly higher SEMA6D expression (Fig.4A, $p < 0.001$). Next, the quantities of SEMA6D in FN-f induced OA chondrocytes were also detected using qRT-PCR. The results showed that the transcriptional level of SEMA6D in FN-f induced OA chondrocytes (control+FN-f) was significantly lower than normal chondrocytes (control) (Fig.4A, $p < 0.05$). Also, OA chondrocytes induced by FN-f with si-SEMA6D and pcDNA3.1-SEMA6D transfection presented conspicuously lower and higher level of SEMA6D, respectively. To lucubrate the functional role of SEMA6D in chondrocytes with active catabolism, we then examined the quantities level of MMP-2 and MMP-13. As shown in Figure.4B-4C was the results of qRT-PCR, which depicted that knockdown of SEMA6D significantly increase the transcriptional level of MMP-2 and MMP-13 in normal chondrocytes, while up-regulation of SEMA6D using pcDNA3.1-SEMA6D reduced the quantities of MMP-2 and MMP-13 ($p < 0.05$). While in FN-f induced OA chondrocytes (control+FN-f), the transcriptional quantities of MMP-2 and MMP-13 have significantly increased ($p < 0.05$), which means FN-f has successfully constructed OA chondrocytes. When given si-SEMA6D transfection to silence SEMA6D in OA chondrocytes, the extent of MMP-2 and MMP-13 presented the highest (Fig.4B-4C, $p < 0.001$), while up-regulation of SEMA6D using pcDNA3.1-SEMA6D resulted in decreasing MMP-2 and MMP-13. Subsequently, western blot assay was performed to further confirm the suppressed function of SEMA6D toward MMP-2 and MMP-13. Similarly, silencing of SEMA6D by si-SEMA6D both in normal and OA chondrocytes led to an increasement of MMP-2 and MMP-13 ($p < 0.01$), while up-regulation of SEMA6D

demonstrated the opposite effect (Fig.4D, $p<0.05$). These results gave a joint clarification that SEMA6D might be a functional factor in preventing further damage of OA cartilage.

SEMA6D promoted anabolic activity via inhibiting the activation of p38 pathway.

Although we have discovered that SEMA6D might serve as a favourable factor in preventing catabolism and accelerating anabolism in chondrocytes, the specific regulatory mechanism of SEMA6D is still under exploration. Based on previous literature study, p38 kinase is associated with the exacerbation of OA via accelerating the synthesis of inflammatory factors and MMPs(26, 27), and mediating chondrocytes apoptosis(28). Similarly, over activation of extracellular regulated kinases (ERK) has been reported as a key factor in interfering the remodeling and proliferation of cartilage cells(29), and activation of ERK pathway would further induce the synthesis of MMP-13. Additionally, it was reported that the interaction of ERK1-Smad1 protein could promote the development of OA(30).

For further investigation of the regulatory pathway of SEMA6D, si-SEMA6D and pcDNA3.1-SEMA6D were transfected to normal chondrocytes and OP1 treated chondrocytes, we then estimated that correlated proteins expression of p38, ERK and Smad1 using western blot assay. As depicted in Figure.5 were the results of western blot assay, which indicated that silencing of SEMA6D using si-SEMA6D significantly improved the extent of phosphorylation of p38 in normal chondrocytes (Fig.5, $p<0.05$), while up-regulation of SEMA6D decreased the quantities of phosphorylation of p38 conspicuously in normal chondrocytes ($p<0.05$) and OP1 treated chondrocytes ($p<0.001$). However, no significant difference existed in the expression quantities of ERK, p-ERK, Smad1 and p-Smad1, which might infer that SEMA6D mainly participated in regulating p38 pathway.

Lucubration of SEMA6D in regulating p38 pathway, we further introduced p38 MAPK inhibitor HY-12839 into the research. Firstly, chondrocytes were divided into two groups, of which one group was given 30 nM of HY-12839 to to block the signaling function of p38 pathway; the other group was given same volume of DMSO. Subsequently, si-SEMA6D, pcDNA3.1-SEMA6D, lip-2000-NC were transfected into chondrocytes. Next, we scrutinized the expression extent of anabolic process associated proteins, including Aggrecan, collagen type II A1 (COL2A1) and ID1 proteins, which have been reported at up-regulation level in OA(31-33). As shown in Figure.6A-6C were the results of qRT-PCR, which demonstrated that up-regulation of SEMA6D using pcDNA3.1-SEMA6D could significantly induce the transcriptional quantities of Aggrecan, COL2A1 and ID1 ($p<0.01$) in chondrocytes without HY-12839 treatment, which suggested that SEMA6D was validated as a promoted factor of chondrocytes anabolism. Additionally, in HY-12839 treated group, the quantities of Aggrecan, COL2A1 and ID1 were up-regulated when compared with control group ($p<0.01$), while overexpression of SEMA6D through pcDNA3.1-SEMA6D induced even higher quantities of Aggrecan, COL2A1 and ID1 ($p<0.001$). Moreover, the results of western blot further verified the p38 suppressing effect of SEMA6D (Fig.6D). According to the consequences of western blot, the expression level of phosphorylated p38 has decreased when given OP1 treated ($p<0.01$), and overexpression of SEMA6D, which suggested that there was lower activation of p38 in chondrocytes with active anabolism. While the expression of Aggrecan, COL2A1 and ID1 significantly increased when there was up-regulation

of SEMA6D in HY-12839 treated chondrocytes ($p < 0.001$). From these results we could conclude that SEMA6D was validated in preventing the course of OA via inhibiting the activation of p38.

