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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Muraki had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Association of vitamin D status with knee pain and radiographic knee osteoarthritis

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SUMMARY

Objective: The objective of the present study was to explore the association of serum vitamin D concentration and polymorphism in the vitamin D receptor (VDR), with knee pain and radiographic knee osteoarthritis (OA) among men and women in a large population-based UK cohort study.
Methods: Seven hundred and eighty-seven participants in the Hertfordshire Cohort Study (399 men, 388 women; mean age 65.6 ± 2.7 years) underwent a questionnaire on knee pain and radiographic knee examination. This study examined the association of Fok1, Cdx2 and Apa1 polymorphism in the gene for the VDR and serum 25(OH)D concentration with knee pain and radiographic knee OA by a generalized estimating equations population averaged logistic regression analysis in the Hertfordshire Cohort Study.
Results: There were no associations of Fok1, Cdx2 and Apa1 polymorphisms of the VDR with knee OA except for Aa for Apa1 compared with AA [Odds ratio (OR) 0.59, 95% confidence interval (CI) 0.36–0.95, $P = 0.031$]. While, ff for Fok1 (OR 1.60, 95% CI 1.07–2.39, $P = 0.022$) and AA for Cdx2 polymorphism (OR 2.21, 95% CI 1.07–4.56, $P = 0.032$) was significantly associated with higher prevalence of knee pain compared with FF for Fok1 and GG for Cdx2, respectively. None of these are statistically significant after adjusting for the three polymorphisms tested. 25(OH)D level was not significantly associated with radiographic knee OA, while, low tertile of 25(OH)D level tended to be associated with knee pain compared with high tertile of 25(OH)D level.
Conclusion: The present cross-sectional study using a large-scale population from the Hertfordshire Cohort study indicated that vitamin D may be associated with pain rather than radiographic change, but the evidence for an association between vitamin D genetic variation and pain in knee OA is very weak in the present study. Further replication of our results will be required to elucidate the association of vitamin D and knee OA.
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Introduction

Knee osteoarthritis (OA) is a major public health issue that causes chronic pain and disability[1–3], although at present the pathogenesis of this condition remains largely unknown. Several environmental factors have been associated with OA, including obesity[4–6], previous injury[7], knee-bending occupations[8,9], and other metabolic factors[10,11]. A previous population-based UK study of twins has also demonstrated a clear genetic influence on radiologic knee OA in women, with up to 65% of the variance being explained by genetic factors[12].

Vitamin D has been shown to stimulate synthesis of proteoglycan by mature articular cartilage *in vitro*[13], and this suggests that vitamin D may directly affect articular cartilage metabolism. Vitamin D receptor (VDR) is found in many types of tissues, including chondrocytes[14,15]. A previous study showed that VDR gene polymorphism was associated with bone[16], although it is still controversial[17]. The relationship between osteoporosis and OA suggests that VDR gene polymorphisms may be associated with both diseases[18]. However, the association of VDR gene polymorphisms with knee OA is

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controversial[19–23]. This may be partly due to different races, differences in environmental factors related to vitamin D metabolism, or the presence of other genetic factors that influence VDR function.

The association of vitamin D level with knee OA is also controversial[24–28]. In previous studies, McAlindon suggested that low serum levels of vitamin D were associated with progression of knee OA[24]. A recent study has also shown that serum 25(OH)D levels were associated with decreased knee cartilage loss[28], but Hunter *et al.* found that there was no significant association between vitamin D levels and knee osteophytes after adjusting for age, body mass index (BMI) and relatedness[25]. The Framingham study also found no association of vitamin D levels with knee OA worsening[26]. This may be partly explained by VDR gene polymorphism, because vitamin D exerts its endocrine and autocrine/paracrine local effects upon binding to and activating its intracellular receptor VDR. In other words, the association of vitamin D level with knee OA may be different by VDR gene polymorphisms, but, to the best of our knowledge, there were no studies investigating the association of vitamin D level with knee OA by VDR gene polymorphisms.

