CCAAT/enhancer binding protein β regulates expression of matrix metalloproteinase-3 in arthritis

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CCAAT/Enhancer Binding Protein β Regulates Expression of Matrix Metalloproteinase-3 in Arthritis.

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ABSTRACT

Objectives: To investigate whether CCAAT/enhancer-binding protein β (C/EBPβ) mediates expression of matrix metalloproteinase-3 (MMP-3) and aggrecanases in arthritis.

Methods: Localization of C/EBPβ and MMP-3 in synovium and cartilage from rheumatoid arthritis and osteoarthritis patients was determined by immunohistochemistry. Cell lines, SW982, C28/I2 and human fibroblast-like synoviocytes stimulated by IL-1β were subjected to western blotting and quantitative polymerase chain reaction. Over-expression of C/EBPβ by adenovirus was performed in cells and organ culture of normal cartilage. Knockdown of C/EBPβ by small interference RNA was performed in cells. Activity of the human MMP-3 and aggrecanase-2 (ADAMTS-5) promoters was analyzed by a luciferase assay. To determine whether C/EBPβ directly binds to the MMP-3 or ADAMTS-5 promoter, a chromatin immunoprecipitation (ChIP) assay was performed.

Results: Immunohistochemistry showed that C/EBPβ and MMP-3 were co-localized in arthritic synovium and cartilage. Western blots revealed increased C/EBPβ expression in cells treated with IL-1β. Expression of MMP-3, MMP-13, and ADAMTS-5 mRNA was significantly increased by the over-expression of C/EBPβ. C/EBPβ stimulated MMP-3 expression and induced matrix degradation in cartilage explants. C/EBPβ knockdown reduced MMP-3 and ADAMTS-5 expression. C/EBPβ stimulated the 2011 bp MMP-3 promoter and the 1768 bp ADAMTS-5 promoter in a dose-dependent manner. Deletion and mutation analysis of the MMP-3 promoter revealed that the C/EBPβ core responsive element was located between -108 bp and -100 bp. A ChIP assay showed that C/EBPβ directly bound to MMP-3 and ADAMTS-5 promoters.

Conclusions: These data demonstrate that C/EBPβ is involved in expression of MMP-3 and ADAMTS-5 in arthritic synovium and cartilage.

INTRODUCTION

Irreversible destruction of cartilage is a feature of arthritis such as rheumatoid arthritis (RA) and osteoarthritis (OA). Inflammation pathways are involved in the catabolic processes of articular cartilage especially in RA and potentially in OA. Proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) play significant roles in mediating inflammation and joint destruction and have been therapeutic targets. Although the levels of proinflammatory cytokines in OA are generally lower than those in RA [1], inflammatory reactions are associated with symptoms of OA especially in the early stages of the disease.[2] Stimulation of these cytokines in arthritis activates numerous transcriptional factors and induces expression of matrix metalloproteinases (MMPs), aggrecanases, and other catabolic factors in the synovium, synovial fluids and cartilage, which adversely affects the joint structures including cartilage.[3, 4]

Among transcriptional factors activated by proinflammatory cytokines, we focused on the functions of the CCAAT/enhancer binding protein (C/EBP) family in arthritis. The C/EBP family contains a basic leucine zipper domain and consists of six family members: C/EBPα, β, δ, ε, γ, and ζ.[5] Functions of the C/EBP family include regulation of numerous cellular processes including differentiation, proliferation, control of metabolism, and inflammation. C/EBPβ and/or δ are induced in response to inflammatory stimulation in various tissues.[6] Previous reports describe that C/EBPβ and δ are expressed in synovial tissues of RA and OA.[7, 8] Recent reports suggest that C/EBPβ may act via three catabolic pathways during the development of arthritis. First, C/EBPβ directly enhances inflammatory enzymes. C/EBPβ and/or δ mediate inflammatory genes such as phospholipase A2 and cyclooxygenase-2 in articular cartilage.[9, 10] Second, C/EBPβ regulates extracellular matrix (ECM) synthesis. C/EBPβ and δ repress type I collagen [11] and activate osteocalcin in osteoblasts.[12] Also, C/EBPβ and δ induced by IL-1β mediate the repression of

cartilage-derived retinoic acid-sensitive protein (CD-RAP) and Col2α1 promoter activity in chondrocytes.[13] Third, C/EBPβ mediates the expression of catabolic enzymes in arthritis. MMP-1 is transactivated by C/EBPβ in chondrocytes through IL-1β-stimulated extracellular signal regulated kinase (ERK) activation.[14] A previous report demonstrated that expression of MMP-13 is mediated by C/EBPβ in human chondrocytes in inflammatory arthritis.[15] These findings suggest that C/EBPβ and δ plays important roles in various catabolic processes in arthritis. Further analysis of the functions of C/EBP is important for understanding the pathogenesis of arthritis.

MMPs are known to be important factors in degrading native collagen and proteoglycans and leading to the development of arthritis. Aggrecanases, also known as A Disintegrin And Metalloproteinase with ThromboSpondin type 1 motifs (ADAMTS), are proteolytic enzymes that are responsible for cleavage of the proteoglycan aggrecan. In humans, aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) are considered to be major contributors to the development of OA.[16, 17] Matrix metalloproteinase 3 (MMP-3) is also highly expressed in synovial cells and chondrocytes of inflammatory arthritis.[18, 19] MMP-3 has a variety of substrate specificities and degrades proteoglycans, collagen types III, IV, IX and XI, gelatin, laminin, and fibronectin.[20] Furthermore, MMP-3 can activate procollagenases MMPs-1, -8, and -13.[21] When OA was experimentally induced in MMP-3 knock-out mice, there was a 67% reduction in the occurrence of severe cartilage damage when compared with control mice.[22] In clinical practice, serum levels of MMP-3 are used as a marker for predicting disease activity and progression in RA and potentially in OA.[23, 24] Thus, MMP-3 plays a pivotal role in the pathological destruction of cartilage in both RA and OA.

In this paper, we investigated the functional involvement of C/EBPβ for regulating catabolic enzymes, specifically MMP-3 and ADAMTS-5, in synovium and

cartilage. Manifestation of the mechanisms related to the regulation of proteolytic enzymes by activation of C/EBPβ may provide new insights into the development of potential therapies.

MATERIALS AND METHODS

Detailed methods are described in the online supplement.

Clinical samples. Tissue samples of articular cartilage and synovium were obtained from patients with RA and OA at the time of total knee arthroplasty (TKA). Subjects included 7 RA patients (mean age, 65.7 ± 12.0 years), who fulfilled the American College of Rheumatology (ACR) diagnostic criteria [25] for RA and 10 OA patients (mean age, 76.0 ± 6.2 years). All studies were carried out under the approval of the University Institutional Ethics Board and in accordance with the Tenets of the Declaration of Helsinki.

