C/EBPβ and RUNX2 cooperate to degrade cartilage with MMP-13 as the target and HIF-2α as the inducer in chondrocytes

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To elucidate the molecular mechanism underlying the endochondral ossification process during the skeletal growth and osteoarthritis (OA) development, we examined the signal network around CCAAT/enhancer-binding protein-β (C/EBPβ, encoded by CEBPB), a potent regulator of this process. Computational predictions and a C/EBP motif-reporter assay identified RUNX2 as the most potent transcriptional partner of C/EBPβ in chondrocytes. C/EBPβ and RUNX2 were induced and co-localized in highly differentiated chondrocytes during the skeletal growth and OA development of mice and humans. The compound knockout of Cebpb and Runx2 in mice caused growth retardation and resistance to OA with decreases in cartilage degradation and matrix metalloproteinase-13 (Mmp-13) expression. C/EBPβ and RUNX2 cooperatively enhanced promoter activity of MMP13 through specific binding to a C/EBP-binding motif and an osteoblast-specific cis-acting element 2 motif as a protein complex. Human genetic studies failed to show the association of human CEBPB gene polymorphisms with knee OA, nor was there a genetic variation around the identified responsive region in the human MMP13 promoter. However, hypoxia-inducible factor-2α (HIF-2α), a functional and genetic regulator of knee OA through promoting endochondral ossification, was identified as a potent and functional inducer of C/EBPβ expression in chondrocytes by the CEBPB promoter assay. Hence, C/EBPβ and RUNX2, with MMP-13 as the target and HIF-2α as the inducer, control cartilage degradation. This molecular network in chondrocytes may represent a therapeutic target for OA.

INTRODUCTION

The endochondral ossification process plays a crucial role in normal skeletal growth (1) and development of osteoarthritis (OA) which is one of the most common joint disorders today (2–7). This process starts with hypertrophic differentiation of chondrocytes expressing type X collagen (COL10A1), followed by cartilage degradation by proteinases like matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin type-1 motifs (ADAMTSs) (8,9). Aiming at elucidation of the molecular mechanism underlying endochondral ossification and identification of therapeutic targets for OA, we previously established a comprehensive screening system of transcription factors that induce chondrocyte hypertrophy using a universal enhancer in the promoter of human COL10A1 gene as the reporter construct (10), and identified CCAAT/enhancer-binding protein-β (C/EBPβ, encoded by CEBPB) as one of the strongest transactivators in chondrocytes (11). C/EBPβ, a member of the leucine zipper family of transcription factors, regulates expression of various genes involved in cell differentiation, proliferation, survival, immune function, female reproduction

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and tumor progression (12,13). Recent studies by others and us have demonstrated that C/EBPβ plays a role in the endochondral ossification process, since the knockout (Cebpβ−/−) mice exhibit growth retardation probably due to impaired hypertrophic differentiation of chondrocytes (11,14,15). However, the growth retardation is mild and temporary for a limited period during embryogenesis, and disappears after birth. We have therefore hypothesized that C/EBPβ is one of the players in a capital molecular network for the endochondral ossification process, and have sought to identify its transcriptional partners, target molecules and upstream signals in chondrocytes during the skeletal growth and OA development.

RESULTS

RUNX2 is identified as a transcriptional partner of C/EBPβ in chondrocytes

To identify transcriptional partners that enhance transactivity of C/EBPβ, we initially performed a screening using an in silico database of protein interactions (STRING), and predicted 178 genes with confidence scores of ≥0.7 as functional partners of human C/EBPβ protein (Supplementary Material, Table S1). Among the genes, we selected 14 genes that satisfied the three criteria: (i) being selected by two or more methods out of seven in the STRING, (ii) transcription factors and (iii) being expressed in chondrocytes (Supplementary Material, Fig. S1). We then performed the second screening of transcription factors using human chondrogenic SW1353 cells co-transfected with a reporter construct containing three consensus C/EBP-binding sequences and expression vectors of the 14 genes selected in the first screening. To exclude the effects of other endogenous C/EBP proteins, we created stable cell lines retrovirally transfected with CEBPβ gene or the small interfering RNA (siRNA) specific for CEBPβ. Among the 14 genes, runt-related transcription factor 2 (RUNX2) and activating transcription factor 4 (ATF4) most strongly induced the transactivity of the baseline (GFP or siGFP-transfected cells) (Fig. 1A). Although the CEBPβ overexpression further enhanced the transactivities of both RUNX2 and ATF4, the CEBPβ knockdown significantly suppressed only RUNX2 transactivity, indicating that RUNX2 is the most potent transcriptional partner of C/EBPβ in chondrocytes. Mammalian two-hybrid assay by transfections of vectors expressing GAL4–RUNX2 and VP16–C/EBPβ fusion proteins with the luciferase reporter vector with GAL4-binding sites into HeLa cells showed the molecular interaction between C/EBPβ and RUNX2 (Fig. 1B).

