Osteoarthritis and Cartilage 19 (2011) 111–117

Osteoarthritis and Cartilage

Requirement of the NF-κB pathway for induction of Wnt-5A by interleukin-1β in condylar chondrocytes of the temporomandibular joint: functional crosstalk between the Wnt-5A and NF-κB signaling pathways

Xian-Peng Ge †, Ye-Hua Gan †, Chen-Guang Zhang ‡, Chun-Yan Zhou ‡, Kang-Tao Ma ‡, Juan-Hong Meng †*, Xu-Chen Ma †*

† Center for Temporomandibular Disorders and Orofacial Pain, Peking University School and Hospital of Stomatology, Beijing 100081, PR China
‡ Department of Biochemistry and Molecular Biology, Peking University School of Basic Medical Sciences, Beijing 100191, PR China

ARTICLE INFO

Article history:
Received 3 June 2010
Accepted 17 October 2010

Keywords:
Wnt-5A
NF-κB
Interleukin-1β
Chondrocyte
Temporomandibular joint

SUMMARY

Objective: We have previously reported that interleukin-1β (IL-1β) up-regulates the expression of Wnt-5A and the activation of Wnt-5A signaling induces matrix metalloproteinase (MMP) through the c-Jun N-terminal kinase pathway in condylar chondrocytes (CCs) of the temporomandibular joint (TMJ). These results suggest that Wnt-5A could play an essential role in IL-1β-mediated cartilage destruction. The objective of this study was to investigate the molecular mechanism underlying IL-1β-induced up-regulation of Wnt-5A in TMJ CCs.

Methods: Primary CCs, limb chondrocytes (LCs) and SW1353 human chondrosarcoma cells were treated with IL-1β in the presence or absent of BAY 11-7082 (an inhibitor of IκBα-phosphorylation). Then, expression of Wnt-5A was estimated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR), Western blotting and immunocytofluorescence. Transient transfection of p65 expression vector and chromatin immunoprecipitation (ChIP) assay was performed to define the effect of p65 on Wnt-5A expression.

Results: IL-1β up-regulated Wnt-5A expression at both the RNA and protein levels in articular chondrocytes. The inhibitor of IκBα-phosphorylation, BAY 11-7082, blocked the induction of Wnt-5A by IL-1β in a dose-dependent manner. Moreover, experiments with overexpression of p65 and ChIP established that induction of Wnt-5A by IL-1β is mediated through the NF-κB pathway, especially the p65 subunit.

Conclusion: These results clarify the molecular mechanism underlying up-regulation of Wnt-5A by IL-1β in chondrocytes, suggesting an important functional crosstalk between Wnt-5A and NF-κB signaling pathways. This finding provides new insights into the involvement of Wnt signaling in the cartilage destruction caused by arthritis.

Introduction

The maintenance of homeostasis in articular cartilage is crucial for the integrity of its structure and function. The fine balance between matrix destruction and repair is regulated by catabolic and anabolic activities of the chondrocytes. In osteoarthritis (OA), this balance is disturbed and tilted toward matrix catabolism, leading to progressive degradation of cartilage[1,2].

Numerous studies have revealed that interleukin-1β (IL-1β) plays a pivotal role in cartilage destruction during the pathophysiological process of OA by promoting the release of matrix degenerative enzymes and inhibiting the synthesis of extracellular matrix proteins by chondrocytes[1]. IL-1β also contributes to the pathogenesis of OA by enhancing the expression of other catabolic cytokines[3] and by inducing apoptosis of chondrocytes *via* overproduction of nitric oxide[4]. Multiple pathways, including the NF-κB pathway, are involved in IL-1β's modulation target gene transcription. Mammalian NF-κB is a set of dimeric DNA binding proteins whose subunits include RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). NF-κB proteins are capable of forming numerous homodimers or heterodimers (for example p65–p50) in complexes with inhibitory IκB and other family members in the cytoplasm. Upon stimulation by cytokines, chemokines and growth

* Address correspondence and reprint requests to: Xu-Chen Ma or Juan-Hong Meng, Center for Temporomandibular Disorders and Orofacial Pain, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China. Tel: 8610-62179977x5345; Fax: 8610-62136628.
E-mail addresses: jhmeng@yahoo.com.cn (J.-H. Meng), kqxcma@bjmu.edu.cn (X.-C. Ma).

1063-4584/$ – see front matter © 2010 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.joca.2010.10.016

factors, IκB is phosphorylated, leading to its proteasome-mediated degradation. In turn, dimeric NF-κB transcription factors, mainly p65–p50, are enabled to translocate to the nucleus to induce target gene expression[5,6].

