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The transcription factor AP-2ε regulates CXCL1 during cartilage development and in osteoarthritis

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SUMMARY

Objective: Recently, the transcription factor AP-2ε was shown to be a regulator of hypertrophy in cartilage and to be differentially expressed in osteoarthritis (OA). However, the only known target gene of AP-2ε up to date is *integrin alpha10*. To better characterize the function of AP-2ε in cartilage we screened for additional target genes.
Design: Promoter analysis, ChIP-assays and electrophoretic mobility shift assay were used to characterize the regulation of a new AP-2ε target gene in detail.
Results: In this study, we determined the chemokine CXCL1, already known to be important in osteoarthritis (OA), as a new target gene of AP-2ε. We could confirm that CXCL1 is expressed in chondrocytes and significantly over-expressed in OA-chondrocytes. Transient transfection of chondrocytes with an AP-2ε expression construct led to a significant increase of the CXCL1 mRNA level in these cells. We identified three potential AP-2 binding sites within the *CXCL1* promoter and performed luciferase assays, indicating that an AP-2 binding motif (AP-2.2) ranging from position −135 to −144 bp relative to the translation start is responsive to AP-2ε. This result was further addressed by site-directed mutagenesis demonstrating that activation of the *CXCL1* promoter by AP-2ε is exclusively dependent on AP-2.2. Chromatin immunoprecipitation and electromobility shift assays confirmed the direct binding of AP-2ε to the *CXCL1* promoter in OA-chondrocytes at this site.
Conclusion: These findings revealed *CXCL1* as a novel target gene of AP-2ε in chondrocytes and support the important role of AP-2ε in cartilage.

Introduction

The family of AP-2 (activating enhancer-binding protein-2) transcription factors consists of five members: AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε. They have a conserved basic-helix-span-helix DNA binding and dimerization domain at the C-terminus and a less conserved proline and glutamine-rich transactivation domain at the N-terminus[1–4]. For the regulation of target gene expression AP-2 proteins bind to the palindromic recognition sequence 5′-GCCN$_{3/4}$GGC-3′ or variations (e.g. 5′-GCCN$_{3/4}$GGG) within multiple gene promoters[5]. *In vitro* and *in vivo* analyses of AP-2 knockout mice demonstrated the importance of *AP-2* genes in numerous physiological processes during development, cell-cycle regulation and cell survival[1,5].

AP-2ε is the last identified AP-2 transcription factor[6,7]. Expression of AP-2ε was first described in the olfactory system[6], in skin and in *in vitro* cultured keratinocytes[7]. In previous studies we could show that AP-2ε is also expressed in chondrocytes[8]. There it regulates the expression of integrin alpha10 which is the predominant type II collagen-binding integrin during cartilage development. Furthermore, we found that AP-2ε plays a role in hypertrophic cartilage during cartilage differentiation and shows increased expression in osteoarthritic cartilage[9]. Additionally, we could identify Sox9 as a direct regulator of AP-2ε expression[9].

The axial skeleton is formed by endochondral bone formation. This complex process starts with the migration of undifferentiated mesenchymal cells to regions which are defined to differentiate into bones. These progenitor cells condense and stick together without increased proliferation[10,11]. They start to produce an extracellular matrix (ECM) containing type I collagen, hyaluronic acid, tenascin and fibronectin[12–14]. Subsequent differentiation of the mesenchymal cells to chondrocytes causes a change of ECM composition. Chondrocytes express cartilage specific type II, type IX and type XI collagen, proteoglycans, aggrecan and cartilage derived

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retinoic acid sensitive protein (CD-RAP) whereas expression of type I collagen stops. After further steps of differentiation chondrocytes become hypertrophic and express increased levels of type X collagen and reduce the expression of type II collagen[15–17]. Finally, osteoblasts infiltrate the cartilage and start to displace it with mineralized bone. Cartilage, which mainly consists of chondrocytes and ECM, serves as a protective layer for the joints. Degradation of the articular cartilage is a major problem in osteoarthritis (OA), a degenerative joint disorder, leading to destruction of the cartilage. The onset of the disease might be triggered by multiple factors such as mechanical overload, defects in the composition of the ECM or altered expression of transcription factors controlling the production of matrix molecules[18].

In OA-chondrocytes several chemokines are up-regulated, including the CXC chemokine CXCL1 (C-X-C motif ligand 1) also known as growth-regulated protein α (GROα)[19]. CXCL1 belongs to the ELR-family of chemokines containing the characteristic amino acid sequence glutamic acid–leucine–arginine immediately before the first cysteine of the CXC motif. Initially, CXCL1 was characterized by its growth stimulatory activity on malignant melanoma cells but several other functions have been described including its association with angiogenesis and inflammation[20]. During cartilage development CXCL1 induces chondrocyte hypertrophy and apoptosis[21,22].