The principal clinical symptom of knee OA is pain[29], but the correlation between pain and radiographic severity is inconsistent[4,30–32]. Fewer studies have addressed factors which might influence knee pain[32–35]; among these, older age, female gender, and physically demanding work, have all been proposed[30–33]. Previous studies, however, have not addressed the role of vitamin D status or fixed genetic variation in the VDR.

The objective of this study was to clarify the association of VDR gene polymorphism with knee pain and radiographic knee OA among men and women in the general population, as well as to examine the association between circulating vitamin D concentration and these indices of OA.

Subjects and methods

Subjects

The Hertfordshire Cohort Study is a population-based cohort study in the UK. Details of the study design have been published previously[36], thus, a brief summary is provided here. The selection procedure was as follows: using the National Health Service Central Registry at Southport, and Hertfordshire Family Health Service Association, we traced men and women who were born during 1931–1939 in Hertfordshire, and still lived there during the period 1998–2003. After obtaining written permission from each subject's general practitioner (GP), we approached each person by letter, asking him or her if they would be willing to be contacted by one of our research nurses. If subjects agreed, a research nurse performed a home visit and administered a structured questionnaire. This included information on socioeconomic status, medical history, drug history, cigarette smoking, alcohol consumption, and reproductive variables in women.

At a subsequent clinic, height was measured to the nearest 0.1 cm using a Harpenden pocket stadiometer (Chasmors Ltd, London, UK) and weight to the nearest 0.1 kg on a SECAfloor scale (Chasmors Ltd). Fasting venous whole-blood samples were taken at this clinic visit. Eligible subjects were then invited to book a return visit for knee radiography. Weightbearing anteroposterior and lateral semiflexed radiographs of both knees were taken at the same hospital using the same radiographic equipment; a standard tube to film distance of 100 cm was used. Radiographs were performed at a median duration of 6 months [interquartile range (IQR) 4.8–7.2] after the clinic visit. Radiographs were graded at the tibiofemoral joints using the Kellgren Lawrence (KL) grade[37]. One trained reader graded the radiographs; KL grade ≥ 2 was the threshold for a definition of knee OA. Subjects were also asked "Have you had any pain in or around your right knee on most days in the last month?" and "Have you had any pain in or around your left knee on most days in the last month?" Knee pain reported in this way was defined as having knee pain. A total of 498 men and 468 women completed a home questionnaire, attended clinic, and underwent knee radiography.

A fasting morning blood sample was obtained from all subjects at the first clinic visit, and the serum separated and stored at −70°C. 25(OH)vitamin D was assayed using a DiaSorin Liason automated chemiluminescent assay with equal specificity for both D2 and D3 (coefficient of variation for vitamin D across the assays was 10–12% for within batch and 10–15% between batch).

Genomic DNA was extracted from whole-blood samples according to standard procedures. VDR genotype was determined by the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis, and three VDR polymorphic sites (Fok1, Cdx2 and Apa1) were analyzed.

Ethical permission for the study was granted by the East and North Hertfordshire Ethical Committees. All participants gave written informed consent.

Statistical analysis

To assess gene polymorphism effects on radiographic knee OA and knee pain, indicator variables were created for Fok1 (FF and ff), Cdx2 (GA and AA) and Apa1 (AA and aa) polymorphism. As both knees have a pain score and a radiographic grade, a generalized estimating equations (GEE) population averaged logistic regression model was used to adjust for clustering of knees within patients. To examine 25(OH)D levels and their association with knee OA and knee pain, we classified subjects into three categories; high tertile (>51.5 nmol/l), middle tertile (35.5–51.5 nmol/l) and low tertile (<35.5 nmol/l). A GEE population averaged logistic regression analysis was used to determine the association of vitamin D level with knee OA and knee pain with and without adjustment for age, gender, BMI, season of the clinic visit and KL grade. To decide whether statically significant associations between VDR polymorphisms and knee outcomes are noteworthy, we used Wacholder's method to calculate the False Positive Report Probability (FPRP) [Wacholder in JNCI 2004]. Data analyses were performed using SAS version 9.0 (SAS Institute, Cary, NC, USA) and Stata version 11.2 (Stata, College Station, TX, USA).