Wnt proteins constitute a large family of secreted glycoprotein ligands that are responsible for important developmental processes and they have been increasingly implicated in adult tissue homeostasis and disease processes[7]. The mammalian genome encodes 19 Wnt proteins and 10 Frizzled (Fz) seven-pass transmembrane receptors. Historically, Wnt proteins have been divided into "canonical" and "noncanonical" classes, which activate β-catenin-dependent and -independent signaling pathways, respectively (see Wnt-homepage: http://www.stanford.edu/~rnusse/wntwindow.html). Recently, the traditional classification has been challenged, as it is clear that the pathway activated by a Wnt isoform can be highly dependent on cellular context and factors such as the complement of cell surface Wnt receptors[8,9].

There is accumulating evidence for a crucial role of Wnt signaling in joint development and postnatal joint homeostasis and remodeling, which points toward a possible association between Wnt signaling and OA[10–12]. A previous study from our group also showed that Wnt signaling may be involved in the destruction of temporomandibular joint (TMJ) condylar cartilage after experimentally-induced OA[13].

Wnt-5A is a representative ligand that activates the β-catenin-independent pathway in Wnt signaling, which has been known to regulate chondrogenesis and cartilage development by promoting chondrocyte differentiation and inhibiting chondrocyte maturation[14–16]. Wnt-5A has been associated with rheumatoid arthritis by induction of some pro-inflammation cytokines/chemokines in fibroblaste-like synoviocytes[17,18]. Although positive expression of Wnt-5A was detected in the articular cartilage of OA[19], the roles of the Wnt ligand in OA have not been well characterized. Recently, several studies have shown that Wnt-5A was involved in IL-1β-induced matrix metalloproteinase (MMP) expression and chondrocyte dedifferentiation in TMJ condylar chondrocytes (CCs) and limb joint chondrocytes, suggesting that Wnt-5A might be involved in cartilage destruction[20,21]. While the up-regulation of Wnt-5A by IL-1β has been reported in chondrocytes in several studies, the mechanism underlying up-regulation of Wnt-5A by IL-1β remains unknown. In the present study, we explored whether up-regulation of Wnt-5A by IL-1β was induced through the NF-κB pathway in the TMJ CCs.

Materials and methods

Cell cultures and treatments

CCs were isolated from TMJ condyles of 2-week-old New Zealand white rabbits (BAOLA, Beijing, China) as described previously[21]. Limb chondrocytes (LCs) were isolated from the hyaline cartilage of hip and shoulder joints by digestion with 0.25% trypsin (Sigma, St. Louis, MO) for 30 minutes (min), followed by digestion with 0.2% collagenase type II (Invitrogen, San Diego, CA) for 4–6 hours (h). Human chondrosarcoma cells (SW1353) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). All cells were cultured in 1:1 mix of Dulbecco's modified Eagle's medium and Ham's F12 Nutrient Mix (DMEM/F12; Gibco, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT), supplemented with 50 units of penicillin/streptomycin.

For cell treatment, cells at 80–90% confluence were washed with phosphate buffered saline (PBS) and were further cultured in serum-free media overnight. IL-1β (10 ng/ml; Sigma) or Wnt-5A (200 ng/ml; R&D Systems, Minneapolis, MN) was added into the media alone or in combination with the specific inhibitor of IκBα phosphorylation, BAY 11-7082[22] (Calbiochem, La Jolla, CA). The concentrations of IL-1β and Wnt-5A used were based on our previous study[21].

RNA isolation and real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated with a Total RNA Isolation kit (SBS Genetech, Beijing, China) according to the manufacturer's instructions and digested with RQ1 RNase-free DNase (Promega, Madison, WI) to remove possible genomic DNA contamination prior to reverse transcription with Moloney murine leukemia virus RT (Invitrogen) for 60 min at 42 °C. Real-time PCR was performed using a 7300 Real-time PCR system (Applied Biosystems, Foster City, CA) and SYBR Green Master Mix (Applied Biosystems). The primers used for real-time RT-PCR were as follows: for rabbit Wnt-5A, forward 5′-GTATCAGGACCACATGCAGTACATC-3′ and reverse 5′-GGAACTGATACTGGCATTCTTTGA-3′, for human Wnt-5A, forward 5′-TAAGCCCAGGAGTTGCTTTG-3′ and reverse 5′-GCAGAGAGGCTG TGCTCCTA-3′[23], and for the internal control 18S RNA, forward 5′-GTAACCCGTTGAACCCCATT-3′ and reverse 5′-CCATCCAATCGG TAGTAGCG-3′. All real-time RT-PCRs were performed in triplicate, and the amplification signal from the target gene was normalized to the 18S RNA signal in the same reaction. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method as earlier described[24].

Western blotting

Cells were lysed with 2% sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2% SDS) supplemented with protease inhibitor cocktail set I (Calbiochem, La Jolla, CA) and phenylmethanesulfonyl fluoride (PMSF, Sigma). The protein concentration was determined by the bicinchoninic acid method using a BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins (50 μg) were fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20. Goat-anti-Wnt-5A (1:1000 dilution, R&D Systems), rabbit anti-p65 (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-p-IκBα (1:500 dilution, Santa Cruz Biotechnology) antibodies were used to detect the proteins. The blots were developed using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection.