In the present study we demonstrate that *CXCL1* is a novel target gene of the transcription factor AP-2ε in hypertrophic chondrocytes and OA-chondrocytes.

Material and methods

Cell culture

Normal chondrocytes were isolated from healthy cartilage which was obtained following the standards of the Ethics Commission of the University of Regensburg or obtained from Cambrex (Iowa, USA) and cultured as suggested as described previously[9]. The proliferating cells are dedifferentiated in culture. To differentiate these cells they were stimulated with TGF-ß1 (10 ng/ml) over 1 week.

Human OA-chondrocytes were prepared from osteoarthritic cartilage slices which were obtained from patients who underwent total knee arthroplasty and have been giving informed consent following the standards of the Ethics Commission of the University of Regensburg. OA severity was evaluated as “end stage” for all individuals undergoing joint replacement surgery according to an arthroscopic score of grade III to IV[23]. Diagnosis of OA was performed by orthopedic surgeons according to classification criteria (clinical, radiographical and biochemical) of the American College of Rheumatology (ACR)[24]. Full thickness cartilage slices were aseptically dissected from healthy aspects of femoral condyles of patients aged 50–76 years. Specifically, prior to isolation of chondrocytes cartilage tissue was first classified macroscopically as either “damaged” or “intact” according to a predefined procedure comprising color, surface integrity and tactile impression tested with a standard scalpel. Accordingly, diseased areas showed a coloration that was rather yellow than white. They showed erosions and discrete protuberances that rendered it impossible to smoothly move the scalpel over the tissue surface. In unaffected regions there was a stronger resistance when cutting deeper into the tissue with the blade of the scalpel, which could not be done in the softened lesional areas[25]. For this study, we have prepared only cartilage slices classified as “damaged” and isolated chondrocytes from those cartilage regions.

Human mesenchymal stem cells (HMSC) from CellSystems (St. Katharinen, Germany) were cultivated in mesenchymal stem cell growth medium (MSCGM) medium (CellSystems) under a humidified atmosphere of 5% CO_2 at 37°C[26]. To stimulate HMSC to either chondrogenic or osteoblastic differentiation cells were seeded in a 15 ml falcon and treated as previously described[9].

All primary cells used (normal chondrocytes, OA-chondrocytes and HMSC) were characterized for expression of Collagen type II (Coll2A1), Collagen type X (Coll10A1), matrix metalloproteinase-13 (MMP-13) and melanoma inhibitory activity (MIA)/CD-RAP to confirm cell-specific expression pattern[25] (Suppl. Fig. 1).

The chondrosarcoma cell line SW1353 was obtained from the American Type Culture Collection (ATCC, #HTB-94). Cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum (Sigma, Deisenhofen, Germany) and split at a 1:5 ratio every 4 days.

RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (PCR)

For isolation of total cellular RNA from cultured cells or from tissue the RNeasy kit (QIAGEN, Hilden, Germany) was used. cDNAs were generated as described previously[9].

For a precise quantification of cDNA expression the real-time PCR LightCycler system (Roche) was used as described previously[27,28]. Specific primer pairs were used for the qRT–PCR analysis of *AP-2ε* and *CXCL1* expression: *AP-2ε-for*: 5′-GAA ATA GGG ACT TAG CTC TTG G-3′ and *AP-2ε-rev*: 5′-CCA AGC CAG ATC CCC AAC TCT G-3′, *CXCL1_for*: 5′-CCC AAG AAC ATC CAA AGT GTG-3′ and *CXCL1_rev*: 5′-GTT GGA TTT GTC ACT GTT CAG C-3′. To calculate expression ratios of the analyzed genes an internal control standard curve of β-actin levels was used.

Plasmid constructs

The AP-2ε expression construct (in pCMX-PL1) was a kind gift from M. Moser (Max-Planck-Institute of Biochemistry, Martinsried, Germany).

To analyze the *CXCL1* promoter for putative transcription factor-binding sites, we screened approximately 1.5 kb of DNA of the upstream regulatory region, using the MATINSPECTOR (Genomatix Software GmbH, Munich, Germany). We determined three potential AP-2 binding sites (AP-2.1/2.2/2.3).