Immunohistochemistry. Immunostaining was performed using primary antibodies for C/EBPβ (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000, C/EBPδ (M-17; Santa Cruz) diluted 1:500, MMP-3 (F-66; Daiichi Fine chemical, Toyama) diluted 1:200, and C-terminal aggrecan neoepitope VDIPEN and NITEGE antibodies, which were exposed following cleavage by MMPs and aggrecanases, respectively (both antibodies kindly provided by Dr. John S. Mort, Shriners Hospital for Children and McGill University), diluted 1:500.[26, 27]

Cell culture. The immortalized human chondrocyte cell line C-28/I2 was cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD) and 1% ITS-(insulin-transferrin-selenium) Universal Culture Supplement Premix reagent (BD Biosciences, San Jose, CA). SW982 cells, a human synovial sarcoma cell line, were grown in RPMI 1640 culture medium containing 10% FBS. Human fibroblast-like synoviocytes

(HFLS) (Cell Applications, Inc, San Diego, CA) that were derived from normal synovial tissue were cultured in DMEM supplemented with 10% FBS.

Western blotting. Whole cell extracts, nuclear or cytoplasmic extracts isolated from treated cells were assayed.

Enzyme-linked immunosorbent assay (ELISA)

Human MMP-3 and IL-6 in the conditioned medium of cells transfected with gene over-expression were detected via ELISA (R&D Systems, Minneapolis, MN)

RNA extraction and real-time reverse transcription (RT)-PCR. Total RNA was extracted from cultured cells and quantitative real-time RT-PCR was performed with the LightCycler2.0 system (Roche) using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan).

Transient transfection and gene over-expression in cell lines. Cells were transfected with an adenovirus expression vector for C/EBPβ-LAP (36 kDa isoform) [28] or a LacZ control for 24 hours. To assess the activity of MMP-3 induced by C/EBPβ, conditioned medium of cells transfected with adenovirus vector for C/EBPβ-LAP or LacZ control for 72 hours was assayed by casein zymography using 12% casein zymogram gels (Invitrogen, Carlsbad, CA).

Gene knockdown for cell lines. Pre-designed small interference RNA (siRNA) for C/EBPβ (siRNA-1 target sequence, 5'-CCCACGUGUAACUGUCAGCtt-3' [sense] and 5'-GCUGACAGUUACACGUGGGtt-3' [antisense]. siRNA-2 target sequence, 5'-GGCCCUGAGUAAUCGCUUAtt-3' [sense], 5'-UAAGCGAUUACUCAGGGCCcg-3' [antisense]) or negative control siRNA were purchased (Ambion). Transfection mixes were prepared using Lipofectamine RNAiMAX (Invitrogen) with control or target-specific siRNA and applied to cells. Cells were cultured for 24 hours after transfection. Transfected SW982, HFLS and C28/I2 cells were treated with 10 ng/ml IL-1β for 24 hours, 10 ng/ml IL-1β for 24 hours, and 2 ng/ml IL-1β for 5 hours, respectively.

Human MMP-3 and ADAMTS-5 promoter reporter constructs. Promoter constructs for human MMP-3 and human ADAMTS-5 were generated by PCR and subcloned into the pGL-4.10 [luc2] vector (Promega, Madison, WI). The 5'-upstream region (-2011 bp) of the human MMP-3 gene was prepared using human genomic DNA as a template and then the -2011 bp MMP-3 promoter constructs (p-full) and promoter deletion constructs (p-del) were generated. There are 3 putative binding sites for C/EBP between - 224 bp and – 56 bp. A 2 bp mutation (m) was made at one site or at both sites (Dm = double mutation) in the p-del2 construct using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). A 2498 bp fragment of the ADAMTS-5 promoter, containing sequences from –1768 to +730 relative to the transcriptional start site, was used as a template to PCR amplify the full-length promoter.

Plasmid transfection and luciferase assay. Cells seeded in 6-well plates were co-transfected with 0.6 μg/well of a series of deletion constructs for the MMP-3 promoter or the -1768 bp ADAMTS-5 promoter construct and pCMV-LAP, an expression vector of rat C/EBPβ-LAP directed by a cytomegalovirus promoter [29], or pMSV-C/EBPδ, an expression vector of rat C/EBPδ [30] using Lipofectamine 2000 (Invitrogen). The transfected cells were serum-starved for 6 hours and then the cultures were changed to medium containing 10% FBS and 1% ITS+ Premix reagent for 18 hours. Luciferase activity was then assayed.

Chromatin Immunoprecipitation (ChIP) Assay. C-28/I2 cells were treated with IL-1β at a concentration of 2 ng/ml in DMEM/F-12 supplemented with 10% FBS and 1% ITS+ Premix reagent and incubated for 24 hours. For immunoprecipitation, anti-C/EBPβ antibodies and normal rabbit IgG were used.

Over-expression in organ culture. Cartilage tissue was obtained from the intercondyle notch of 3 young adult patients who underwent anterior cruciate ligament (ACL)

reconstruction. The cartilage tissue was cultured with an adenovirus expression vector for C/EBPβ-LAP or LacZ control for 24 hours.

Statistical Analysis. For *in vitro* investigations, non-parametric comparisons were performed using a Mann-Whitney U-test. P values less than 0.05 were considered significant.

RESULTS

Colocalization of C/EBPβ and MMP-3 in synovium and cartilage from arthritis patients. We initially examined C/EBPβ and MMP-3 expression in synovial tissue and articular cartilage from OA and RA patients by immunohistochemistry. C/EBPβ and MMP-3 were expressed in OA and RA synovial tissue (figure 1). The distribution of C/EBPβ in synovial tissue was mainly in the synovial lining layer rather than in the sublining layer. MMP-3 had a similar distribution. In degenerative RA cartilage, C/EBPβ and MMP-3 were expressed in a proportion of the transitional and deep zones (figure 2). In mild OA cartilage, C/EBPβ and MMP-3 were predominantly distributed in the superficial zone. In areas of severe OA, chondrocytes that formed clusters in the deep zones were immunostained by C/EBPβ and MMP-3. Normal cartilage sections had little or no expression of C/EBPβ or MMP-3. VDIPEN epitopes were also positive in the peripheral area of cells expressing MMP-3 and C/EBPβ in both OA and RA cartilage. In addition, CEBPδ was expressed with a similar distribution in these tissues samples. The colocalization of C/EBPβ, -δ and MMP-3 in arthritis synovium and cartilage suggests that C/EBPβ and -δ are involved in the regulation of MMP-3 expression.

