Table III
Association of VDR gene polymorphisms and knee pain

	Total	Number (%) with knee pain	Crude OR (95% CI)	*P*-value	Adjusted OR* (95% CI)	*P*-value	Power†	FPRP prior probability	
								0.1	0.01
Fok1									
FF	328	115 (35.1)	1.00		1.00				
Ff	333	139 (41.7)	1.19 (0.89, 1.61)	0.244	1.14 (0.84, 1.56)	0.398	0.71	0.83	0.98
ff	108	51 (47.2)	1.50 (1.00, 2.24)	0.052	1.60 (1.07, 2.39)	0.022	0.40	0.33	0.84
Cdx2									
GG	491	189 (38.5)	1.00		1.00				
AG	248	94 (37.9)	1.05 (0.78, 1.42)	0.733	0.99 (0.73, 1.34)	0.936	0.71	0.92	0.99
AA	29	15 (51.7)	2.20 (1.08, 4.47)	0.03	2.21 (1.07, 4.56)	0.032	0.14	0.67	0.96
Apa1									
AA	213	87 (40.8)	1.00		1.00				
Aa	388	151 (38.9)	0.90 (0.65, 1.23)	0.5	0.93 (0.67, 1.30)	0.678	0.62	0.91	0.99
aa	166	64 (38.6)	0.97 (0.65, 1.43)	0.864	0.92 (0.61, 1.40)	0.71	0.45	0.93	0.99

Of 787 subjects, genotyping was completed for 769, 768 and 767 with Fok1, Csk2 and Apa1 polymorphism of the VDR, respectively.
* As both knees have a pain score, GEE population averaged logistic regression analysis after adjustment for age, gender, BMI and KL grade was used to calculate adjusted OR.
† To detect an OR of 1.5, we are looking for a difference in proportions of 39.3% vs 49.3% for knee pain.

articular cartilage *in vitro*[13], and this suggests that vitamin D may directly affect articular cartilage metabolism. Further, *in vitro* experiments confirmed that loss of VDR in chondrocytes reduced osteoclastogenesis by inducing receptor activator of NF-κB ligand (RANKL) expression[38], indicating that polymorphism of the VDR may affect osteophyte formation. In addition, the VDR gene has a thymine to cytosine single nucleotide polymorphism (SNP) at the Fok1 restriction site in the first of two potential start (ATG) codons located in the 50 region, resulting in a VDR protein that is shorter by three amino acids[39]. The F allele lacks the first ATG; thus, translation starts at the second ATG, instead of the first ATG, where translation of the f allele starts[40]. Most data indicate that the F allele is more effective than the f allele in transactivation of the 1,25-dihydroxyvitamin D signal[41]. However, a meta-analysis studying the association between VDR polymorphisms and OA[42] found no associations between VDR variation and OA. The ongoing GWAS studies on OA did not also find the foci polymorphism[43,44]. In the present study, the best *P*-value is only 0.022 which would be at least 0.066 when adjusted. Given the lack of a replication cohort, the evidence for an association between vitamin D genetic variation and pain in knee OA is very weak. In addition, considering that the sample size is modest for association studies in general, and more specifically for genetic association studies, the significant association of VDR gene polymorphism with radiographic knee OA in the present study may be due to random error. Additional and larger studies will be required, and, longitudinal studies may also determine whether this locus has any influence on the progression of joint damage at the knee.

IOF Working Group suggests that 75 nmol/L is the appropriate target level of serum 25(OH)D for individuals[45]. Vitamin D insufficiency, defined as 25(OH)D levels <75 nmol/L is prevalent worldwide[46], and the present study also showed that 604/683 (88.4%) had vitamin D insufficiency defined as <75 nmol/L. While, the association of serum vitamin D level and radiographic knee OA is controversial[24–27], McAlindon suggested that subjects with low serum levels of vitamin D are approximately three times more likely to have progression of established knee OA than subjects with high serum levels[24], but the number of subjects with progressive knee OA were comparably small in the study. Hunter *et al.* found that there was evidence of decreased vitamin D levels in subjects with knee osteophytes compared to those without osteophyte, but after adjusting for age, BMI and relatedness, the significant differences disappeared[25]. While, the Framingham study also found no association of vitamin D levels with knee OA worsening, defined as joint space loss on radiography or as worsening cartilage score on magnetic resonance imaging (MRI)[26]. In the present study, contrary to VDR gene polymorphisms, there were no significant association between vitamin D level and radiographic knee OA. Further, there were no differences in association of vitamin D level with radiographic knee OA among VDR gene polymorphisms.

Like radiographic knee OA, a Fok1 polymorphism of the VDR was significantly associated with knee pain in the present study. Further, knee pain also tended to be associated with vitamin D level, although it was not associated with radiographic knee OA. The correlation with the radiographic severity of knee OA is controversial[4,30–32]. In our previous study, 10% of men and 20% of women without radiographic knee OA had knee pain, and approximately 50% of men and 40% of women with severe radiographic knee OA had no knee pain in the elderly[4]. This indicates

Table IV
Association of 25(OH)D level with radiographic knee OA and knee pain

	Radiographic knee OA					Knee pain				
	n (%)	Crude OR (95% CI)	*P*-value	Adjusted OR* (95% CI)	*P*-value	*n* (%)	Crude OR (95% CI)	*P*-value	Adjusted OR† (95% CI)	*P*-value
25(OH)D level		0.99 (0.90, 1.10)	0.889	1.03 (0.92, 1.16)	0.627		–	–	–	–
Tertile 3 (51.2–147)	30/225 (13.3)	–	–	–	–	79/225 (35.1)	1.00		1.00	
Tertile 2 (35.9–51)	41/229 (17.9)	–	–	–	–	89/229 (38.9)	1.10 (0.77, 1.58)	0.598	1.04 (0.70, 1.56)	0.832
Tertile 1 (17–35.8)	36/229 (15.7)	–	–	–	–	105/229 (45.9)	1.48 (1.04, 2.10)	0.031	1.47 (0.95, 2.25)	0.08

OR of continuous vitamin D is for a 10-unit increase. For knee pain effect of vitamin D level was non-linear, so stratified into tertiles. Of 787 subjects, 25(OH)D was measured in 683 subjects.
* As both knees have and a radiographic grade, GEE population averaged logistic regression analysis after adjustment for age, gender, BMI and season of the clinic visit was used to calculate adjusted OR.
† As both knees have a pain score, GEE population averaged logistic regression analysis after adjustment for age, gender, BMI, season of the clinic visit and KL grade was used to calculate adjusted OR.

that there may be other factors associated with knee pain rather than radiographic knee OA, but there were few studies regarding factors associated with knee pain. Previous studies have shown that age, female sex and physical demanding work were associated with knee pain[32–35], but these factors were also reported as those associated with radiographic knee OA[4,9]. In the present study, vitamin D level tended to be associated with knee pain without association with radiographic knee OA, indicating that the association of vitamin D level with knee pain may be independent of radiographic knee OA. In fact, the result was almost similar after adjustment for radiographic knee OA, although it did not reach significance. Previous study has shown that vitamin D deficiency was related to quadriceps weakness[47], which is strongly associated with knee pain and disability in the community, even when activation and psychological factors are taken into account[48]. This may partly explain the association of vitamin D level and knee pain.

There are several limitations in the present study. First, the sample size was modest for association studies in general, and more specifically for genetic association studies. Further, we did not make multiple testing adjustments in the present study. In addition, studies reporting biomarker associations and, even more so, genetic associations have suffered from the report of false positives and the best way of addressing this is by testing these associations in independent cohorts and replicating the results. Thus the association of VDR gene polymorphisms with knee pain may be due to random error. However, FPRP values were low for association of Apa1 (Aa) on radiographic knee OA, and Fok1 (ff) for knee pain, suggesting these associations may be noteworthy, thus, these may merit replication in further studies. Second, we did not analyze Bsm and Taq, although these SNP are near Apa1. Third, 25(OH)D should have different association with different feature of ROA such as joint space narrowing or osteophytosis, but we did not analyze the association of joint space narrowing or osteophytosis with 25(OH)D or VDR polymorphisms.