To obtain the different promoter constructs used for luciferase assays human genomic regions of the *CXCL1* promoter were amplified by PCR with specific primer pairs and cloned into luciferase expression vectors. The following promoter constructs were generated: *CXCL1prom128* (−17 to −128), *CXCL1prom299* (−17 to −299), *CXCL1prom1448* (−17 to −1448) in pGL3basic and *CXCL1enh* (−1213 to −1448) in pGL3prom.

Mutation of the potential AP-2 binding sites within the *CXCL1* promoter constructs was carried out with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, USA) according to the manufacturer’s instructions. For mutation of the first binding site (AP-2.1) the following primers were used: *CXCL1prom_AP-2.1mut_for*: 5′-GAG ACA TCT GCC CCT GCA GCA GAA GCC TCT G-3′ and *CXCL1prom_AP-2.1mut_rev: 5′*-CAG AGG CTT CTG CTG CAG GGG CAG ATG TCT C-3′. For mutation of the second binding site (AP-2.2) the following primers were used: *CXCL1prom_AP-2.2mut_for: 5′*-GGA ATT TCC CTG GTA CGG GGG CTC CGG G-3′ and *CXCL1prom_AP-2.2mut_rev: 5′*-CCC GGA GCC CCC GTA CCA GGG AAA TTC C-3′.

Transient transfection and luciferase assay

Transient transfection of primary chondrocytes with the AP-2ε expression construct was performed using the Amaxa Nucleofector

System (Amaxa GmbH, Cologne, Germany) with the Amaxa Human Chondrocyte Nucleofector Kit according to the manufacturer's instructions (program: U24). Cells were harvested 24 h later, and RNA was isolated.

DNA transfection of the SW1353 cells was performed using Lipofectamine plus (Invitrogen, Carlsbad, CA, USA) as described previously[9].

Luciferase assays were performed as follows. Cells were cultured in six-well plates for 24 h and then treated with cationic lipid/plasmid DNA suspension: 0.5 μg of luciferase reporter plasmid (*CXCL1* promoter constructs or an empty pGL3basic vector as a control), 0.1 μg of the internal control plasmid pRL-TK and optionally 0.05 μg AP-2ε expression plasmid or pCMX-PL1. 24 h after the transfection cells were harvested and the lysate was analyzed for luciferase activity with a luminometer using Promega dual-luciferase assay reagent (Promega Corporation, Madison, WI, USA). At least three independent transfection experiments were performed for each construct.

Preparation of nuclear extracts

Nuclear extracts from osteoarthritic chondrocytes were prepared by the method of Dignam *et al.*[29].

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously[9] and based on the binding of nuclear AP-2ε to ^{32}P-labeled oligonucleotides containing an AP-2ε binding site within the promoter of the analyzed AP-2ε target gene *CXCL1*. The following double-stranded oligomeric binding site for AP-2ε was generated: *CXCL1_oligo*: 5′-AATTTCCCTGGCCCGGGGGCT
CCGGGCTT-3′. This fragment corresponds to a promoter region −154 to −126 upstream of the ATG of *CXCL1* spanning the second AP-2ε binding site (AP-2.2). To demonstrate specificity of binding of AP-2ε a specific antiserum against AP-2ε[30] and, as a control, an unrelated antibody was used.

Chromatin immunoprecipitation assay (ChIP)

The ChIP assay was performed using the ChIP-IT™ Express kit following the manufacturer's instructions (Active Motif, Carlsbad, USA) and as previously described[9]. Chromatin isolation was performed with chondrocytes from OA patients and samples were immunoprecipitated with a specific primary AP-2ε antiserum[30]. An RNA polymerase II antibody was used as a positive control, and an IgG antibody as a negative control, following the protocol provided with the control kit (ChIP-IT control Kit-human; Active Motif). Four DNA templates from the ChIP experiments were used for analysis by PCR: input DNA (1:5), DNA isolated by RNA polymerase II ChIP (Pol II), by the negative control IgG ChIP (IgG), by the AP-2ε ChIP (AP-2ε). A control reaction with no DNA template was also performed (H_2O). Three sets of specific primer pairs were used for the PCR reaction: The glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the negative control primer pairs provided in the kit and a primer pair spanning the second AP-2ε binding site (AP-2.2) within the promoter of *CXCL1*: *CXCL1_prom2_for*: 5′-CCC AAG CGC TCC ACC CTG-3′ and *CXCL1_prom2_rev*: 5′-CTC TCC GAG ATC CGC GAA C-3′ (145 bp fragment). PCR fragments were analyzed on an 1.5% agarose gel.