Expression of C/EBPβ and MMP-3 in cell lines after treatment with IL-1β. We set out to determine whether IL-1β could promote C/EBPβ protein and IL-6, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 gene expression in cell lines. Western blots

revealed that stimulation with IL-1β induced expression of both major isoforms (LAP; 36 kDa and LIP; 20 kDa) of C/EBPβ in nuclear extracts (figure S1A). Quantitative RT-PCR showed that MMP-3 and MMP-13 mRNA was strongly enhanced in cells treated with IL-1β (figure S1B). Since C/EBPβ is known to induce IL-6 expression in response to IL-1[31], we also confirmed the stimulation of IL-6 mRNA expression in these cells, which suggested the induced C/EBPβ is active in these cells. ADAMTS-5 was up-regulated approximately 2-fold in these cells. ADAMTS-4 was not significantly up-regulated in SW982 and C28/I2 cells, but was increased 3-fold in HFLS cells.

Over-expression of C/EBPβ stimulates expression of MMP-3, MMP-13 and ADAMTS-5. We next examined cells transfected with an adenovirus vector expressing C/EBPβ-LAP or LacZ control for 24 hours. Western blots showed exogenous over-expression of C/EBPβ-LAP in whole extracts isolated from transfected cells (figure 3A). The up-regulation of IL-6 expression was confirmed in all cells. The mRNA expression of MMP-3 and MMP-13 was also significantly increased in all cells. ADAMTS-5 mRNA expression was increased 5-fold by C/EBPβ over-expression, but ADAMTS-4 mRNA was not increased. In addition, ELISA analysis in the culture medium revealed the cells over-expressed CEBPβ-LAP released more amount of total MMP-3 and IL-6 than that of control (figure 3B). Casein zymography revealed that MMP-3 was induced in an active form by the over-expression of C/EBPβ (figure 3C).

C/EBPβ knockdown by siRNA reduced MMP-3 and ADAMTS-5 expression. We next assayed siRNA transfected cells targeting C/EBPβ mRNA. Transfected cells were cultured with IL-1β. Nuclear extracts and mRNA expression of C/EBPβ were effectively reduced by both siRNA-1 and siRNA-2 C/EBPβ transfection (figure 4A). C/EBPβ knockdown with siRNA-1 caused a significant decrease of IL-6 mRNA expression (figure 4B). MMP-3 mRNA expression was remarkably reduced in all cells. MMP-13 expression

was repressed in C-28/I2 cells and HFLS cells. ADAMTS-5 expression was significantly reduced in C28/I2 cells. C/EBPβ knockdown with siRNA-2 caused similar results (data not shown). The knockdown of C/EBPβ by siRNA reduced the expression of IL-6 and these enzymes in a dose dependent manner (figure S2). We also performed experiments with CEBPδ knockdown. Cells transfected with the siRNA-CEBPδ showed reduction of IL-6 and MMP3 mRNA (figure S3).

C/EBPβ functions as an activator of the MMP-3 and ADAMTS-5 promoter. We further analyzed the *in vitro* promoter activity of MMP-3 and ADAMTS-5 using C28/I2 cells. A luciferase reporter gene construct containing 2011 bp from the human MMP-3 promoter or 2498 bp from the human ADAMTS-5 promoter was co-transfected with the C/EBPβ pCMV-LAP expression vector into C28/I2 cells. Promoter activities for both MMP-3 and ADAMTS-5 were up-regulated in a dose-dependent manner (figure 5A). Similar promoter activity was exhibited in SW982 cells (data not shown). C/EBPδ expression vector also activated MMP-3 and ADAMTS-5 promoters in a dose dependent manner, whereas the stimulating effects were weaker than that of C/EBPβ.

Localization of the C/EBPβ binding site in the MMP-3 promoter. To identify the C/EBPβ responsive element in the MMP-3 promoter, deletion and mutation analysis was performed. We generated a series of 5'-promoter deletion constructs (figure 5B), which were co-transfected with C/EBPβ pCMV-LAP into C28/I2 cells. Construct p-del3 demonstrated reduced luciferase activity (figure 5C). These results showed that the putative C/EBPβ binding site is located between -224 bp and -56 bp of the MMP-3 promoter.

To determine the core binding site, we performed site directed mutagenesis, which created two mutations changing GNAA into GNCC. Analysis of the sequence between -224 bp and -56 bp indicated the presence of three C/EBPβ consensus binding motifs T(T/G)NNGNAA(T/G). We created 3 single mutation constructs and 1 double

mutation construct in the p-del2 MMP-3 promoter. The promoter activities of p-del2-m3 and p-del2–D(m2+m3) equally decreased by 25%, indicating that the core binding site of C/EBPβ is located between -108 bp and -100 bp of the human MMP-3 promoter (figure 5D). A ChIP assay was performed using primers in the human MMP-3 and ADAMTS-5 promoter sequences, which amplified sites including the C/EBPβ consensus binding motifs. This analysis indicated that C/EBPβ binds to the MMP-3 and the ADAMTS-5 promoter region in C28I2 cells treated with IL-1β (figure 5E).

C/EBPβ induces MMP-3 with a degrading activity in explants of cartilage tissue. We next used adenoviruses carrying LacZ or C/EBPβ-LAP to infect explants of normal cartilage tissues. Immunohistochemical staining confirmed an increased expression of C/EBPβ induced by the infection (figure 6A). Over-expression of C/EBPβ-LAP markedly induced MMP-3 expression in cartilage tissues. In addition, anti-VDIPEN-epitopes intensely stained cartilage tissues infected with C/EBPβ-LAP (figure 6B). Furthermore, anti-NITEGE-epitopes, which are generated following cleavage by aggrecanases such as ADAMTS-4 and ADAMTS-5, also stained C/EBPβ-LAP-infected cartilage (figure 6B). These results suggest that over-expression of C/EBPβ promotes the degradation of aggrecan by stimulating the expression of active proteolytic enzymes such as MMP-3, MMP-13 and ADAMTS-5.