When we compared expression patterns of C/EBPβ and RUNX2 in cultures of mouse primary chondrocytes, mouse chondrogenic ATDC5 cells and SW1353 cells, both expressions similarly increased according to differentiation of all cells (Fig. 1C). In the growth plate cartilage of adult mice, C/EBPβ and Runx2 were co-localized in highly differentiated chondrocytes of hypertrophic and later differentiation stages during expression of Col10a1 and Mmp-13 (Fig. 1D). Subcellularly, C/EBPβ and RUNX2 were co-localized inside the nucleus, especially at the nucleoli speckles including active transcription sites (Fig. 1E).

C/EBPβ and RUNX2 cooperatively control skeletal growth

To determine the involvement of C/EBPβ and RUNX2 in the skeletal growth, we generated their compound-knockout mice by appropriate mating. Because the Runx2 homozygous-knockout (Runx2−/−) mice died just after birth, we used the heterozygous-knockout (Runx2+/−) mice. Cebp−/− and Cebp−/−:Runx2−/− mice were born at much lower frequencies than the expected Mendelian ratio and short-lived even after birth. Although Cebp−/− mice were normal, Cebp−/− mice exhibited mild and temporary dwarfism only for a limited period during embryogenesis, and grew normally after birth, as we previously reported (11). Runx2−/− and Cebp−/−:Runx2−/− mice did not show a significant growth retardation; however, Cebp−/−:Runx2−/− mice showed more severe dwarfism than their Cebp−/− littermates during the perinatal periods (Fig. 2A and Supplementary Material, Fig. S2A) and remained smaller than the other genotype littermates even 12 weeks after birth (Fig. 2B). Cleidocranial dysplasia with hypoplastic clavicle and open fontanelle were also enhanced by the compound insufficiency (Fig. 2A and Supplementary Material, Fig. S2B and C). The percentage of the proliferative zone relative to the total limb length was increased in Cebp−/−, Cebp−/−:Runx2−/− and Cebp−/−:Runx2−/− littermates, indicating a delay of chondrocyte hypertrophy by the Cebp insufficiency (Fig. 2C and D and Supplementary Material, Fig. S3A and B), as previously reported (11,14). The percentage of the proliferative zone and the start of chondrocyte hypertrophy were similar between Cebp−/− and Cebp−/−:Runx2−/− littermates; however, that of the hypertrophic zone with Col10a1 expression was elongated and that of the bone area was markedly decreased in the Cebp−/−:Runx2−/−+ littermates. Since this impairment was associated with a decrease in Mmp-13 expression, C/EBPβ and RUNX2 seem to cooperatively promote matrix degradation through the Mmp-13 induction (Fig. 2E and Supplementary Material, Fig. S4). Although the Mmp-3 expression was also considerably decreased in the Cebp−/−:Runx2−/−+ limbs, this may be due to the effect of deficiency of both alleles of Runx2 insulincaused impairment of steps later than chondrocyte hypertrophy under the Cebp deficiency. Since this impairment was associated with a decrease in Mmp-13 expression, C/EBPβ and RUNX2 seem to cooperatively promote matrix degradation through the Mmp-13 induction (Fig. 2E and Supplementary Material, Fig. S4). Although the Mmp-3 expression was also considerably decreased in the Cebp−/−:Runx2−/−+ limbs, this may be due to the effect of deficiency of both alleles of Runx2 because it was similarly decreased in the Cebp−/−+ limbs (Supplementary Material, Fig. S4). Expressions of other proteinases like Mmp-9, Adamts4 and Adamts5, as well as vascular endothelial growth factor (Vegf), an essential protein for vascularility, were little affected by the Cebp or Runx2 insufficiency (Supplementary Material, Fig. S4). When we examined the histological phenotypes of the compound deficient mice after birth, the hypertrophic zone seemed to have gradually been restored to normal, and by 16 weeks of age the growth plate phenotype in Cebp−/−:Runx2−/−+ mice was ameliorated (Supplementary Material, Fig. S3A–C).

