International Immunopharmacology 69 (2019) 88–94

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Semaphorin 4A acts in a feed-forward loop with NF-κB pathway to exacerbate catabolic effect of IL-1β on chondrocytes

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ARTICLE INFO

Keywords:
Semaphorin 4A
NF-κB
Osteoarthritis
IL-1β
Chondrocyte catabolism

ABSTRACT

Inflammation is fundamental in osteoarthritis (OA) pathogenesis. Semaphorin 4A (Sema4A) has been implicated in immune-associated diseases, however, its role in OA remains unclear. In this study, we show that Sema4A is upregulated in knee OA articular cartilage as well as in chondrocytes exposed to IL-1β treatment in vitro. Moreover, IL-1β-induced Sema4A upregulation is abrogated in the presence of BAY 11-7082, a specific inhibitor of NF-κB pathway, suggesting that the activation of NF-κB is required for Sema4A upregulation under this pathological condition. Intriguingly, Sema4A in turn activates NF-κB through facilitating Rac1/AKT-dependent IκBα phosphorylation and subsequent degradation. Functionally, Sema4A aggravates the catabolic effect of IL-1β on chondrocytes, which can be largely attributed to exacerbated NF-κB activation, since NF-κB inhibition remarkably abolishes this effect. In conclusion, our study suggests that Sema4A is a novel regulator of NF-κB-dependent catabolic events in chondrocytes, which may underlie OA pathogenesis.

1. Introduction

Osteoarthritis (OA) is the most common arthritis and one of the leading causes of pain and physical disability worldwide [1]. OA is characterized by a progressive loss of articular cartilage accompanied by morphological and functional changes in the whole joint, including synovium, periarticular ligaments and subchondral bone [2]. At present, no effective therapies are available for modifying the processes that underlie the pathogenesis of OA. It is now widely accepted that OA is a whole-organ disease with inflammation driving many pathological events [3]. Among the several pathways and regulators mediating the OA-associated inflammation, the transcription factor nuclear factor kappa B (NF-κB) is deemed to play a prominent role [4].

Under unstimulated conditions, NF-κB binds with the inhibitory protein IκB and is retained in the cytosol in an inactive form. Whereas, when activated by stimuli such as proinflammatory cytokines and matrix degradation enzymes, a cascade of biochemical reactions is initiated and leads to the phosphorylation and subsequent degradation of IκB, thereby resulting in the release of active NF-κB, which translocates to the nucleus to induce the expression of target genes [5]. NF-κB pathway is activated in OA chondrocytes and is essential to induce various proinflammatory mediators and catabolic factors, including IL-1β, TNF-α, matrix metalloproteinases (MMPs) and inducible nitric oxide synthase (iNOS), etc., which in turn further activate this pathway [4,6]. Through exerting various proinflammatory and catabolic effects, NF-κB pathway deeply participates in OA pathophysiology. Therefore, it's proposed that targeting NF-κB pathway may allow us to develop novel therapeutic interventions for minimizing OA progression [6–8].

In recent years, accumulating evidence suggests that semaphorin 4A (Sema4A), a class IV transmembrane semaphorin, plays a crucial role in fine tuning of immune processes and is involved in various inflammatory diseases including allergic asthma, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and inflammatory bowel disease [9]. However, to date, the role and mechanism of Sema4A in OA are not characterized. In the present study, we reveal the association of Sema4A with OA, and uncover the unrecognized interplay between Sema4A and NF-κB pathway that functions to exacerbate the catabolic effect of IL-1β on chondrocytes.

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https://doi.org/10.1016/j.intimp.2019.01.006
Received 5 October 2018; Received in revised form 15 December 2018; Accepted 4 January 2019
Available online 25 January 2019

2. Materials and methods

2.1. Antibodies and reagents

The antibodies and reagents were obtained from the following sources: anti-Sem4A (abcam, ab70178), anti-phospho-p65 (S536) (abcam, 86299), anti-p65 (abcam, ab32536), anti-phospho-IκBα (Ser32) (Cell Signaling, 2859), anti-IκBα (Cell Signaling, 9242), anti-phospho-Akt (Ser473) (Cell Signaling, 9271), anti-Akt (Cell Signaling, 9272), anti-phospho-Rac1 (Ser71) (Cell Signaling, 2461), anti-Rac1 (abcam, ab33186), anti-NOS2 (Novus Biologicals, NB300-605), anti-COX2 (Santa Cruz, sc-1745), anti-MMP3 (abcam, ab52915), anti-MMP13 (abcam, ab39012), anti-β-Actin (Novus Biologicals, NB600-501), anti-GAPDH (Santa Cruz, sc-32233), anti-Histone 3 (Cell Signaling, 9715), goat anti-rabbit IgG-HRP (abcam, ab6721), goat anti-mouse IgG-HRP (abcam, ab6789), goat anti-rabbit IgG-peroxidase (Millipore, AP132P), BAY 11-7082 (TOCRIS, 1744), LY294002 (APExBIO, A8250), wortmannin (Selleck, S2758) and recombinant mouse IL-1β was purchased from BioLegend.