In conclusion, the present cross-sectional study using a large-scale population from the Hertfordshire Cohort study revealed that a Fok1 and Cdx2 polymorphism of the VDR were significantly associated with knee pain, but not with radiographic knee OA. There were no associations between radiographic knee OA and vitamin D level, but it tended to be associated with knee pain. Further replication of our results will be required to elucidate the association of vitamin D and knee OA.

Author contributions

All authors have made substantial contributions to all three of sections (1), (2) and (3) below:

(1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data,

(2) drafting the article or revising it critically for important intellectual content,

(3) final approval of the version to be submitted.

Conflict of interest

There are no conflicts of interest.

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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 (*Cebpb*$^{-/-}$) 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 *CEBPB* gene or the small interfering RNA (siRNA) specific for *CEBPB*. 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 si*GFP*-transfected cells) (Fig. 1A). Although the *CEBPB* overexpression further enhanced the transactivities of both RUNX2 and ATF4, the *CEBPB* 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 nuclear 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. *Cebpb*$^{-/-}$ and *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ mice were born at much lower frequencies than the expected Mendelian ratio and short-lived even after birth. Although *Cebpb*$^{+/-}$ mice were normal, *Cebpb*$^{-/-}$ 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 *Cebpb*$^{+/-}$;*Runx2*$^{+/-}$ mice did not show a significant growth retardation; however, *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ mice showed more severe dwarfism than their *Cebpb*$^{-/-}$ 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 *Cebpb*$^{-/-}$, *Cebpb*$^{+/-}$;*Runx2*$^{+/-}$ and *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ littermates, indicating a delay of chondrocyte hypertrophy by the *Cebpb* 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 *Cebpb*$^{-/-}$ and *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ littermates; however, that of the hypertrophic zone with Col10a1 expression was elongated and that of the bone area was markedly decreased in the *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ limbs (Fig. 2C–E and Supplementary Material, Fig. S3A and B), demonstrating that *Runx2* insufficiency caused impairment of steps later than chondrocyte hypertrophy under the *Cebpb* 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 *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ limbs, this may be due to the effect of deficiency of both alleles of *Cebpb* because it was similarly decreased in the *Cebpb*$^{-/-}$ 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 vascularity, were little affected by the *Cebpb* 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 *Cebpb*$^{-/-}$;*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

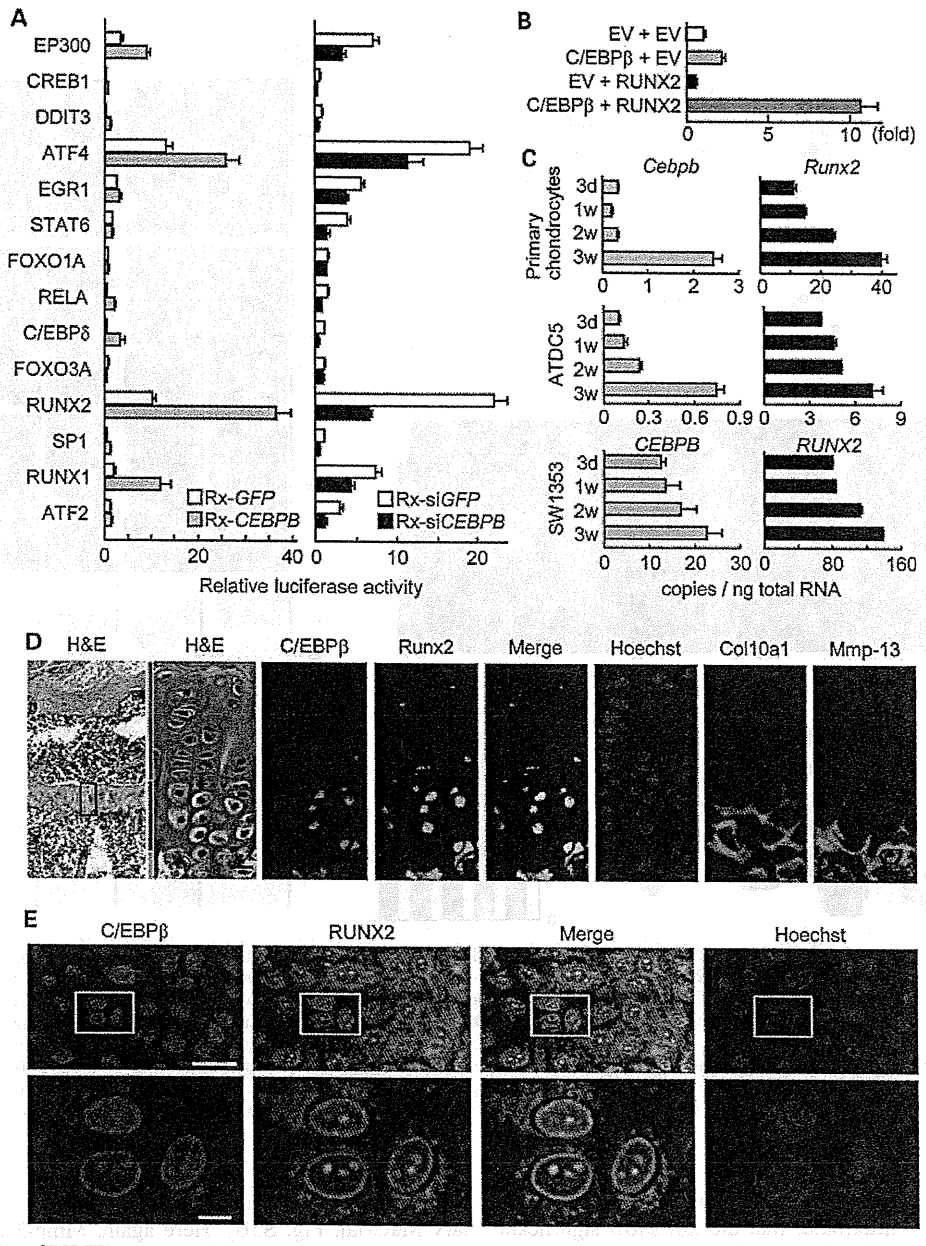

Figure 1. Identification of RUNX2 as a transcriptional partner of C/EBPβ in chondrocytes. (**A**) Luciferase activities after transfections of 14 selected transcription factors into human chondrogenic SW1353 cells with a reporter construct containing three consensus C/EBP-binding sequences, which are retrovirally transfected with *CEBPB* (Rx-*CEBPB*) or the siRNA (Rx-si*CEBPB*), when compared with the respective control (Rx-*GFP* or Rx-si*GFP*). Experiments were done in triplicate with data shown as means ± SEM. (**B**) 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. Experiments were done in triplicate with data shown as means ± SEM of relative fold increase in luciferase activity when compared with EV + EV (which was arbitrarily set to 1). (**C**) The time course of mRNA levels of *Cebpb* and *Runx2* during differentiation of mouse primary chondrocytes, ATDC5 cells and SW1353 cells cultured for 3 weeks. Experiments were done in triplicate with data shown as means ± SEM. (**D**) Hematoxylin-eosin (H&E) and immunostaining with antibodies to C/EBPβ (red), Runx2 (green), the merged images (yellow), Col10a1, Mmp-13 and Hoechst nuclear staining (blue) in the growth plate cartilage of proximal tibias of 16-week-old mice. The boxed area in the left H&E-stained image indicates the regions shown in the right-enlarged images. Red, blue and green bars indicate layers of proliferative and hypertrophic zones and bone area, respectively. Scale bar, 20 μm. (**E**) Subcellular localization of C/EBPβ (red), RUNX2 (green) and the merged images (yellow) in SW1353 cells. Boxed areas in top images indicate the regions shown in the respective bottom-enlarged images. Scale bars, 50 μm (top) and 10 μm (bottom).