Statistical analysis

Results are expressed as mean ± SD (range) or percent. Comparison between groups was made using the Student's paired *t*-test. A P value < 0.05 was considered statistically significant. All calculations were performed using the GraphPad Prism software (GraphPad software Inc, San Diego, USA).

Results

To determine the role of AP-2ε in cartilage differentiation, especially in hypertrophic chondrocytes, we searched for genes harbouring binding sites for AP-2 within their promoter sequences and showing an increased expression in hypertrophic and osteoarthritic chondrocytes. After an extensive literature study we used the MatInspector Software of Genomatix to perform promoter analysis for several candidate genes. This screening is based on known consensus binding sites for transcription factors. We analyzed several candidate genes and finally focused on the chemokine CXCL1. To confirm the expression of CXCL1 in healthy and osteoarthritic chondrocytes we performed quantitative real-time PCR. As expected, CXCL1 is expressed in normal chondrocytes. In OA-chondrocytes the expression is significantly increased up to 43.3-fold ($P = 0.0084$). To further analyze the influence of AP-2ε on CXCL1 expression we transiently transfected chondrocytes with an AP-2ε expression construct. Forced AP-2ε expression leads to significant 1.9-fold increase ($P = 0.049$) of CXCL1 expression in these cells (data not shown). Those results indicate that *CXCL1* could be activated by the transcription factor AP-2ε.

To further analyze whether AP-2ε and CXCL1 are co-expressed during chondrocyte differentiation, we used an *in vitro* model system for HMSC differentiation into chondrocytes established in our lab. Marker genes for different stages of chondrogenesis like *Coll2A1*, *Coll10A1*, *CD-RAP* and *aggrecan* were analyzed to demonstrate differentiation stages[9]. Using this model system the expression of AP-2ε and CXCL1 mRNA was followed by quantitative RT-PCR over 40 days. Interestingly, the expression of both molecules increased coordinately during late chondrogenic differentiation starting at day 15 (data not shown).

To get further insight into the regulatory mechanisms leading to up-regulation of CXCL1 in osteoarthritic chondrocytes we studied the promoter of *CXCL1*. We focused on 1500 bp upstream of the translation start site. Analysis of this region with the MatInspector software offered three potential AP-2 binding motifs located at base pair −1390 to −1398 (AP-2.1), −135 to −144 (AP-2.2) and −59 to −67 (AP-2.3) relative to the translation start (ATG) (Fig. 1).

To test whether AP-2ε can activate the *CXCL1* promoter we performed luciferase assays with different promoter constructs. First a *CXCL1* promoter construct ranging from position −17 to −1448 bp including all three predicted AP-2 binding sites was cloned into a reporter gene plasmid containing a promoter-less luciferase gene (*CXCL1prom1448*). SW1353 cells were transiently transfected with this plasmid and luciferase activity was measured. Compared to the control (pGL3basic) *CXCL1prom1448* shows strongly increased promoter activity (Fig. 2). Co-transfection of an AP-2ε expression plasmid results in a 2.5-fold increase of luciferase activity of *CXCL1prom1448* while the control plasmid is not activated. This assay confirms that the 1448 bp region of the *CXCL1* promoter is indeed responsive to AP-2ε.

To test whether one, two or all three of the identified AP-2 binding sites are involved in this activation process we designed the following promoter constructs: *CXCL1prom128* (−17 to −128) which contains only AP-2.3 and *CXCL1prom299* (−17 to −299) including AP-2.2 and AP-2.3. *CXCL1prom128* exhibits far less promoter activity compared to the 1448 construct, whereas *CXCL1prom299* is even slightly more active (Fig. 2). Forced AP-2ε expression does not increase the activity of the 128 construct implying that AP-2.3 is no binding site of AP-2ε. On the other hand co-transfection of AP-2ε does increase the activity of the 299