2.2. Clinical OA cartilage samples

Clinical OA knee articular cartilage samples were obtained from 21 OA patients during their knee replacement at the First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine. The OA symptom of all patients met the American College of Rheumatology classification criteria for OA diagnosis [10]. The normal knee articular cartilage samples were obtained from 25 patients who underwent amputation from accidents and had no medical history of OA. The informed consents were obtained from all enrolled subjects. The clinical study design and sampling processes were conducted in accordance with the rules approved by the Medical Ethical Committee of the First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine.

2.3. Immunohistochemistry

Immunohistochemistry of knee articular cartilage tissues was performed as adapted to a previous study [11]. In brief, paraffin-embedded sections with 5 μm thickness were deparaffinized and antigen retrieval was conducted boiling in 10 mM citrate buffer (pH 6.0) for 10 min. Sections were blocked in 3% H_20_2 for another 10 min and further blocked with 5% goat serum for 1 h at room temperature. Sections were incubated with IgG isotype control antibody or primary anti-Sema4A (1:100 dilution) overnight in a humid box at 4 °C. After repeated rinse, peroxidase-conjugated secondary antibodies (1:1000 dilution) were added onto the sections for 1 hr incubation. After mild rinse, sections were stained with diaminobenzidine for 2–3 min to reveal immunoreactivity, and then counterstained with hematoxylin for another 1 min. Eventually, sections were visualized under a light microscope. IHC scores from 20 stochastic regions were collected and calculated by the equation of [(value of weak − intensity pixels) (value of moderate − intensity pixels) (value of strong − intensity pixels)] / total value of pixels [12].

2.4. Animals, chondrocyte isolation and in vitro culture

Male C57BL/6J mice with 10 weeks of age were used as donors of chondrocytes. Mice were fed under the specific pathogen-free conditions. All experimental procedures of mice complied with the protocols approved by the Institutional Animal Care and Use Committees of the First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine for animal welfare. For the isolation and in vitro culture of primary articular chondrocytes from mice, experiments were conducted as previously documented [13]. In brief, after being dissected from the knee joint surface and rinsed in sterile PBS in a biosafety cabinet, the mouse cartilage tissue samples were cut into small pieces using a sterile scalpel blade, and then incubated in the solution containing 2 mg/ml pronase (Sigma) for 2 h at constant 37 °C, followed by being digested overnight in 1 mg/ml collagenase II (Sigma) at 37 °C with constant agitation. The cell suspension products were filtered through a 70 μm size cell strainer (BD) for purification. Chondrocytes with a single-cell status were seeded in a monolayer in a culture plate with a density of 25,000 cells/cm^2. The complete DMEM/F12 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Invitrogen) and 1% L-glutamine (Invitrogen) were added in culture plates for culturing chondrocytes. The culture plates were maintained in a humidified incubator with conditions of constant 37 °C and 5% CO_2. Chondrocytes were passaged by 1:3 ratio when density reached 80% confluency.

2.5. Chondrocyte treatment and transfection

The mouse recombinant Sema4A fused with His tag at C-terminal (mR-Sema4A) was produced in HEK293 cells. One day before treatment, chondrocytes were seeded with 30% confluent. The titrated concentrations of mouse recombinant IL-1β, 10 μM BAY 11-7082, 25 μM LY294002, 100 nM wortmannin or mouse recombinant Sema4A (mR-Sema4A) was added into the medium for treating chondrocytes. For mouse Rac1 knockdown, siRNA specifically targeting luciferase control (siCtrl), Rac1 (siRac1) or Sema4A (siSema4A) was transfected into chondrocytes using Lipofectamine RNAiMAX reagent (ThermoFisher Scientific, 12778150) at a final concentration of 20 nM according to manufactures instructions. siRNAs were purchased from Sigma-Aldrich. Two or three days after transfection, chondrocytes were harvested and the efficiency of overexpression or knockdown was assessed by Western blot or qRT-PCR analysis.

2.6. Protein isolation and Western blot analysis

The ultrasonicated cartilage tissues or in vitro cultured chondrocytes were collected and homogenized in RIPA lysis buffer (strong) (Beyotime, P0013B) supplemented with protease inhibitor cocktail (Beyotime, P1008) in ice-cold bath for 25 min, the tubes were flicked every 5 min. The final cell lysates were centrifuged at 13000 × rpm for 10 min at 4 °C. The bottom pellets were discarded and the supernatants were collected in a new tube place on ice. Protein concentration was determined using BCA method according to reagent kit (Pierce, 23225). 5 × SDS loading buffer was added into protein samples (1:4 ratio) and then denatured for 5 min at 95 °C in a metal bath. Loading samples were immediately used for Western blot or stored at −80 °C for further experiments. Western blot analysis was performed as described previously [14]. In brief, equal amount of total proteins were loaded and run in 10% or 12% SDS-PAGE. After being transferred onto Immobilon-P PVDF membranes (Millipore, IPVH00010), PVDF membranes were blocked with 5% skim milk soluted in TBST for 1 h at room temperature. PVDF membranes were then incubated in sequence with primary and secondary antibodies (1:1000 dilution). The protein bands on PVDF membranes were finally visualized with ECL detection reagents (Sigma-Aldrich, GERPN2209). The band intensity was quantified and analyzed using ImageJ software when necessary.