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

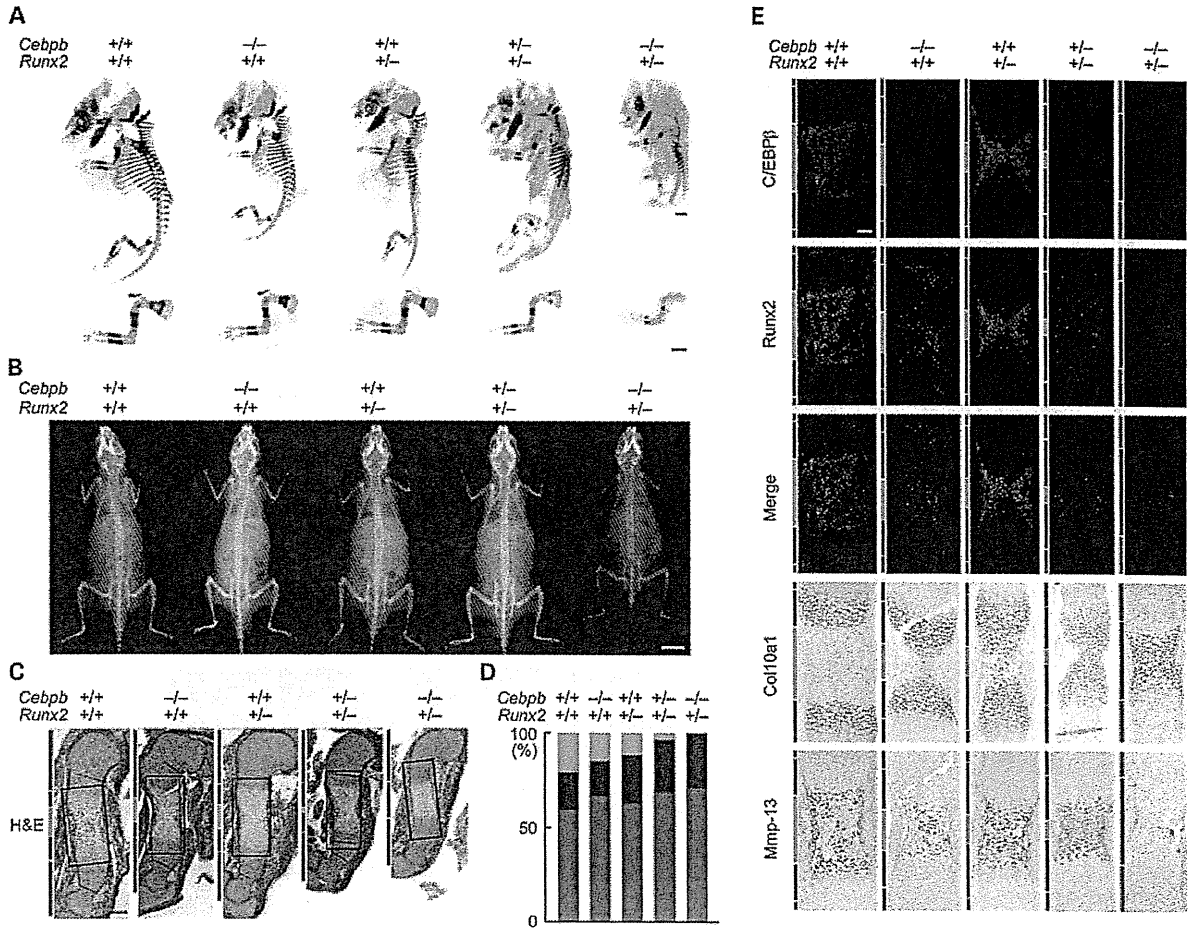

Figure 2. Skeletal findings of five genotype littermates. (**A**) Double staining with Alizarin red and Alcian blue of the whole skeletons (top) and forelimbs and clavicles (bottom) of wild-type (*Cebpb*$^{+/+}$;*Runx2*$^{+/+}$), *Cebpb* homozygous-knockout (*Cebpb*$^{-/-}$), *Runx2* heterozygous-knockout (*Runx2*$^{+/-}$), *Cebpb* heterozygous- and *Runx2* heterozygous-knockout (*Cebpb*$^{+/-}$;*Runx2*$^{+/-}$) and *Cebpb* homozygous- and *Runx2* heterozygous-knockout (*Cebpb*$^{-/-}$;*Runx2*$^{+/-}$) littermates (E14.5). Scale bars, 1 mm. (**B**) Plain radiographs of the whole skeletons of five genotype littermates at 12 weeks of age. Scale bar, 1 cm. (**C**) H&E staining of the humerus of five genotype littermates (E14.5). The boxed areas indicate the regions shown in the enlarged immunostaining images in (**E**). Red, blue and green bars to the left indicate layers of proliferative and hypertrophic zones and bone area, respectively. Scale bar, 200 μm. (**D**) Percentage of the length of proliferative zone (red), hypertrophic zone (blue) and bone area (green) over the total humeral length of the five genotype littermates. (**E**) Immunostaining with antibodies to C/EBPβ (red), Runx2 (green), the merged images (yellow), Col10a1 and Mmp-13 in the boxed areas above. Scale bar, 100 μm.

OA development among wild-type, *Cebpb*$^{+/-}$, *Runx2*$^{+/-}$, and *Cebpb*$^{+/-}$;*Runx2*$^{+/-}$ littermates that did not show significant skeletal abnormality under physiological conditions (Fig. 2B and Supplementary Material, Fig. S5A). We did not use *Cebpb*$^{-/-}$ or *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ 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*$^{+/-}$ or *Runx2*$^{+/-}$ mice showed moderate suppression of OA development, as we previously reported (4,11), and the *Cebpb*$^{+/-}$;*Runx2*$^{+/-}$ 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*$^{+/-}$;*Runx2*$^{+/-}$ and *Cebpb*$^{+/-}$ 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/EBPβ 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).