Results

Of 984 subjects, 170 (17.3%) provided incomplete pain questionnaires. A further 19 (1.9%) lacked genotypic information. We also excluded eight subjects with total knee arthroplasty, leaving 787 (399 males and 388 females) participants in this analysis. Comparison between the 787 subjects with complete information and those without complete information revealed no statistically significant differences in mean age (responders 65.6 years, nonresponders 65.7 years; $P=0.66$), sex (responders 80.3% women, nonresponders 79.8% women; $P=0.87$), BMI (responders 27.0, nonresponders 27.4; $P=0.19$) or prevalence of knee OA (responders 15.2% women, nonresponders 16.3% women; $P=0.74$). The characteristics of these participants are shown in Table I. The men were slightly younger than the women, and they had a lower mean BMI; serum vitamin D concentration was also significantly higher among men than women. There were no significant differences in mean values [IQR] of 25(OH)D concentration (nmol/l) among VDR gene polymorphisms of Fok1 [FF 45.5 (30.0–56.0), Ff 47.1 (31.5–56.8), ff 51.3 (31.0–69.0)], Cdx2 [GG 45.9 (30.6–56.0), AG 47.5 (31.2–60.2), AA 54.1 (36.7–68.0)] and Apa1 [AA 48.4 (32.9–61.3), Aa 47.5 (30.0–59.1), aa 42.7 (31.0–50.9)]. There were no significant

Table I
Characteristics of participants

	Overall	Men	Women	*P*-value
Number of subjects	787	399	388	
Age, years	65.6 (2.7)	64.8 (2.6)	66.4 (2.6)	<0.001
BMI, kg/m^2	27.0 (4.3)	26.8 (3.6)	27.2 (4.9)	0.22
25(OH)D level, nmol/l* means, (IQR)	42.5 (30.8, 57.3)	44.4 (34.7, 64.2)	41.0 (28.3, 54.1)	<0.001
Radiographic knee OA, *n*, (%)	120 (15.3)	70 (17.5)	50 (12.9)	0.069
Knee pain, *n*, (%)	309 (39.3)	147 (36.8)	162 (41.8)	0.16

Except where indicated otherwise, values represent means (standard deviation). The differences in age, BMI and 25(OH)D level between men and women were examined by the non-paired Student's *t*-test. The differences in prevalence of radiographic knee OA and knee pain between men and women were examined by chi square test.

* Of 787 subjects, 25(OH)D was measured in 683 subjects.

differences in the prevalence of radiographic knee OA and knee pain between genders. Of 120 subjects with radiographic knee OA, 79 (65.8%) had knee pain, while, of 667 subjects without radiographic knee OA, 230 (34.5%) had knee pain. Knee pain was significantly associated with radiographic knee OA after adjustment for age, gender and BMI [Odds ratio (OR); 3.03, 95% confidence interval (CI); 1.98–4.68].

We examined the association of VDR gene polymorphisms and radiographic knee OA (Table II). There were no associations of Fok1, Cdx2 and Apa1 polymorphisms of the VDR with knee OA except for Aa for Apa1 compared with AA after adjustment for age, gender and BMI, and FPRP values were low for association of Apa1 (Aa) on radiographic knee OA suggesting this association may be noteworthy. We also examined the associations of the alleles with knee OA. f for Fok1 tended to associate with higher prevalence of knee OA than F ($P = 0.06$). The alleles for Cdx2 and Apa1 were not significantly associated with knee OA ($P = 0.94$ and 0.64).

We also examined the association of VDR gene polymorphisms and knee pain (Table III). Unlike radiographic knee OA, Fok1 and Cdx2 polymorphism was significantly associated with prevalence of knee pain after adjustment for age, gender BMI and KL grade, and FPRP values were low for association of Fok1 (ff) for knee pain, suggesting this association may be noteworthy. There were no associations of Apa1 polymorphisms with knee pain. When analyzed in men and women separately, Fok1 polymorphism was significantly associated with knee pain after adjustment for age, BMI and KL grade in women (Ff: OR; 1.17, 95% CI; 0.75–1.81, $P = 0.486$, ff: OR; 2.46, 95% CI; 1.38–4.39, $P = 0.002$, compared with FF), while, not in men (Ff: OR; 1.10, 95% CI; 0.71–1.73, $P = 0.649$, ff: OR; 1.01, 95% CI; 0.58–1.76, $P = 0.98$, compared with FF). We also examined the associations of the alleles with knee pain. f for Fok1 had significantly associated with higher prevalence than F ($P = 0.01$). The alleles for Cdx2 and Apa1 were not significantly associated with knee pain ($P = 0.49$ and 0.64, respectively).