2.7. mRNA extraction and RT-qPCR analysis

For extracting total mRNA of tissue samples, the cartilage tissues were initially grinded into homogenates under liquid nitrogen using pestle and mortar. The total mRNA of these tissue homogenates or in vitro cultured chondrocytes was extracted using RNeasy Mini Kit (Qiagen, 74106). The total mRNA level was quantified using NanoDrop 2000c (ThermoFisher Scientific), and 2 μg of total mRNA were reverted into cDNA using RevertAid First strand cDNA Synthesis kit (ThermoFisher Scientific, K1622) based on manufacturer's instructions.

RT-qPCR analysis was performed using these templates combined with FastStart SYBR Green Master kit (Sigma-Aldrich) and ABI7500 Real-Time PCR systems (Applied Biosystems). Human or mouse *ACTB* gene was used as an endogenous control throughout. Data were analyzed using the comparative Ct method and expressed as mean ± S.D. The used primer pairs are listed below: Human Sema4A forward 5′-AGGA TCCTTCAGTTCTGGCC-3′, reverse 5′-GTAAGGGAAGCAGGAGGGAG-3′; Mouse Sema4A forward 5′-ACGGCTACTCATACCCTGTG-3′, reverse 5′-CGATGAGAAAATGGGGCCAG-3′; Human β-Actin forward 5′-TTCG AGCAAGAGATGGCCA-3′, reverse 5′-AGGTAGTTTCGTGGATGCCA-3′; Mouse β-Actin forward 5′-GCTGTATTCCCCTCCATCGT-3′, reverse 5′-CTTCTCCATGTCGTCCCAGT-3′; Mouse Nos2 forward 5′-TCTTGGAG CGAGTTGTGGAT-3′, reverse 5′-TGACACAAGGCCTCCAATCT-3′; Mouse Nos2 forward 5′-AAGACGCCACATCCCCTATT-3′, reverse 5′-GAATGCG TAGAGAGGGGAGA-3′; Mouse Mmp3 forward 5′-GGGTTGGAGATGAC AGGGAA-3′, reverse 5′-GGAGAAAGTGAGTGGGGTCA-3′; Mouse Mmp13 forward 5′-ATCACCTGATTCTTGCGTGC-3′, reverse 5′-ATCTG TGCTCATCTGTGGCT-3′.

2.8. Statistical analysis

Data represent at least three independent experiments and are expressed as mean ± s.d. The statistical significance was calculated using unpaired two-tailed Student's *t*-test. A value of $P < 0.05$ was considered with a statistical significance.

3. Results

3.1. Sema4A expression is upregulated in knee OA articular cartilage

Initially, we examined whether Sema4A has clinical relevance to OA. To this end, we compared its expression in knee cartilage samples collected from healthy individuals (normal) and patients with OA. The data from qRT-PCR analysis showed that the mRNA level of Sema4A was significantly higher in OA cartilage than that of normal cartilage (Fig. 1A, $P < 0.01$). Consistent with this result, the protein level of Sema4A was also increased in OA cartilage as compared with normal counterparts (Fig. 1B). Moreover, the upregulation of Sema4A in OA cartilage was further confirmed by the immunohistochemistry analysis on the cartilage tissue sections (Fig. 1C). In sum, these data indicate that compared with normal cartilage, Sema4A expression is upregulated in OA cartilage, suggesting a clinical relevance of Sema4A to OA pathogenesis.

3.2. NF-κB activation is required for Sema4A upregulation in chondrocytes exposed to IL-1β

Next, we studied Sema4A in chondrocytes under a pathological condition in vitro, i.e., exposed to IL-1β [15]. We found that Sema4A expression in chondrocytes was increased by IL-1β treatment in a dose-dependent manner at both mRNA (Fig. 2A) and protein levels (Fig. 2B). As known, IL-1β is often overproduced by synovial cells and chondrocytes in OA cartilage, and imposes the inflammatory and catabolic effects that are critical for OA pathogenesis [16–18]. Therefore, we suspect that the upregulation of Sema4A in OA cartilage may be associated with the stimulation of IL-1β present in focal sites. In chondrocytes, NF-κB is a cardinal regulator that is activated by IL-1β stimulation and mediates the biological effects [19–21]. We then asked whether NF-κB activation is responsible for Sema4A upregulation when treated with IL-1β. BAY 11-7082, an inhibitor of NF-κB activity [22], was applied to inhibit IL-1β-induced NF-κB activation in chondrocytes. Interestingly, the results showed that the upregulation of Sema4A by IL-1β stimulation was abrogated when NF-κB activity was inhibited by BAY 11-7082, as evidenced by the failure of induced expression of p-p65 (Fig. 2C–D). Taken together, these results show that Sema4A is upregulated in chondrocytes exposed to IL-1β, in which the activation of NF-κB plays a crucial role.