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-1500   CTAAATGCA GGTGGCAGCC TTGCCTGTGT TCTCTGCTTG GCTAGGAACA CAGGTCTTAC
-1440   CTATTGAGCT GGGCTGTGTA GAACTCTGTT GTGGAGACAT CTGCCCCTGG GGCAGAAGCC
                                                    AP-2.1
-1380   TCTGCTTTTT CCCCCTCCTC CCATCTTACT CCATGTCTCA GAGAGCTCTG AATCCCACTT
-1320   GGAGAATCAC ACTTAAACCC TCTAAAAACC TAATGATGAA TAAAAATAAG TTCTCTAGAA
-1260   CTTCTGGAGA AAAAAGTAAT AAAGCTACCA GGTTAAATGA CTGAAATTCC TGAGAGAAAA
-1200   CAACATGTGT GTGTTTCTCT AGAAAGGGGG CCCAATACTG AATACCAGGA AGTCCTATAG
-1140   TAAATGGAAT GTGACTCTAT GTGGGATCCG GCGTTCCTAT TTCATCCGAA TGCATGTCTG
-1080   CTGCTTCAGT GGGAAGGGTG CTTGCACACC AGGTACCCAC TCCCTGGTGT CATGTGCTAT
-1020   GCAGTCCAAA GACAGAACCA GGAATGGTGA GCCCATGAGC CTGCTGGACC CAGCCCCTCC
-960    GAGGTCCGGA GTGACAACCA GTGCCGTATT TCTAGATCAA ACCTGAACCC CTCCTACAGG
-900    GAAAAGATTT CCAGGGGATT TTGAAAGTTC CAACATTTTA CAGGGAAGAA GGAAGATAAG
-840    CAGGATATGA AAGAAGAGTT CATGTTATAC AGCCCTGGCT TCCACTGACG CTAACACTGG
-780    ATTCAGCTTT TGACACTGAT AATCTGTTGC CACCAAATGG AAAACGTAAA CAAGATATTC
-720    TAAGTGTGGT TAGAGAATAT GCAACACAAG GAACAAGCAG AACATTCTTC TCTGGAATCT
-660    GACATAATGG ACTGTACTTT CACAGACAGC ACTGATGTTA GATGTACGTG AAATAGGCTA
-600    AACTGAAAAT AAGAAAGGCT GAGGCAGAGA GGATAATATA GCTCCAGCCT ATCTCCCAGC
-540    ACCTTGTTAA TTTCTCTCAA TCTCCAGCCA CAAATCCGAG ACACAACGCT CTTCCTCCAA
-480    AGAGGTCGCG CCTTCTCTGT GGTGGTTCTC AGGGATCCGC CCCAGCTCCT TCTCCGTTCC
-420    CAGCCCCACA CACTGGGATC ACCAGGCACC CAAGATCCCA CCTCTCAGGT GGTATCTTCA
-360    GCGCAGGCTG CCACTCAGCC CCCCTCCAGG GATCTGGGGC AGAAGGCGAA TATCCCAGAG
-300    TCTCAGAGTC CACAGGAGTT ACTCTGAAGG GCGAGGCGCG GGCTGCATCA GTGGACCCCC
-240    ACACCCCACC CGCACCCCAA GCGCTCCACC CTGGGGGCGG GGCCGTCGCC TTCCTTCCGG
-180    ACTCGGGATC GATCTGGAAC TCCGGGAATT TCCCTGGCCC GGGGGCTCCG GGCTTTCCAG
                                                    AP-2.2
-120    CCCCAACCAT GCATAAAAGG GGTTCGCGGA TCTCGGAGAG CCACAGAGCC CGGGCCGCAG
                  TATA Box                                      AP-2.3
-60     GCACCTCCTC GCCAGCTCTT CCGCTCCTCT CACAGCCGCC AGACCCGCCT GCTGAGCCCC
        ATG
```

Fig. 1. Schematical overview of the *CXCL1* promoter.
Putative AP-2 binding sites are marked within the sequence.

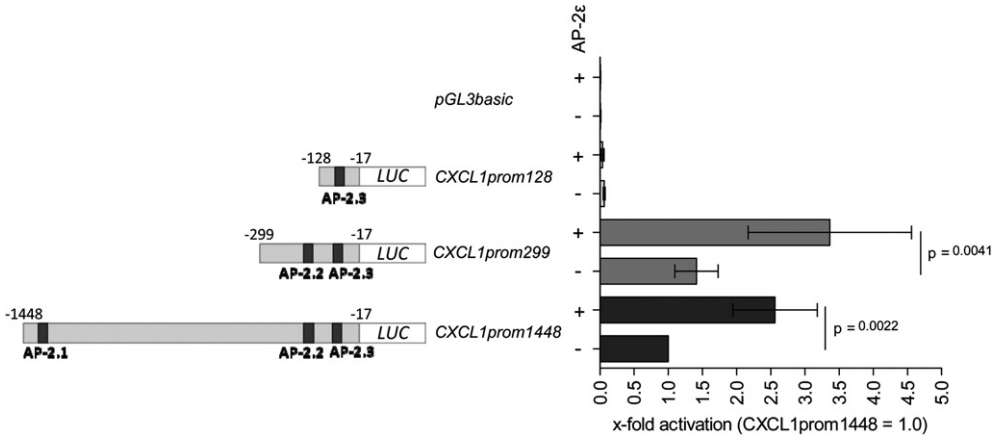

Fig. 2. Regulation of the *CXCL1* promoter by AP-2ε.
Promoter constructs ranging from −17 up to −128, −299 and −1448, respectively, were sub-cloned into pGL3basic, and promoter activity was analyzed in SW1353 cells. Additionally, an expression construct for AP-2ε was co-transfected into SW1353 cells and promoter activity was measured. *CXCL1prom1448* is set as one. Data are given as means. 95% confidence intervals were used to represent uncertainty of estimates.
All experiments were repeated at least three times.