We next examined the association of 25(OH)D level and knee OA (Table IV). GEE logistic regression analysis showed that 25(OH)D level was not significantly associated with radiographic knee OA. For knee pain effect of vitamin D level was non-linear, so we classified subjects into three groups; high tertile (>51.5 nmol/l, $n = 225$), middle tertile (35.5–51.5 nmol/l, $n = 229$) and low tertile (<35.5 nmol/l, $n = 229$); low tertile of 25(OH)D level tended to be associated with knee pain compared with high tertile of 25(OH)D level after adjustment for age, gender, BMI, season of the clinic visit and KL grade (Table IV).

Discussion

This is the first study to examine the association of radiographic knee OA and knee pain with vitamin D level and VDR gene polymorphism at the same time. A Fok1 polymorphism of the VDR was significantly associated with radiographic knee OA and knee pain. There were no associations between radiographic knee OA and 25(OH) D level, while 25(OH)D level tended to be associated with knee pain.

The association of VDR gene polymorphism with OA is controversial[19–23]. In previous studies, a nested case-control study in Britain showed the 'T' allele was associated with knee OA in women[19]. The Rotterdam Study showed that the 'bAT' haplotype was associated with reduced prevalence of OA[20]. While, The Framingham study found no evidence for an association of the VDR gene with knee OA[23]. In a case-control study in Japan, there was also no significant association between VDR gene polymorphism and knee OA[21], although cases were sampled from hospital attenders in the study and controls did not undergo X-rays, causing the inevitable selection bias to occur. This inconsistency may also be due to differences in the relative importance of this gene in different races, differences in environmental factors related to vitamin D metabolism, or the presence of other genetic factors that influence VDR function. Further, the association of genetic factors with knee OA is diminishing later in life due to the effects of lifestyle factors, thus it may be difficult to find out their association in the elderly. In the present study, a Fok1 polymorphism of the VDR was significantly associated with radiographic knee OA. Vitamin D has been shown to stimulate synthesis of proteoglycan by mature

Table II
Association of VDR gene polymorphisms and radiographic knee OA

	Total	Number (%) with knee OA	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)*	*P*-value	Power†	FPRP prior probability	
								0.1	0.01
Fok1									
FF	328	39 (11.9)	1.00		1.00				
Ff	333	60 (18.0)	1.50 (0.96, 2.34)	0.072	1.54 (0.96, 2.46)	0.071	0.47	0.58	0.94
ff	108	18 (16.7)	1.38 (0.75, 2.57)	0.301	1.56 (0.82, 2.94)	0.173	0.27	0.85	0.98
Cdx2									
GG	491	75 (15.3)	1.00		1.00				
AG	248	37 (14.9)	1.03 (0.66, 1.59)	0.903	0.94 (0.60, 1.48)	0.781	0.49	0.93	0.99
AA	29	5 (17.2)	1.30 (0.48, 3.57)	0.605	1.09 (0.43, 2.74)	0.858	0.11	0.99	1.00
Apa1									
AA	213	36 (16.9)	1.00		1.00				
Aa	388	51 (13.1)	0.64 (0.40, 1.03)	0.068	0.59 (0.36, 0.95)	0.031	0.39	0.42	0.89
aa	166	31 (18.7)	1.12 (0.65, 1.91)	0.687	1.04 (0.60, 1.81)	0.884	0.28	0.97	1.00

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 radiographic grade, GEE population averaged logistic regression analysis after adjustment for age, gender and BMI was used to calculate adjusted OR.

† To detect an OR of 1.5, we are looking for a difference in proportions of 15.3% vs 21.3% for radiographic knee OA.