To confirm the SEMA6D's effect in improving osteoarthritis, immunofluorescence staining was performed to further examine the expression of COL2A1. As depicted in Figure.7 were the immunofluorescence results, from which we could infer a conclusion, that is, up-regulation of SEMA6D brought by pcDNA3.1-SEMA6D could increase the expression level of COL2A1 in normal chondrocytes. Also, in chondrocytes with active anabolic metabolism brought by OP1, overexpression of SEMA6D improved the quantities of COL2A1 conspicuously. These results further verified that SEMA6D was capable to improve the course of OA.

Discussion

Osteoarthritis (OA) is a common disease of locomotor system characterized by destruction of chondrocytes, and its incidence rate is positively correlated with age(34). To date, the exact pathogeny and pathogenesis still remain unclear. Nevertheless, as the research development of the regulatory function of microRNAs in various types of diseases, the pathological association of miRNAs and OA has become a research hotspot, and accumulating researches reported that miRNAs function as key factor in mediating the development of OA. Simultaneously, our previous studies discovered that miR-7 was significantly overexpressed in OA chondrocytes, and served as a promoting factor in mediating the course of OA(12). For further exploration of the down-stream regulatory pathway, multiple databases including Targetscan, starBase v3.0 and miRanda were utilized to calculate the target gene of miR-7, of which SEMA6D was a target gene with highest score. Bioinformatics prediction manifested that there is an overlapping sequence between miR-7 and SEMA6D, which indicated an interaction possibility of miR-7 and SEMA6D. Therefore, we conducted dual-luciferase assay and confirmed the direct interaction of miR-7 and SEMA6D (Fig.1A). Further investigation performed by qRT-PCR revealed that SEMA6D was negatively regulated by miR-7 (Fig.1B).

Next, clinical section of OA patients and OA mice were collected for immunohistochemical (IHC) staining, to estimate the expression level of SEMA6D. The results of IHC showed that the expression level of SEMA6D was higher in normal human cartilage tissues than OA tissues. Similar phenomenon could be found in mice. To investigate the regulatory function of miR-7 in vivo, miR-7 mimics and inhibitor were given to OA mice, respectively. The results of IHC demonstrated that up-regulation of miR-7 resulted in lower expression level of SEMA6D, while blockage of miR-7 presented the opposite (Fig.2A). QRT-PCR was also used to confirm the lower-than-usual expression level of SEMA6D in OA tissues (Fig.2B).

Although SEMA6D has been found as a factor in regulating the course of OA, the exact function of SEMA6D still need to be understood. It is reported that the pathogenesis of OA is the imbalance of catabolic and anabolic activity of chondrocytes (17, 18). Hence, we used the reported FN-f and OP1 to induce active catabolism and anabolism, respectively. The quantities of miR-7 and SEMA6D were studied and we found that miR-7 was up-regulated in FN-f treated chondrocytes, while the quantities of SEMA6D

decreased (Fig.3A-3B). The expression level of miR-7 and SEMA6D presented dose dependent. Contrarily, SEMA6D was overexpressed in OP1 treated chondrocytes and miR-7 was down-regulated (Fig.2D-2E). Western blot further confirmed this phenomenon (Fig.2D and Fig.2F). These consequences probably suggested that SEMA6D participated in regulating the catabolism and anabolism of chondrocytes.

Subsequently, si-SEMA6D and pcDNA3.1-SEMA6D were transfected into chondrocytes to further study the exact function of SEMA6D. Results showed that up-regulation of SEMA6D led to lower quantities of MMP-2 and MMP-13 both in normal chondrocytes and in FN-f induced OA chondrocytes, while knockdown of SEMA6D had the opposite results (Fig.4A-4D), which might indicate that SEMA6D could help prevent the development of OA. Next, we estimated the expression nuance of OA pathologically correlated pathway to find the regulatory pathway of SEMA6D and we found that p38 was a responsive target of SEMA6D. Hence, HY-12839, a p38 inhibitor, was given to chondrocytes for further exploration of the relation between SEMA6D and p38 pathway. Results demonstrated that SEMA6D indeed exerted anabolism promoted function via hindering the activation of p38 pathway (Fig.6 and Fig.7).

From above studies we can concluded that SEMA6D served as a suppressor of OA and miR-7/SEMA6D axis could regulating the development of OA via mediating p38 pathway.

Conclusions

The discovery of present research illustrated that SEMA6D is a negatively regulatory factor of miR-7 and a pivotal mediator of the catabolism and anabolism of chondrocytes. SEMA6D exerts its function via inhibiting the activation of p38 pathway.

Methods And Materials

Cell culture

Human normal chondrocyte C28/I2 cell line was incubated with 90% high glucose DMED (HyClone, USA) with 10% FBS (Gibco, USA) at a condition of 37 °C and 5% CO₂.

Dual luciferase activity

To construct the reporter plasmids of wild-type (WT) and mutated (Mut) SEMA6D, pGL3 vector (Promega Corporation, Madison, USA) and the synthetic SEMA6D with wild-type (WT) or mutated (Mut) region were adopted. Subsequently, the reporter plasmids and miR-7 mimic were co-transfected into chondrocytes through Lipofectamine 2000. MiR-NC was used as negative control. Next, dual-luciferase Reporter Gene Assay System was conducted to estimate the Renilla and Firefly luciferase activities after 24 h.

Cell transfections

To construct chondrocytes with different expression quantities of SEMA6D, small interfering RNA-SEMA6D and pcDNA3.1-SEMA6D (Genechem, Shanghai, China) were synthesized and transfected into

chondrocytes utilizing Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer's protocol to induce silence and up-regulation of SEMA6D, respectively. Lip2000-NC was used as negative control.

