Published in final edited form as:
Osteoarthritis Cartilage. 2013 November ; 21(11): 1766–1773. doi:10.1016/j.joca.2013.08.010.

Interleukin 6 Mediates Select Effects of Notch in Chondrocytes

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Abstract

Objective—Notch receptors determine cell fate by regulating transcription, an event mediated by the Notch intracellular domain (NICD), which is generated by proteolysis brought about by Notch-ligand interactions. Since Notch activation or exposure to interleukin (Il)6 have similar effects in chondrocytes, we explored whether Il6 contributes to the mechanisms of Notch action in these cells.

Method—NICD was overexpressed in primary chondrocytes from *Rosa*^{Notch} mice, where the *Rosa26* promoter precedes a *loxP*-flanked STOP cassette followed by the NICD coding sequence. Cells were infected with adenoviral vectors expressing Cre to induce NICD or green fluorescent protein as control. Gene expression was determined by quantitative reverse-transcription polymerase chain reaction. Il6 protein concentration in the culture media was determined by enzyme-linked immunosorbent assay. To test the mechanisms of Notch action on *Il6* expression, cells were transfected with a fragment of the *Il6* promoter or control vector pGL3, or transcriptionally arrested with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Il6 was inhibited with a neutralizing antibody, whereas a normal immunoglobulin G was used as control.

Results—NICD induced Il6 mRNA and protein, and transactivated the *Il6* promoter without affecting *Il6* mRNA stability. Il6 neutralization had no impact on gene expression under basal conditions, and did not modify the effects of NICD on sex determining region-Y-related high mobility group-box gene 9, collagen type II α1 and collagen type X α1 expression. Conversely, Il6 neutralization opposed aggrecan (*Acan*) suppression and prevented matrix metalloprotease (*Mmp*)*13* induction by NICD.

Conclusion—Il6 mediates suppression of *Acan* and induction of *Mmp13* expression by Notch in chondrocytes.

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COMPETING INTERESTS STATEMENT

We have nothing to disclose.

AUTHOR CONTRIBUTIONS

E.C. obtained funding, provided substantial contributions to the design of the study, revised the manuscript critically for important intellectual content and approved the final version to be submitted. S.Z. obtained funding, designed the study, acquired and analyzed data, drafted and revised the article and approved the final version to be submitted. S.Z. takes responsibility for the integrity of the work of the work as a whole, from inception to finished article.

Keywords

Notch; Mmp13; Il6; Col10a1; Acan; chondrocytes

INTRODUCTION

The Notch1 to Notch4 receptors, and the jagged (Jag)1 and 2, and delta-like (Dll)1, 3 and 4 ligands are transmembrane proteins that relay signals between adjacent cells, thereby determining cell fate and function [1]. Interactions of Notch with Jag or Dll result in sequential proteolytic cleavages by extracellular metalloproteases and by the γ-secretase protein complex, leading to the release of the intracellular domain of Notch (NICD) to the cytoplasm [2]. NICD translocates to the nucleus and associates with the DNA binding protein Epstein-Barr virus latency C-promoter binding factor 1, suppressor of hairless, and lag1 (CSL), also known as Rbpjκ in mice, and with mastermind-like proteins. Subsequently, the transcriptional repressors associated with Rbpjκ are displaced by transcriptional activators, resulting in the induction of Notch target genes, such as those encoding for hairy enhancer of split (Hes) and Hes related with YRPW-motif (Hey) [3,4].

Chondrocytes are cartilage forming cells of mesenchymal origin, and proliferation and differentiation of growth plate chondrocytes results in linear growth of long bones [5]. Chondrocytes in articular cartilage derive from mesenchymal precursors found at the articular surface, and under physiological conditions, these cells express proteoglycan (*Prg*)*4*, sex determining region-Y-related high mobility group-box gene (*Sox*)*9*, aggrecan (*Acan*) and collagen type II α1 (*Col2a1*) and preserve the function of the extracellular matrix [6,7]. Changes in the synovial fluid concentration of Prg4, and suppressed *Sox9*, *Acan* and *Col2a1* transcript levels associated to increased expression of collagen type X α1 (*Col10a1*) by articular chondrocytes, are observed when cartilage integrity is compromised [8,9,10,11]. These events are coupled to the induction of gene markers of matrix degradation, such as a disintegrin-like and metallopeptidase with thrombospondin type 1 motif (*Adamts*)*4* and matrix metalloprotease (*Mmp*)*13*, and to the expression of mediators of inflammation, such as interleukin (Il)6 [12,13,14].

Although Notch signaling suppresses chondrogenesis and differentiation of hypertrophic chondrocytes, under selected conditions, overexpression of NICD in chondrocytes induces *Col10a1* and *Mmp13* transcripts [15,16,17,18,19,20,21,22]. Increased expression of components of the Notch signaling pathway and nuclear localization of the intracellular domains of NOTCH1 and NOTCH2 are observed in human osteoarthritic chondrocytes, indicating that activation of Notch signaling is associated with cartilage degeneration [23,24,25]. Accordingly, inactivation of *Rbpjκ* in chondrocytes or suppression of Notch by a γ-secretase inhibitor prevents surgically induced cartilage degeneration, suggesting that activation of Notch signaling is detrimental to the integrity of articular cartilage [25]. However, the mechanisms that mediate the effects of Notch on chondrocyte function are understood partially.