Immunocytofluorescence

Cells were cultured on glass coverslips and fixed with cold 50% *methanol*/50% *acetone* at 4 °C for 15 min. The cells were permeabilized with 0.1% Triton X-100 for 10 min × three times and blocked with 5% bovine serum albumin in PBS for 30 min. The cells were then incubated with goat-anti-Wnt-5A antibody (1:60 dilutions, R&D Systems) at 4 °C overnight, followed by fluorescein isothiocyanate-conjugated secondary antibodies for 1 h. The nuclei were counterstained with Hoechst 33342 in PBS for 5 min. The coverslips were mounted on slides with 70% glycerol in PBS and examined with an Olympus FV1000 confocal microscope.

Transfection experiments

The mammalian expression vector for NF-κB p65 (pRK-p65) was a generous gift from Dr. Hongbing Shu (Wuhan University College of Life Science, Wuhan, China)[25]. The primary CCs, LCs and SW1353 cells at 80–90% confluence were transfected with the vectors using Vigofect (Vigorous, Beijing, China), which is a mixture of non-lipid cations, according to the manufacturer's instructions. Transfected

cells were maintained in complete DMEM/F12 for 48 h and used for further analyses.

Luciferase assay

To confirm the suppressive effect of BAY 11-7082 on NF-κB activity, CCs growing in 24-well tissue culture plates were transfected with the NF-κB-Luc reporter plasmid for 6 h without serum and then incubated in complete DMEM/F12 for 24 h; they were then treated with different concentrations of BAY 11-7082 for another 24 h. To assay the effectiveness of overexpression of p65, CCs were cotransfected with NF-κB-Luc and pRK-p65 and then incubated in complete DMEM/F12 for 48 h. Transfection efficiency was normalized by cotransfection with 10 ng pRL-Renilla. Cells were harvested in 120 μl of Reporter Lysis Buffer (Vigorous), and activities of the firefly luciferase and the control Renilla luciferase were simultaneously assayed as described in the dual luciferase reporter assay system (Vigorous). Firefly luciferase activity was normalized to the Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed in SW1353 cells essentially as described previously[26–28]. The NF-κB p65 antibody (Santa Cruz Biotechnology) was used for the immunoprecipitation of cross-linked chromatin. Primers used for the amplification of the Wnt-5A promoter were as follows: primer 1, 5′-AATAAAGGTTTGTGG TTGGGTA-3′ (forward) and 5′-AAGGCAGTTCGTGTAGAGGAT-3′ (reverse); primer 2, 5′-AAGGTCTTTTGCACAATCACG-3′ (forward) and 5′-CGCAGGCA ACTGTTCCAC-3′ (reverse); and primer 3, 5′-CCAGCAAATGGGACTCGG-3′ (forward) and 5′-AAGCGGGAAAG CAACACT-3′ (reverse).

Enzyme-linked immunosorbent assay (ELISA)

The culture media from the primary CCs were collected after the cells were treated with Wnt-5A for 12, 24 or 48 h, centrifuged at 2000×*g* for 10 min to discard the debris, and stored at −70 °C until use. IL-1β and MMP-1 were detected in the supernatants using the ELISA kit for IL-1β (Junyao Biotechnology, Beijing, China) or for MMP-1 (ESBLab, Sacramento, CA), according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated three times with independent cultures and similar results were obtained. Data are presented as the mean and 95% confidence interval (CI). Statistical significance was determined by the two-tailed unpaired Student's *t*-test. *P* values less than 0.05 were considered significant.

A Fold change in Wnt-5A gene expression

Cells	$2^{-\Delta\Delta Ct}$		P-value
	Control	IL-1β (10 ng/ml)	vs control
CC	1	2.42 (1.62, 3.22)	0.0016
LC	1	3.36 (2.16, 4.57)	0.0011
SW1353	1	3.21 (2.29, 4.13)	0.0003