3.3. Sema4A activates NF-κB through facilitating Rac1/AKT-dependent IκBα phosphorylation

Due to its expression and function in both nervous and immune systems, Sema4A is termed neuroimmune semaphorin [23]. Unexpectedly, we found that the level of p-p65 was increased in chondrocytes exposed to mouse recombinant Sema4A (mR-Sema4A) (Fig. 3A). Additionally, mR-Sema4A treatment induced the nuclear

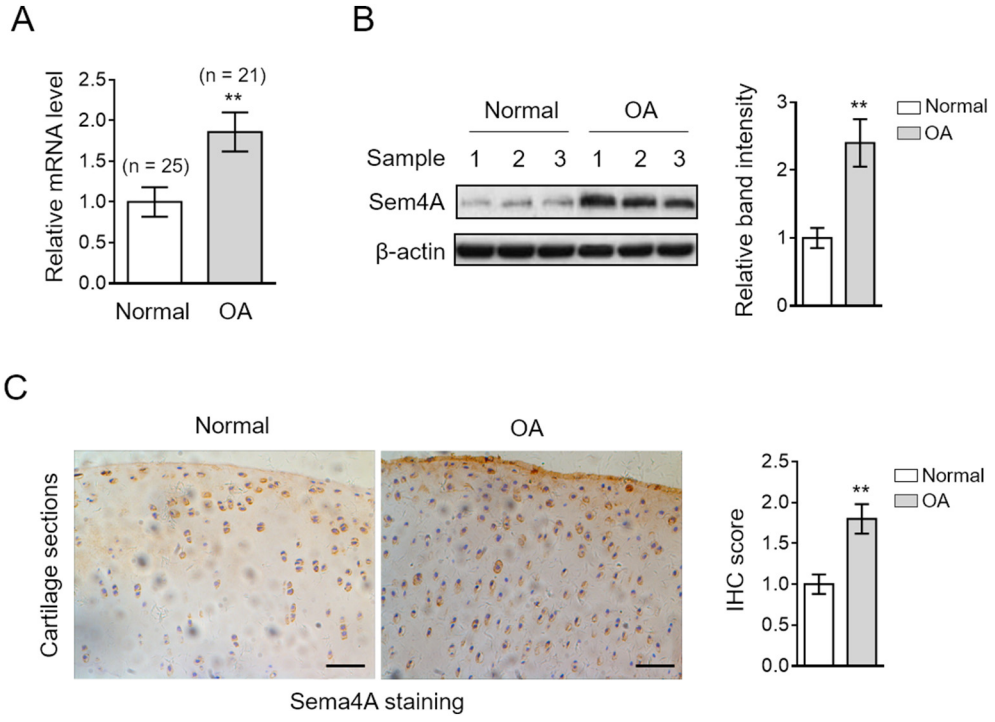

Fig. 1. Sema4A expression is upregulated in knee OA articular cartilage.
(A–C) Human knee cartilage samples were collected from healthy subjects (normal) (n = 25) and OA patients (n = 21). (A) The mRNA level of Sema4A was quantified by qRT-PCR analysis. The results are represented as relative to normal. (B) The protein level of Sema4A was measured by Western blotting. The representative images (left) and band intensity analysis (right) are shown. (C) Sema4A was detected with IHC staining, and IHC score analysis is depicted right. Scale bar, 50 μM. β-Actin was used as a reference or loading control. Data are mean ± s.d. Student's *t*-test. **, $P < 0.01$.