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.

Acknowledgments

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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

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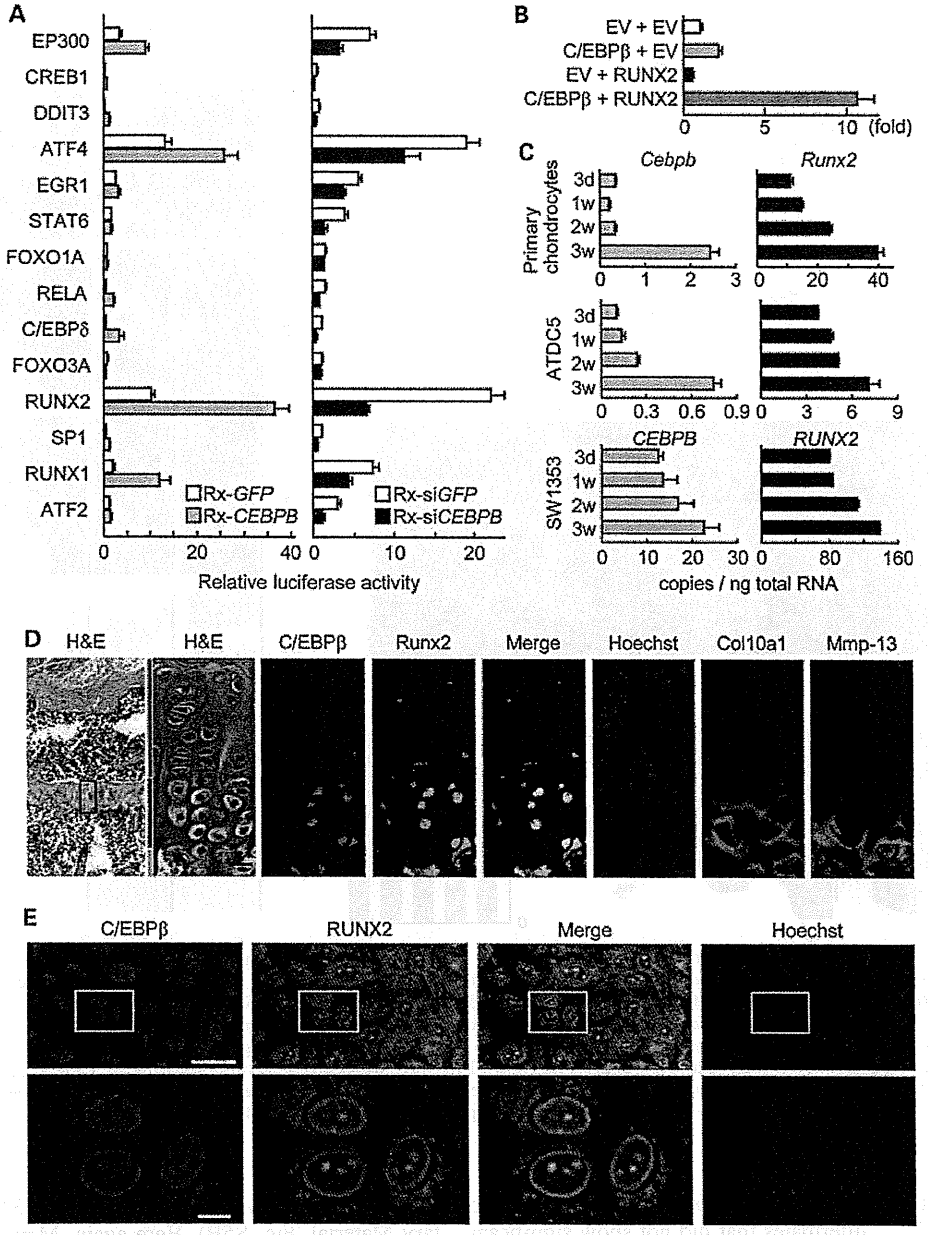