Notch induces Il6 expression in cells of mesenchymal origin, and exposure of bovine, rabbit, and human chondrocytes to Il6 phenocopies selected effects of Notch in murine chondrocytes [26,27,28,29]. In this study, we investigated whether Notch regulates Il6 expression in chondrocytes and whether Il6 mediates the effects of Notch in these cells. To this end, primary chondrocyte-enriched cells were harvested from $Rosa^{Notch}$ mice where Cre recombination induces the expression of NICD following excision of a *loxP*-flanked STOP cassette, placed between the *Rosa26* promoter and the NICD coding sequence [30].

METHODS

Primary chondrocyte-enriched cell cultures

Rosa^{Notch} mice, generated by D.A. Melton (Harvard University, Cambridge, MA), were obtained from Jackson Laboratories (Bar Harbor, ME) [30]. Chondrocyte-enriched cells were obtained from 3 to 4 day old male and female littermate *Rosa*^{Notch} or wild-type C57BL/6 mice and cultured independently in order to retain the individual identity of the donor. The distal epiphysis of the femur, tibia, humerus, ulna and radius, and the proximal epiphysis of the tibia and ulna were dissected under a Unitron Z850 stereo microscope (Unitron, Commack, NY), and trabecular bone removed to limit contamination from osteoblastic cells. Cartilage was transferred to high glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, New York) and digested with 0.25% trypsin in 0.9 mM EDTA (Life Technologies) for 40 min at 37 C with continuous mixing. Subsequently, cartilage was exposed to DMEM containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and 200 U/ml of type II collagenase from *Clostridium histolyticum* (Worthington Biochemical Corporation, Lakewood, NJ) for 2 to 4 h at 37 C with continuous mixing. Following digestion, tissue debris was removed by straining through a 70 μm membrane and chondrocyte-enriched cells were collected by centrifugation at 500 g for 5 min. Cells were seeded at a density of 50,000 cells/cm^2 and cultured in DMEM supplemented with 10% FBS at 37 C in a humidified 5% CO_2 incubator [21,31]. Experimental protocols were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

Adenoviral infection

Primary chondrocyte-enriched cells from *Rosa*^{Notch} mice were transferred to DMEM containing 2% FBS for 1 h and exposed overnight to 100 multiplicity of infection of replication defective recombinant adenoviruses. An adenoviral vector expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter (Ad-CMV-Cre, Vector Biolabs, Philadelphia, PA) was used to excise the STOP cassette and allow NICD expression. An adenoviral vector where the CMV promoter directs expression of green fluorescent protein (GFP; Ad-CMV-GFP, Vector Biolabs) was used as control. Following infection, primary chondrocyte-enriched cells were allowed to recover for 24 to 48 h and cultured in the presence of DMEM containing 10% FBS [21]. At confluence, cultures were exposed to 100 μg/ml ascorbic acid (Sigma-Aldrich, Saint Louis, MO) to prevent loss of chondrocyte phenotype and promote acquisition of a mature chondrocyte phenotype [32]. Activation of Notch signaling in *Rosa*^{Notch} cells expressing Cre recombinase, and the effects of Notch on the chondrocyte phenotype, were confirmed in independent cultures from male or female mice and the effects of Notch were not sexually dimorphic (data not shown) [21,33,34,35,36]. Therefore, observations from cell cultures obtained from mice of either sex were pooled for analysis of results.

Cytochemical staining, enzyme-linked immunosorbent assay (ELISA) and Il6 neutralization

To determine formation of chondrogenic and mineralized nodules, cultures from *Rosa*^{Notch} mice were fixed for 10 min at room temperature in 3.7% formaldehyde in phosphate buffered saline and subsequently stained with 1% alcian blue in 3% acetic acid, or 2% alizarin red in H_2O (all from Sigma-Aldrich). Images were acquired with a Coolpix 995 digital camera mounted on an Eclipse TS100 inverted microscope equipped with contrast phase lenses (all from Nikon Inc., Melville, NY).

To explore whether NICD induces Il6 protein levels, confluent *Rosa*^{Notch} chondrocyte-enriched cells were cultured for 3 days before exposure for 24 h to DMEM containing 100

μg/ml ascorbic acid. Il6 concentration in the medium was measured with a mouse Il6 ELISA kit, in accordance to the manufacturer's instruction (BD Biosciences, San Jose, California).

In selected experiments, *Rosa*^{Notch} chondrocyte-enriched cells were exposed to DMEM for 6 h and subsequently exposed DMEM containing 1 μg/ml neutralizing murine monoclonal immunoglobulin G (IgG) against Il6 (clone B-E8; EMD Millipore, Billerica, MA) or control murine normal IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Transient transfections