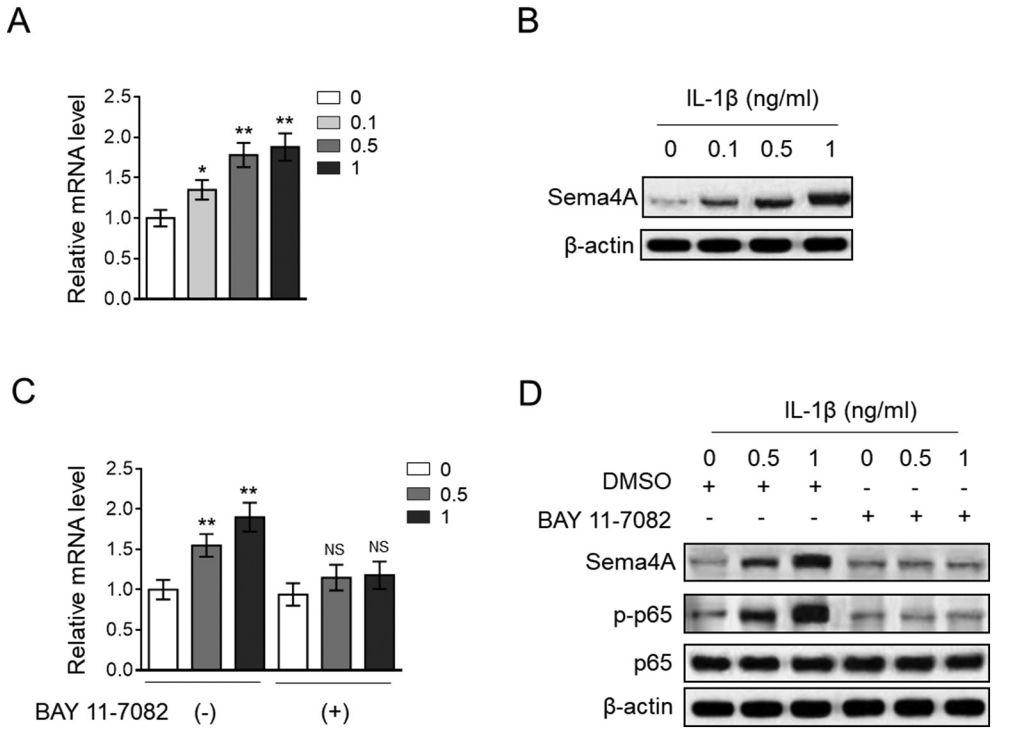

Fig. 2. IL-1β-induced NF-κB activation up-regulates Sema4A in chondrocytes.
(A–B) Relative mRNA level (A) and protein level (B) of Sema4A in primary mouse articular chondrocytes treated with or without increasing concentrations of IL-1β for 24 h (n = 3). (C–D) Primary mouse articular chondrocytes were cultured in the presence or absence of 10 μM BAY 11-7082, and further treated with or without increasing concentrations of IL-1β as indicated for 24 h (n = 3). The relative mRNA level (C) and protein level (D) of Sema4A was determined. β-Actin was used as a reference or loading control. Data are mean ± s.d. Student's *t*-test. *, $P < 0.01$; **, $P < 0.01$; NS, not significant.

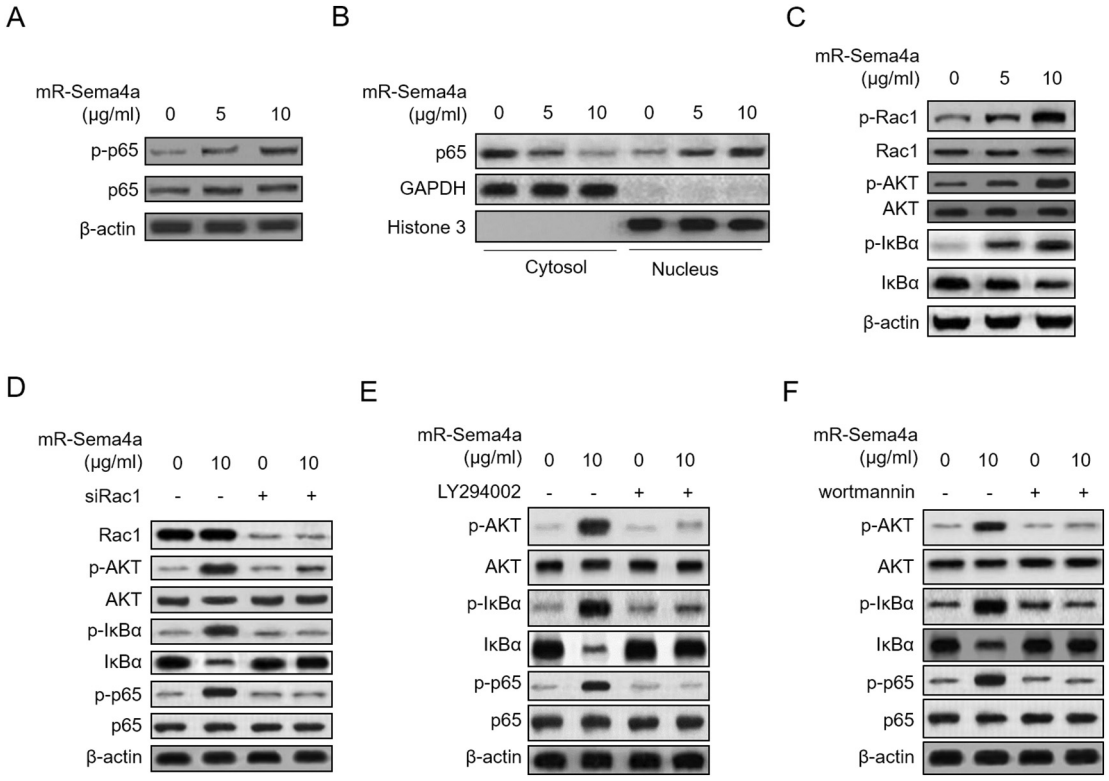

Fig. 3. Sema4A activates NF-κB through promoting Rac1/AKT pathway-dependent IκBα phosphorylation.
(A) Primary mouse articular chondrocytes were treated with mouse recombinant Sema4A (mR-Sema4A) as indicated for 24 h, the protein expression of p-p65 and p65 was analyzed by Western blotting. (B) Primary mouse articular chondrocytes were treated as in (A), the protein level of p65 in cytosol and nucleus was analyzed by Western blotting. (C) Primary mouse articular chondrocytes were treated as in (A), the protein expression of p-AKT, AKT, p-Rac1, Rac1, p-IκBα and IκBα was analyzed by Western blotting. (D) Primary mouse articular chondrocytes were transfected with siRNA targeting luciferase control (siCtrl) or Rac1 (siRac1) for 48 h and further treated with mR-Sema4A for 24 h, the protein expression of p-AKT, AKT, p-Rac1, Rac1, p-IκBα, IκBα, p-p65 and p65 was analyzed by Western blotting. (E–F) Primary mouse articular chondrocytes were treated with mR-Sema4A in the presence or absence of 25 μM LY294002 (E) or 100 nM wortmannin (F) as indicated for 24 h, the protein expression of p-AKT, AKT, p-IκBα, IκBα, p-p65 and p65 was analyzed by Western blotting. β-Actin was used as a loading control. Experiments were conducted at least 3 times independently and representative images are shown.