C/EBPβ and RUNX2 cooperatively control OA development

In addition to the physiological role in the skeletal growth, we next examined the contribution of C/EBPβ and RUNX2 to OA development. Since this impairment was associated with a decrease in Mmp-13 expression, C/EBPβ and RUNX2 seem to cooperatively promote matrix degradation through the Mmp-13 induction (Fig. 2E and Supplementary Material, Fig. S4). Although the Mmp-3 expression was also considerably decreased in the Cebp−/−:Runx2−/−+ limbs, this may be due to the effect of deficiency of both alleles of Runx2 because it was similarly decreased in the Cebp−/−+ limbs (Supplementary Material, Fig. S4). Expressions of other proteinases like Mmp-9, Adamts4 and Adamts5, as well as vascular endothelial growth factor (Vegf), an essential protein for vascularility, were little affected by the Cebp or Runx2 insufficiency (Supplementary Material, Fig. S4). When we examined the histological phenotypes of the compound deficient mice after birth, the hypertrophic zone seemed to have gradually been restored to normal, and by 16 weeks of age the growth plate phenotype in Cebp−/−:Runx2−/−+ mice was ameliorated (Supplementary Material, Fig. S3A–C).
development in surgical and age-related OA models. In a surgical model by inducing instability to the knee joints of 8-week-old mice (4,5), C/EBPβ and Runx2 were co-expressed in chondrocytes of the superficial joint cartilage of wild-type mice with OA development for 8 weeks after surgery (Fig. 3A). To know the functional involvement, we compared
OA development among wild-type, Cebpb\textsuperscript{+/+}, Runx2\textsuperscript{+/+}, and Cebpb\textsuperscript{+/-};Runx2\textsuperscript{+/-} littermates that did not show significant skeletal abnormality under physiological conditions (Fig. 2B and Supplementary Material, Fig. S5A). We did not use Cebpb\textsuperscript{-/-} or Cebpb\textsuperscript{-/-};Runx2\textsuperscript{-/-} mice in this experiment since their skeletons were originally small, the joint shape was abnormal and the activity was low, so that mechanical stress caused by the joint instability was not assumed to be comparable with that of wild-type mice. The Cebpb\textsuperscript{+/-} or Runx2\textsuperscript{+/-} mice showed moderate suppression of OA development, as we previously reported (4,11), and the Cebpb\textsuperscript{+/-};Runx2\textsuperscript{+/-} mice exhibited greater suppression (Fig. 3A), which was confirmed by quantification with the OARSI histopathology grading systems (16,17) (Fig. 3B). When compared with knockout of either one allele of Cebpb or Runx2, the compound insufficiency caused a considerable decrease in Mmp-13, but not Col10a1, Mmp-9, Adamts4, Adamts5 or Vegf (Fig. 3A and Supplementary Material, Fig. S5B). Here again, Mmp-3 expression was similarly decreased in the Cebpb\textsuperscript{+/-};Runx2\textsuperscript{+/-} and Cebpb\textsuperscript{+/-} cartilages, suggesting the effect of deficiency of one allele of Cebpb (Supplementary Material, Fig. S5B). In an age-related OA model using 1-year-old mice of four genotypes under physiological conditions, knockout of either one allele of Cebpb or Runx2 also caused moderate suppression of OA development and the compound insufficiency caused greater and significant suppression with a decrease in Mmp-13 expression (Fig. 3C and D).

In surgical human knee joint specimens, C/EBP\(\beta\) and RUNX2 were co-expressed in the tibial cartilage with severe degradation (modified Mankin score = 13), although little detected in that with mild degradation (modified Mankin score = 4) (Fig. 3E and Supplementary Material, Fig. S5C).
Figure 3. Contribution of C/EBPβ and RUNX2 to OA development. (A) Safranin O staining and immunostaining with antibodies to C/EBPβ (red), Runx2 (green), the merged images (yellow), Col10a1, Mmp-13 and Hoechst nuclear staining of joint cartilage 0–8 weeks after surgical OA induction in the knee joints of 8-week-old wild-type (+/+-), Cebpb+/-, Runx2+/- and Cebpb+/-;Runx2+/- littermates. Boxed areas in each Safranin O-stained image indicate the regions shown in the enlarged immunofluorescent images below. Scale bars, 200 µm (top) and 100 µm (bottom). (B) Quantification of OA development on the femoral and tibial cartilage by OARSI histopathology grading systems in the knee joints of the four genotypes above. Data are expressed as means ± SEM. n = 8, *P < 0.05 and #P < 0.01 versus wild-type. (C) Safranin O staining and immunostaining with antibodies to Col10a1 and Mmp-13 in the knee joints of 1-year-old littermates of four genotypes. Boxed areas in each Safranin O-stained image indicate the regions shown in the enlarged immunofluorescent images below. Scale bars, 200 µm (top) and 100 µm (bottom). (D) Quantification of OA development as above in 1-year-old. n = 8, *P < 0.05 versus wild-type. (E) H&E staining, Safranin O staining and immunostaining with antibodies to C/EBPβ (red), RUNX2 (green) and the merged images (yellow) in human tibial cartilages of mild (modified Mankin score = 4) and severe degradation (13) stages obtained as surgical specimens of total knee arthroplasty. Scale bars, 50 µm.
To further investigate a possible association of the CELBPB gene with knee OA in humans, we searched a Japanese population-based cohort of the ROAD study (18) for sequence variations in the exon and the 5′-end flanking region of the CELBPB gene and identified two polymorphisms with minor allele frequencies >0.1: rs35698361 (GC and TT for major and minor alleles, respectively, at –422 to –421 from transcription start site (TSS); minor allele frequency = 0.251) and rs4253439 (C and T for major and minor alleles, respectively, at +636 from TSS; minor allele frequency = 0.346) (Table 1 and Supplementary Material, Fig. S6). However, a case-control comparison of allelic frequencies and their haplotype frequencies between 188 subjects with knee OA and 232 controls showed no significant association of these polymorphisms with knee OA (P > 0.05), implicating that CELBPB may not regulate human OA development by its own gene level.