DISCUSSION

In this study, we demonstrated that the transcription factor C/EBPβ promotes MMP-3 expression in arthritic synovial membrane and cartilage tissue. Alteration of MMP-3 expression by gain and loss of function of C/EBPβ was most significant, suggesting that C/EBPβ is one of the main regulators of MMP-3 expression. Furthermore, this study also suggests that C/EBPβ regulates, at least in part, ADAMTS-5, which is also a crucial enzyme

in degrading cartilage in OA. These findings suggest that C/EBPβ plays crucial roles in cartilage degradation through production of various catabolic enzymes in synovium and cartilage in RA and OA. In addition, we performed experiments in relation to CEBPδ. Promoter assay and treatment with siRNA for C/EBPδ indicated that C/EBPδ is also involved in cartilage degradation by production of IL-6, MMP-3 and ADAMTS-5, whereas the contribution of C/EBPδ may be weaker than that of C/EBPβ. C/EBPδ may play compensatory role for C/EBPβ in the catabolic processes in arthritis.

In inflammatory arthritis, particularly in RA, synovitis is thought to be a key player in pathogenesis. In contrast, the pathogenesis of OA may originate from cartilage and the cartilage degradation may induce secondary synovitis. However, recently the concept of synovial inflammation contributing to OA pathology particularly in early disease has been emphasized.[4] Immunohistochemical staining in this study showed that C/EBPβ was expressed in synovial tissues regardless of arthritis type (RA or OA). These findings suggest that C/EBPβ is a common proinflammatory factor in arthritic synovial tissues, which profoundly affects degradation of cartilage. As previously described, MMP-3 is highly expressed in both RA and OA synovium, which is a potent activator of tissue procollagenases. Aforementioned in introduction, MMP-3 knock-out mice demonstrating the importance of this matrix-degrading enzyme in an animal model of OA.[22] However, the relevance of MMP-3 in RA is far to be clear since the same MMP-3 knock-out mice normally develop arthritis.[32] This report indicated that MMP-3 is one of important catabolic factors, whereas only inhibition of MMP-3 is insufficient to inhibit the cartilage degradation. Joint destruction results from multiple matrix-degrading enzymes such as other MMPs and ADAMTSs. MMP-13 and ADAMTS-5 also exist in the synovial tissue of arthritis.[33-35] Therefore, the regulation of these catabolic factors by C/EBPβ in the synovium may be important for the pathogenesis of RA and OA.

In addition to the findings in synovium, we identified that C/EBPβ is involved in the expression of MMP-3, MMP-13 and ADAMTS-5 in chondrocytes. It is reported that MMP-3 is strongly expressed in early degenerative cartilage, while expression of MMP-3 is reduced in late stage OA. MMP-13 and ADAMTS-5 are up-regulated in late stage OA cartilage.[36] These observations raise the possibility that C/EBPβ function may depend on OA stage. Inflammatory signals might occur as a result of cartilage damage induced by other factors such as mechanical stress. The activation of C/EBPβ initiated by inflammatory signals in synovium and cartilage may subsequently mediate various catabolic processes including up-regulation of MMP-1, MMP-3, MMP-13 and ADAMTS-5. Activated C/EBPβ also mediates the phenotype change of chondrocytes into hypertrophic cells, which express MMP-13, vascular endothelial growth factor (VEGF) and type X collagen, as recent papers have reported.[37, 38] Therefore, regardless of pathogenesis, C/EBPβ plays crucial roles in degeneration of cartilage in OA. We suggest that C/EBPβ mediates MMP-3 activity in early disease via an inflammatory signal pathway. With progression of OA, C/EBPβ function may stimulate a different pathway involved in hypertrophic differentiation of chondrocytes and regulation of MMP-13 and ADAMTS-5.

Our data indicate that C/EBPβ regulates ADAMTS-5 expression particularly in chondrocytes. The molecular mechanism involving expression of ADAMTS-5 remains unclear. Examination of the human ADAMTS-5 promoter suggests that it is regulated by runt-related transcription factor-2 (RUNX2),[39] which is a protein known to be expressed in OA. RUNX2 is required for chondrocyte hypertrophy in the development of OA.[40] C/EBPβ also promotes hypertrophic differentiation of chondrocytes.[37] There is increasing evidence to suggest that C/EBPβ acts as a co-activator of RUNX2 on several down-stream targets. Indeed, the osteocalcin promoter is up-regulated by co-activation of C/EBPβ and RUNX2.[12] We found potential C/EBPβ binding motifs in the ADAMTS-5 promoter

(-1517 to -1509), which is located next to the RUNX2 responsive motifs previously reported.[39] In fact, the CHIP assay showed that C/EBPβ binds the ADAMTS-5 promoter region between -1648 bp and -1365 bp. Therefore, it is conceivable that C/EBPβ interacts with RUNX2 and synergistically activates ADAMTS-5 expression in arthritis leading to degradation of cartilage.

In conclusion, C/EBPβ is considered to play two major roles in arthritis. One role is the regulation of an inflammatory signal pathway. C/EBPβ regulates MMP-1, MMP-3, MMP-13 and partially ADAMTS-5 in synovium and chondrocytes in arthritis during the process of inflammation. The other role promotes hypertrophic differentiation of chondrocytes. MMP-13 is a major collagenase in arthritis and is a hypertrophic marker of chondrocytes. Therefore, C/EBPβ may regulate MMP-3, MMP-13 and ADAMTS-5 in the process of hypertrophy. The data presented here indicate that C/EBPβ mediates multiple factors, which reinforces the idea that C/EBPβ is one of the most important inflammatory signals in arthritis.

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FIGURE LEGENDS

Figure 1. Expression and distribution of C/EBPβ, C/EBPδ, MMP-3 in arthritic synovium by immunohistochemistry. **A**, Immunohistochemistry of OA synovium. **B**, RA synovium. Synovium sections were stained with hematoxylin counterstain. **IgG**; staining with normal IgG antibodies, **C/EBPβ**; anti-C/EBPβ antibodies, **C/EBPδ**; anti-C/EBPδ antibodies, **MMP-3**; anti-MMP-3 antibodies in synovium. Original magnification 200× in **A** and **B,**

scale bar, 100 μm. C/EBP = CCAAT/enhancer-binding protein; MMP = matrix metalloproteinase; RA = rheumatoid arthritis; OA = osteoarthritis.

Figure 2. Expression and distribution of C/EBPβ, C/EBPδ, MMP-3 and VDIPEN in arthritic articular cartilage by immunohistochemistry. **VDIPEN;** anti-VDIPEN (a metalloproteinase-generated neoepitope) antibodies. Immunohistochemistry were performed in OA normal cartilage, mild OA cartilage**,** severe OA cartilage**,** RA cartilage. Cartilage sections were stained with methyl green counterstain. Original magnification 200×**,** scale bar, 100 μm.