translocation of p65 (Fig. 3B), indicating that mR-Sema4A activates NF-κB pathway in chondrocytes. Semaphorin signaling regulates the activity of Rho family proteins, which are upstream modulators of Akt pathway [24–26]. Besides, Rac1, the Ras-related C3 botulinum toxin substrate 1, is an important member of the Rho family and Rac1/Akt pathway was reported to be associated with NF-κB activation [26]. We found that mR-Sema4A treatment activated Rac1/Akt pathway in chondrocytes (Fig. 3C), as shown by increased phosphorylation of Rac1 and Akt. The activation of this pathway seems relevant to NF-κB activation, since mR-Sema4A treatment concomitantly resulted in increased IκBα phosphorylation and decreased protein level of basal IκBα (Fig. 3C), two essential biochemical events for activating NF-κB. To verify whether Rac1/Akt pathway activation accounts for mR-Sema4A-induced NF-κB activation, we depleted Rac1 expression by transfecting chondrocytes with siRNA targeting Rac1. As expected, mR-Sema4A-induced Rac1/Akt pathway activation was blocked when Rac1 was depleted, and meanwhile, the increased phosphorylation of IκBα and decreased IκBα protein level were completely recovered without Rac1/Akt pathway activation (Fig. 3D). The IκBα phosphorylation and reduction determine the activation NF-κB in this scenario, as the increased phosphorylation of p65 also vanished accordingly (Fig. 3D). Furthermore, similar results were obtained when chondrocytes were treated with LY294002 (Fig. 3E) or wortmannin (Fig. 3F), two potent inhibitors of PI3K/Akt pathway [27,28], indicating that the activated Akt is responsible for Sema4A-induced NF-κB activation. Collectively, these lines of evidence suggest that Sema4A induces IκBα phosphorylation in a Rac1/AKT pathway-dependent manner, whereby activating NF-κB in chondrocytes.

3.4. NF-κB activation contributes to Sema4A-aggravated catabolic effect of IL-1β on chondrocytes

To learn more about the role of Sema4A associated with OA pathogenesis, we explored whether it has effect on the IL-1β-treated chondrocytes. The catabolic effect of IL-1β treatment on chondrocytes was confirmed by the increased expression of protein markers including NOS2, COX2, MMP3, MMP13 at both mRNA (Fig. 4A) and protein (Fig. 4B) levels. Remarkably, mR-Sema4A treatment further increased the expression of these marker in chondrocytes exposed to IL-1β, however, without IL-1β treatment, mR-Sema4A only slightly increased the protein expression of these markers (Fig. 4A–B), suggesting that Sema4A only aggravates the IL-1β-induced catabolic effect on chondrocytes.

As shown in Fig. 3, Sema4A activates NF-κB in chondrocytes. As NF-κB is a major mediator of IL-1β effect on chondrocytes [21,29,30], we asked whether Sema4A-induced NF-κB activation is associated with the aggravated IL-1β catabolic effect. To address it, we again used BAY 11-7082 to inhibit NF-κB activity. The results showed that under the treatment of IL-1β, in line with the inhibited NF-κB activity by the addition of BAY 11-7082, the mR-Sema4A-increased expression of catabolic markers was significantly attenuated at both mRNA (Fig. 4C) and protein (Fig. 4D) levels, although did not totally recovered to the basal group. Moreover, since Sema4A induces NF-κB activation through the Rac1/AKT pathway, we found that wortmannin treatment similarly diminished mR-Sema4A-increased expression of catabolic markers in IL-1β-stimulated chondrocytes (Fig. 4E). Altogether, these data illustrate that Sema4-induced NF-κB activation is a critical mechanism that accounts for the promotive role of Sema4A in the IL-1β-induced catabolic effect on chondrocytes (Fig. 5).

At last, to verify whether the endogenous Sema4A promotes IL-1β-induced chondrocyte catabolism, we depleted Sema4A expression via siRNA transfection in chondrocytes stimulated with or without IL-1β, and then checked the expression of catabolic factors via qRT-PCR analysis. The result showed that compared with siRNA control, IL-1β-induced expression of catabolic factors was significantly diminished, although only partially, when transfected with Sema4A siRNA (Fig. 4F). This evidence further points to a positive role of Sema4A involved in IL-1β catabolic effect on chondrocytes (Fig. 5).

4. Discussion

Currently, the pathogenesis of OA remains obscure, and no drugs are available for exerting a disease-modifying effect that can effectively prevent the continuous degeneration of cartilage [31]. However, increasing insight into the inflammatory processes that underlie OA pathogenesis holds great promise for developing novel and disease-modifying therapies, and indeed, several anti-inflammatory treatments have shown considerable efficacy in animal OA models [3]. Among these promising targets, NF-κB pathway attracts much attention due to its pivotal role in the regulation of inflammation processes [6,8,32]. Nonetheless, the molecular mechanisms of the regulation of NF-κB pathway in the context of OA pathological condition are not fully delineated, which impedes the formulation of rational therapies targeting this pathway.