To assess whether NICD regulates *Il6* transcription in chondrocytes, chondrocyte-enriched cells from *Rosa*^{Notch} mice were transiently transfected with a 1.3 kilobase (kb) fragment of the murine *Il6* promoter cloned into pGL3 basic, upstream of the luciferase coding sequence (Il6-Luc; D.L. Allen, University of Colorado Boulder, Boulder, CO) [37]. To induce Notch signaling in wild-type chondrocyte-enriched cells, a 2.4 kb DNA fragment containing the murine NICD coding sequence (J.S. Nye, Columbia University, New York City, NY) was cloned in the pcDNA 3.1 expression vector (Life Technologies, Carlsbad, CA) downstream of the CMV promoter to generate pcDNA-NICD [38]. To determine the effects of NICD on Notch transactivation in wild-type chondrocytes, cells were transiently transfected with pcDNA-NICD or with pcDNA3.1, as control, and co-transfected with a construct where six multimerized dimeric CSL binding sites linked to the β-globin basal promoter govern luciferase expression (12xCSL-Luc; L.J. Strobl, Munich, Germany)[39]. To confirm the effects of NICD on the transactivation of the *Il6* promoter, wild-type chondrocytes transiently transfected with pcDNA-NICD or with pcDNA3.1 were co-transfected with Il6-Luc. To correct for transfection efficiency, cells were co-transfected with a construct where the CMV promoter directs β-galactosidase expression (Clontech, Mountain View, CA). *Rosa*^{Notch} chondrocytes were exposed to a 3 μl/2 μg mix of FuGene6 (Roche, Indianapolis, IN) and DNA for 16 h before adenoviral infection with Ad-CMV-Cre or Ad-CMV-GFP. Wild-type chondrocyte cultures at 70% confluence were exposed to a 3 μl/2 μg mix of X-tremeGENE 9 (Roche) and DNA for 16 h. Cells were harvested after 48 h in luciferase extraction buffer (Roche), and luciferase and β-galactosidase activities were measured on an Optocomp luminometer (MGM Instruments, Hamden, CT), according to manufacturer's instructions (Roche).

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA from chondrocyte-enriched cells from *Rosa*^{Notch} mice was extracted with the RNeasy mini kit, according to manufacturer's instructions (Qiagen, Valencia, CA) and changes in gene expression determined by qRT-PCR. Half to one μg of total RNA were reverse-transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA), according to manufacturer's instructions, and amplified in the presence of specific primers (Table 1; all from IDT), and iQ SYBR Green Supermix (BioRad) at 60 C for 35 cycles. mRNA copy number was estimated by comparison to a 10-fold serial dilution of cDNA for *Acan, Adamts4*, *Col10a1, Il6*, *Mmp13* and *Sox9* (all from Thermo Scientific, Rockford, IL), *Col2a1, Hes1*, ribosomal protein l (*Rpl*)*38* (all from American Type Culture Collection, Manassas, VA), *Prg4* (Source BioScience, Nottingham, United Kingdom), *Hey1* and *Hey2* (both from T. Iso, University of Southern California, Los Angeles, CA) and Hey-like (*HeyL*; from D. Srivastava, University of Texas Southwestern Medical Center, Dallas, TX) [4,40,41,42]. Reactions were conducted in a CFX96 real time PCR detection system (BioRad), fluorescence monitored during every PCR cycle at the annealing step and specificity of the reaction assessed by analysis of melting curves.

RNA decay experiments

To establish the effects of NICD on the stability of *Il6* mRNA, RNA polymerase 2 was inhibited in confluent chondrocyte-enriched cell cultures from *Rosa*^{Notch} mice by exposure to 300 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, BioMol, Plymouth Meeting, PA). *Il6* mRNA levels at various times following the exposure to DRB were determined in total RNA extracts by qRT-PCR analysis. The slopes of mRNA decay were established by fitting the log10 of the percentage of *Il6* transcript levels corrected for *Rpl38* expression over corrected *Il6* transcripts before exposure to DRB against time by linear regression.

Statistical analysis

Estimates and associated uncertainty are expressed as means and 95% confidence interval, respectively. Each observation represents an independent culture from individual mice and is the average of 2 technical replicates. Normality of data distribution was determined with 95% confidence by the Shapiro-Wilk test. Significance of statistical differences was tested for pairwise comparisons by the Student's *t*-test with a $p < 0.05$ and for multiple comparisons by applying the Bonferroni correction with a $p < 0.0250$ [43]. Statistical differences between slopes of regression lines of corrected Il6 mRNA levels against time were determined by analysis of covariance [44]. Statistical analyses were performed using Microsoft Office Excel 2003 SP2 (Microsoft, Redmond, WA) or PASW software version 18.0.0 (IBM, Armonk, NY).

RESULTS

NICD induces Il6 by a transcriptional mechanism in chondrocyte-enriched cells

Exposure to Il6 recapitulates select effects of Notch in chondrocytes, and we tested whether Notch signaling regulates Il6 expression in chondrocyte-enriched cells from *Rosa*^{Notch} mice infected with Ad-CMV-Cre to induce NICD or with control Ad-CMV-GFP [26,27,28,29]. Analysis of mRNA levels by qRT-PCR revealed that activation of Notch signaling induced a 7 fold increase in *Il6* expression (Fig. 1A). To establish whether induction of *Il6* transcripts by Notch translates into increased Il6 production, levels of Il6 protein were measured by ELISA in the supernatant of *Rosa*^{Notch} chondrocytes. Il6 concentration in the culture medium of controls and of cells overexpressing NICD was about 20 pg/ml and 290 pg/ml, respectively (Fig. 1B), demonstrating that Notch caused a 15 fold increase of Il6 protein levels and confirming that activation of Notch signaling in chondrocytes induces Il6.