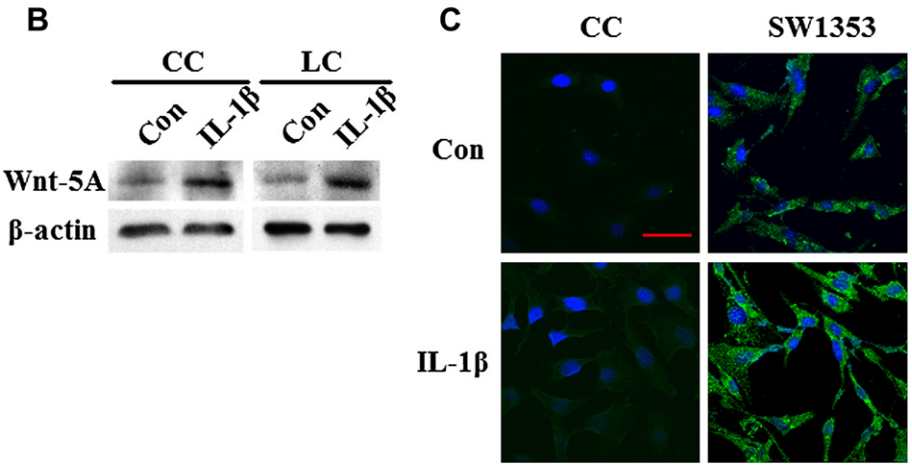


Fig. 1. Up-regulation of Wnt-5A by IL-1β in chondrocytes. (A) Wnt-5A mRNA was increased in CCs from rabbit TMJ, LCs from shoulder and hip joints and SW1353 human chondrosarcoma cells after treatment with IL-1β (10 ng/ml) for 24 h. The Wnt-5A mRNA expression was determined by real-time RT-PCR. Data presented were the mean and 95% CI of three independent experiments. (B) The expression of Wnt-5A in CCs and LCs was analyzed by Western blotting after treatment with IL-1β (10 ng/ml) for 48 h. (C) The intensity of immunofluorescence staining for Wnt-5A protein in CCs and SW1353 significantly increased after treatment with IL-1β for 24 h (Scale bar = 50 μm). Con = control.

Results

Up-regulation of Wnt-5A by IL-1β in CCs

Induction of Wnt-5A expression in response to IL-1β stimuli was observed in the primary CCs from TMJ and in the primary LCs by real-time RT-PCR and Western blotting [Fig. 1(A, B)]. The up-regulation of Wnt-5A mRNA expression by IL-1β was also shown in SW1353 human chondrosarcoma cells by real-time RT-PCR [Fig. 1(A)].

Immunocytofluorescence showed that the Wnt-5A immunostaining was significantly increased in the cytoplasm of the CCs and SW1353 cells after treatment with IL-1β for 24 h, showing the concordance of the effect of both the mRNA and protein levels [Fig. 1(C)].

Blocking IL-1β induction of Wnt-5A with IκBα phosphorylation inhibitor

To explore whether the NF-κB pathway was involved in the induction of Wnt-5A by IL-1β, the primary chondrocytes were treated with IL-1β in the presence of a highly specific pharmacologic inhibitor of IκBα phosphorylation (BAY 11-7082). The efficacy of BAY 11-7082 was confirmed with an NF-κB-dependent luciferase reporter assay [Fig. 2(A)] or by examining the phosphorylation of IκBα with Western blotting analysis [Fig. 2(B)]. The induction of Wnt-5A expression by IL-1β in CCs and LCs was dose-dependently blocked by the IκBα specific inhibitor determined by real-time RT-PCR and Western blotting [Fig. 2(C, D)]. Similar results were also observed in SW1353 cells [Fig. 2(C)].

Induction of Wnt-5A expression by overexpression of NF-κB p65

To further confirm the involvement of NF-κB in the induction of Wnt-5A by IL-1β, the primary chondrocytes were transiently transfected with a vector containing the NF-κB p65 gene. The transfection efficiency for primary chondrocytes with Vigofect was confirmed using a green fluorescence protein (GFP)-containing plasmid [Fig. 3(A)]. The effectiveness of p65 transfection was confirmed by luciferase assay [Fig. 3(B)]. Real-time RT-PCR and Western blotting results showed that overexpression of p65 in CCs and LCs significantly induced Wnt-5A expression compared with transfection of an empty vector [Fig. 3(C, D)]. Similar results were also observed in SW1353 cells [Fig. 3(C)].

Recruitment of NF-κB p65 to Wnt-5A promoter after IL-1β treatment

According to the sequences of the Wnt-5A promoter in human, mouse and rat, there is a conserved NF-κB p65 binding consensus located 50 bp upstream of the transcription start site of Wnt-5A in all three species [Fig. 4(A)]. To examine whether p65 could be recruited to the promoter of Wnt-5A after IL-1β stimuli, a ChIP assay was conducted in SW1353 cells treated with IL-1β for 90 min. The results showed that p65 could directly bind to the Wnt-5A promoter after IL-1β treatment [Fig. 4(B)].