Immunofluorescence examination

To further estimate the expression quantities of SEMA6D in different group of chondrocytes, cells were cultured to reach a confluence of roughly 80% after transfection. PBS was used to wash cells three times and cells were fixed by cold methanol for 20 min. 0.1% Triton X-100 was utilized to permeate the fixed cells. Subsequently, the primary antibody of COL2A1 was used to treat cells at 4°C for 24 h. A secondary Alexa Fluor® 594-conjugated antibody was added and maintained at 25°C for another 1 h. DAPI was utilized to stain nucleus for 5 min. Finally, the fluorescence intensity was estimated by Leica fluorescence microscope.

Tissues sample of OA mice and human cartilage and its HE staining and immunohistochemistry

The study has been authorized by the Ethics Committee of the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University (Changzhou, China). All participants have signed the informed consent. The OA articular cartilage tissues and normal cartilage tissues were resected from the Department of Orthopedics of the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University (Changzhou, China). Male mice purchased from Qinglongshan (Nanjing, China) were used to construct OA animal model for further experiments. IL-1 β (Pepro Tech, Rocky Hill, NJ, USA) was used to induce OA in mice. Subsequently, knee articulation tissues were resected from mice and were fixed with 4% paraformaldehyde, followed by dehydration, verification, paraffin embedding and baking to produce paraffin slices. The manufacturing process of human cartilage paraffin slices was consistent with mice paraffin slices. Then, samples were dyed using hematoxylin for 5 min and followed by water washing. Slices were stained using eosin for another 5 min, and then xylene was used to dehydrate the slices. Microscopic imaging system was utilized to examine and photograph the slices. For immunohistochemistry assay, paraffin slices were blocked using hydrogen peroxide for 20 min and followed by pepsin treatment. 5% BSA was used to block slices for 1 h at 25°C. The primary antibodies were incubated with the slices overnight and the slices were then incubated with secondary antibodies for 1 h at room temperature. 3,3'-diaminobenzidine was used as chromogenic reagent.

Western blot assay

Cells in exponential growth period were collected and washed with PBS three times. Cells were then lysed using lysis buffer on ice for 30 min. BCA kit was used to calculate the total proteins concentration. Loading buffer was added into proteins solution and boiled for 5 min. After separated on SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes and were then incubated with primary antibodies (including SEMA6D, MMP-2, Smad1, p-Smad1, ERK, p-ERK, p38, p-p38, Aggrecan, COL2A1 and ID1) and subsequently secondary antibodies. GAPDH was adopted as a negative control. Finally, to observe the intensity of proteins' strap, an enhanced chemiluminescence reagent (Thermo Scientific,

Waltham, MA, USA) was used. The grey intensity was analyzed via image J and Graphpad prism software.

Quantitative reverse-transcription PCR (qRT-PCR)

TRIzol Reagent was used for the extraction of total RNA from chondrocytes. Chloroform incubation was conducted for 15 min at room temperature and isopropyl alcohol was added. Then centrifugation was performed to obtain RNA from the precipitation. The expression of miR-7, SEMA6D, Aggrecan, COL2A1, ID1 and Smand1 were analyzed through PrimeScript RT reagent Kit and SYBR Prime Script RT-PCR Kits according to manufacturer's guideline. The results were analyzed through $2^{-\Delta\Delta Ct}$. GAPDH was the internal control. The primer sequences were: hAggrecan, forward: 5'-GACTTCCGCTGGTCAGATGG-3', reverse: 5'-CGTTTGTAGGTGGTGGCTGTG-3'; ID1, forward: 5'-GTGCCTAAGGAGCCTGGAAA-3', reverse: 5'-TTCAGCGACACAAGATGCGA-3'; MMP13, forward: 5'-CATGAGTTCGGCCACTCCTT-3', reverse: 5'-CCTGGACCATAGAGAGACTGGA-3'; miR-7, forward: 5'-TGGAAGACTAGTGATTTTGT-3', reverse: 5'-CCAGTCTCAGGGTCCGAGGTATTC-3'.

Construction of active catabolism and anabolism chondrocytes.

For lucubration of the specific role of SEMA6D in chondrocytes, fibronzetin fragment (FN-f) and osteopenia protein-1 (OP1) were utilized to induce catabolism and anabolism in chondrocytes, respectively. FN-f with different concentration (0, 0.5, 1.0, 2.0 μ M) was used to seek the optimal concentration. Also, 0, 50, 100, 150 ng/ml of OP1 were used to find favorable concentration.

Statistical analyses

The experiment data were statistically analyzed through SPSS 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad 7 (GraphPad Software, San Diego, CA, USA). Student's t test was used to compare between-group statistical difference, and multiple groups were compared using one-way analyses of variance.

Abbreviations

OA: osteoarthritis; SEMA6D: Semaphorin 6D; si-SEMA6D: small interfering RNA of SEMA6D; FN-f: fibronectin fragment; OP1: osteogenic protein 1; MMP: matrix metalloprotein; ERK: extracellular regulated protein kinases; COL2A1: collagen type II A1; p-p38: phosphorylated p38.

Declarations

Authors' contributions

XZ designed and exerted the project. JZ, YZ and ZY gleaned information. XZ, YZ, JZ, ZY and HY conducted the experiments. ZY and YZ are responsible for data statistics. YH and NX wrote the article. HY and JL revised the manuscript.

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Competing interests

There are no competing interests between authors.

Consent for publication

Not applicable.

Data availability

The research data generated during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The research was approved by the local ethics committee and all participants signed the informed consent.