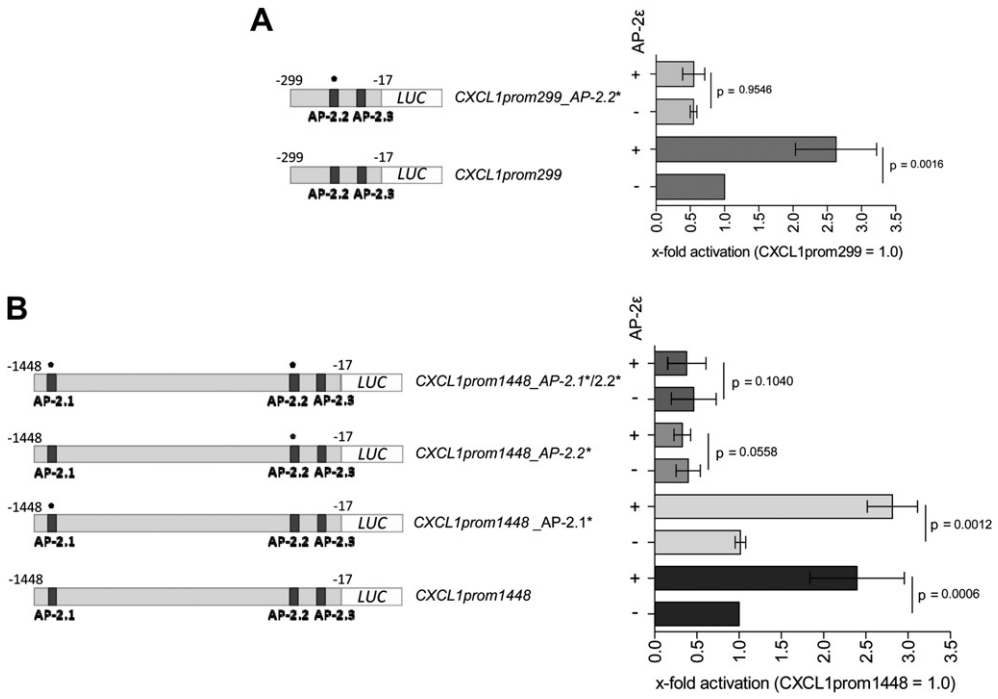

Fig. 3. Mutagenesis of AP-2 binding sites in the *CXCL1* promoter.
CXCL1 promoter constructs with or without mutated AP-2 binding sites were sub-cloned into pGL3basic, and promoter activity was analyzed in SW1353 cells. Additionally, an expression construct for AP-2ε was co-transfected into SW1353 cells, together with the *CXCL1* promoter constructs, and promoter activity was measured. A: Analysis of the 299 promoter construct containing a mutated AP-2.2 site. *CXCL1prom299* is set as one B: Analysis of the 1448 promoter construct containing a mutated AP-2.1 and/or AP-2.2 site. *CXCL1prom1448* is set as one. Data are given as means. 95% confidence intervals were used to represent uncertainty of estimates. All experiments were repeated at least three times.

construct, suggesting that AP-2.2 might indeed be an AP-2ε binding site (Fig. 2).

To address this question we mutated AP-2.2 in *CXCL1prom299*, and AP-2.1 and/or AP-2.2 in *CXCL1prom1448* and measured luciferase activity. The mutation of AP-2.2 in the 299 construct (*CXCL1prom299_AP-2.2**) leads to decreased promoter activity compared to the wild-type 299 construct [Fig. 3(A)]. Even more interesting, co-transfection of an expression plasmid for AP-2ε results in no increase of promoter activity when AP.2.2 is mutated whereas the wild-type 299 construct is significantly induced by AP-2ε. This demonstrates that AP-2ε activates the *CXCL1* promoter at AP-2.2.

Mutation of AP-2.1 ranging from base pair −1390 to −1398 in the 1448 construct (*CXCL1prom1448_AP-2.1**) does not lower the luciferase activity of this construct compared to the wild-type 1448 construct. Co-transfection of AP-2ε still significantly increases promoter activity by about 2.7-fold [Fig. 3(B)]. On the other hand, individual mutation of AP-2.2 (*CXCL1prom1448_AP-2.2**) as well as in conjunction with AP-2.1 (*CXCL1prom1448_AP-2.1*/2.2**) strongly reduces promoter activity and leads to a promoter sequence that cannot be induced by AP-2ε anymore [Fig. 3(B)].

To confirm that AP-2.1 is not responsive to AP-2ε we analyzed the site independently of AP-2.2. Therefore, we cloned a 235 bp fragment ranging from base pair −1213 to −1448 into a reporter gene plasmid containing a luciferase gene with a SV40 promoter (*CXCL1enh*) and carried out luciferase assays once more. As can be seen in Fig. 4 the control vector and *CXCL1enh,* respectively, are not induced by AP-2ε.

To demonstrate the direct binding of AP-2ε to the AP-2.2 site in the *CXCL1* promoter we performed electromobility shift assays with

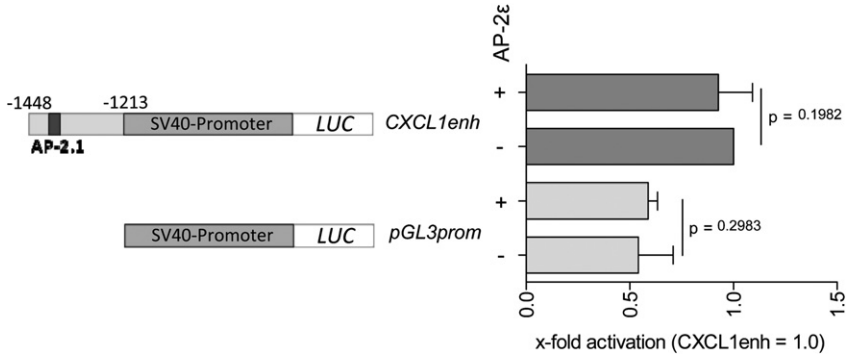

Fig. 4. Regulation of the distal *CXCL1* promoter region by AP-2ε.