To study the mechanisms that mediate induction of *Il6* by Notch, the effects of Notch activation on the transactivation of a transiently transfected 1.3 kb fragment of the Il6 promoter (Il6-Luc) or on control pGL3 vector were tested in chondrocyte-enriched cells from *Rosa*^{Notch} littermate mice. Activity of pGL3 was not induced by Notch in comparison to controls, indicating that pGL3 does not contain regulatory DNA elements responsive to Notch signaling in chondrocytes. In control cells, activity of Il6-Luc was about 100-fold greater than the activity of pGL3, suggesting that under control conditions the *Il6* promoter is transactivated in chondrocytes (Fig. 1C). In accordance with the stimulatory effects of Notch on *Il6* mRNA levels, Il6-Luc was transactivated in the context of Notch induction. The extent of this effect was less pronounced than the induction of Il6 mRNA and protein levels by Notch, indicating that additional regulatory regions present in the intact *Il6* locus determine the magnitude of *Il6* induction by Notch. To assess whether Notch regulates the stability of *Il6* transcripts, *Il6* expression was determined by qRT-PCR in *Rosa*^{Notch} chondrocyte-enriched cells exposed to DRB, an inhibitor of mRNA transcription. The estimated half-life of the *Il6* transcript was 1.2 h, and Notch did not affect the rate of *Il6*

mRNA decay (Fig. 1D), indicating that post-transcriptional mechanisms do not mediate the effects of Notch on *Il6* expression.

To induce Notch activity in chondrocytes by alternate mechanisms, chondrocyte-enriched cells from wild-type C57BL/6 mice were transiently transfected with pcDNA-NICD or with control pcDNA3.1. To determine whether pcDNA-NICD activates the Notch signaling pathway, cells were co-transfected with pGL3 or with the 12xCSL-Luc reporter. Transient NICD overexpression had no impact on pGL3 activity whereas it induced 12xCSL-Luc transactivation, confirming activation of Notch signaling (Fig. 2). Under basal conditions, Il6-Luc activity was about 8 fold higher than pGL3 activity, whereas in the context of Notch induction Il6-Luc transactivation was about 4 fold higher than in the presence of pcDNA. These results suggest that the Il6 promoter has basal activity in chondrocytes and confirm that Notch induces Il6 expression by transcriptional mechanisms.

Il6 neutralization opposes the effects of NICD on Mmp13 and Acan expression

Exposure of chondrocytes to Il6 phenocopies selected effects of Notch signaling, and we asked whether Il6 mediates the effects of Notch on the expression of chondrocyte gene markers [27,28,29]. To investigate this possibility, chondrocyte-enriched cells from *Rosa*^{Notch} mice were exposed to a neutralizing murine monoclonal IgG raised against Il6 or murine IgG, either in the context of Notch activation or under basal conditions.

Notch induced *Hey1*, *Hey2* and *HeyL*, and to a lesser extent, *Hes1* transcripts, either in cells exposed to the neutralizing Il6 antibody or to normal IgG (Fig. 3), demonstrating that inhibition of Il6 does not alter Notch transactivation in chondrocyte-enriched cells. In agreement with previous results, Notch activation suppressed *Sox9* and *Col2a1* mRNA levels and induced *Col10a1* expression in cells exposed to normal IgG [20,21,45]. Similar results were obtained in the presence of the Il6 neutralizing antibody, indicating that Il6 is not required for these effects of Notch in chondrocytes. Levels of *Il6* mRNA were not affected by the neutralizing Il6 antibody, either under basal conditions or in the context of NICD overexpression, excluding the possibility that the neutralizing Il6 antibody precludes induction of *Il6* expression by Notch (Fig. 4). Inhibition of Il6 in the absence of Notch had no impact on Sox9, Col2a1 and Col10a1 expression, suggesting that basal Il6 levels are not required for expression of these chondrocyte gene markers.

Although Notch activation had no effect on *Prg4* transcripts, Notch suppressed *Acan* mRNA levels and this effect was opposed partially by Il6 neutralization, indicating that Notch inhibits expression of selected gene markers of chondrocyte function and this effect is mediated in part by Il6. Notch activation did not affect *Ad amts4* expression, whereas it increased *Mmp13* transcripts levels, confirming that under selected conditions NICD overexpression induces gene markers of cartilage matrix degradation (Fig 5) [20,21]. Neutralization of Il6 opposed the stimulatory effects of Notch on *Mmp13* expression, indicating that Il6 is required for this effect of Notch in chondrocytes (Fig. 5). Il6 neutralization in cells expressing GFP did not affect *Prg4*, *Acan*, *Adamts4* and *Mmp13* transcript levels, confirming that Il6 is dispensable for expression of selected chondrocyte gene markers.

DISCUSSION

In this study, we explored the mechanisms that mediate the regulation of gene expression by the Notch signaling pathway in chondrocytes. Several studies have established Notch as a suppressor of chondrogenesis *in vivo*, and this effect is consistent with the role of Notch as an inhibitor of mesenchymal cell differentiation [1]. However, the role of Notch signaling in differentiated cells of the chondrocyte lineage is controversial, since both suppression of

hypertrophic differentiation and elongation of the hypertrophic zone of the growth plate have been described as effects of NICD overexpression under the control of the *Col2a1* promoter [18,20,22]. The validity of the *Rosa*^{Notch} system to study the effects of Notch signaling in primary chondrocyte-enriched cell cultures was confirmed by the induction of Notch target genes following infection with Ad-CMV-Cre (data not shown). In agreement with previous findings, Notch induced expression of *Hey1*, *2* and *L* to a greater extent than *Hes1* (data not shown) [21].