In the present study, we show that Sema4A, previously known as a neuroimmune semaphorin, is a novel regulator of NF-κB pathway in chondrocytes that activates NF-κB through promoting the phosphorylation IκBα in a Rac1/AKT-dependent manner. Interestingly, we also report that NF-κB activation in turn is required for Sema4A upregulation in chondrocytes exposed to IL-1β. Lastly, we reveal that Sema4A has a functional role in aggravating IL-1β proarthritic effect, and NF-κB inhibition attenuates it. Thus, this study suggests a reciprocal regulation between Sema4A and NF-κB in chondrocytes (Fig. 5), which may provide a useful hint for the understanding of the role of Sema4A in OA pathogenesis and also offer a potential therapeutic target for OA treatment.

One recent study has shown that Sema4A expression was significantly higher in synovial tissues of RA patients compared with those of OA patients, and that Sema4A expression was positively correlated with disease severity [33]. Besides, Sema4A also plays a role in other chronic inflammatory diseases [9]. Here, we found that compared with normal knee cartilage, Sema4A had higher expression level in OA knee cartilage. To our knowledge, this is the first report that suggests a clinical relevance of Sema4A upregulation to knee OA. However, further studies with larger clinical sample size are needed to verify this association and examine whether Sema4A could also predict OA severity and whether it shows similar expression change in different types of OA occurring in other anatomical sites. Utilizing chondrocytes cultured in vitro, we found that IL-1β treatment induced Sema4A expression. Given the critical role of IL-1β in the development and progression of OA [34], we suspect it's very possible that the upregulation of Sema4A expression in knee OA cartilage is associated with IL-1β stimulation at OA focal sites. Mechanistically, in chondrocytes, the induction of Sema4A expression by IL-1β stimulation is strictly dependent on NF-κB activation, as its inhibition completely abrogates Sema4A induction. It has been demonstrated that lipopolysaccharide (LPS) stimulation increased NF-κB recruitment to the promoter region of Sema4A gene, which is responsible for Sema4A induction in synovial fibroblasts [33], suggesting that Sema4A might be a direct target downstream of NF-κB pathway. Together with our findings, we propose that upon treatment of inflammatory stimuli, Sema4A, as a common downstream target of NF-κB pathway, may be transcriptionally induced in the context of various inflammatory diseases. Whether this is the case deserves further investigations.

It has been reported that the Rac1/Akt/NF-κB pathway plays an important role in EphrinA2-promoted tumorigenicity of liver cancer [26]. Moreover, Sema3C promotes the survival and tumorigenicity of glioma stem cells through activating Rac1/NF-κB pathway [35]. We uncover that Sema4A promotes Rac1/AKT-dependent IκBα phosphorylation and activates NF-κB in chondrocytes. Therefore, our study extends the function of Rac1 to chondrocyte biology, and might also suggest that the activation of this pathway may serve as a prosurvival