IL-1β secretion not affected by Wnt-5A in CCs

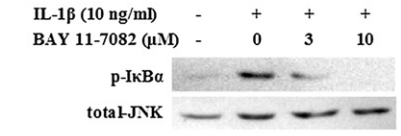
To investigate whether there was positive feedback between Wnt-5A and IL-1β, the level of IL-1β was determined by ELISA in the

A

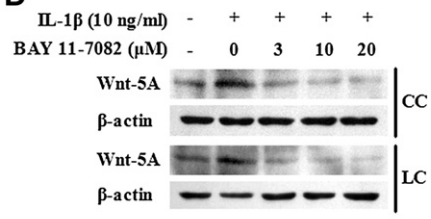
Inhibition of NF-κB activity by BAY 11-7082

NF-κB-Luc	BAY 11-7082 (μM)	Luciferase activity ratio	P-value vs 0 μM
+	0	0.82 (0.67, 0.96)	-
+	3	0.56 (0.21, 0.91)	0.0438
+	10	0.44 (0.35, 0.52)	0.0006
+	20	0.28 (0.20, 0.37)	0.0002

B



D



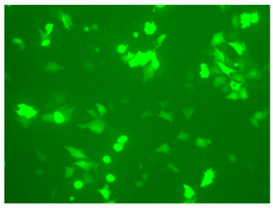
C

Fold change in Wnt-5A gene expression

IL-1β	BAY 11-7082 (μM)	CC		LC		SW1353	
		$2^{-\Delta\Delta Ct}$	P-value	$2^{-\Delta\Delta Ct}$	P-value	$2^{-\Delta\Delta C}$	P-value
-	-	1	-	1	-	1	-
+	0	2.49 (2.04, 2.94)	0.0001	3.25 (1.94, 4.56)	0.0018	3.26 (1.57, 4.94)	0.0045
+	3	1.65 (0.19, 3.11)	0.0776*	1.50 (0.80, 2.20)	0.0071*	1.27 (0.54, 2.00)	0.0096*
+	10	1.06 (0.33, 1.80)	0.0020*	0.72 (0.05, 1.48)	0.0020*	0.82 (0.37, 1.27)	0.0038*
+	20	0.55 (0.42, 0.68)	<0.0001*	0.42 (0.08, 0.76)	0.0008*	0.61 (0.31, 0.91)	0.0026*

Fig. 2. Blocking IL-1β induction of Wnt-5A with IκBα phosphorylation inhibitor. (A) The suppressive effect of BAY 11-7082 on NF-κB activity was confirmed with an NF-κB-Luc reporter assay. (B) The efficacy of BAY 11-7082 was confirmed by examining the phosphorylation of IκBα with Western blotting analysis. (C) BAY 11-7082 dose-dependently blocked the induction of Wnt-5A by IL-1β in chondrocytes. CCs, LCs and SW1353 cells were stimulated with IL-1β (10 ng/ml) for 24 h in the presence of BAY 11-7082 or dimethyl sulfoxide as solvent control. Wnt-5A mRNA was analyzed by real-time RT-PCR. (D) The expression of Wnt-5A protein in CCs and LCs was analyzed by Western blotting after treatment with IL-1β (10 ng/ml) for 48 h. Data presented were the mean and 95% CI of three independent experiments. * = comparison vs "0 μM".

A

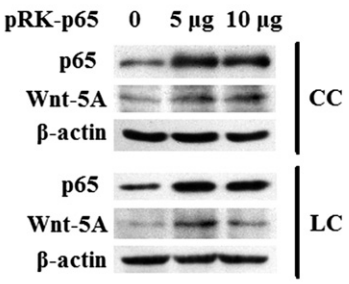


B

Activation of NF-κB activity by transfecting pRK-p65

Plasmids	Luciferase activity ratio	P-value
Control + NF-κB-Luc	0.12 (0.10, 0.14)	-
pRK-p65 + NF-κB-Luc	0.72 (0.42, 1.02)	0.0010

D



C Fold change in Wnt-5A gene expression

Plasmids	CC		LC		SW1353	
	$2^{-\Delta\Delta Ct}$	P-value	$2^{-\Delta\Delta Ct}$	P-value	$2^{-\Delta\Delta Ct}$	P-value
Control	1	-	1	-	1	-
pRK-p65 (5 μg)	2.64 (2.10, 3.19)	<0.0001	4.40 (3.65, 5.14)	<0.0001	1.96 (1.50, 2.43)	0.0008
pRK-p65 (10 μg)	4.14 (2.64, 5.64)	<0.0001	3.98 (3.20, 4.76)	<0.0001	2.57 (1.48, 3.67)	0.0036

Fig. 3. Induction of Wnt-5A expression by overexpression of NF-κB p65. (A) The transfection efficiency for primary CCs with VigFect was confirmed using a GFP-containing plasmid. The image was taken 48 h after trasfection. (B) The effectiveness of overexpression of p65 was confirmed by luciferase assay after cotransfection of pRK-p65 and NF-κB-Luc. (C) CCs of TMJ, LCs and SW1353 cells were transfected with empty vectors or vectors containing p65 (pRK-p65). Wnt-5A expression was analyzed by real-time RT-PCR. (D) The protein levels of p65 and Wnt-5A after transfection of pRK-p65 in CCs and LCs were determined by Western blotting. Data presented were the mean and 95% CI of three independent experiments.