Figure 3. A, Effect of C/EBPβ over-expression on IL-6, MMPs, and ADAMTS expression in cells. SW982, C28/I2 and HFLS cells were transfected with adenovirus expression vector for C/EBPβ-LAP or LacZ (negative control) and cultured for 24 hours. Whole cell extracts isolated from transfected cells were assayed by western blotting to confirm exogenous C/EBPβ-LAP expression. Over-expression of C/EBPβ-LAP stimulates IL-6, MMP-3, MMP-13, and ADAMTS-5 mRNA expression as investigated by quantitative real time RT-PCR in three cell lines. IL-6 = interleukin-6; ADAMTS = a disintegrin and metalloproteinase with thrombospondin type 1 motif; HFLS = human fibroblast like synoviocytes. * $p<0.05$ versus control; Mann-Whitney U test.

B, Enzyme-linked immunosorbent assay (ELISA) for total MMP-3 and IL-6 with conditioned medium from C28/I2 or SW982 cells transfected with adenovirus expression vector for LacZ or LAP. Total MMP-3 and IL-6 were detected in cultured medium for 72 hours and 48 hours, respectively. * $p<0.05$ versus control; Mann-Whitney U test.

C, Casein zymography for MMP-3 with conditioned medium from SW982 or C28/I2 cells transfected with adenovirus expression vector for LacZ or LAP and cultured for 72 hours.

Figure 4. Effect of C/EBPβ knockdown on IL-6, MMPs, and ADAMTS expression in cells. Small interference RNA (siRNAs) targeting C/EBPβ or a negative control siRNA were transfected into SW982, HFLS and C28/I2 cells. Transfected cells were cultured with or without 10 ng/ml IL-1β for 24 hours, 10 ng/ml IL-1β for 24 hours, 2 ng/ml IL-1β for 5 hours, respectively. **A,** Effectiveness of C/EBPβ knockdown was ascertained by western blots and quantitative RT-PCR in three cell lines. **B,** RNA from cells transfected with control siRNA or siRNAs specific for C/EBPβ were analyzed for alterations in IL-6, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 gene expression by quantitative RT-PCR. Expression levels are presented relative to cells transfected with control siRNA. * $p<0.05$ versus control; Mann-Whitney U test.

Figure 5. A, Transactivation of the human MMP-3 promoter or human ADAMTS-5 promoter by C/EBPβ or C/EBPδ. The 2011 bp MMP-3 or the 2498 bp ADAMTS-5 promoter-luciferase reporter vectors were co-transfected into C28/I2 cells with pCMV-LAP or pMSV-C/EBPδ (0, 0.1, 0.4, 0.8, and 1.2 μg). The renilla luciferase normalization vector expressing and GFP, which was used to adjust the total amount of transfected DNA. Relative luciferase activity was assayed 24 hours post-transfection. **B,** Deletion promoter analysis of the human MMP-3 promoter. Schematic of the 5'-deletion constructs of the MMP-3 promoter (p-del), which were subcloned into the pGL-4.10 [luc2] vector. **C,** The MMP-3 promoter deletion constructs were co-transfected with pCMV-LAP or GFP into C28/I2 cells. Relative luciferase activity was assayed 24 hours post-transfection. **D,** Site-directed mutagenesis of the human MMP-3 promoter and luciferase reporter assay. The promoter

deletion construct, p-del2, contains three potential binding sites for C/EBPβ. A 2 bp mutation (m) was made at one site (m1, -m2, -m3) or at both sites (Dm = double mutation), D (m2+m3), of the p-del2 construct. Mutant constructs were co-transfected with pCMV-LAP or GFP into C28/I2 cells and the relative luciferase activity was assayed 24 hours post-transfection. pCMV-LAP = an expression vector of rat C/EBPβ-LAP. pMSV-C/EBPδ = an expression vector of rat C/EBPδ. GFP = green fluorescent protein. **E,** C/EBPβ binds to the human MMP-3 and ADAMTS-5 promoter in C28/I2 cells. After treatment with IL-1β for 24 hours, a Chromatin immunoprecipitation assay (ChIP) was performed using C/EBPβ specific antibodies or control IgG. The C/EBPβ bound MMP-3 promoter or C/EBPβ-ADAMTS-5 promoter complex were amplified by semi-quantitative RT-PCR with primers for the MMP-3 promoter (from -214 bp to +19 bp), a negative MMP-3 control (from -1727 bp to -1487 bp), the ADAMTS-5 promoter (from -1648 bp to -1365 bp), or a negative ADAMTS-5 control (from -2044 bp to -1887 bp). Similar concentrations of input DNA was used before immunoprecipitation and contained random DNA sequence. The PCR products were amplified for 33 cycles.

Figure 6. Normal cartilage was transfected with control (LacZ) or CMV-LAP (C/EBPβ) for 24 hours. Organ cultured cartilage tissues were immunostained. **A**, anti-IgG, C/EBPβ, and MMP-3 antibodies, **B,** anti-VDIPEN (a mettaloproteinase-generated neoepitope) epitope and NITEGE (an aggrecan C-terminal neoepitope) epitope antibodies. Cartilage sections were stained with methyl green counterstain. Original magnification 200× in **A**, scale bar, 100 μm and 400× in **B**, scale bar, 50 μm.