Figure 1. Identification of RUNX2 as a transcriptional partner of C/EBPβ in chondrocytes. (**A**) Luciferase activities after transfections of 14 selected transcription factors into human chondrogenic SW1353 cells with a reporter construct containing three consensus C/EBP-binding sequences, which are 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

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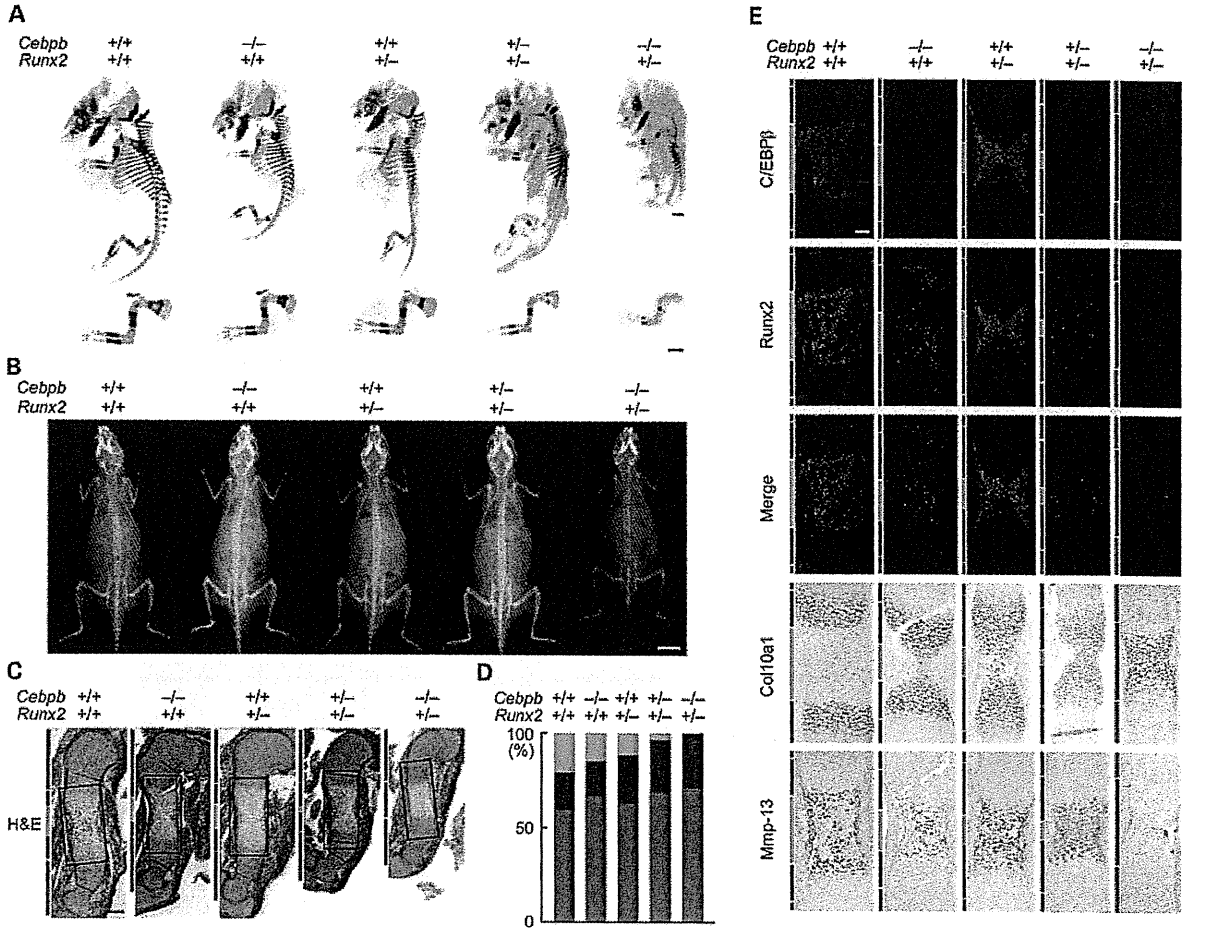

Figure 2. Skeletal findings of five genotype littermates. (**A**) Double staining with Alizarin red and Alcian blue of the whole skeletons (top) and forelimbs and clavicles (bottom) of wild-type (*Cebpb*$^{+/+}$;*Runx2*$^{+/+}$), *Cebpb* homozygous-knockout (*Cebpb*$^{-/-}$), *Runx2* heterozygous-knockout (*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).