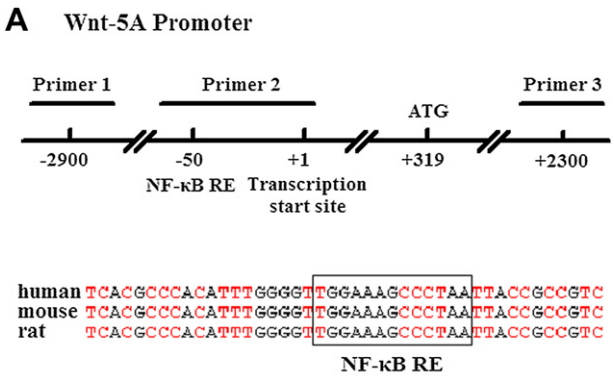


Fig. 4. Recruitment of NF-κB p65 to Wnt-5A promoter after IL-1β treatment. (A) The consensus binding sequence for NF-κB (TTAGGGCTTTCCA) is located 50 bp upstream of the transcription start site of the Wnt-5A promoter (top). The binding sequence for NF-κB in the Wnt-5A promoter is conserved (bottom). (B) ChIP analysis showed that p65 could be recruited to the Wnt-5A promoter after treatment with IL-1β (10 ng/ml) for 90 min (Primer 2). ChIP analysis with control primers against distinct promoter regions revealed no recruitment of p65 (Primer 1 and Primer 3). Input = chromatin input before immunoprecipitation, Anti-p65 = immunoprecipitated chromatin with anti-p65 antibody, IgG = immunoprecipitated chromatin with control IgG.

culture media of the primary CCs treated with Wnt-5A for different times. MMP-1 in the culture media, which served as the positive control, increased time-dependently after treatment with Wnt-5A. In contrast, IL-1β in the culture media was not affected by the treatment with Wnt-5A (Table I).

Discussion

In the present study, we showed that the induction of Wnt-5A expression by IL-1β in chondrocytes was mediated through the NF-κB pathway. To the best of our knowledge, this is the first report to demonstrate that the NF-κB pathway is involved in IL-1β induction of Wnt-5A in chondrocytes. We are also the first to demonstrate that IL-1β could induce Wnt-5A expression in human chondrosarcoma cells (SW1353). Although Wnts are secreted proteins, the cytoplasmic staining for Wnt-5A in the SW1353 cells is clear, which could be explained by that SW1353 is a chondrosarcoma cell line, and up-regulation of Wnt-5A expression has been associated with various cancers with clear cytoplasmic Wnt-5A staining[29,30]. Further experiments are needed to confirm the expression of Wnt-5A in the cell line.

NF-κB has been shown to play a crucial role in the process of OA, leading to cartilage destruction and articular damage[31]. In articular chondrocytes, NF-κB activation mediates responses to important proinflammatory cytokines [for example, IL-1β and tumor necrosis

Table I
The levels of MMP-1 and IL-1β in the culture media were determined by ELISA after treatment with Wnt-5A (200 ng/ml) for different times. Data presented were the mean and 95% CI of three independent experiments

Wnt-5A (200 ng/ml) Time (h)	MMP-1		IL-1β	
	Absorbance (A)	*P*-value	Absorbance (A)	*P*-value
0	0.15 (0.12, 0.18)	–	0.13 (0.03, 0.22)	–
12	0.20 (0.13, 0.28)	0.0463	0.14 (0.05, 0.23)	0.6682
24	0.24 (0.17, 0.32)	0.0105	0.12 (0.10. 0.15)	0.7658
48	0.28 (0.16, 0.39)	0.0098	0.12 (0.09, 0.15)	0.7184

factor-α (TNF-α)], including a decrease in the expression of chondrocyte specific genes (collagen type II, link protein gene)[32], and an increase in the expression of MMPs (MMP-1, MMP-3, MMP-13)[33], cytokines (IL-6, IL-8) and chemokines[34]. NF-κB is also involved in the regulation of apoptosis in articular chondrocytes, primarily exerting anti-apoptosis effects[31,35]. In the current study, we showed that BAY 11-7082, a specific inhibitor of IκBα-phosphorylation, abrogated the induction of Wnt-5A by IL-1β. Indeed, the inhibitor also blocks the up-regulation of Wnt-5A by TNF-α in human macrophages[22]. Furthermore, activation of NF-κB by overexpression of p65 up-regulated the expression of Wnt-5A and p65 could be recruited to the promoter of Wnt-5A after IL-1β treatment. These results suggest that Wnt-5A may be an essential target of NF-κB activation in chondrocytes.