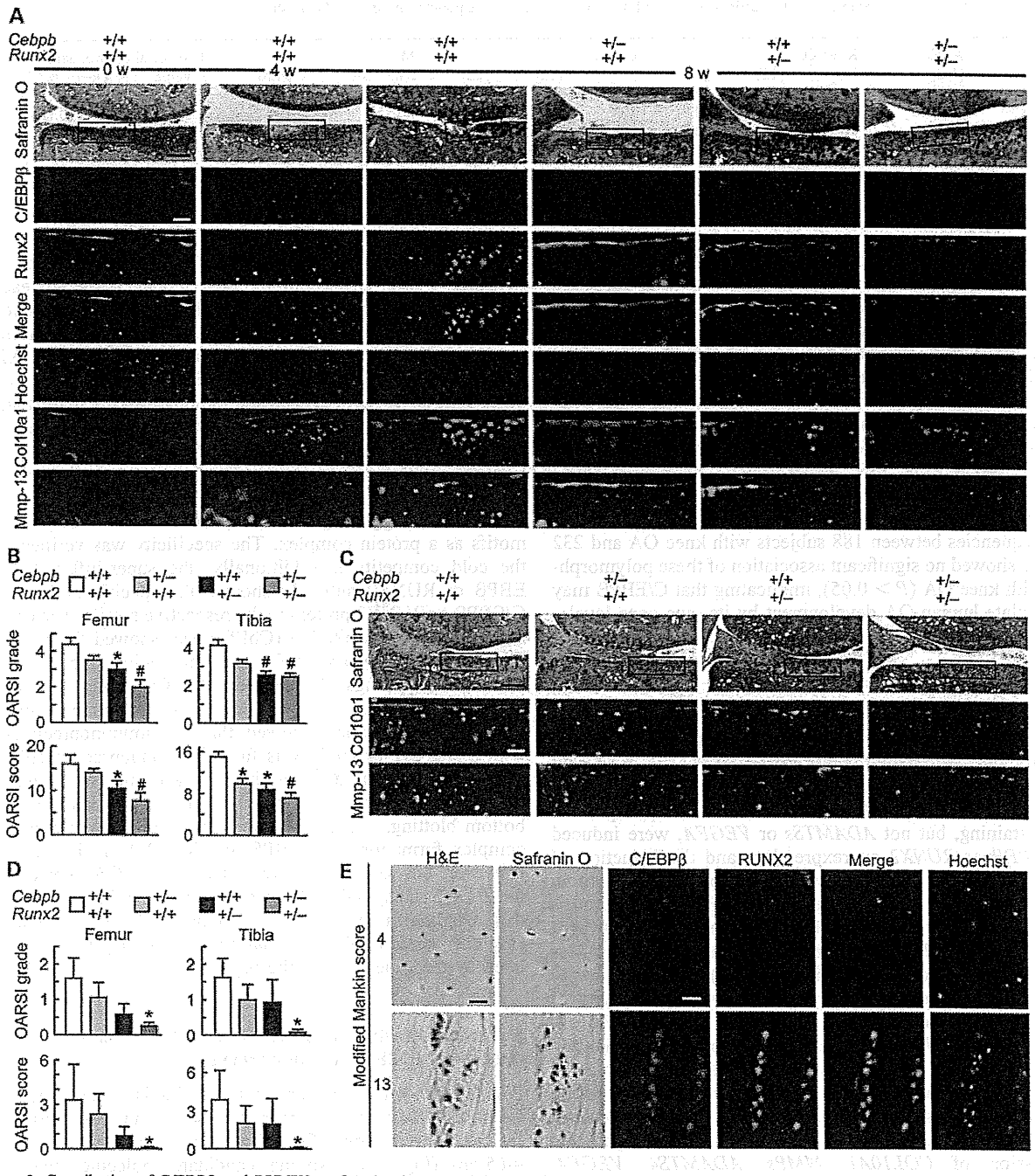

Figure 3. Contribution of C/EBPβ and RUNX2 to OA development. (**A**) Safranin O staining and immunostaining with antibodies to C/EBPβ (red), Runx2 (green), the merged images (yellow), Col10a1, Mmp-13 and Hoechst nuclear staining of joint cartilage 0–8 weeks after surgical OA induction in the knee joints 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.

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

SNP	Allele [1/2]	Knee OA [11]	[12]	[22]	Control [11]	[12]	[22]	MAF Knee OA	Control	Test for allele frequency[a] *P* value	OR (95%CI)
rs35698361	[GC/TT]	106	68	14	129	91	12	0.255	0.248	0.803	1.04 (0.76–1.42)
rs4253439	[C/T]	91	74	23	96	101	35	0.319	0.369	0.134	0.80 (0.60–1.07)

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.
[a]Pearson's χ^2-test.

To further investigate a possible association of the *CEBPB* 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 *CEBPB* 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 C/EBPβ 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 *CEBPB, RUNX2,* or their combination. *COL10A1* and *MMPs* as well as alkaline phosphatase (ALP) staining, but not *ADAMTSs* or *VEGFA*, were induced by *CEBPB* 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 *CEBPB* 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 *CEBPB, 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 *CEBPB* 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 box (SOX), RUNX, myocyte enhancer factor-2C (MEF2C), v-rel reticuloendotheliosis viral oncogene homolog A (RELA), 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

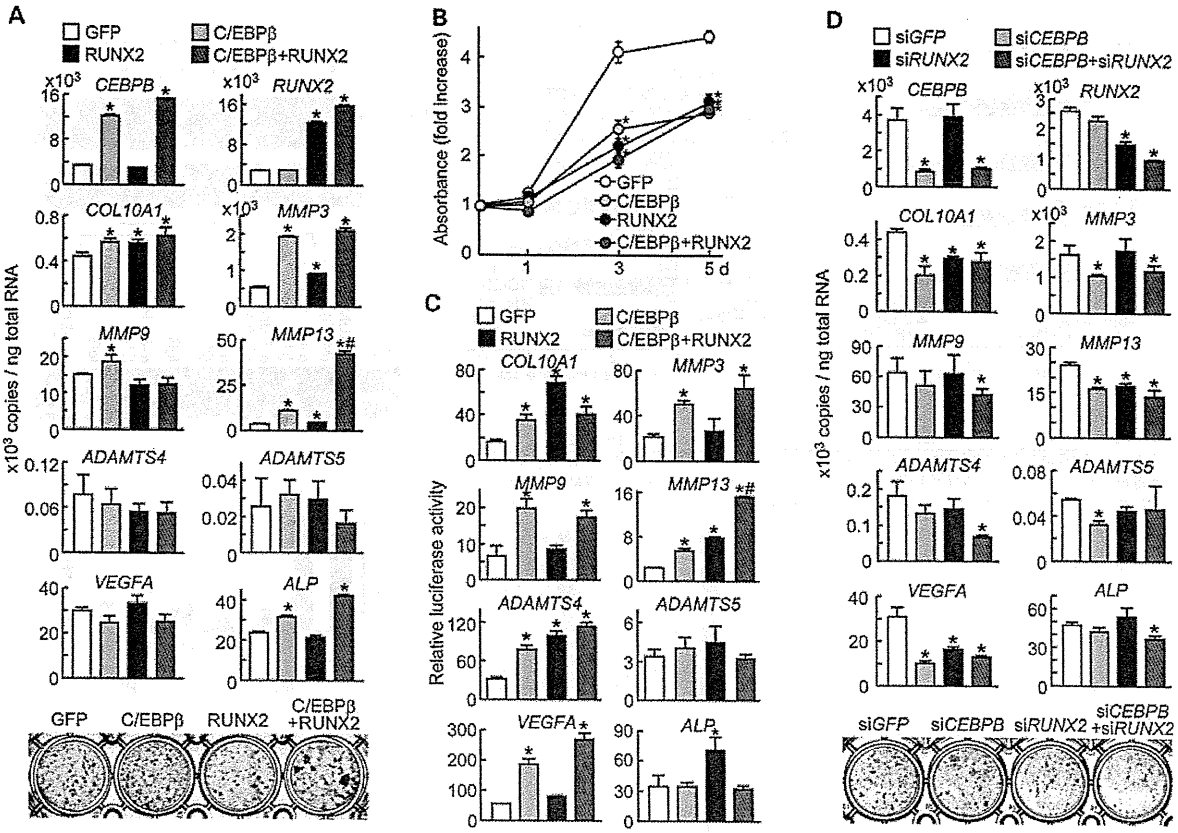

Figure 4. Effects of gain and loss of functions of C/EBPβ and RUNX2 on endochondral ossification parameters in cultures of chondrocytes. (**A**) mRNA levels of *CEBPB*, *RUNX2*, *COL10A1*, *MMP3*, *9*, *13*, *ADAMTS4*, *5*, *VEGFA* and *ALP* (graphs) and ALP staining (bottom) in stable lines of SW1353 cells retrovirally transfected with *CEBPB*, *RUNX2*, their combination, or the control *GFP*. (**B**) Growth curves by the CCK-8 assay of stable lines of SW1353 cells retrovirally transfected with the genes above. $^{*}P < 0.01$ versus *GFP* (**C**) Promoter activities by luciferase assays of *COL10A1*, *MMP3*, *9*, *13*, *ADAMTS4*, *5*, *VEGFA* and *ALP* by transfections of *CEBPB*, *RUNX2*, their combination or the control *GFP* in SW1353 cells co-transfected with reporter constructs containing respective proximal promoter fragments (~1–3 kb). (**D**) mRNA levels of the factors above and ALP staining in stable lines of SW1353 cells retrovirally transfected with siRNA specific for *CEBPB*, *RUNX2*, their combination or the control *GFP*. All experiments were done in triplicate with data shown as means ± SEM. $^{*}P < 0.05$ versus *GFP* or si*GFP*, $^{\#}P < 0.05$ versus both *CEBPB* alone and *RUNX2* alone.