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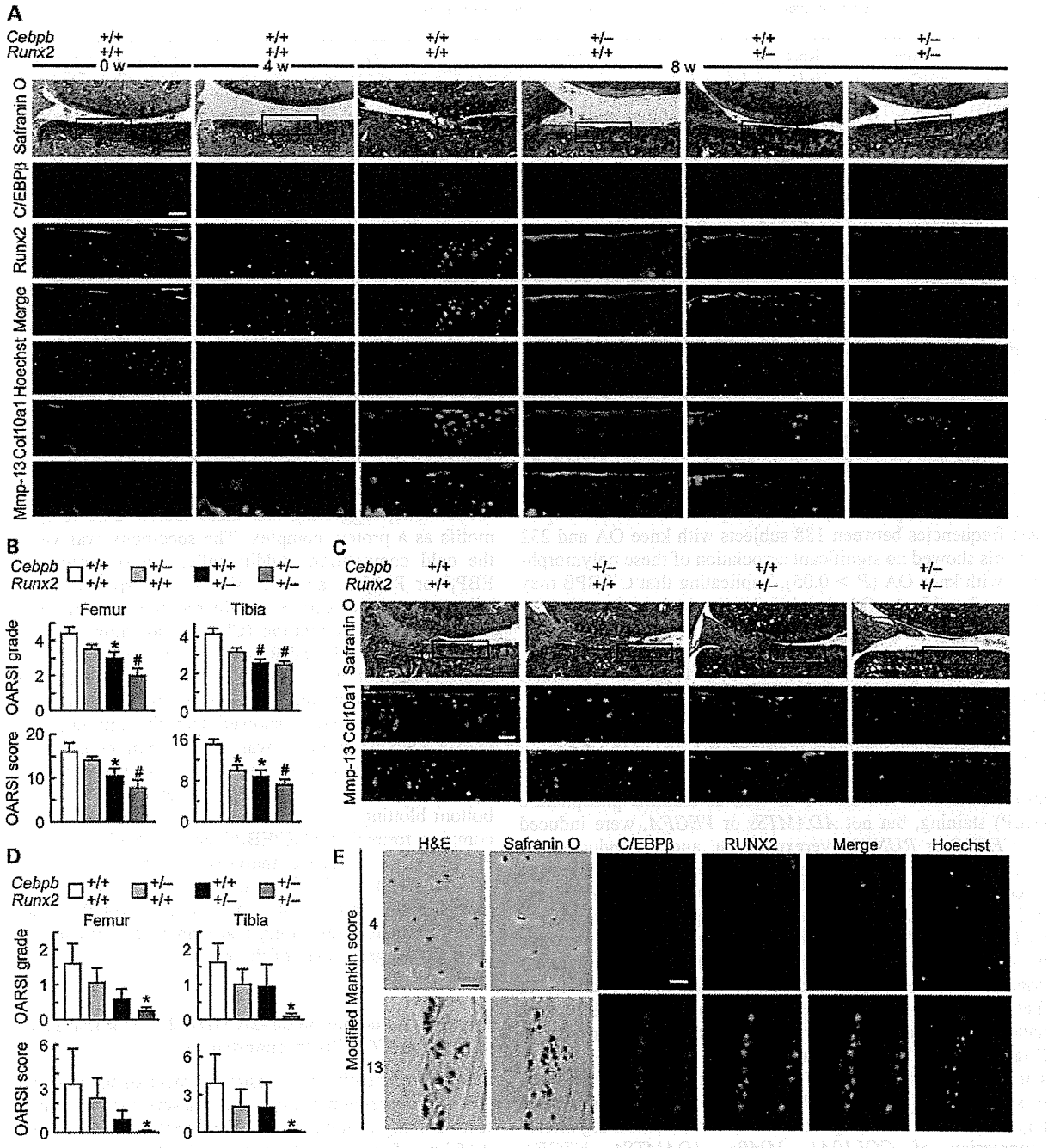