We confirmed that induction of Notch signaling in primary chondrocytes, irrespective of donor sex, inhibits progression to a mature chondrocyte phenotype while inducing expression of *Col10a1* and *Mmp13* [20,21]. In previous work we reported that chondrocyte-enriched cell cultures present minimal expression of osteoblast gene markers, suggesting presence of osteoblastic cells [21]. Under selected conditions, osteoblasts express *Mmp13*, raising the possibility that increased *Mmp13* transcripts levels exhibited by chondrocyte-enriched cell cultures overexpressing NICD is due to presence of contaminating osteoblasts [46]. However, Notch activation did not increase *Mmp13* expression in primary osteoblast cultures (data not shown), indicating that the effect is selective to cells of the chondrocyte lineage. NICD overexpression led to the induction of Il6 and suppression of *Acan* mRNA levels, but did not affect *Prg4* or *Adamts4* expression. These findings indicate that Notch activation in murine chondrocytes affects selected gene markers of chondrocyte function and cartilage matrix degradation. It is important to mention that the model culture used contains both articular and growth plate chondrocytes, limiting our ability to discern the cell population that is responding to the Notch signal.

Notch transactivated the *Il6* promoter and induced release of Il6 without affecting the stability of the *Il6* mRNA, demonstrating that Notch induces Il6 expression by transcriptional mechanisms. Activity of the *Il6* promoter and expression of Il6 were observed in control cultures, and this is consistent with work from other laboratories reporting expression of Il6 by chondrocytes under basal conditions [47]. However, concentration of Il6 in the supernatant of control cells was minimal and the neutralizing Il6 antibody had no impact on chondrocyte gene markers expression in the absence of Notch, suggesting that basal levels of Il6 are dispensable for chondrocyte function. Induction of *Il6* by Notch is in agreement with previous studies carried out in bone marrow stromal cells, macrophages and breast cancer cells, and confirms that *Il6* is a direct target gene of Notch signaling [26,48,49].

Activation of the Notch signaling pathway was not altered by Il6 neutralization, excluding the possibility that the effects of Il6 inhibition in the context of Notch induction are secondary to suppression of Notch signaling. Although Il6 inhibited *Sox9* and *Col2a1* expression, Il6 neutralization did not prevent suppression of both genes by Notch in *Rosa*^{Notch} chondrocyte-enriched cells, and this is in agreement with previous studies demonstrating that HEY1 and HES1 mediate the inhibitory effects of NICD on *COL2A1* expression in human chondrocytes [27,28,29,45]. The effects of Il6 on *Col10a1* expression were not reported, and induction of *Col10a1* by Notch was not affected by Il6 neutralization, raising the possibility that *Col10a1*, similarly to *Hey* genes and *Hes1*, is a direct target of Notch signaling. In agreement with the effects of Il6 on *Mmp13* in chondrocytes, Il6 neutralization precluded *Mmp13* induction by Notch, indicating that Il6 mediates this effect of Notch. Conversely, Il6 neutralization partially opposed suppression of *Acan* transcripts by Notch in chondrocytes [27,28,29]. HEY1 overexpression inhibits *ACAN* expression in human bone marrow-derived chondrocytes, suggesting that suppression of *Acan* mRNA levels by Notch in murine cells is mediated by induction of both *Hey1* and *Il6* [45].

In conclusion, Il6 mediates induction of *Mmp13* expression by Notch and contributes to the inhibitory effect of Notch on *Acan* mRNA levels in cells of the chondrocyte lineage.

Acknowledgments

The authors thank Drs. D.A. Melton for *Rosa*^{Notch} mice, D.L. Allen for *Il6* promoter fragment, J.S. Nye for NICD cDNA and L.J. Strobl for 12xCSL-Luc. The authors also thank D. Bridgewater for technical assistance and M. Yurczak for secretarial assistance.

ROLE OF THE FUNDING SOURCE

This work was supported by Grant DK045227 from the National Institute of Diabetes and Digestive and Kidney Diseases (E.C.), and Research Fellowship 5371 from the Arthritis Foundation (S.Z.). Sponsors of this study had no role in the study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Abbreviation list

Acan	aggrecan
Ad	adenovirus
Adamts	a disintegrin-like and metallopeptidase with thrombospondin type 1 motif
CMV	cytomegalovirus
Col2a1	collagen type II α1
Col10a1	collagen type X α1
CSL	Epstein-Barr virus latency C promoter binding factor 1/suppressor of hairless/lag 1
Dll	delta-like
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
Fwd	forward
GFP	green fluorescent protein
Hes	hairy enhancer of split
Hey	hes related with YRPW motif
HeyL	Hey-like
IDT	Integrated DNA Technologies
Il6	interleukin 6
IgG	immunoglobulin G
Jag	jagged
kb	kilobase
luc	luciferase
Mmp	matrix metalloprotease
NICD	Notch intracellular domain
PCR	polymerase chain reaction

Prg	proteoglycan
qRT-PCR	quantitative reverse transcription-PCR
Rev	reverse
Rpl38	ribosomal protein l38
SEM	standard error of the mean
Sox	sex determining region-Y-related high mobility group-box gene