C/EBPβ and RUNX2 transactivate MMP-13 as a protein complex in chondrocytes

To examine the downstream target of C/EBPβ and RUNX2 in chondrocytes, we created stable lines of SW1353 cells with retroviral overexpression of CELBPB, RUNX2, or their combination. COL10A1 and MMPs as well as alkaline phosphatase (ALP) staining, but not ADAMTS4 or VEGFA, were induced by CELBPB or RUNX2 overexpression, and the induction of MMP13 alone was significantly enhanced by the combination as compared to the single overexpression (Fig. 4A). Although cell proliferation was inhibited by the single overexpression of CELBPB or RUNX2, as we reported previously (11), this was not enhanced by their combination (Fig. 4B). In addition to endogenous expression, we examined the promoter activity of these genes by the luciferase assay, and confirmed the enhancement of the MMP13 transactivation by the combination (Fig. 4C). For the loss-of-function analysis, we have created stable lines of SW1353 cells with retroviral overexpression of specific siRNAs for CELBPB, RUNX2 or their combination, and found that the compound knockdown caused significant suppression of COL10A1, MMPs, ADAMTS4, VEGFA, and ALP (Fig. 4D). These lines of evidence indicate that C/EBPβ and RUNX2 may cooperatively promote cartilage degradation during the skeletal growth and OA development mainly through stimulation of the MMP-13 expression.

We further examined the mechanism underlying the transactivation of MMP13 by the combination of CELBPB and RUNX2. Deletion analyses of the 1 kb fragment of the MMP13 promoter predicted the core responsive element to be located between –144 and –89 bp (Fig. 5A), which contains a C/EBP-binding motif (−103/−97) and a RUNX-binding motif (osteoblast-specific cis-acting element 2 (OSE2); –138/−132). Site-directed mutagenesis in each motif caused significant suppression of the promoter activation by C/EBPβ and RUNX2, respectively, and that in both motifs caused further suppression of the activation by RUNX2, C/EBPβ, and their combination (Fig. 5B). Electrophoretic mobility shift assay (EMSA) showed the binding of C/EBPβ and RUNX2 proteins with the oligonucleotide including the region containing C/EBP and OSE2 motifs (Fig. 5C). The binding of C/EBPβ or RUNX2 was blocked only when mutations were created in both motifs, but not by mutagenesis in either motif alone, suggesting that these factors bind to respective motifs as a protein complex. The specificity was verified by the cold competition. Additionally, the supershift with C/EBPβ or RUNX2 antibody showed the specific binding of C/EBPβ or RUNX2 protein to the respective motifs. The chromatin immunoprecipitation (ChIP) assay showed the in vivo binding of C/EBPβ and RUNX2 to this region (Fig. 5D, top and middle blottings). Furthermore, the ChIP assay followed by release of the immune complexes and reimmunoprecipitation (ChIP-reIP assay) showed that the immunoprecipitate with a RUNX2 antibody was further immunoprecipitated by a C/EBPβ antibody and amplified by a primer set spanning the binding region (Fig. 5D, extreme right lane of the bottom blotting, and E, extreme right graph), confirming the complex formation of C/EBPβ and RUNX2 on this region. However, our sequence analyses using knee OA subjects in the ROAD study failed to detect genetic variations around this binding region in the human MMP13 promoter (Fig. 5F), again implicating that human OA may not be regulated by the gene level of the region.