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Figures

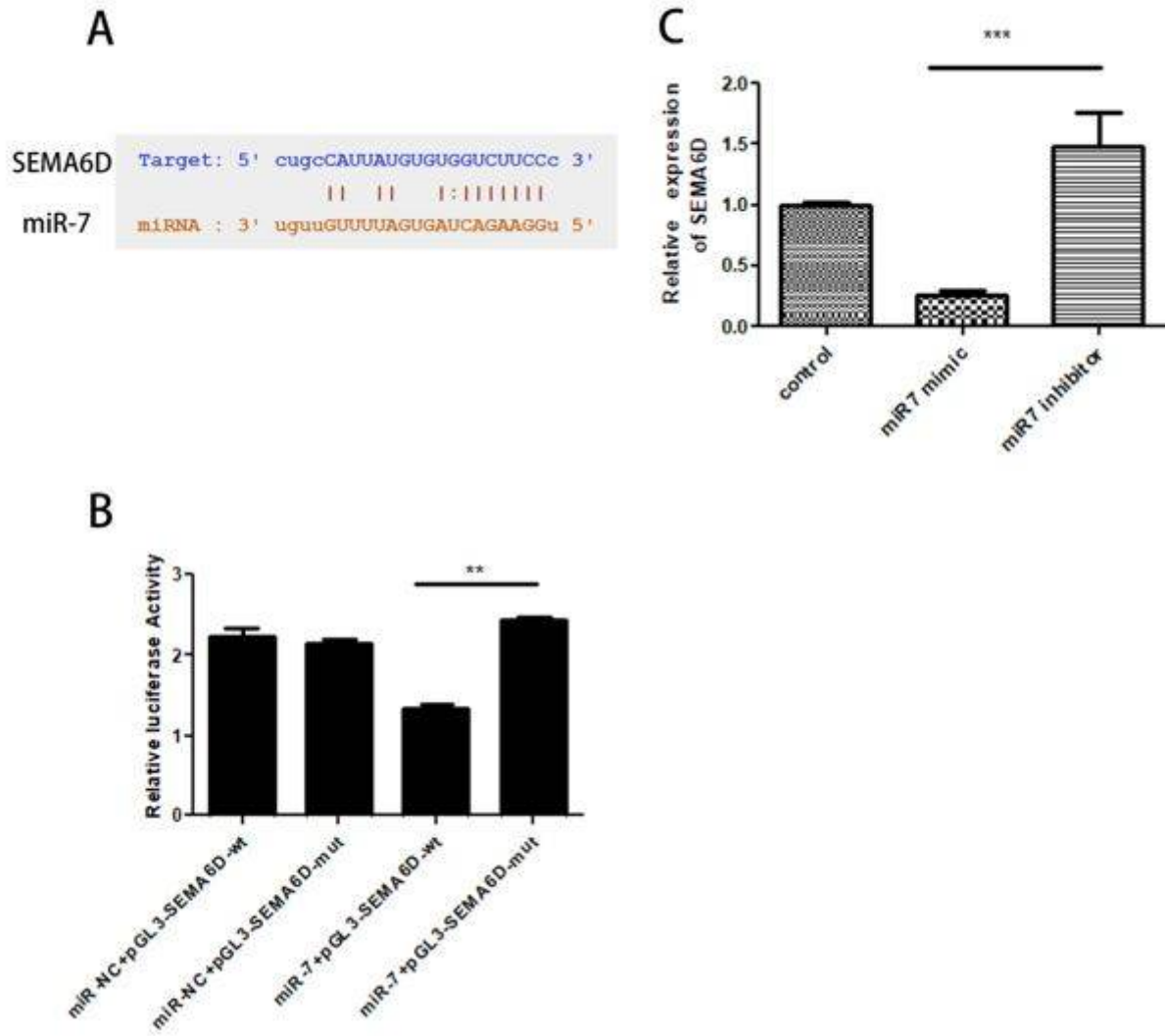


Figure 1

SEMA6D was recognized as a negatively regulatory target of miR-7. A. Bioinformatics prediction showed an overlapping sequence between miR-7 and SEMA6D. B. The result of dual-luciferase assay demonstrated the existence of direct interaction between miR-7 and SEMA6D. C. QRT-PCR assay indicated that the quantities of SEMA6D was down-regulated when there was overexpression of miR-7, and SEMA6D increased when miR-7 was down-regulated. ** means $p < 0.01$ between groups. *** means $p < 0.001$ between groups.