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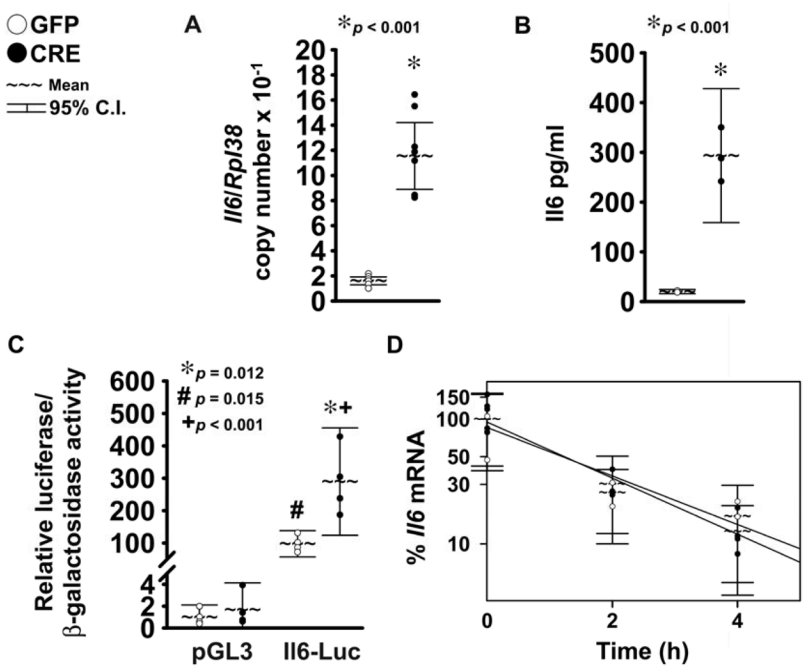

Figure 1. NICD induces Il6 expression by transcriptional mechanisms

Primary *Rosa*^{Notch} chondrocyte-enriched cells were infected with Ad-CMV-Cre (CRE, full circles) to induce NICD or with control Ad-CMV-GFP (GFP, empty circles). In panel A, total RNA was extracted 3 days after adenoviral infection, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Il6* copy number over *Rpl38* copy number. Observations represent cultures from individual mice, n = 8. * indicates differences between CRE and GFP. In panel B, Il6 concentration in the culture medium was determined by ELISA. Observations represent cultures from individual mice, n = 3. * indicates a difference between CRE and GFP. In panel C, cells were transfected with the pGL3 luciferase expression vector or with a fragment of the *Il6* promoter cloned in pGL3 (Il6-Luc), and a β-galactosidase expression vector 24 h before adenoviral infection and harvested after 48 h. Data are expressed as luciferase activity corrected for β-galactosidase activity, relative to corrected luciferase activity in control cells transfected with pGL3. Observations represent cultures from individual mice n = 4. * indicates differences between CRE and GFP. # indicates a difference between Il6-Luc and pGL3 activity in cells expressing GFP. + indicates a difference between Il6-Luc and pGL3 activity in cells expressing CRE. In panel D, osteoblasts were transcriptionally arrested with DRB (time 0), and total RNA harvested at the indicated times was amplified by qRT-PCR. Data are percentage of *Il6* mRNA copy number corrected for *Rpl38* copy number, relative to corrected expression of *Il6* at the time of exposure to DRB, plotted versus time (h). Intercept and slope of regression lines were calculated by linear regression of corrected *Il6* expression over time. Circles represent means ± SEM for 4 cultures from individual mice.

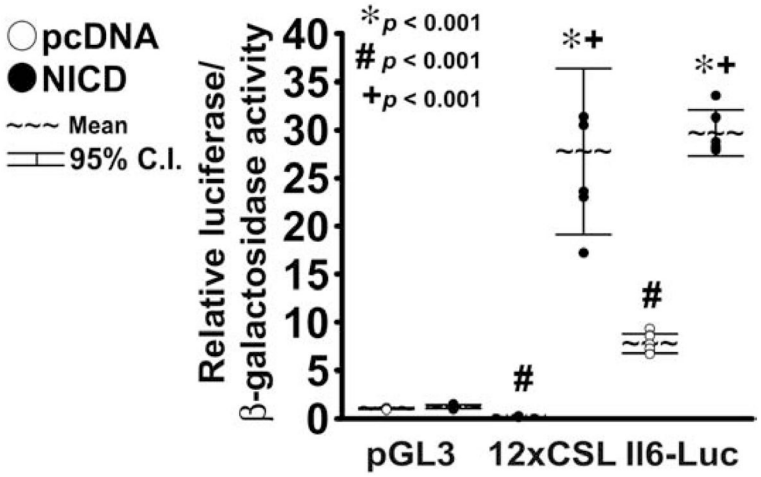

Figure 2. Transient overexpression of NICD transactivates the *Il6* promoter

Primary chondrocyte-enriched cells from littermate wild-type C57BL/6mice were transfected with pcDNA-NICD (NICD, full circles) to induce NICD or with control pcDNA3.1 (pcDNA, empty circles). Cells were co-transfected with the pGL3 luciferase expression vector, the 12-CSL-Luc reporter (12xCSL) or a fragment of the *Il6* promoter cloned in pGL3 (Il6-Luc), and a β-galactosidase expression vector. Chondrocytes were harvested after 48 h. Data are expressed as luciferase activity corrected for β-galactosidase activity, relative to corrected luciferase activity in control cells transfected with pGL3. Observations represent cultures from individual mice n = 6. * indicates differences between NICD and pcDNA. # indicates a difference between 12xCSL, or Il6-Luc, and pGL3 activity in cells transfected with pcDNA. + indicates a difference between 12xCSL, or Il6-Luc, and pGL3 activity in cells transfected with NICD.