### Hypoxia-inducible factor-2α (HIF-2α) is a transcriptional inducer of C/EBPβ in chondrocytes

Finally, to identify the upstream mechanism that regulates C/EBPβ expression, we performed a screening of transcription factors using a human CEBPB promoter fragment (−740 to +65 bp) (Fig. 6A). Among candidate molecules that are known to regulate chondrocyte differentiation, such as sex-determining region Y (SOX), RUNX, myocyte enhancer factor-2C (MEF2C), v-rel reticuloendotheliosis viral oncogene homolog A (REL), HIF, other C/EBPs, ATF, specificity protein-1 (SP1), intercellular domain of Notch1 (Notch1-ICD), recombination signal-binding protein for immunoglobulin kappa J region (RBP-J) and hairy and enhancer of split 1 (HES1), we found that HIF-2α (encoded by EPAS1) showed the strongest activation. Deletion analyses predicted the core responsive element to be located between −103 and −46 bp

### Table 1. Association of polymorphisms in the CELBPB locus with knee OA in a Japanese population of the ROAD study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Knee OA</th>
<th>Control</th>
<th>MAF</th>
<th>Test for allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs35698361</td>
<td>GC/TT</td>
<td>106</td>
<td>86</td>
<td>129</td>
<td>91</td>
</tr>
<tr>
<td>rs4253439</td>
<td>C/T</td>
<td>91</td>
<td>74</td>
<td>14</td>
<td>23</td>
</tr>
</tbody>
</table>

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

*Pearson’s χ²-test.
which contains a hypoxia-responsive element (HRE) motif (~69/–61). Site-directed mutagenesis in this motif caused a significant suppression of the promoter activation by HIF-2α (Fig. 6C). EMSA showed the binding of HIF-2α protein with this HRE region in the CEBPβ promoter, and the complex specificity was confirmed by the cold competition and by the supershift with an antibody to HIF-2α (Fig. 6D). In cultured primary chondrocytes, the Cebpb expression was enhanced by retroviral overexpression of HIF-2α and suppressed by that of the dominant-negative mutant (DN-HIF-2α) (Fig. 6E). We then looked at the C/EBPβ expression in the limb cartilage and OA joint cartilage of Epas1+/−/− mice, since Epas1−/− mice died at the early embryonic stage, as reported previously (7). The Epas1 haploinsufficiency caused a decrease in C/EBPβ expression in the limb cartilage of embryos (Fig. 6F). Furthermore, as we previously reported (7), the haploinsufficiency caused a resistance to cartilage degradation in the knee joint after surgical OA induction, which was associated with a decrease in C/EBPβ expression in the joint cartilage (Fig. 6G).

DISCUSSION

Although the previous studies have identified C/EBPβ as a potent transcription factor for endochondral ossification, the knockout in mice (Cebpb−/−) caused only a mild and transient impairment of the skeletal growth (11,14,15). This was thought to be owing to a compensatory mechanism by other C/EBP family members like C/EBPδ which is the principal partner for heterodimer formation and has the most similar function to C/EBPβ in mesenchymal cells (19,20). However, the C/EBPδ expression was much weaker than C/EBPβ in skeletal tissues, and was not altered in the Cebpb−/− mice (11,21), denying this possibility. Instead, we have identified RUNX2 as the most potent transcriptional partner of C/EBPβ. The compound knockout of Cebpb and Runx2 (Cebpb−/−;Runx2+/− and Cebpb+/−;Runx2−/−) affects cartilage degradation which is known to be the most critical step in the endochondral ossification process (22,23). We show that MMP-13 is the direct transcriptional target of C/EBPβ and RUNX2. Although we were unable to identify
any abnormality of growth plates even in Cebpb−/−; Runx2+/− mice at the age of 16 weeks (Supplementary Material, Fig. S3C), this is not surprising because the growth plates in Mmp13−/− mice have a lengthened hypertrophic zone from embryonic stages but the phenotype is gradually ameliorated after birth (23). The Runx2+/− mice are known to show a complete lack of Mmp-13 expression in cartilage (24,25), while Cebpb−/− mice show the suppression, but not abrogation (Fig. 2E) (11). Furthermore, the C/EBPβ overexpression markedly enhances the MMP-13 expression in combination with RUNX2 (Fig. 4A). These indicate that RUNX2 is indispensable to switch on the MMP13 transcription, whereas C/EBPβ modulates the MMP-13 expression level in the presence of RUNX2 during the skeletal growth. The insufficient suppression of MMP-13 expression by partial insufficiency of both C/EBPβ and Runx2 in the Cebpb−/−;Runx2+/− limb cartilage (Fig. 2E) and in cultured chondrocytes transfected with the specific siRNAs (Fig. 4D) may be due to the remainder of the basal expression by RUNX2 and its enhancement by C/EBPβ. This insufficient suppression of Mmp-13 expression (Fig. 2E) and more profound effect of impaired transition to hypertrophic differentiation (11) might be the cause of the apparently shortened hypertrophic zone of growth plates in Cebpb−/− mice at E14.5 (Fig. 2D). In addition, we could
not deny the possibility that C/EBPβ and RUNX2 have other target molecules, as the Cebpb<sup>−/−</sup>;Runx2<sup>−/−</sup> mice exhibited dwarfism even after birth (Fig. 2B), differently from Mmp13<sup>−/−</sup> mice. In fact, the previous studies showed the co-operative regulation of osteocalcin in osteoblasts by C/EBPβ and RUNX2 (14,20), which was supported by our current examination that the phenotype of cleidocranial dysplasia in Runx2<sup>−/−</sup> mice was enhanced under the Cebpb insufficiency (Fig. 2A and Supplementary Material, Fig. S2B and C).