(Fig. 6B), 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 *CEBPB* 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

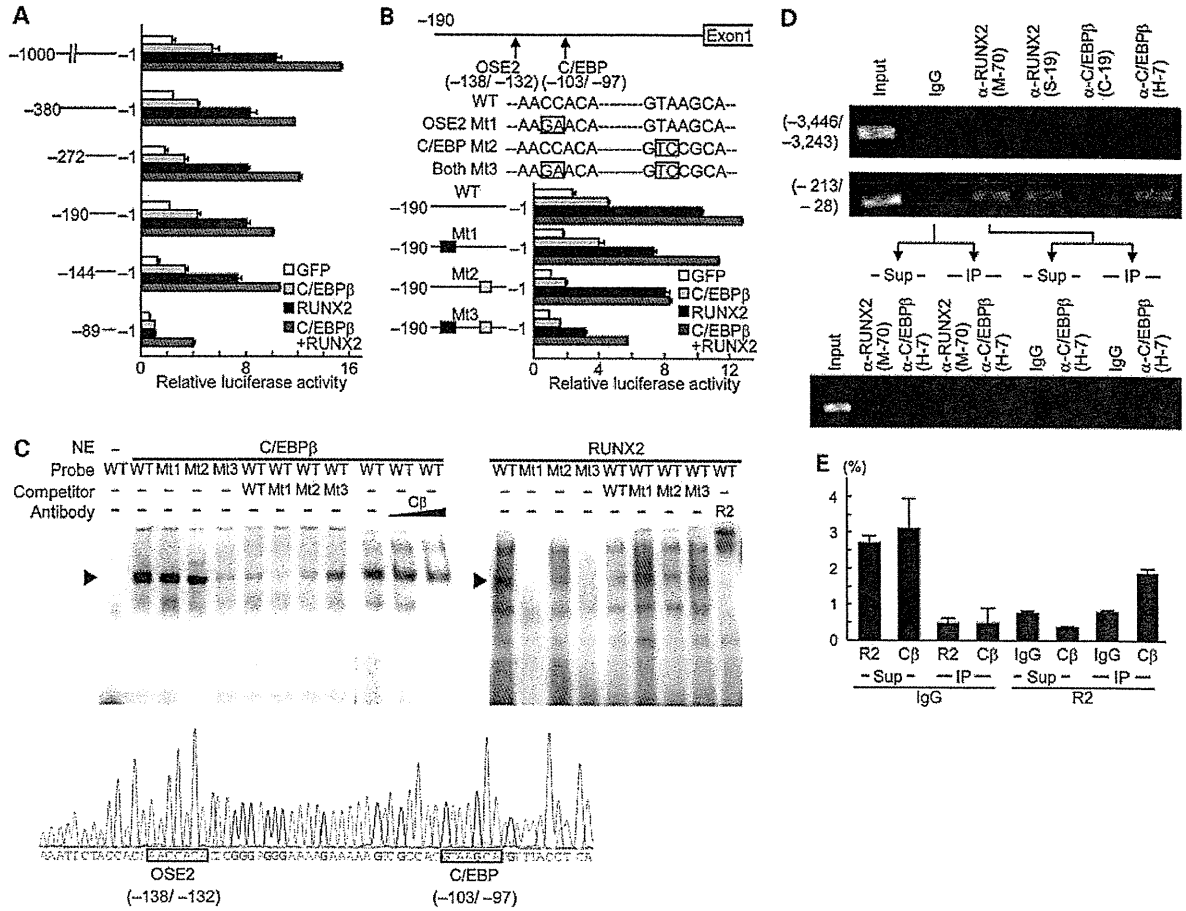

Figure 5. Transcriptional regulation of MMP13 by C/EBPβ and RUNX2. (**A**) Deletion analysis using luciferase-reporter constructs containing the 5′-flanking sequence from −1000 to −1 bp of the *MMP13* gene and a series of deletion fragments in SW1353 cells transfected with *CEBPB, RUNX2*, their combination or the control *GFP*. Experiments were done in triplicate with data shown as means ± SEM. (**B**) Site-directed mutagenesis analysis using luciferase-reporter constructs containing −190/−1 of the *MMP13* gene in the SW1353 cells above. Mutations were created in the OSE2 motif (Mt1), C/EBP-binding motif (Mt2) or both (Mt3). (**C**) EMSA for specific binding (arrowhead) of the wild-type (WT) oligonucleotide probe containing C/EBP and OSE2 motifs above or the mutated probes (Mt1, Mt2 and Mt3) with nuclear extract (NE) of COS-7 cells overexpressing C/EBPβ (left) or RUNX2 (right). The cold competition with a 50-fold excess of unlabeled WT or the mutated probes, and the supershift by an antibody to C/EBPβ (Cβ) or RUNX2 (R2) are presented. (**D**) ChIP (top and middle) and ChIP-reIP (bottom) assays. The ChIP assay was performed using cell lysates of SW1353 cells that were amplified by a primer set spanning the identified region (middle: −213/−28 bp) or not spanning the region (top: −3,446/−3,243 bp) before (input) and after immunoprecipitation with antibodies to RUNX2 (α-RUNX2: M-70 and S-19), C/EBPβ (α-C/EBPβ: C-19 and H-7) or non-immune IgG (IgG). For the ChIP-reIP assay, immunoprecipitates (IP) with non-immune IgG or anti-RUNX2 in the lysates above and their supernatants (Sup) were sequentially applied for another ChIP analysis. (**E**) Quantification of the ChIP-reIP above by the real-time polymerase chain reaction (RT-PCR) analysis using antibodies to C/EBPβ (Cβ), RUNX2 (R2), or non-immune IgG. Experiments were done in triplicate with data shown as means ± SEM of the percentage of the input. (**F**) Sequence analyses around the RUNX2 (OSE2; −138/−132) and C/EBPβ (C/EBP; −103/−97)-binding regions identified by the luciferase assay in the human MMP13 gene of 96 case and control subjects in the ROAD study.