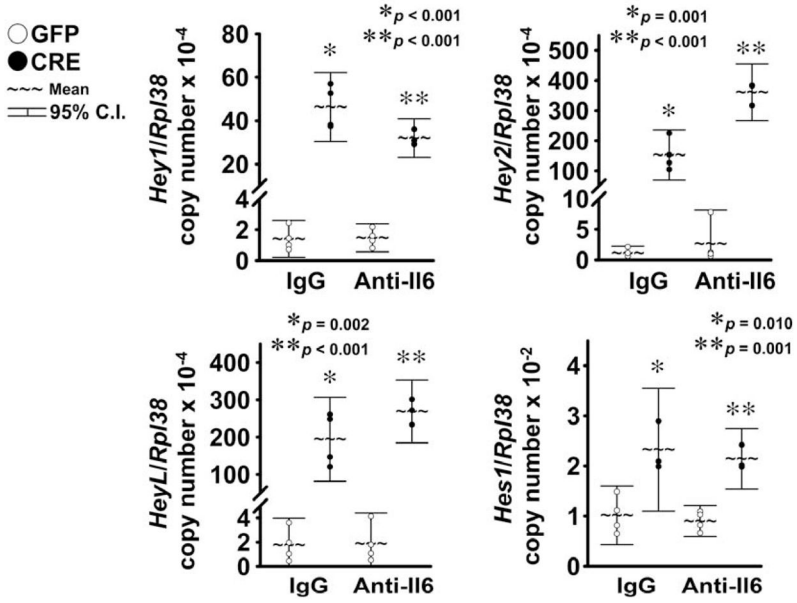

Figure 3. Il6 neutralization does not oppose induction of Notch target genes by NICD

Primary *Rosa*^{Notch} chondrocyte-enriched cells were infected with Ad-CMV-Cre (CRE, full circles) to induce NICD or with control Ad-CMV-GFP (GFP, empty circles). Cells were cultured for 3 days under conditions favoring chondrocyte maturation and serum removed for 6 h, before exposure to murine Il6 antibody (Anti-Il6) or control normal murine immunoglobulin G (IgG). Total RNA was extracted 24 h after exposure to antibodies, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Hey1*, *Hey2*, *HeyL* and *Hes1* copy number over *Rpl38* copy number. Observations represent cultures from individual mice n = 3–4. * indicates differences between CRE and GFP in the presence of IgG. ** indicates differences between CRE and GFP in the presence of Anti-Il6.

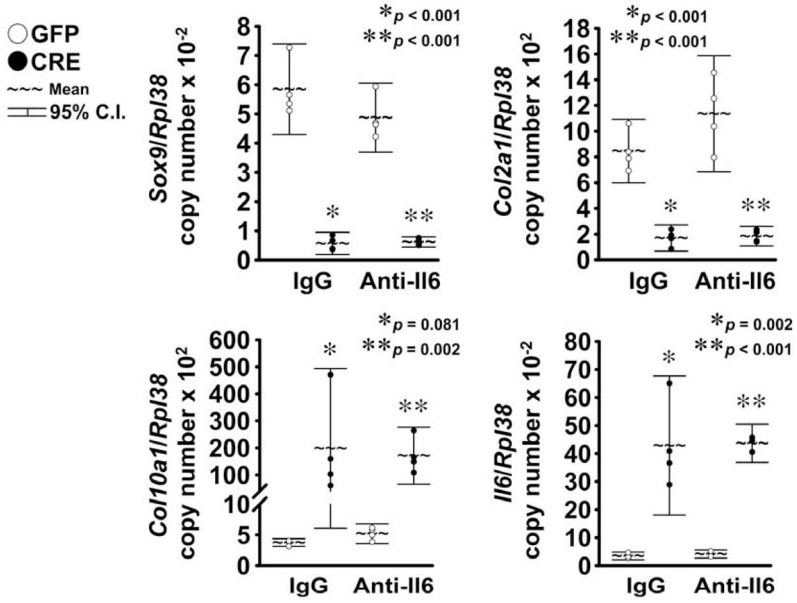

Figure 4. Il6 neutralization does not affect *Sox9* and *Col2a1* suppression and *Col10a1* and *Il6* induction by NICD

Primary *Rosa*^{Notch} chondrocyte-enriched cells were infected with Ad-CMV-Cre (CRE, full circles) to induce NICD or with control Ad-CMV-GFP (GFP, empty circles). Cells were cultured for 3 days under conditions favoring chondrocyte maturation and serum removed for 6 h, before exposure to murine Il6 antibody (Anti-Il6) or control normal murine immunoglobulin G (IgG). Total RNA was extracted 24 h after exposure to antibodies, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Sox9*, *Col2a1*, *Col10a1* and *Il6* copy number over *Rpl38* copy number. Observations represent cultures from individual mice n = 3–4. * indicates differences between CRE and GFP in the presence of IgG. ** indicates differences between CRE and GFP in the presence of Anti-Il6.