There is an abundance of data from developmental and cellular models suggesting that the Wnt signaling pathway may interact with several other pathways, such as the bone morphogenetic protein (BMP) pathway, Hedgehog (Hh) pathway, Notch pathway and fibroblast growth factor (FGF) pathway[36]. Several studies have also demonstrated that NF-κB could interplay with β-catenin and GSK-3β, two key components of the canonical Wnt signaling pathway[37–41]. In addition to these studies, we have provided data in this study to show an important functional crosstalk between Wnt-5A and NF-κB signaling pathways in chondrocytes.

Although Wnt-5A has been shown to stimulate the release of IL-1β in human macrophages[42], our results showed that the release of IL-1β was not influenced by Wnt-5A in the CCs. Therefore, Wnt-5A promoting the release of IL-1β might be in a cell-type specific manner.

In conclusion, we have shown that the induction of Wnt-5A expression by IL-1β is through the NF-κB pathway in chondrocytes, suggesting an important functional crosstalk between Wnt-5A and NF-κB signaling pathways. This result provides new insight into the involvement of Wnt signaling in the destruction of cartilage in arthritis.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Xuchen Ma and Dr. Juanhong Meng had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ge, Xuchen Ma, Meng, Kangtao Ma, Zhou.

Acquisition of data. Ge, Gan, Zhang.

Analysis and interpretation of data. Ge, Xuchen Ma, Meng, Gan.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Dr Hongbing Shu for providing the p65 expression vector and Xinghua Liu, Yi Liu, Zhiling Zhang, Ting Guo, and Jiaji Wang for technical assistance. This research was supported by the National Natural Science Foundation of China (Grant Number: 30772439).

References

1. Hashimoto M, Nakasa T, Hikata T, Asahara H. Molecular network of cartilage homeostasis and osteoarthritis. Med Res Rev 2008;28(3):464–81.
2. Bauge C, Attia J, Leclercq S, Pujol JP, Galera P, Boumediene K. Interleukin-1beta up-regulation of Smad7 via NF-kappaB activation in human chondrocytes. Arthritis Rheum 2008;58(1):221–6.
3. Fan Z, Bau B, Yang H, Aigner T. IL-1beta induction of IL-6 and LIF in normal articular human chondrocytes involves the ERK, p38 and NFkappaB signaling pathways. Cytokine 2004;28(1):17–24.
4. Maier R, Bilbe G, Rediske J, Lotz M. Inducible nitric oxide synthase from human articular chondrocytes: cDNA cloning and analysis of mRNA expression. Biochim Biophys Acta 1994;1208(1):145–50.
5. Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol 2002;2(10):725–34.
6. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell 2002;109(2):S81–96.
7. Nusse R. Wnt signaling in disease and in development. Cell Res 2005;15(1):28–32.
8. Chien AJ, Conrad WH, Moon RT. A Wnt survival guide: from flies to human disease. J Invest Dermatol 2009;129(7):1614–27.
9. van Amerongen R, Mikels A, Nusse R. Alternative wnt signaling is initiated by distinct receptors. Sci Signal 2008;1(35):re9.
10. Corr M. Wnt-beta-catenin signaling in the pathogenesis of osteoarthritis. Nat Clin Pract Rheumatol 2008;4(10):550–6.
11. Luyten FP, Tylzanowski P, Lories RJ. Wnt signaling and osteoarthritis. Bone 2009;44(4):522–7.
12. Lodewyckx L, Lories RJ. WNT Signaling in osteoarthritis and osteoporosis: what is the biological significance for the clinician? Curr Rheumatol Rep 2009;11(1):23–30.
13. Meng J, Ma X, Ma D, Xu C. Microarray analysis of differential gene expression in temporomandibular joint condylar cartilage after experimentally induced osteoarthritis. Osteoarthritis Cartilage 2005;13(12):1115–25.
14. Church V, Nohno T, Linker C, Marcelle C, Francis-West P. Wnt regulation of chondrocyte differentiation. J Cell Sci 2002;115(Pt 24):4809–18.
15. Kawakami Y, Wada N, Nishimatsu SI, Ishikawa T, Noji S, Nohno T. Involvement of Wnt-5a in chondrogenic pattern formation in the chick limb bud. Dev Growth Differ 1999;41(1):29–40.
16. Yang Y, Topol L, Lee H, Wu J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development 2003;130(5):1003–15.
17. Sen M, Chamorro M, Reifert J, Corr M, Carson DA. Blockade of Wnt-5A/frizzled 5 signaling inhibits rheumatoid synoviocyte activation. Arthritis Rheum 2001;44(4):772–81.
18. Sen M. Wnt signalling in rheumatoid arthritis. Rheumatology (Oxford) 2005;44(6):708–13.
19. Nakamura Y, Nawata M, Wakitani S. Expression profiles and functional analyses of Wnt-related genes in human joint disorders. Am J Pathol 2005;167(1):97–105.
20. Ryu JH, Chun JS. Opposing roles of WNT-5A and WNT-11 in interleukin-1beta regulation of type II collagen expression in articular chondrocytes. J Biol Chem 2006;281(31):22039–47.
21. Ge X, Ma X, Meng J, Zhang C, Ma K, Zhou C. Role of Wnt-5A in interleukin-1beta-induced matrix metalloproteinase expression in rabbit temporomandibular joint condylar chondrocytes. Arthritis Rheum 2009;60(9):2714–22.
22. Blumenthal A, Ehlers S, Lauber J, Buer J, Lange C, Goldmann T, *et al*. The Wingless homolog WNT5A and its receptor Frizzled-5 regulate inflammatory responses of human mononuclear cells induced by microbial stimulation. Blood 2006;108(3):965–73.
23. Dissanayake SK, Olkhanud PB, O'Connell MP, Carter A, French AD, Camilli TC, *et al*. Wnt5A regulates expression of