The transactivation of MMP13 by C/EBPβ and RUNX2 is through their specific binding to a C/EBP-binding motif and an OSE2 motif, respectively, in the promoter. Although the identified OSE2 motif is the consensus site for RUNX2 binding in the MMP13 gene as shown by previous studies (25,26), the identified C/EBP-binding motif is different from that reported in a previous study which predicted a more distal region between −981 bp and −936 bp containing two C/EBP-binding motifs, but not a RUNX-binding motif, in particular chondrocytes of inflammatory arthritis (27). Considering much weaker activation by C/EBPβ alone than in combination with RUNX2 on the 1 kb MMP13 promoter containing the region (Fig. 4C), and only a slight decrease in the promoter activity between −1000 and −380 bp (Fig. 5A), this distal region may be responsible mainly for MMP-13 expression under inflammatory stimulations like rheumatoid arthritis.
however, there is about 30 bp distance between the C/EBP and OSE2 motifs, suggesting a conformational change of DNA or involvement of intervening proteins. Our screening also identified ATF4 as another possible transcriptional partner of C/EBPβ, but the transactvity on the C/EBP-binding motif was not suppressed by the CEBPB knockdown (Fig. 1A). While ATF4 is reported to be a key partner of C/EBPβ in osteoblasts by binding to the OSE1 motif (14), there is no OSE1 motif or other possible binding motif of ATF4 around the identified region in the MMP13 promoter.

Instead, HIF-2α may possibly function as the intervening protein, since there is an HRE motif (−106/−101) between the C/EBP-binding and OSE2 motifs in this region. This motif is just what we have identified as the core responsive element to HIF-2α in the MMP13 promoter. Also, our present and previous studies have found that HIF-2α directly binds to and activates the promoters of CEBPB and RUNX2 (Fig. 6) and RUNX2 (29), indicating that HIF-2α activates the MMP13 promoter directly and indirectly. HIF-2α is also a potent trans-activator of various key factors for the endochondral ossification process: COL10A1, MMP-3, -9, VEGF, Indian hedgehog and parathyroid hormone 1 receptor (7), so that HIF-2α may extensively control the sequential steps of this process. The present human genetic studies have failed to show the association of human CEBPB gene polymorphisms with knee OA (Table 1 and Supplementary Material, Fig. S6), nor was there a genetic variation around the identified responsive region in the human MMP13 promoter (Fig. 5F). In addition, our preliminary genome-wide association studies using the ROAD cohorts have failed to detect a significant association of single nucleotide polymorphisms (SNPs) in the human RUNX2 gene or in MMP13 gene with knee OA (data not shown), meaning that C/EBPβ, RUNX2 or MMP-13 may not clinically regulate the OA development by its own gene level. Contrarily, a functional SNP in the human EPAS1 gene which is related to the promoter activity is associated with knee OA (7). Hence, clinically the genetic variation of HIF-2α might possibly control the expression or activity of C/EBPβ and RUNX2, which then regulates the MMP13 trans-activation during OA development.

Taken together, the present study on a molecular network around C/EBPβ in chondrocytes has identified RUNX2 as the transcriptional partner, MMP-13 as the target and HIF-2α as the inducer during endochondral ossification, implicating that these may possibly represent therapeutic targets of OA. In fact, their knockout mice exhibit enhancement of cartilage degradation (4,7,11,30,31). Although ADAMTS5 is known to be another key regulator of OA development in the mouse models (32,33), ADAMTS4 and ADAMTS5 are little regulated by C/EBPβ or RUNX2 (Fig. 4 and Supplementary Material, Figs. S4 and S5B), indicating an independent pathway. The Cebpb+/−:Runx2+/− mice show much greater resistance to OA development than Cebpb+/− or Runx2+/− mice in the surgical and age-related models (Fig. 3), and little affected the skeletal growth (Fig. 2). Hence, the C/EBPβ and RUNX2 complex may represent a rational therapeutic target for OA with minimal effects on physiological skeletal homeostasis. Establishment of an effective and selective delivery system to chondrocytes, or identification of related extracellular signals that might be easier to target will be the next task to realize a disease-modifying treatment of OA.