Figure 1

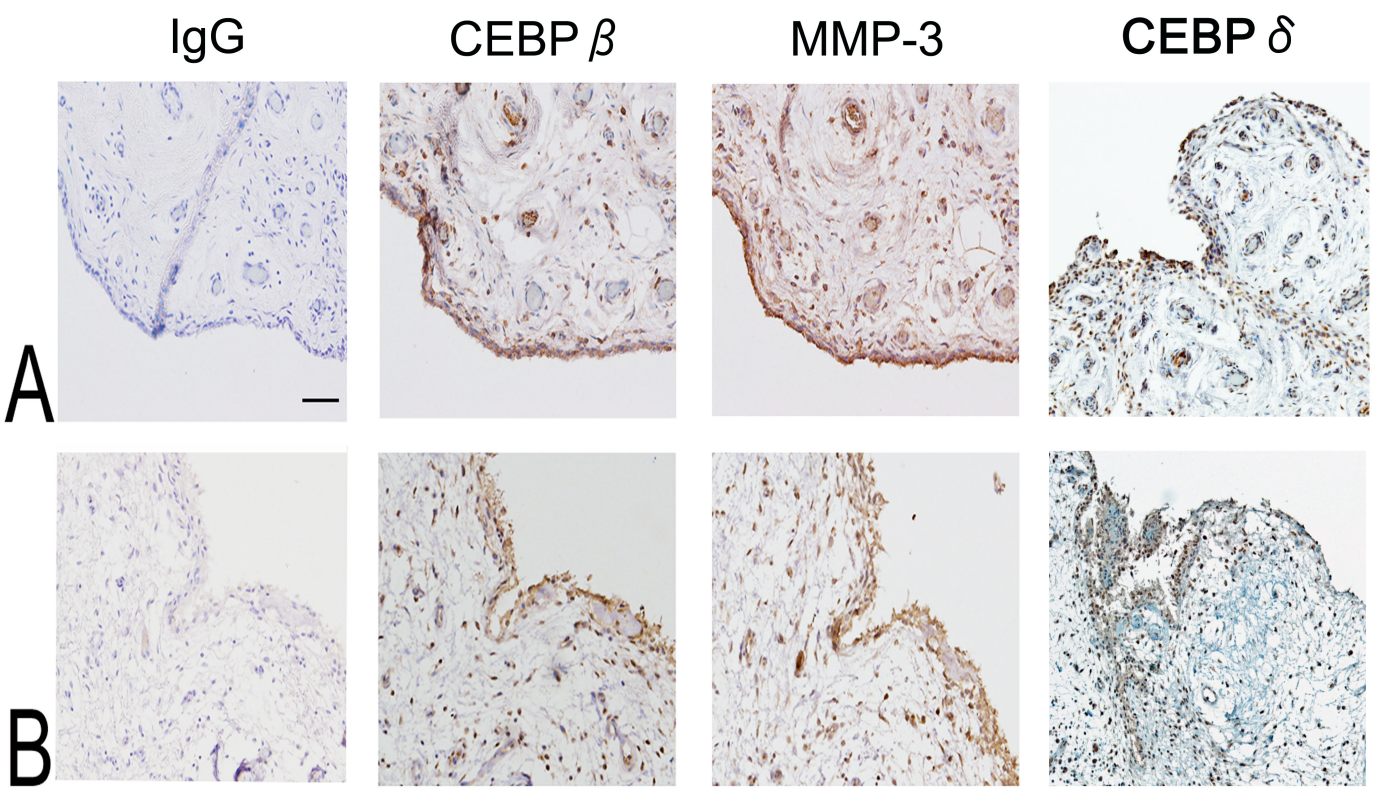

Figure 2

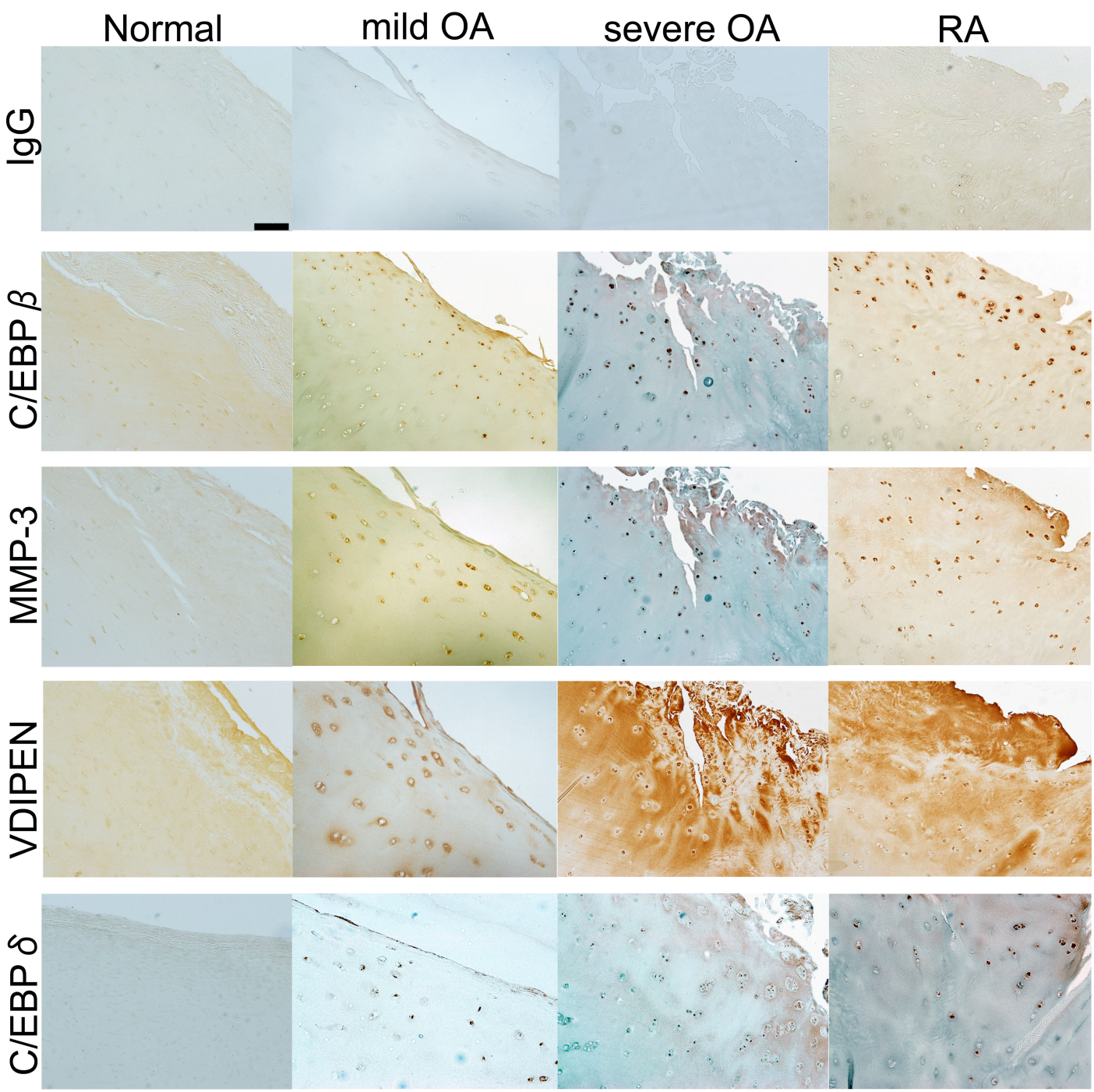

Figure 3

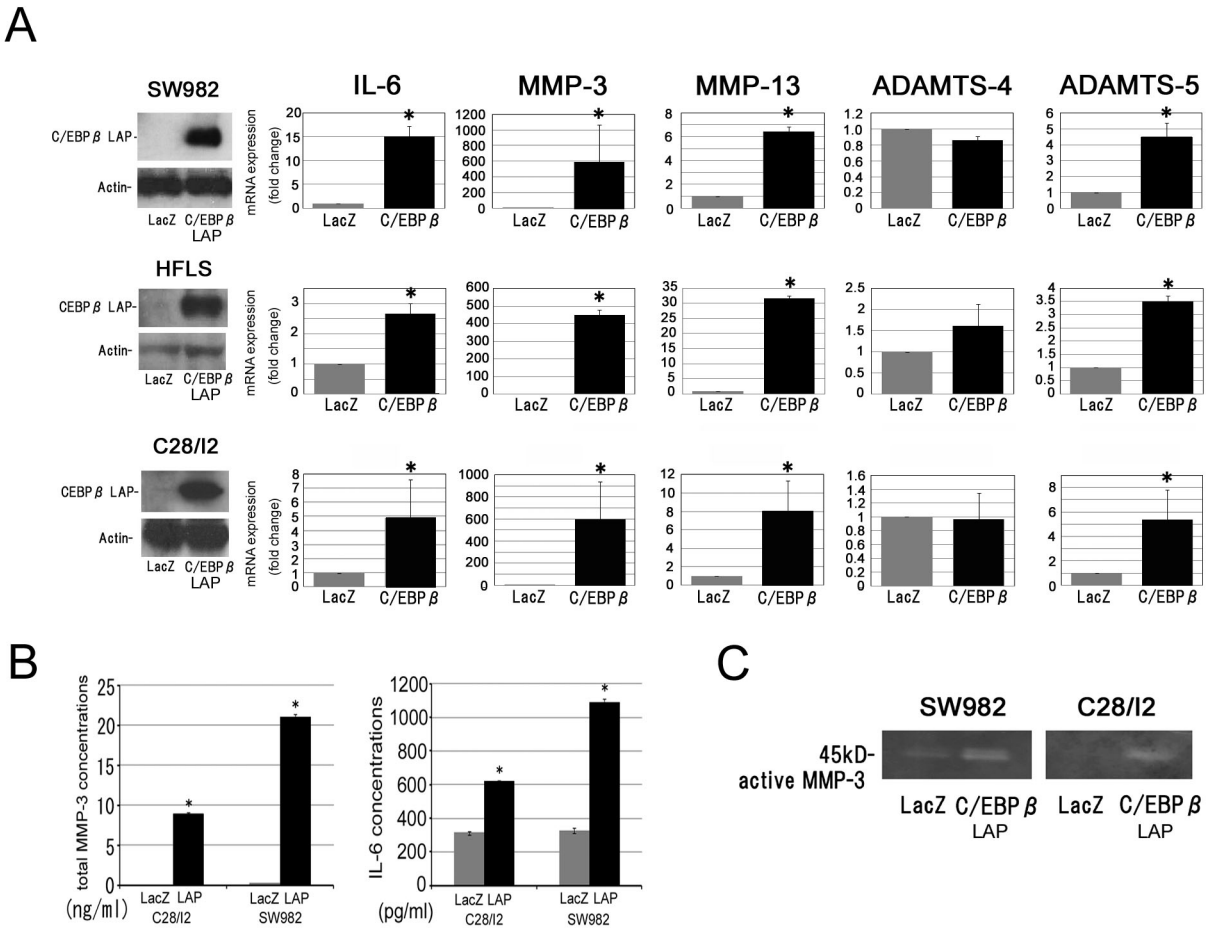
A
SW982
HFLS
C28/I2
C/EBPβ LAP-
CEBPβ LAP-
Actin-
LacZ
C/EBPβ LAP
mRNA expression (fold change)
IL-6
MMP-3
MMP-13
ADAMTS-4
ADAMTS-5
B
total MMP-3 concentrations