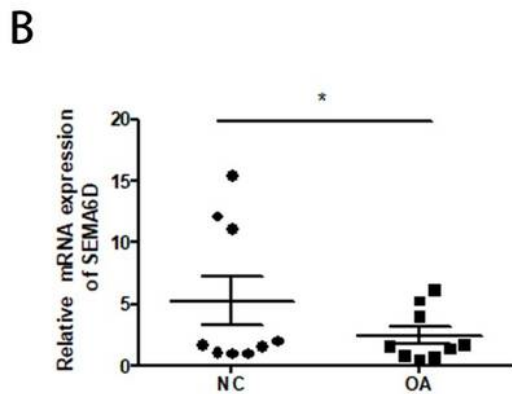
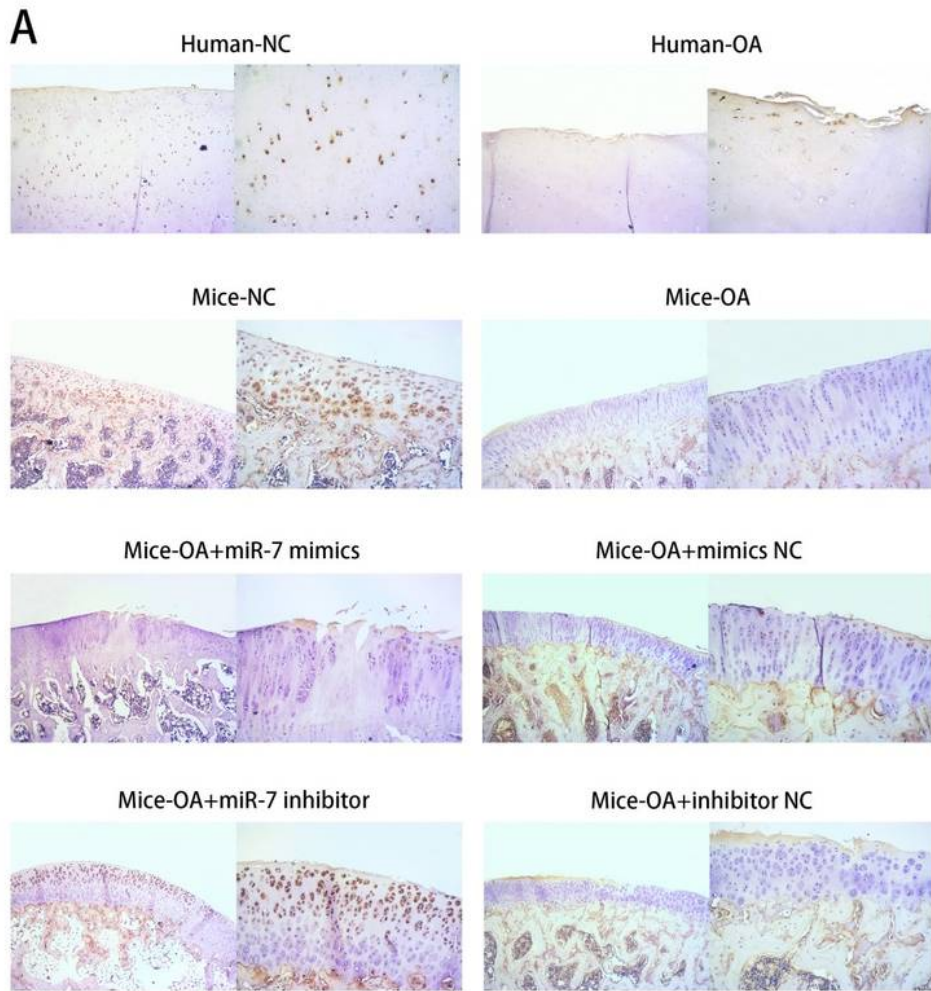


Figure 2

SEMA6D was negatively regulated by miR-7 in vivo. A. The IHC results of SEMA6D in human and mice cartilage tissues in different groups. B. QRT-PCR indicated that SEMA6D was up-regulated in normal cartilage tissues compared to OA tissues. * $p < 0.05$.