MATERIALS AND METHODS

Computational predictions

We used database and online resource STRING ver8.3 (http://string.embl.de/, last accessed on November 24, 2011) generalizing access to protein interaction data, by integrating known and predicted interactions from a variety of sources.

Construction of expression vectors

We prepared expression vectors for the luciferase assay in pCMV-HA (Clontech) and siRNA vectors for the human CEBPB and RUNX2 gene (NM_005194.2: nucleotides 1633–1653, and NM_001024630.3: nucleotides 4311–4331, respectively) in piGENEhU6 vectors (iGENE Therapeutics). We created the dominant-negative HIF-2α mutant as described previously (34). We generated retroviral vectors using pMx vectors as described previously (35) and adeno-virus vectors by the AdenoX Expression system (Clontech), and we verified all vectors by DNA sequencing.

Mice

Cebpb- and Runx2-mutant mice were gifts from Shizuo Akira (Osaka University) and Toshihisa Komori (Nagasaki University), respectively (36,37). We purchased Epas1-mutant mice (38) from the Jackson Laboratory. We performed all experiments according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo. In each experiment, we compared genotypes of male littermates that were maintained in a C57BL/6 background.

Cell cultures

We cultured SW1353 cells (American Type Culture Collection) and ATDC5 cells (Riken BRC) in Dulbecco’s modified Eagle medium: nutrient mixture F-12 (DMEM/F12) (1:1) with 10 and 5% fetal bovine serum (FBS), respectively. We cultured ATDC5 cells for 3 weeks with insulin to induce hypertrophic differentiation. We isolated primary chondrocytes from the ribs of mouse embryos, and cultured them in a monolayer for 1 week in DMEM with 10% FBS. We assessed cell proliferation using a CCK-8 Assay Kit (Dojindo) and ALP activity as previously described (11). For immunocytochemistry, after fixation of 3.7% formalin, we incubated the cells with antibodies to C/EBPβ (C-19; Santa Cruz Biotechnology Inc.), RUNX2 (27-K; ibid). We used a secondary antibody conjugated with Alexa Fluor 568 (Invitrogen) for C/EBPβ, and a CSA II Biotin-Free Catalyzed Amplification System (DAKO) for RUNX2, and applied Hoechst 33258 nuclear stain (Invitrogen) for counterstaining.
Mammalian two-hybrid assay
We performed the mammalian two-hybrid assay using the Checkmate mammalian two-hybrid system (Promega) and the PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink).

Luciferase assay
We purchased pC/EBP-Luc construct from Stratagene. We prepared the COL10A1 promoter region (from −1,028 to +127 bp relative to the TSS), MMP3 (−1,551 to +39), MMP9 (−1,775 to +17), MMP13 (−1,000 to −1), ADAMTS4 (−2,406 to +27), ADAMTS5 (−1,242 to +27), VEGFA (−1,000 to −1), ALP (−3,000 to +3,000) and CEBPB (−740 to +65) by polymerase chain reaction (PCR) using human genomic DNA as the template, and we cloned them into the pGL3-Basic vector or pGL4.10 [luc2] vector (Promega). We created deletion and mutation constructs by PCR, performed luciferase assays with the PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink) and showed the data as the ratio of the firefly activities to the Renilla activities.

Histological analysis
We performed double staining of skeletons of mouse embryos or neonates with a solution containing Alizarin red S and Alcian blue 8GX (Sigma) after fixation in 95% ethanol and acetone. We performed H&E and Safranin O stainings according to standard protocols after fixation in 4% paraformaldehyde buffered with PBS. For immunohistochemistry, we incubated the sections with antibodies to C/EBPβ (C-19; Santa Cruz Biotechnology Inc.), Runx2 (27-K; Santa Cruz Biotechnology Inc.), Vegf (A-20; Santa Cruz Biotechnology Inc.), Mmp-3 (AA07; Santa Cruz Biotechnology Inc.), Mmp-9 (H-129; Santa Cruz Biotechnology Inc.), Adamts4 (H-74; Santa Cruz Biotechnology Inc.) and Adamts5 (H-200; Santa Cruz Biotechnology Inc.), Col10a1 (LSL) and Mmp-13 (Chemicon) diluted 1:500 in blocking reagent. For immunofluorescence, we used a secondary antibody conjugated with Alexa Fluor 568 (Invitrogen) for C/EBPβ, and a CSA II Biotin-Free Catalyzed Amplification System (DAKO) for other molecules, and applied Hoechst 33258 nuclear stain (Invitrogen) for counterstaining. For immunoperoxidase methods in Col10a1 and Mmp-13 detection, we also used the CSAII System, and applied methylgreen for counterstaining. Images of the sections were taken at room temperature with a BZ-8000 microscope (Keyence) and BZ Viewer software (ibid) by using a Plan Apo 10x NA 0.45 objective lens (Nikon). The contrast of the images was enhanced using BZ Analyzer (Keyence) for better rendering without altering the relationship of the target to the control images.