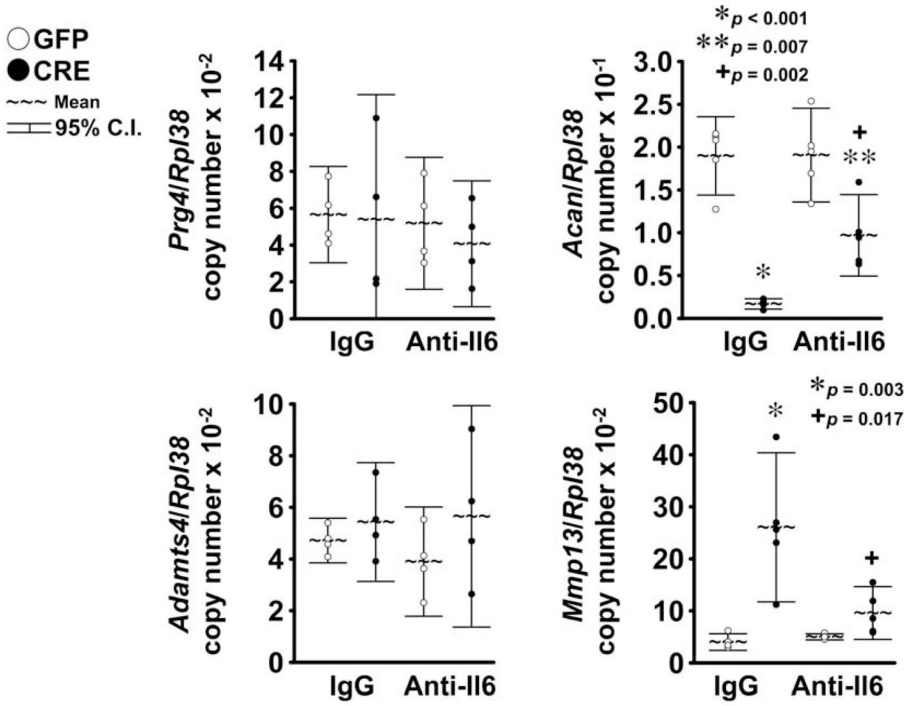

Figure 5. Il6 neutralization opposes the effects of NICD on *Acan* and *Mmp13* expression

Primary *Rosa*^{Notch} chondrocyte-enriched cells were infected with Ad-CMV-Cre (CRE, full circles) to induce NICD or with control Ad-CMV-GFP (GFP, empty circles). Cells were cultured for 3 days under conditions favoring chondrocyte maturation and serum removed for 6 h, before exposure to murine Il6 antibody (Anti-Il6) or control normal murine immunoglobulin G (IgG). Total RNA was extracted 24 h after exposure to antibodies, and changes in gene expression analyzed by qRT-PCR. Data are expressed as ratio of *Prg4*, *Acan*, *Adamts4* and *Mmp13* copy number over *Rpl38* copy number. Observations represent cultures from individual mice n = 3–5. * indicates differences between CRE and GFP in the presence of IgG. ** indicates differences between CRE and GFP in the presence of Anti-Il6. + indicates a difference between IgG and Anti-Il6 in the context of CRE infection.

Table 1

Primers used for qRT-PCR

Forward (Fwd) and reverse (Rev) primers used to determine changes in gene expression by qRT-PCR. GenBank accession numbers for the transcript variants recognized by primer pairs are indicated.

Gene	Strand	Primer Sequence	GenBank Accession Number
Acan	Fwd	5′-ATGGTCCTTCTATGACATACACTCCCCG-3′	NM_007424
	Rev	5′-TTGTTACAGCGCCACCAAGG-3′	
Adamts4	Fwd	5′-GATGTGTGCAAGCTTACCT-3′	NM_172845
	Rev	5′-CATCCGTAACCTTTGGAGA-3′	
Col2a1	Fwd	5′-GACCCAAACACTTTCCAACCGCAGT-3′	NM_031163; NM_003396
	Rev	5′-TCATCAGGTCAGGTCAGCCATT-3′	
Col10a1	Fwd	5′-CAGGCTTTCTGGGATGCCGCTTGT-3′	NM_009925
	Rev	5′-GGGCACCTACTGCTGGGTAA-3′	
Hes1	Fwd	5′-ACCAAAGACGGCCTCTGAGCACAGAAAGT-3′	NM_008235
	Rev	5′-ATTCTTGCCCTTCGCCTCTT-3′	
Hey1	Fwd	5′-ATCTCAACAACTACGCATCCCAGC-3′	NM_010423
	Rev	5′-GTGTGGGTGATGTCCGAAGG-3′	
Hey2	Fwd	5′-AGCGAGAACAATTACCCTGGGCAC-3′	NM_013904
	Rev	5′-GAGGTAGTTGTCGGTGAATTGG-3′	
HeyL	Fwd	5′-CAGTAGCCTTTCTGAATTGCGAC-3′	NM_013905
	Rev	5′-GCTTGGAGGAGCCCTGTTTCT-3′	
Il6	Fwd	5′-CGGCCTTCCCTACTTCACAAGTCCG-3′	NM_031168
	Rev	5′-CAGGTCTGTTGGGAGTGGTATCC-3′	
Mmp13	Fwd	5′-GGAAGACCTTGTGTTTGCAGAGC-3′	NM_008607
	Rev	5′-CACTGTAGACTTCTTCAGGATTCCCG-3′	
Prg4	Fwd	5′-CGCCTTTTCCAAAGATCAATACTA-3′	NM_021400; NM_001110146
	Rev	5′-GTGGTAATTGCTCTTGCTGTT-3′	
Rpl38	Fwd	5′-AGAACAAGGATAATGTGAAGTTCAAGGTTC-3′	NM_001048057; NM_001048058; NM_023372
	Rev	5′-CTGCTTCAGCTTCTCTGCCTTT-3′	
Sox9	Fwd	5′-CCTACTACAGTCACGCAGCCG-3′	NM_011448
	Rev	5′-GGGTTCATGTAAGTGAAGGTGGA-3′	