A *CXCL1* promoter construct (*CXCL1enh*) from position −1213 to −1448 bp containing the potential AP-2 binding site AP-2.1 was sub-cloned into pGL3prom, and promoter activity was analyzed in SW1353 cells. Additionally, an expression construct for AP-2ε was co-transfected into SW1353 cells and promoter activity was measured. *CXCL1enh* is set as one. Data are given as means. 95% confidence intervals were used to represent uncertainty of estimates. All experiments were repeated at least three times.

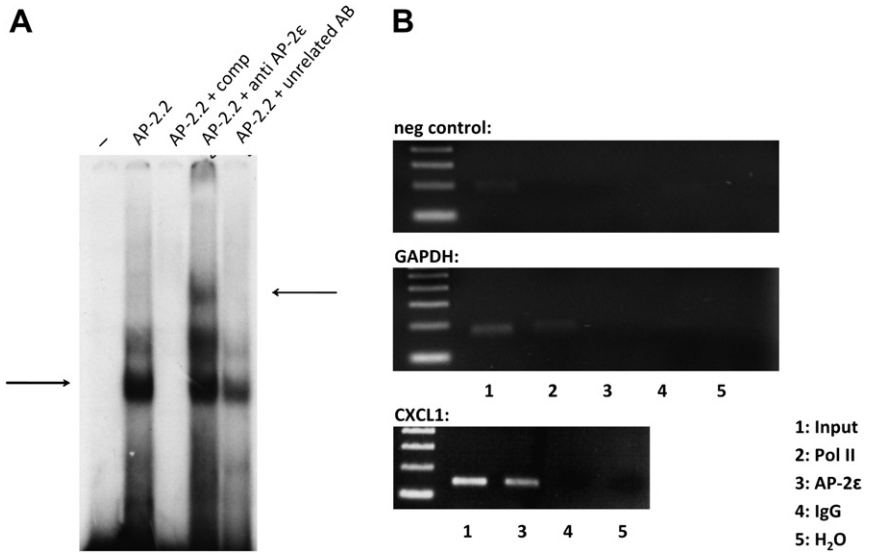

Fig. 5. Direct binding of AP-2ε to the *CXCL1* promoter. A: Electromobility shift assay to demonstrate the binding of AP-2ε to the *CXCL1* promoter. AP-2ε binding was shown using labeled oligonucleotides spanning the second AP-2 binding site AP-2.2 (lane 2) incubated with nuclear extract of osteoarthritic chondrocytes. For competition experiments unlabeled oligonucleotides (lane 3) were used in excess. A specific AP-2ε antibody was used to confirm the specificity of AP-2ε binding (lane 4) whereas no binding to an unrelated antibody was observed (lane 5). B: A ChIP assay demonstrates the direct binding of AP-2ε to the *CXCL1* promoter. DNA samples of ChIP reactions (Pol II, IgG, and AP-2ε) and input DNA were used in PCRs with different primer pairs (GAPDH, negative control primers, AP-2ε). All PCR fragments could be detected in the input DNA sample. A PCR product was generated using the AP-2ε ChIP DNA (CXCL1, lane 3).

a labeled oligonucleotide spanning the second potential AP-2 binding site (AP-2.2). Figure 5(A) depicts that incubation of oligonucleotide AP-2.2 with nuclear extract of OA-chondrocytes leads to the generation of a complex (lane 2). Incubation with an excess of unlabeled oligonucleotides (comp) could completely displace this complex (lane 3). The specificity of AP-2ε binding was confirmed by incubation with a specific AP-2ε antibody (lane 4) leading to the generation of a supershifted complex whereas using an unrelated antibody no supershift was observed. These data demonstrate that the transcription factor AP-2ε binds to the AP-2 binding site AP-2.2 within the *CXCL1* promoter.

Additionally, the direct binding of AP-2ε to the *CXCL1* promoter was supported by chromatin immunoprecipitation assays with chromatin isolated from osteoarthritic chondrocytes. Again, binding of AP-2ε to the AP-2.2 binding site of the *CXCL1* promoter could be revealed [Fig. 5(B)].

Discussion

Recently, we could show that the transcription factor AP-2ε is a positive regulator of integrin alpha10 expression in chondrocytes[8]. The aim of this study was to specify the processes during cartilage development induced by AP-2ε. Therefore, we looked for other AP-2ε target genes in chondrocytes besides *integrin alpha10*. Using this strategy, we hoped to learn more about the functional relevance of AP-2ε for chondrogenesis and the development of the cartilage disease OA.