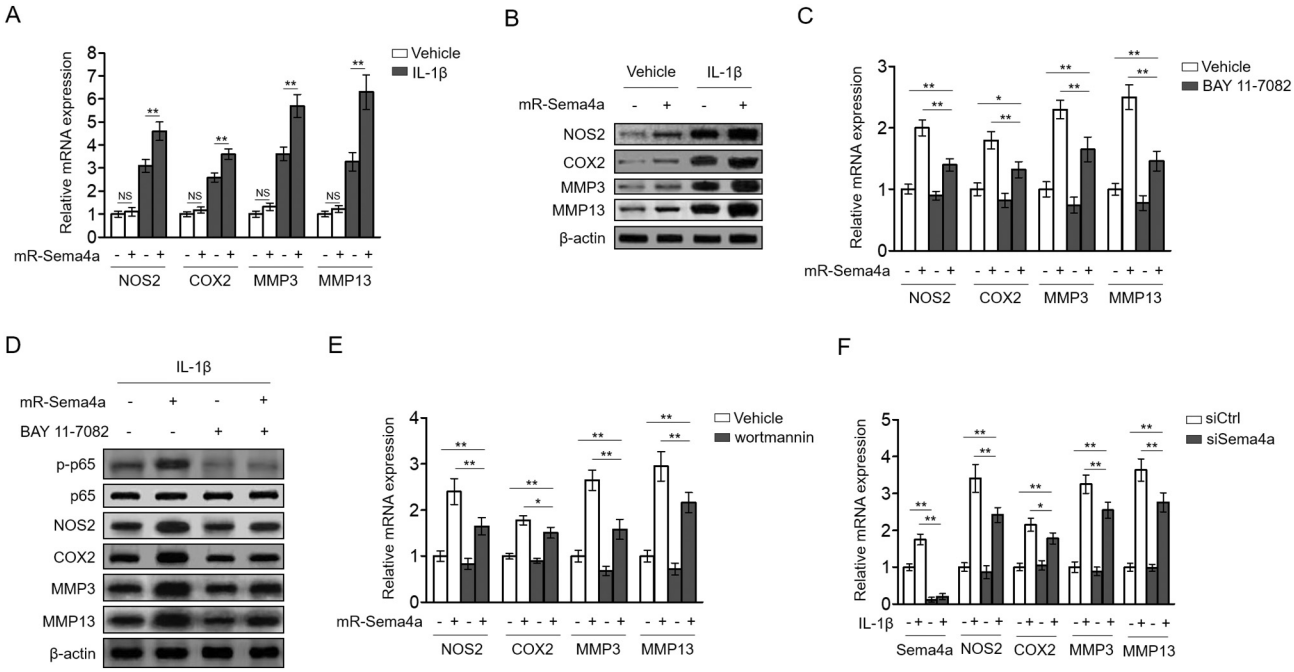

Fig. 4. Exacerbated NF-κB activation contributes to Sema4A-enhanced catabolic effect of IL-1β on chondrocytes.
(A–B) mRNA level (A) and protein level (B) of NOS2, COX2, MMP3 and MMP13 in primary mouse articular chondrocytes treated with mR-Sema4A in combination with or without 1 ng/ml IL-1β for 24 h. (C–D) mRNA level (C) and protein level (D) of NOS2, COX2, MMP3 and MMP13 in IL-1β-incubated primary mouse articular chondrocytes treated with mR-Sema4A in combination with or without 10 μM BAY 11-7082 for 24 h. (E) mRNA level of NOS2, COX2, MMP3 and MMP13 in IL-1β-incubated primary mouse articular chondrocytes treated with mR-Sema4A in combination with or without 100 nM wortmannin for 24 h. (F) Primary mouse articular chondrocytes were transfected with siRNA targeting luciferase control (siCtrl) or Sema4a (siSema4a) for 48 h and further treated with or without 1 ng/ml IL-1β for 24 h. The mRNA level of Sema4a, NOS2, COX2, MMP3 and MMP13 was determined by qRT-PCR analysis. β-Actin was used as a reference or loading control. Experiments were conducted at least 3 times independently. Data are mean ± s.d. Student's *t*-test. **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

signal for chondrocytes when exposed to excessive exogenous Sema4A.

IL-1β is known to cause pain and even disability in OA patients by promoting the catabolic events in the bone joint [36]. IL-1β aggravates degradation processes in OA cartilage by upregulating expressing of MMPs and accelerates clinical manifestations through inducing the production of inflammatory mediators such as nitric oxide synthase (NOS2) and cyclooxygenase-2 (COX2) [30]. Using an in vitro model in which cultured primary chondrocytes were stimulated with IL-1β, Sema4A was found to aggravate the catabolic effect of IL-1β on chondrocytes. In light of the finding that Sema4A is upregulated in OA cartilage, this functional study suggests that Sema4A may have a detrimental role in the progression of OA. If it is true, the feed-forward regulation between Sema4A and IL-1β-activated NF-κB may make the OA pathological condition even worse. However, solid evidence from clinical studies and animal models is warranted to address whether Sema4A indeed imposes a detrimental role in OA pathogenesis.

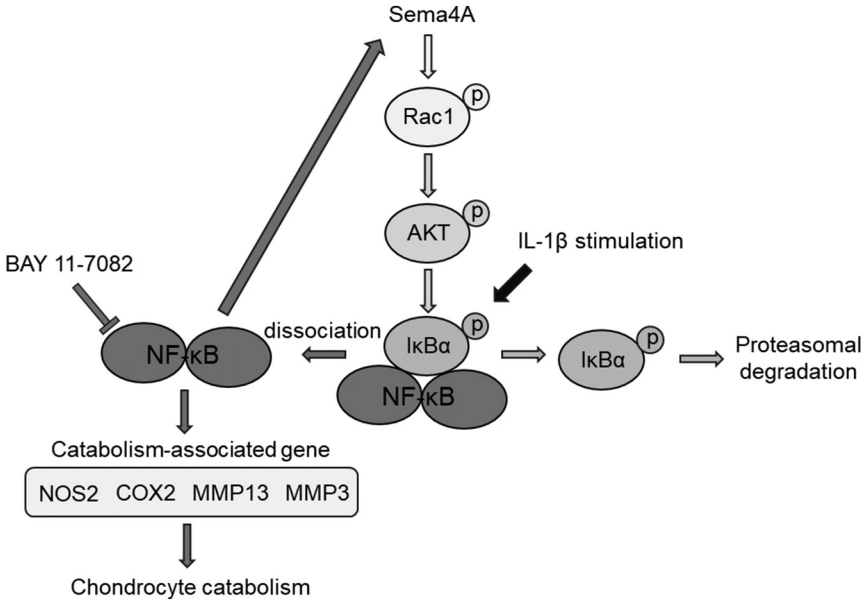

Fig. 5. Interaction between Sema4A and NF-κB pathway promotes IL-1β-induced catabolic effect on chondrocytes. IL-1β-induced NF-κB activation upregulates Sema4A expression. On the other hand, Sema4A activates NF-κB through promoting Rac1/AKT pathway-dependent IκBα phosphorylation and subsequent degradation. This positive feed-back loop enhances NF-κB activation and expression of catabolism-associated genes, thus aggravating the catabolic effect of IL-1β on chondrocytes.

Funding

This work was supported by grants from the project of the National Natural Science Foundation of China (grant nos. 81302994, 81473697, 81574002 and 81273778), GZUCM Science Fund for Creative Research Groups (2016KYTD10), and GZUCM Torch Program (A1-AFD015142Z08).

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