Introduction
Cartilage-derived retinoic acid-sensitve protein (CD-RAP) is a small secreted matrix protein expressed in developing and adult cartilage and by chondrocytes in culture. It was originally cloned as a mRNA co-regulated with Col2a1 in cartilage and provides an excellent model for cartilage gene transcription (1). We have shown that the expression of CD-RAP is repressed by Interleukin-1β (IL-1β) (2) and that C/EBPβ plays an important role in the IL-1β-induced repression of cartilage-specific proteins (3). Co-activator CREB-binding protein (CBP) and p300 are structurally and functionally similar transcriptional co-regulators that modulate in the activities of many different transcription factors including C/EBPβ. Importantly, mutations in the gene encoding CBP, were found to cause Rubinstein-Taybi syndrome characterized by skeletal abnormalities (4). Therefore, we hypothesize that CBP/p300 will effect chondrocyte gene expression. We show that CBP/p300 stimulates CD-RAP gene expression by at least two mechanisms; first, direct binding to C/EBP eliminating its repression capability and second, by an additional mechanism independent of C/EBPβ.

Method
Transient transfection assay. To investigate the effect of CBP/p300 on the CD-RAP promoter, DNA transfections of RCS cells were performed using Fugene6 transfection reagent (Roche, Indianapolis, IN, USA). 2.2 kbp of the CD-RAP promoter was subcloned into the luciferase reporter plasmid pGL-3 basic (5). Isoforms of C/EBPβ (CMV-LAP and CMV-LIP), CMV-CBP or CMV-p300 was co-transfected with CMV-β-galactosidase internal control. The luciferase activities were assayed and normalized to β-galactosidase.

Electrophoretic mobility shift assay. To investigate the effect of CBP/p300 on DNA binding activity of C/EBPβ, the electrophoretic mobility shift assay (EMSA) was performed. Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA, USA) and complementary oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-32P]dATP. Oligonucleotide P1 spanning -2117-bp to -2069-bp of CD-RAP promoter covering HMG-like sites 1 to 3 and a potential C/EBPβ site was used as a competitor. C/EBPβ (its isoforms C/EBPβ-LIP and C/EBPβ-LAP), CBP and p300 proteins were synthesized by in vitro translation.

Results
CBP/p300 enhances the CD-RAP promoter and overcomes repression by C/EBPβ. Transient transfection assay revealed CBP/p300 enhanced the CD-RAP promoter activity in a dose-dependent manner, although C/EBPβ itself repressed it (Fig. 1). p300 expression restored the promoter activity which was repressed by C/EBPβ (data not shown).

Discussion
Transcriptional co-activators such as CBP and p300 function as important elements in the transcription network, linking individual transactivators via protein-protein interactions to the basal transcriptional machinery. CBP/p300 appears to be a particularly versatile coactivator because they interact with several different transactivators. Our results demonstrate that CBP/p300 stimulates gene transcription by binding and sequestering the repressor protein C/EBPβ. Our results also show additional stimulation by CBP/p300 that cannot be explained solely by inhibition of C/EBPβ. Recently CBP/p300 was found to function as a co-activator of Sox9 in cartilage tissue specific expression and chondrocyte differentiation (6). That is, CBP/p300 enhances Sox9 dependent Col2a1 promoter activity and disruption of the CBP/Sox9 complex inhibits Col2a1 mRNA expression. As Sox9 is also critical for CD-RAP gene expression, we propose that CBP/p300 interacts with Sox9 and then enhances the promoter through Sox9 binding site (Fig. 3). These results suggest CBP/p300 is an important cofactor in chondrocyte specific gene expression via regulating C/EBPβ transcriptional activity. Understanding the molecular mechanism of how co-activator CBP/p300 effects matrix gene expression may present a clue to the treatment of articular joint disease such as osteoarthritis in adult humans.

Fig. 1. C/EBPβ represses the activity of CD-RAP promoter while CBP/p300 enhances it in a dose-dependent manner. Relative luciferase activities of RCS cells transfected with luciferase reporter vectors containing 2.2 kbp promoter. CMV-C/EBP β, CMV-p300 and CMV-CBP were co-transfected at three degrees. Each expression plasmid and reporter plasmid were standardized individually at a ratio of 1:1, and the total amount of DNA per well was adjusted to 1.0 µg by the addition of a mock DNA plasmid.

Fig. 2. Inhibition of C/EBPβ binding to DNA by CBP/p300 by EMSA. C/EBPβ-LIP or C/EBPβ-LAP bound to γ-32PdATP-labeled oligonucleotide P1 in the presence of increasing amounts of in vitro translated CBP or p300 protein.

Fig. 3. Proposed models of CD-RAP Promoter Regulation. A) CBP/p300 interacts with Sox9 and then binds to the CD-RAP promoter. B) C/EBPβ alone represses. C) CBP/p300 interacts with C/EBPβ and Sox9, inhibiting binding of C/EBPβ and enhances Sox9 binding site.

References
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