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

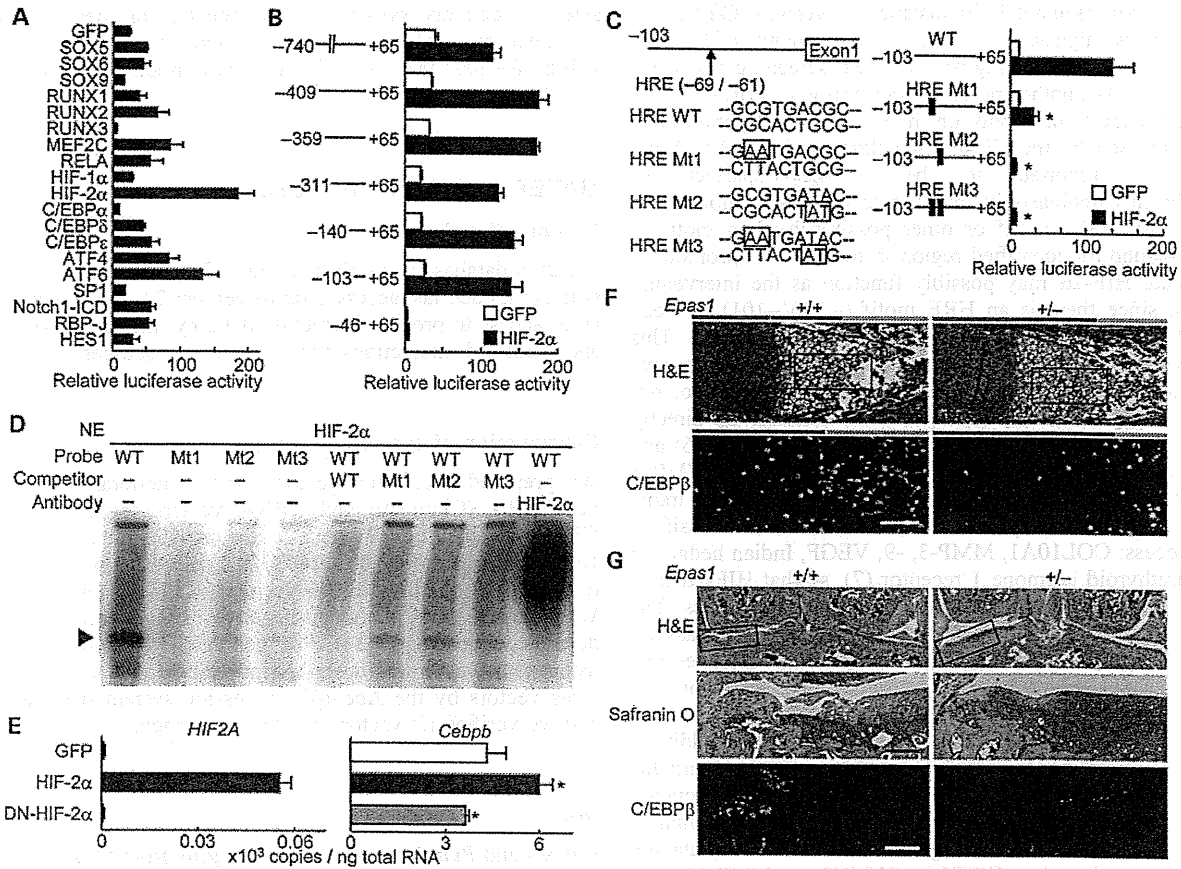

Figure 6. Upstream mechanism that regulates C/EBPβ. (**A**) Luciferase activities after transfection of putative chondrocyte-related transcription factors into SW1353 cells with a reporter construct containing a fragment (−740 to +65 bp) of the *CEBPB* gene. Experiments were done in triplicate with data shown as means ± SEM. (**B**) Deletion analysis using luciferase-reporter constructs containing a series of deletion fragments the *CEBPB* gene in SW1353 cells transfected with HIF-2α or the control GFP. (**C**) Site-directed mutagenesis analysis using luciferase-reporter constructs containing −103/+65 of the *CEBPB* gene with mutations (Mt1, Mt2 and Mt3) in the HRE motif in the cells above. Experiments were done in triplicate with data shown as means ± SEM. $^*P < 0.01$ versus wild-type (WT) with HIF-2α. (**D**) EMSA for specific binding (arrowhead) of the WT oligonucleotide probe containing the HRE or the mutated probes above with nuclear extract of COS-7 cells overexpressing HIF-2α. The cold competition with a 50-fold excess of unlabeled WT or the mutated probes, and the supershift by an antibody to HIF-2α are presented. (**E**) mRNA levels of *Cebpb* in mouse primary chondrocytes with retroviral overexpression of HIF-2α, dominant-negative mutant of HIF-2α (DN-HIF-2α), or the control GFP after 1-week cultures. Experiments were done in triplicate with data shown as means ± SEM.$^*P < 0.05$ versus GFP. (**F**) H&E staining and C/EBPβ immunostaining in tibial limb cartilages of wild-type (+/+) and *Epas1*$^{+/-}$ littermates (E17.5). Red, blue and green bars indicate layers of proliferative and hypertrophic zones and bone area, respectively. Scale bars, 100 μm. (**G**) H&E, Safranin O stainings and C/EBPβ immunostaining of joints cartilage of +/+ and *Epas1*$^{+/-}$ littermates 8 weeks after surgical OA induction. Scale bars, 100 μm. Boxed areas in each H&E-stained image indicate the regions shown in the enlarged images below.

not deny the possibility that C/EBPβ and RUNX2 have other target molecules, as the *Cebpb*$^{-/-}$;*Runx2*$^{+/-}$ mice exhibited dwarfism even after birth (Fig. 2B), differently from *Mmp13*$^{-/-}$ mice. In fact, the previous studies showed the cooperative 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*$^{+/-}$ 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 articular 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. According to a crystallization analysis (28), C/EBPβ and RUNX2 are likely to form a complex by binding of basic leucine zipper domain and Runt domain, respectively;

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 transactivity 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 (7). Also, our present and previous studies have found that HIF-2α directly binds to and activates the promoters of *CEBPB* (Fig. 6) and *RUNX2* (29), indicating that HIF-2α activates the *MMP13* promoter directly and indirectly. HIF-2α is also a potent transactivator 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* transactivation 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 resistance to OA development in the experimental models, and transgenic mice overexpressing *Mmp13* in joint cartilage 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 adenovirus 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* (−1551 to +39), *MMP9* (−1775 to +17), *MMP13* (−1000 to −1), *ADAMTS4* (−2406 to +27), *ADAMTS5* (−1242 to +27), *VEGFA* (−1000 to −1), *ALP* (−3000 to +3000) 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 99.5% 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 abovementioned ChIP assay analysis on immunoprecipitates 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 χ^2 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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Prevalence and correlates of regional pain and associated disability in Japanese workers

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ABSTRACT

Objectives To assess the prevalence and correlates of regional pain and associated disability in four groups of Japanese workers.

Methods As part of a large international survey of musculoskeletal symptoms (the CUPID study), nurses, office workers, sales/marketing personnel and transportation operatives in Japan completed a self-administered questionnaire (response rate 83%) covering experience of pain in six anatomical regions, associated disability and sickness absence, and various possible occupational and psychosocial risk factors for these outcomes. Associations with risk factors were assessed by logistic regression.

Results Analysis was based on 2290 subjects. Rates of regional pain were generally less than in the UK, with a particularly low prevalence of wrist/hand pain among office workers (6% in past month). The strongest and most consistent risk factor for regional pain in the past month was tendency to somatise (ORs (95% CIs) for report of ≥2 versus 0 distressing somatic symptoms 3.1 (2.4 to 4.0) for low back pain, 2.8 (2.1 to 3.8) for shoulder pain, and 2.5 (1.6 to 4.1) for wrist/hand pain). Sickness absence for regional pain complaints in the past year was reported by 5% of participants, the major risk factor for this outcome being absence during the same period for other medical reasons (OR 3.7, 95% CI 2.4 to 5.8).

Conclusions Japanese office workers have markedly lower rates of wrist/hand pain than their UK counterparts. In Japan, as in Western Europe, somatising tendency is a major risk factor for regional pain. Sickness absence attributed to regional pain complaints appears to be much less common in Japan than in the UK, and to be driven principally by a general propensity to take sickness absence.

What this paper adds

- Japanese office workers have markedly lower rates of wrist/hand pain than office workers in the UK.
- In Japan, as in Western Europe, somatising tendency is a major risk factor for musculoskeletal complaints.
- Sickness absence attributed to musculoskeletal disorders appears to be much less common in Japan than in the UK.
- Our findings add weight to a growing body of evidence that the prevalence of musculoskeletal symptoms and resultant disability and sickness absence varies markedly between countries.
- Strategies to control work-related musculoskeletal disorders should take into account the factors that underlie these differences, which may include culturally determined health beliefs and expectations.

INTRODUCTION

Musculoskeletal pain, especially in the back, neck and upper limbs, is a common complaint in many developed countries, and an important cause of disability and work incapacity. It is often attributed to strain from forceful or repetitive occupational activities, and epidemiological research has demonstrated fairly consistent associations of low back pain with work involving heavy lifting and/or repeated bending of the trunk,[1] and of painful disorders of the forearm with work that entails repetitive movements of the wrist or hand.[2]

However, regional pain complaints and associated disabilities are not a simple consequence of physical stresses to tissues. There is strong evidence that they are influenced also by psychological factors such as low mood and a general tendency to worry about common somatic symptoms (somatising tendency).[3 4] In addition, culturally determined health beliefs could also have an important role, and may explain large variations in the incidence and prevalence of pain and disability that have been observed between countries,[5 6] and within countries over time.[5] It is important to understand the contribution of these psychosocial influences if preventive measures are to be optimised.

To help advance knowledge in this area, a multicentre international study, CUPID (Cultural and Psychosocial Influences on Disability), has been established. The study, which is being carried out in 19 countries (both developing and developed) from six continents, involves a baseline cross-sectional survey that will allow comparison of rates of regional pain and associated disability in samples of workers who carry out similar physical activities but in widely different cultural environments. This is followed by a longitudinal component, which explores predictors of persistent and newly incident pain.

In this paper, we report findings from the initial cross-sectional survey that was carried out in Japan as part of the CUPID study, and draw comparisons with experience in the UK.

METHOD

The survey focused on four occupational groups—nurses, office workers, sales/marketing personnel and transportation operatives. All participants worked in or near Tokyo. The nurses were employed

at Tokyo University Hospital, the office workers in administrative and clerical jobs at the same hospital and at four pharmaceutical companies and a private trading company, the sales/marketing personnel at six pharmaceutical companies, and the transportation operatives (mainly lorry drivers and loaders) at two companies transporting baggage and mail.