OA experiment
We performed the surgical procedure to create an experimental OA model on 8-week-old male mice as reported previously (4,5) and we analyzed them 8 weeks after surgery. We also used the age-related OA model on 1-year-old mice bred under physiological conditions. We quantified OA severity by the OARSI histopathology grading system (0–6 for grade and 0–24 for score) (16,17), which was assessed by a single observer who was blinded to the experimental group.

Real-time RT–PCR
We extracted total RNA from SW1353 cells cultured for 2 weeks after confluency using standard protocols. We performed real-time RT–PCR with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using FastStart Universal SYBR Green Master (Roche) with GAPDH as the internal control. We ran all reactions in triplicate. Primer sequence information is available upon request.

Electrophoretic mobility shift assay
We prepared nuclear extracts from COS-7 cells adenovirally transfected with C/EBPβ, RUNX2 or HIF-2α, and we performed the EMSA with the DIG Gel Shift Kit (Roche). Regions of the oligonucleotide probe were as follows: MMP13, from −150 to −90 bp relative to the TSS; CEBPB, −85 to −35. For competition analysis, we used 50-fold excess of unlabeled competitor probe containing the binding reaction. For the supershift experiment, we added 1 μl of an antibody to C/EBPβ (C-19; Santa Cruz Biotechnology Inc.), RUNX2 (M-70; Santa Cruz Biotechnology Inc.) or HIF-2α (H-310; Santa Cruz Biotechnology Inc.).

ChIP and ChIP-reIP assay
We performed the ChIP assay in SW1353 cells with a OneDay ChIP kit (Diagenode). For immunoprecipitation, we used antibodies to RUNX2 (M-70 and S-19; Santa Cruz Biotechnology Inc.), C/EBPβ (C-19 and H-7; Santa Cruz Biotechnology Inc.) and the normal rabbit immunoglobulin G (IgG) (Diagenode). Primer sets, one spanning and the other not spanning the identified responsive element, are ranged from −213 to −28 and from −3446 to −3243, respectively. We further performed the ChIP-reIP assay by the sequential application of the above-mentioned ChIP assay on the sequential precipitates with the normal rabbit IgG or anti-RUNX2 in the cell lysates and their supernatants. For the quantification, we performed real-time PCR with the ABI Prism 7000 Sequence Detection System (Applied Biosystems) as the abovementioned.

Human samples
We obtained human samples from individuals undergoing total knee arthroplasty after obtaining written informed consent as approved by the Ethics Committee of the University of Tokyo. We histologically assessed cartilage samples by the modified Mankin scoring system (39,40).

Human genetic studies
We recruited individuals over 50 years of age with (n = 188; mean age, 76.9; range, 59–88) and without (n = 232; 76.4; 62–87) knee OA in a population-based cohort of the ROAD study (18). We diagnosed OA on the basis of radiographic findings by the Kellgren–Lawrence grading system (41): the knee OA population included individuals with Grades 3 and
4 and the control population with Grades 0 and 1. After obtaining written informed consent as approved by the Ethics Committee, we extracted genomic DNA from peripheral blood leukocytes of individuals using standard protocols. We searched polymorphisms around the CEBPB gene using the dbSNP-database (http://www.ncbi.nlm.nih.gov/SNP/) last accessed on November 24, 2011), and we genotyped an identified MNP (rs35698361) by direct DNA sequencing using a sequence primer ranging from −607 to −588 bp relative to the TSS, and the other identified SNP (rs4253439) by PCR-restriction fragment length polymorphism using BmgT120I (Takara Bio) as the enzyme. We also genotyped the region around the identified C/EBP-β and RUNX2 binding sites in the human MMP13 promoter of 96 case and control subjects randomly selected from the ROAD study by direct DNA sequencing using a reverse sequence primer ranging from −48 to −29 bp relative to the TSS. We confirmed that the P value of the Hardy–Weinberg equilibrium test in the control population was >0.01.

Statistical analysis

We reported all data as means ± SEM of at least three independent experiments, each performed in triplicate. We compared means of groups by ANOVA, and determined the significance of differences by post-hoc testing using Tukey’s method in parametric values and Steel’s method in non-parametric values. In the case control association study, we evaluated genotypic and allelic models by the χ² test for the Hardy–Weinberg equilibrium using spreadsheet software (Excel). P values <0.05 were considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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