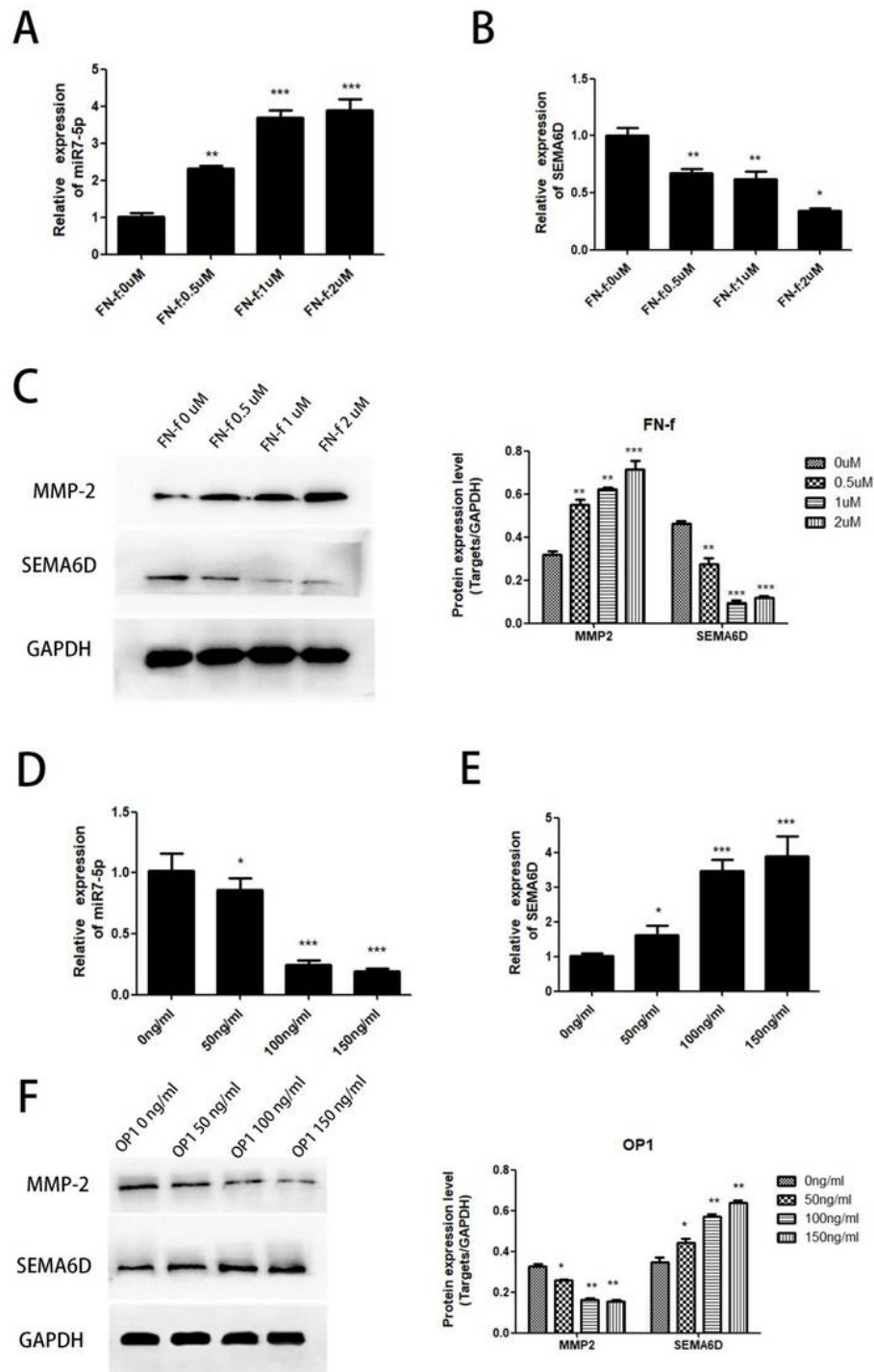


Figure 3

Investigation of SEMA6D in chondrocytes with active catabolism or anabolism. A-B. Chondrocytes were stimulated with different concentration of FN-f, and the quantities of miR-7 and SEMA6D were measured by qRT-PCR. C. The expression level of MMP-2 and SEMA6D were estimated using western blot after given different concentration of FN-f. D-E. Chondrocytes were stimulated with different concentration of OP1, and the quantities of miR-7 and SEMA6D were measured by qRT-PCR. F. The expression level of

MMP-2 and SEMA6D were estimated using western blot after given different concentration of OP1. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with control group.

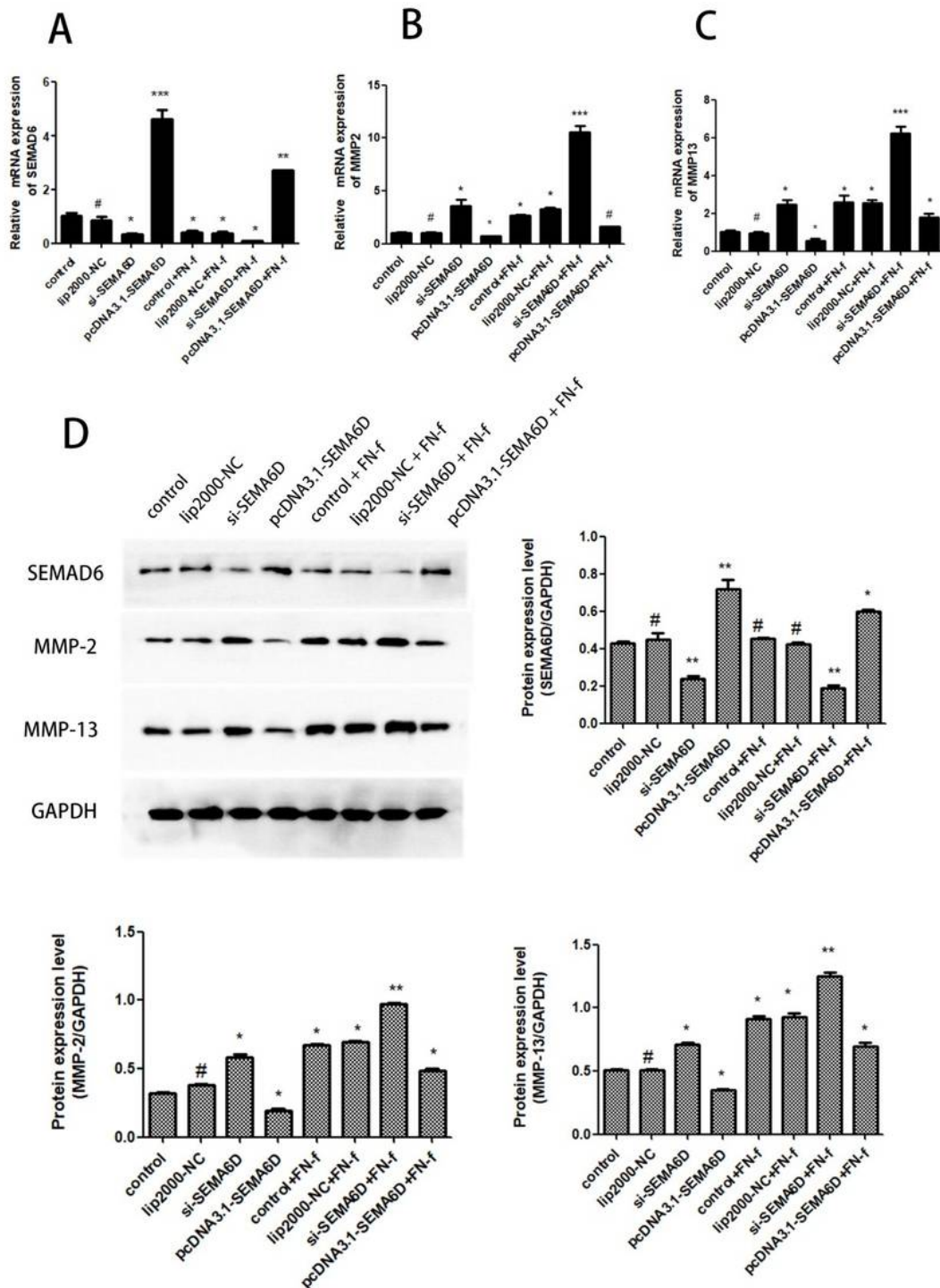


Figure 4

SEMA6D reversed the catabolism caused by FN-f. A. The transfection effect of si-SEMA6D and pcDNA3.1-SEMA6D was verified by QRT-PCR. B-C. Down-regulation of SEMA6D resulted in overexpression of MMP-2 and MMP-13 both in normal chondrocytes and OP1 treated chondrocytes. D. Western blot was

performed to measure the expression of MMP-2, MMP-13 and SEMA6D. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # means no significant difference compared with control group.