Within each participating organisation, a manager agreed to act as a coordinator for data collection. The coordinator distributed a self-administered questionnaire to all employees in relevant jobs, with a covering letter from the survey team. Completed questionnaires were then returned to the survey team via the coordinator. A total of 3187 questionnaires were distributed to 1074 nurses, 425 office workers, 380 sales/marketing personnel and 1308 transportation operatives. No reminders were sent to non-responders.

The questionnaire was a Japanese translation of the survey instrument that is being used throughout the CUPID study. The accuracy of the translation was checked by independent back-translation to English and comparison with the original. Amendments were then made as necessary. Among other things, the questionnaire asked about demographic characteristics, hours of work and duration of employment in current job, whether the job involved certain specified activities in an average working day, job satisfaction, mental health, indicators of tendency to somatise, experience of pain during the past month and past year at each of six anatomical sites (low back, neck, shoulder, elbow, wrist/hand and knee), disability for specified everyday tasks arising from such pain, and absence from work in the past year because of musculoskeletal pain or for other reasons. Mental health (mood) was assessed from the relevant subscale from the SF-36 questionnaire,[7] and was graded to three levels defined by approximate thirds of the distribution of scores in all subjects combined. Somatising tendency was assessed using a subset of items from the Brief Symptom Inventory,[8] and was graded according to the number of symptoms (out of a total of seven) that were reported as causing at least moderate concern in the past week.

Data from the completed questionnaires were entered onto computer, and after checks for errors, were analysed using SPSS V.15 and STATA V.10 software. Because a major focus of the study was pain and disability during the past year, subjects were excluded from the main analysis if they had worked in their current job for less than a year.

In addition to the compilation of simple descriptive statistics, logistic regression was used to explore associations with regional pain (classified in various ways) and associated disability and sickness absence. Pain at an anatomical site was considered disabling if during the past month it had made at least one of the everyday activities specified in the questionnaire difficult or impossible. These activities were: getting dressed (all sites of pain), doing normal household jobs (all sites of pain), cutting toe nails (low back), combing or brushing hair (shoulder), bathing/showering (shoulder), opening bottles, jars or taps (elbow and wrist/hand), writing (wrist/hand), locking and unlocking doors (wrist/hand), walking up and down stairs (knee) and walking on level ground (knee). When looking at associations with occupational activities, we defined for each site of pain an activity in an average working day that could cause physical stress to local tissues. These activities were: lifting weights of ≥25 kg by hand (low back); work with the hands above shoulder height for ≥1 h in total (neck and shoulders); repeated bending and straightening of the elbow for ≥1 h in total (elbow); use of a keyboard or other repetitive movements of the wrist/fingers for ≥4 h in total (wrist/hand); and kneeling or squatting for ≥1 h in total (knees). Associations in the logistic regression analyses were summarised by ORs with associated 95% CIs.

RESULTS

Questionnaires were returned by 2651 (83%) of the workers to whom they were issued, but 285 were excluded from analysis because the individual had been in his/her current job for less than a year, and a further 76 because of missing information on age (52), sex (1) or both (23). Of the remaining 2290 subjects, 599 were nurses, 316 were office workers, 355 were sales/marketing personnel and 1020 were transportation operatives, representing 56%, 74%, 93% and 78% of those mailed in the respective occupational groups.

Table 1 summarises various characteristics of the participants. Most of the nurses were women, whereas almost all of the sales/marketing personnel and transportation operatives were men. The majority of subjects were employed full-time, including 30% of the sample (mostly sales/marketing personnel and transportation operatives) who indicated that they worked for more than 60 h per week. Reported occupational activities were much as would be expected, with a high frequency of keyboard use by office workers (89%). Transportation operatives and nurses had the highest prevalence of heavy lifting (83% and 66%, respectively) and of repeated bending and straightening of the elbow (78% and 72%). Rates of job satisfaction were relatively low in office workers (28%) and sales/marketing personnel (31%). Poor mental health and tendency to somatise were most common among nurses. In the study sample overall, the somatic symptoms most frequently reported as distressing were nausea or upset stomach (14%), weakness (12%) and faintness or dizziness (8%).

Table 2 shows the prevalence of pain at different anatomical sites in the study sample as a whole. The lower back was the site most commonly affected by pain, with a prevalence of 28% in the past month. Next most common were pain in the neck (21% in the past month) and shoulder (17%). In comparison, pain in the elbow and wrist/hand was much less frequent. The sites most commonly affected by disabling pain in the past month were the lower back (11%) and knee (8%). Only 4% of subjects had been absent from work during the past year because of low back pain, and absence because of pain in the elbow or wrist/hand was extremely rare.

The prevalence of regional pain by occupational group is summarised in table 3 (data for men and women separately are given in online supplementary tables 1 and 2). At almost all anatomical sites, pain in the past month was most common in nurses or transportation operatives, and least frequent in sales/marketing personnel. However, office workers had the highest prevalence of sickness absence in the past year attributed to regional pain (11%). A total of 251 subjects (11%) reported pain in the past month at three or more anatomical sites, 744 (32%) reported disabling pain at one or more sites during the past month, and 125 (5%) indicated that they had taken sickness absence during the past year because of regional pain.

Table 4 gives results from logistic regression analyses exploring risk factors for pain at different anatomical sites. For each site, two outcomes were examined—any pain in the past month and disabling pain in the past month—the comparator in both cases being no pain at the site in the past month. All analyses were adjusted for sex, age, mental health and occupational group. Significant associations with locally stressful physical activities were observed for pain in the low back (lifting ≥25 kg), wrist/hand (use of keyboard or repeated movements of hands/fingers for ≥4 h) and knee (kneeling or squatting for ≥1 h). However,

Table 1 Characteristics of participants by occupational group

Characteristic	Nurses (n=599)		Office workers (n=316)		Sales/ marketing personnel (n=355)		Transportation operatives (n=1020)		Total (n=2290)	
	n	%	n	%	n	%	n	%	n	%
Sex										
Male	20	3.3	181	57.3	331	93.2	1016	99.6	1548	67.6
Female	579	96.7	135	42.7	24	6.8	4	0.4	742	32.4
Age (years)										
19–29	253	42	14	4	103	29	214	21	584	26
30–39	193	32	112	35	178	50	415	41	898	39
40–49	81	14	101	32	63	18	278	27	523	23
50–64	72	12	89	28	11	3	113	11	285	12
Hours worked per week										
Up to 20	30	5	35	11	30	8	142	14	237	10
21–40	248	41	114	36	33	9	97	10	492	21
41–60	286	48	148	47	188	53	214	30	836	37
≥61	20	3	15	5	103	29	552	54	690	30
Missing	15	3	4	1	1	0.2	15	1	35	2
Occupational activities in an average working day										
Use of keyboard ≥4 h	142	24	281	89	99	28	25	2	547	24
Other repeated movements of wrist/fingers ≥4 h	144	24	44	14	36	10	336	33	560	24
Repeated bending and straightening of elbow for ≥1 h in total	434	72	74	23	107	30	795	78	1410	62
Work with hands above shoulder height ≥1 h in total	73	12	5	2	15	4	343	34	436	19
Lifting weights of ≥25 kg by hand	398	66	10	3	33	9	849	83	1290	56
Kneeling or squatting ≥1 h in total	289	48	7	2	43	12	534	52	873	38
Satisfied with current job										
Yes	329	55	91	28	108	31	589	58	1117	49
Mental health										
Good	164	27	142	45	119	34	297	29	722	32
Intermediate	190	32	85	27	121	34	331	32	727	32
Poor	234	39	84	27	110	31	371	36	799	35
Somatising tendency (number of symptoms in past week causing at least moderate concern)										
0	170	28	141	45	146	41	516	51	973	42
1	237	40	107	34	121	34	278	28	743	32
≥2	183	31	66	21	86	24	213	21	548	24

the strongest and most consistent associations were with somatising tendency. For disabling pain in the low back, neck and shoulder, the ORs for report of ≥2 versus 0 distressing somatic symptoms were all 4.5 or higher. Associations with poor mental health (not shown) were much weaker than with somatising tendency, and not statistically significant.