(ng/ml)
IL-6 concentrations
(pg/ml)
LacZ LAP C28/I2
LacZ LAP SW982
C
SW982
C28/I2
45kD-
active MMP-3

Figure 4

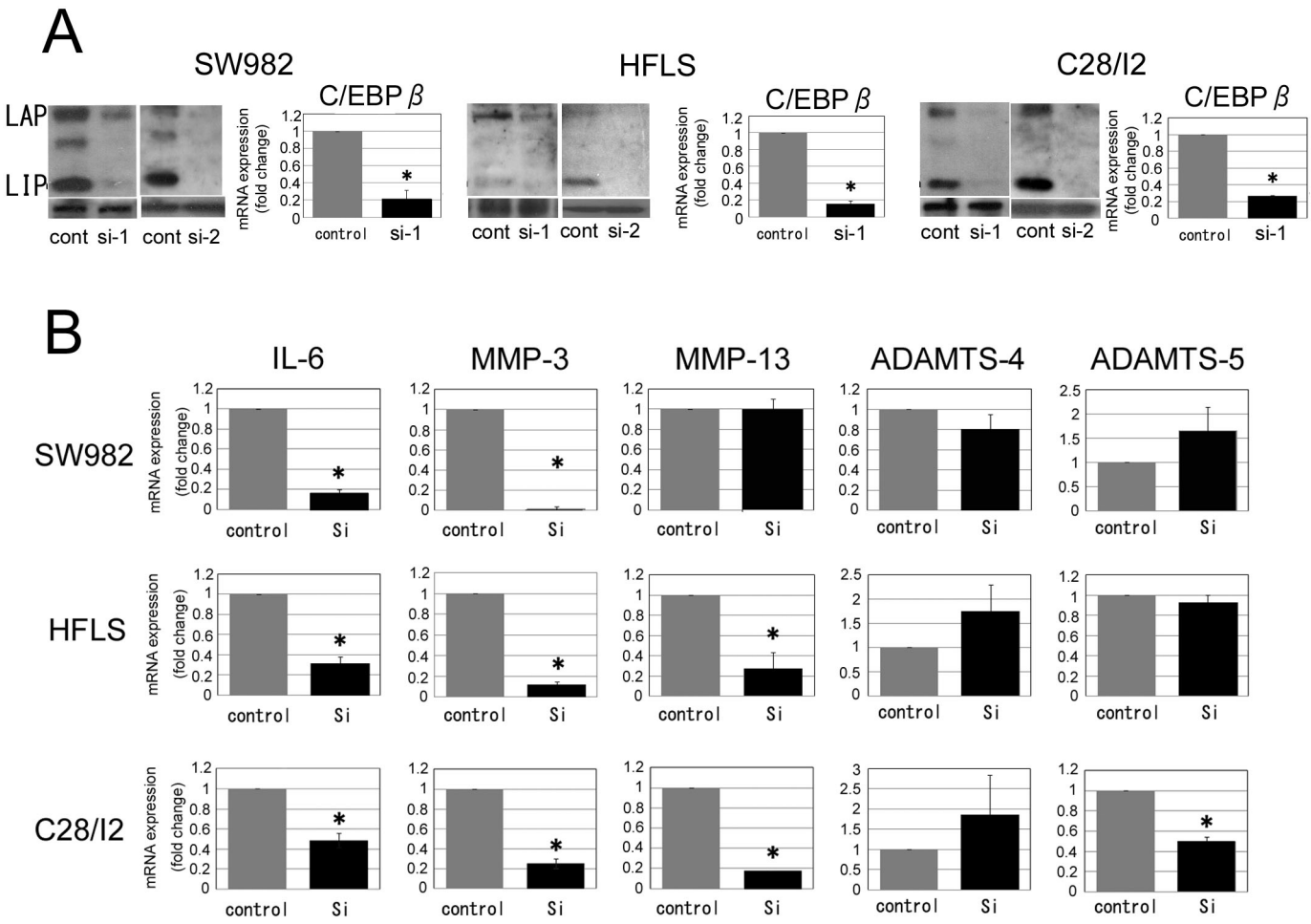
A
SW982
HFLS
C28/I2
C/EBP β
LAP
LIP
cont si-1 cont si-2
mRNA expression (fold change)
control
si-1
B
IL-6
MMP-3
MMP-13
ADAMTS-4
ADAMTS-5
SW982
HFLS
C28/I2
control
Si