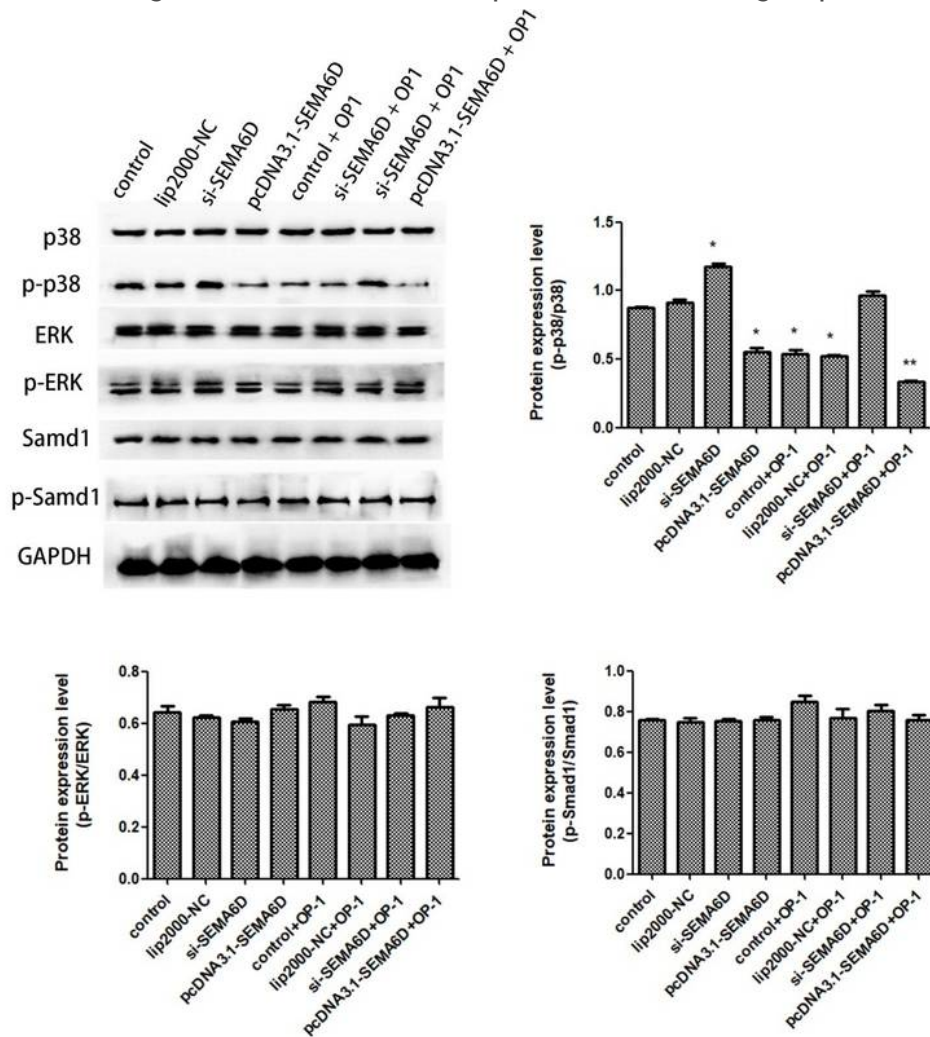


Figure 5

Exploration of the regulatory pathway of SEMA6D. Results of western blot showed that p38 presented responsive alteration when chondrocytes were given SEMA6D interference, while ERK and Smad1 did not show any change. ** $p < 0.01$, * $p < 0.05$ compared with control group.