tumor-associated antigens in melanoma via changes in signal transducers and activators of transcription 3 phosphorylation. Cancer Res 2008;68(24):10205–14.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods 2001;25(4):402–8.
25. Huang J, Teng L, Li L, Liu T, Li L, Chen D, *et al*. ZNF216 Is an A20-like and IkappaB kinase gamma-interacting inhibitor of NFkappaB activation. J Biol Chem 2004;279(16):16847–53.
26. Gan Y, Shen YH, Wang J, Wang X, Utama B, Wang J, *et al*. Role of histone deacetylation in cell-specific expression of endothelial nitric-oxide synthase. J Biol Chem 2005;280(16):16467–75.
27. Zhang CG, Jia ZQ, Li BH, Zhang H, Liu YN, Chen P, *et al*. Beta-Catenin/TCF/LEF1 can directly regulate phenylephrine-induced cell hypertrophy and Anf transcription in cardiomyocytes. Biochem Biophys Res Commun 2009;390(2):258–62.
28. Zhang H, Yi X, Sun X, Yin N, Shi B, Wu H, *et al*. Differential gene regulation by the SRC family of coactivators. Genes Dev 2004;18(14):1753–65.
29. Da Forno PD, Pringle JH, Hutchinson P, Osborn J, Huang Q, Potter L, *et al*. WNT5A expression increases during melanoma progression and correlates with outcome. Clin Cancer Res 2008;14(18):5825–32.
30. Yamamoto H, Oue N, Sato A, Hasegawa Y, Yamamoto H, Matsubara A, *et al*. Wnt5a signaling is involved in the aggressiveness of prostate cancer and expression of metalloproteinase. Oncogene 2010;29(14):2036–46.
31. Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage 2006;14(9):839–48.
32. Seguin CA, Bernier SM. TNFalpha suppresses link protein and type II collagen expression in chondrocytes: role of MEK1/2 and NF-kappaB signaling pathways. J Cell Physiol 2003;197(3):356–69.
33. Vincenti MP, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res 2002;4(3):157–64.
34. Pulai JI, Chen H, Im HJ, Kumar S, Hanning C, Hegde PS, *et al*. NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. J Immunol 2005;174(9):5781–8.
35. Relic B, Bentires-Alj M, Ribbens C, Franchimont N, Guerne PA, Benoit V, *et al*. TNF-alpha protects human primary articular chondrocytes from nitric oxide-induced apoptosis via nuclear factor-kappaB. Lab Invest 2002;82(12):1661–72.
36. Chen Y, Alman BA. Wnt pathway, an essential role in bone regeneration. J Cell Biochem 2009;106(3):353–62.
37. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature 2000;406 (6791):86–90.
38. Umar S, Sarkar S, Wang Y, Singh P. Functional cross-talk between beta-catenin and NFkappaB signaling pathways in colonic crypts of mice in response to progastrin. J Biol Chem 2009;284(33):22274–84.
39. Cho M, Gwak J, Park S, Won J, Kim DE, Yea SS, *et al*. Diclofenac attenuates Wnt/beta-catenin signaling in colon cancer cells by activation of NF-kappaB. FEBS Lett 2005;579(20):4213–8.
40. Sun J, Hobert ME, Duan Y, Rao AS, He TC, Chang EB, *et al*. Crosstalk between NF-kappaB and beta-catenin pathways in bacterial-colonized intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 2005;289(1):G129–37.
41. Carayol N, Wang CY. IKKalpha stabilizes cytosolic beta-catenin by inhibiting both canonical and non-canonical degradation pathways. Cell Signal 2006;18(11):1941–6.
42. Pereira C, Schaer DJ, Bachli EB, Kurrer MO, Schoedon G. Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the antiinflammatory action of activated protein C and interleukin-10. Arterioscler Thromb Vasc Biol 2008;28(3):504–10.