Figure 5

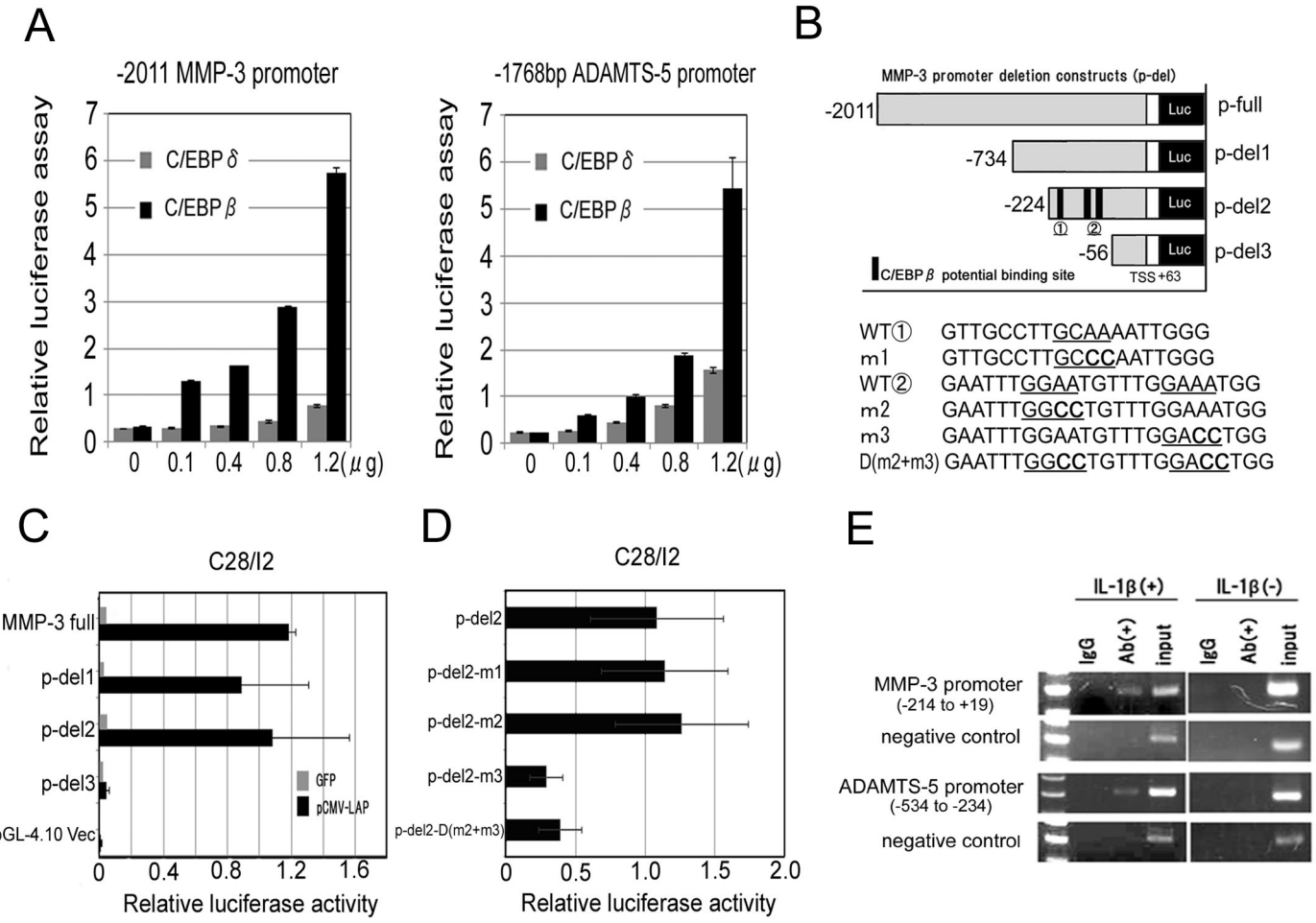
A
-2011 MMP-3 promoter
-1768bp ADAMTS-5 promoter
Relative luciferase assay
C/EBP δ
C/EBP β
0 0.1 0.4 0.8 1.2(μg)
B
MMP-3 promoter deletion constructs (p-del)
-2011
-734
-224
-56
Luc
p-full
p-del1
p-del2
p-del3
C/EBP β potential binding site
TSS +63
WT① GTTGCCTTGCAAAATTGGG
m1 GTTGCCTTGCCCAATTGGG
WT② GAATTTGGAATGTTTGGAAATGG
m2 GAATTTGGCCTGTTTGGAAATGG
m3 GAATTTGGAATGTTTGGACCTGG
D(m2+m3) GAATTTGGCCTGTTTGGACCTGG
C
C28/I2
MMP-3 full
p-del1
p-del2
p-del3
pGL-4.10 Vec
GFP
pCMV-LAP
0 0.4 0.8 1.2 1.6
Relative luciferase activity
D
C28/I2
p-del2
p-del2-m1
p-del2-m2
p-del2-m3
p-del2-D(m2+m3)
0 0.5 1.0 1.5 2.0
Relative luciferase activity
E
IL-1β(+)
IL-1β(-)
IgG
Ab(+)
input
MMP-3 promoter
(-214 to +19)
negative control
ADAMTS-5 promoter
(-534 to -234)
negative control

Figure 6