Table 5 presents findings from two regression analyses, one for the risk of pain in the past month at three or more anatomical sites, and the other for disabling pain at one or more anatomical sites in the past month. In each case, the comparator was no pain at any site in the past month. Both variables were strongly associated with somatising tendency and showed a clear, progressive increase in risk in relation to the number of stressful physical activities reported. In addition, both were more frequent at older ages. Associations with poor mental health and job dissatisfaction were much weaker.

In contrast, sickness absence because of regional pain in the past year was unrelated to occupational physical activities and showed no clear association with somatising tendency (table 6). It was, however, strongly associated with sickness absence during the past year for other reasons (OR 3.7, 95% CI 2.4 to 5.8), which was reported by 16% of participants.

DISCUSSION

In this cross-sectional survey of Japanese workers, rates of regional pain were generally lower than have been reported in

Table 2 Prevalence of regional pain by anatomical site

Anatomical site	Any pain in past month		Disabling pain in past month*		Any pain in past year		Pain for ≥1 month in past year†		Pain causing absence from work in past year	
	n	%	n	%	n	%	n	%	n	%
Low back	636	28	255	11	1075	47	293	13	101	4
Neck	484	21	91	4	735	32	209	9	40	2
Shoulder	382	17	107	5	549	24	193	8	25	1
Elbow	123	5	39	2	170	7	36	2	7	0.3
Wrist/hand	161	7	72	3	236	10	69	3	9	0.4
Knee	285	12	181	8	429	19	116	5	27	1

*For definition of disabling pain, please see text.
†Pain for at least 1 month in total.

Table 3 Prevalence of regional pain by occupational group

Category of pain	Nurses		Office workers		Sales/marketing personnel		Transportation operatives	
	n	%	n	%	n	%	n	%
Low back pain in past month	182	30	68	22	68	19	318	31
Neck pain in past month	184	31	85	27	63	18	152	15
Shoulder pain in past month	132	22	61	19	47	13	142	14
Elbow pain in past month	16	3	13	4	11	3	83	8
Wrist/hand pain in past month	39	7	19	6	15	4	88	9
Knee pain in past month	74	12	36	11	34	10	141	14
Pain at ≥3 sites in past month	80	13	34	11	16	5	121	12
Disabling pain at any site in past month*	220	37	79	25	65	18	380	37
Pain at any site causing absence from work in past year	15	3	34	11	13	4	63	6

*For definition of disabling pain, please see text.

the UK, with a particularly low frequency of pain in the wrist and hand. The prevalence of sickness absence attributed to regional pain was also substantially lower than in the UK. Pain at most sites was more common in workers who indicated that they were exposed to stressful physical activities in their job, but the strongest and most consistent risk factor for regional pain and associated disability was somatising tendency. In contrast, risk of sickness absence because of regional pain was related not to physical activities or somatising tendency, but to absence from work because of other health problems.

The occupational groups that were studied cannot necessarily be regarded as representative of the general population of working age in Japan. Nevertheless, they encompass a range of occupational tasks, both manual and non-manual, and provide useful insights into patterns of musculoskeletal symptoms and disability in a cultural environment that is notably different from that in, say, Western Europe. Furthermore, the high response rate that was achieved makes it likely that the samples of workers who participated were fairly typical of the occupational groups from which they were drawn.

A concern always in international studies of this type is that the meaning of questions may be distorted in translation between languages. Thus, care was taken to check the accuracy of the Japanese questionnaire by back-translation to English. It remains possible that a term such as "pain" is understood somewhat differently in Japan. However, this should not affect the relative frequency of the symptom at different anatomical sites, and is less likely to have been a problem in relation to more objective outcomes such as sickness absence.

Another possible source of error was incomplete recall of symptoms, particularly if they last occurred many months before the questionnaire was completed. For this reason, we based most of our analysis on pain and disability that was reported in the past month. An exception was sickness absence, for which a longer time period was required to give meaningful numbers of cases. However, we would expect spells of sickness absence to be more memorable than more minor episodes of pain.

The prevalence of pain at most of the anatomical sites considered was somewhat lower than has been recorded in UK workers who were surveyed using similar questions.[6] For example, low back pain in the past month was reported by 28% of the Japanese workers as compared with 28% in a sample of white UK office workers and 37% in a group of white UK manual workers, while the corresponding figures were 21% versus 26% and 23% for neck pain, 17% versus 20% and 24% for shoulder pain, and 5% versus 10% and 9% for elbow pain. More remarkable, however, is the much lower prevalence of wrist/hand pain in Japanese workers (7% vs 30% and 23%). This lower prevalence extended to Japanese office workers (6% with wrist/hand pain), most of whom were regular users of computer keyboards. The difference in the prevalence of wrist/hand pain

Table 4 Risk factors for regional pain in past month

Risk factor	Low back		Neck		Shoulder		Elbow		Wrist/hand		Knee	
	n	OR* (95% CI)	n	OR* (95% CI)	n	OR* (95% CI)	n	OR* (95% CI)	n	OR* (95% CI)	n	OR* (95% CI)
Any pain in past month†												
Physical activity‡	421	1.9 (1.4 to 2.5)	87	1.2 (0.9 to 1.6)	76	1.2 (0.9 to 1.7)	81	1.2 (0.8 to 2.0)	86	1.9 (1.3 to 2.6)	144	2.0 (1.5 to 2.7)
Somatising tendency§												
0	348	1	240	1	98	1	71	1	90	1	160	1
1	113	1.7 (1.3 to 2.3)	106	2.3 (1.8 to 3.1)	77	1.9 (1.4 to 2.6)	16	1.2 (0.7 to 2.1)	29	1.6 (1.0 to 2.5)	52	1.7 (1.2 to 2.4)
≥2	158	3.1 (2.4 to 4.0)	125	3.2 (2.4 to 4.2)	97	2.8 (2.1 to 3.8)	31	2.5 (1.6 to 4.1)	38	2.2 (1.4 to 3.3)	71	2.6 (1.9 to 3.6)
Job dissatisfaction	260	1.3 (1.0 to 1.6)	225	1.1 (0.8 to 1.4)	201	1.1 (0.9 to 1.5)	68	1.1 (0.7 to 1.7)	64	1.5 (1.0 to 2.1)	133	1.1 (0.8 to 1.4)
Disabling pain in past month†												
Physical activity‡	180	2.2 (1.5 to 3.4)	24	1.6 (0.9 to 2.7)	24	1.1 (0.6 to 1.8)	24	0.7 (0.3 to 1.4)	39	1.8 (1.1 to 3.0)	95	2.0 (1.4 to 2.9)
Somatising tendency§												
0	128	1	37	1	39	1	20	1	32	1	90	1
1	38	1.6 (1.0 to 2.4)	17	2.3 (1.2 to 4.2)	19	2.5 (1.4 to 4.5)	4	1.0 (0.3 to 2.9)	18	2.7 (1.4 to 4.9)	38	2.1 (1.4 to 3.2)
≥2	82	4.5 (3.2 to 6.4)	33	5.0 (2.9 to 8.4)	45	7.2 (4.4 to 11.8)	14	3.9 (1.8 to 8.2)	21	3.4 (1.8 to 6.3)	51	3.3 (2.2 to 4.9)
Job dissatisfaction	157	1.5 (1.1 to 2.0)	36	1.1 (0.7 to 1.8)	38	1.2 (0.8 to 2.0)	18	0.7 (0.3 to 1.3)	27	1.5 (0.9 to 2.7)	79	1.1 (0.8 to 1.6)

*For each anatomical site and pain outcome, ORs were derived from a logistic regression model that included all of the risk factors presented together with sex, age (in four strata), mental health (in three strata) and occupational group.
†Risks are relative to no pain at site in past month.
‡Stressful occupational activity in an average working day defined as lifting weights of ≥25 kg by hand (low back), work with the hands above shoulder height for ≥1 h (neck and shoulder), repeated bending and straightening of elbow for ≥1 h (elbow), use of a keyboard or repeated movements of hands/fingers for ≥4 h (wrist/hand), kneeling or squatting for ≥1 h (knee).
§Number of somatic symptoms causing at least moderate concern in past week.