The promoter of the chemokine *CXCL1* contains three potential AP-2 consensus binding sites (AP-2.1/2.2/2.3). We revealed that only the AP-2 binding site at position −135 to −144 bp (AP-2.2) is important. Our results, therefore, demonstrate that the predicted binding site AP-2.2 is necessary and sufficient for the activation of the *CXCL1* promoter by AP-2ε.

Performing electromobility shift assays and ChIP assays the direct binding of AP-2ε to the *CXCL1* promoter at AP-2.2 could be confirmed. Enhanced expression of AP-2ε in chondrocytes leads to an increased expression of CXCL1 in these cells. The results shown here point out that the transcription factor AP-2ε is a positive regulator of *CXCL1* in chondrocytes.

CXCL1 is already known to be expressed in chondrocytes and also the functional role of CXCL1 during chondrocyte differentiation is well analyzed[21]. Together with IL-8 this chemokine induces hypertrophic differentiation of articular chondrocytes and calcification through p38 MAPK activation and transglutaminase 2[21]. This leads to an induction of apoptosis which is dependent on additional signals from the ECM and matrix degrading activity[31,22]. Furthermore, in OA, chondrocyte hypertrophy and apoptosis can cause dysregulation of matrix repair by alteration in collagen subtype and MMP expression, and in cell viability[32,33].

The expression of AP-2ε is induced by the transcription factor Sox9 in the beginning of hypertrophic cartilage development[9]. This study now demonstrates that AP-2ε activates CXCL1 expression. This induction of CXCL1 expression *via* AP-2ε leads to differentiation and calcification of articular chondrocytes and in case of OA can force matrix degradation. Further studies are necessary to confirm the functional role of AP-2ε as a regulator of more target genes important for late chondrocyte differentiation. In summary, our data clearly show that the transcription factor AP-2ε activates CXCL1 expression in chondrocytes and osteoarthritic chondrocytes through direct binding to the *CXCL1* promoter.

Author contributions

A.W. and S.N. conducted all experimental work in the study. A.K.B. and A.W. designed the study. A.K.B. supervised the project. All authors contributed to analysis and interpretation of the data and in writing the manuscript.

Conflict of interest

There is no conflict of interest.

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Supplementary material

Supplementary data associated with this article can be found in online version at doi:10.1016/j.joca.2010.11.011.

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