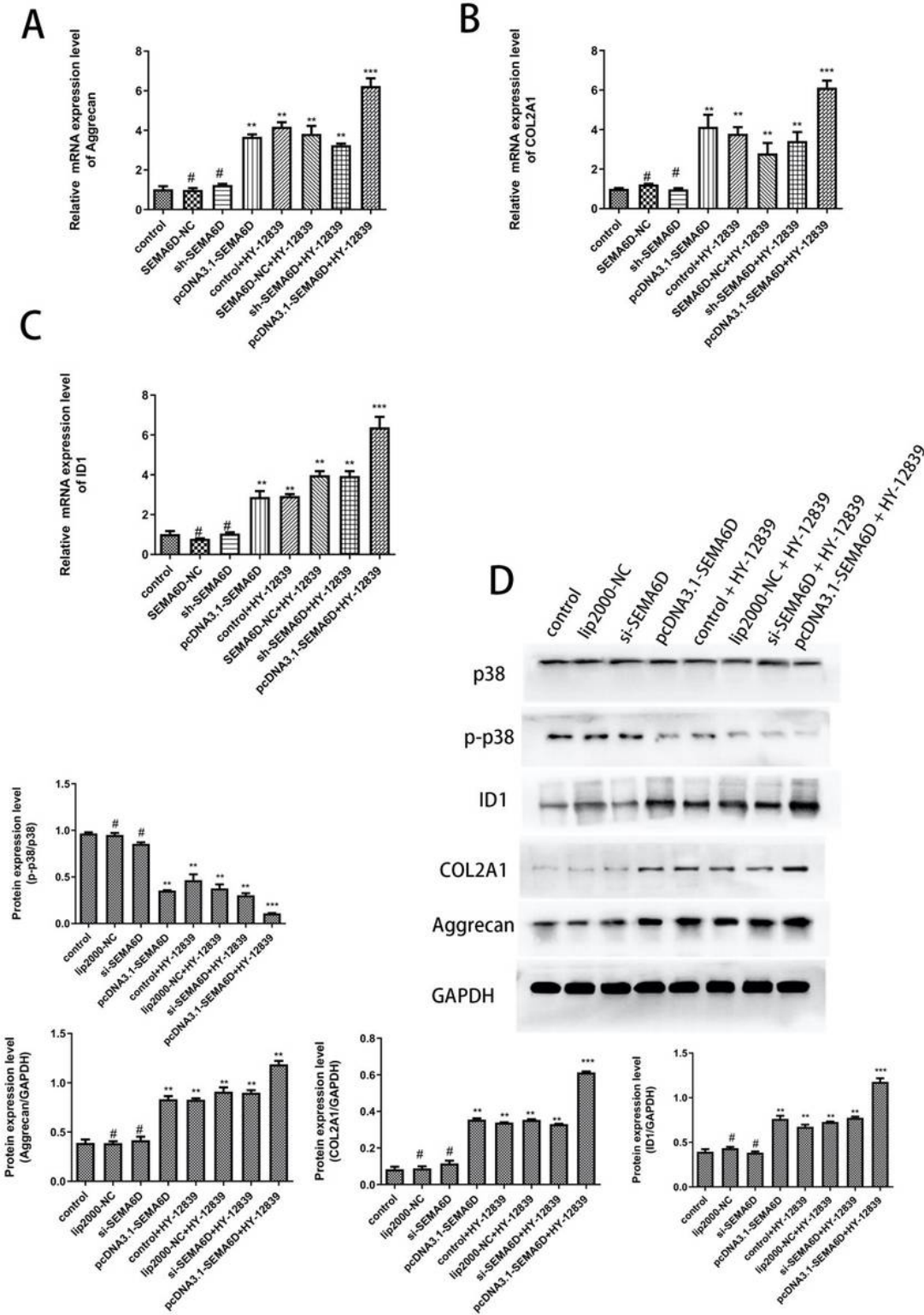


Figure 6

SEMA6D exerted regulatory function in OA via inhibiting p38 pathway. A-C. The results of qRT-PCR of anabolism related proteins including Aggrecan, ID1 and COL2A1. D. The expression level of anabolism related proteins including Aggrecan, ID1 and COL2A1 were measured by western blot. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # means no significant difference compared with control group.

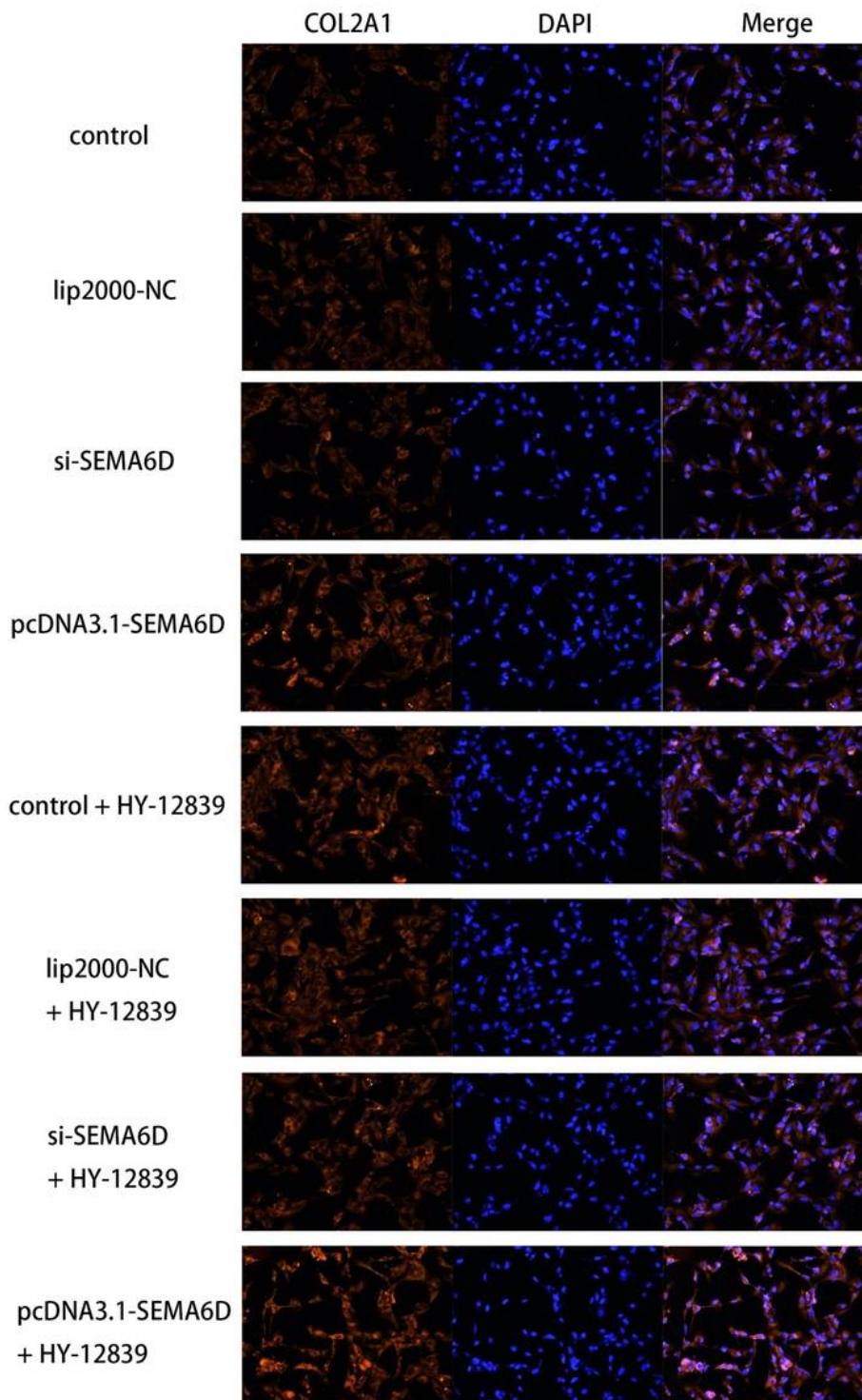


Figure 7

The expression level of COL2A1 was measured by immunofluorescence staining.