Figure 3. Contribution of C/EBPβ and RUNX2 to OA development. (**A**) Safranin O staining and immunostaining with antibodies to C/EBPβ (red), Runx2 (green), the merged images (yellow), Col10a1, Mmp-13 and Hoechst nuclear staining of joint cartilage 0–8 weeks after surgical OA induction in the knee joints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

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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

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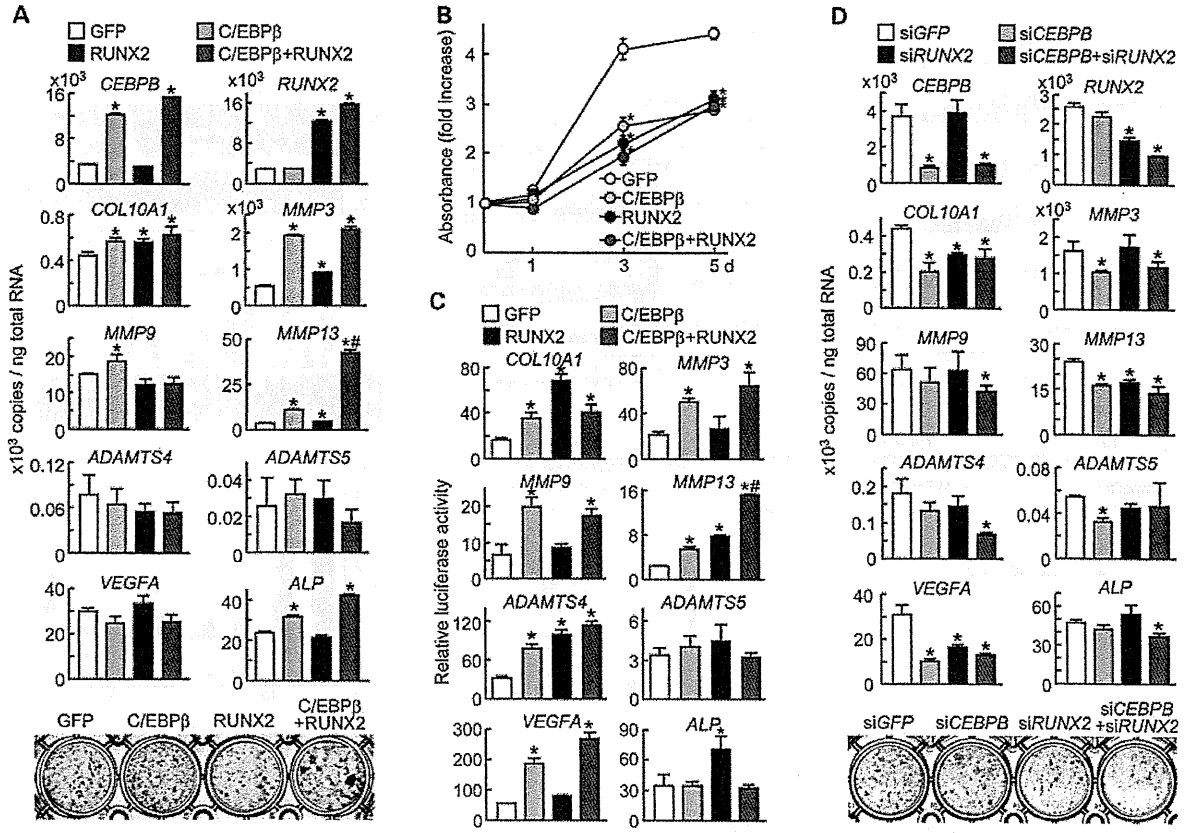

Figure 4. Effects of gain and loss of functions of C/EBPβ and RUNX2 on endochondral ossification parameters in cultures of chondrocytes. (**A**) mRNA levels of *CEBPB, RUNX2, COL10A1, MMP3, 9, 13, ADAMTS4, 5, VEGFA* and *ALP* (graphs) and ALP staining (bottom) in stable lines of SW1353 cells retrovirally 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

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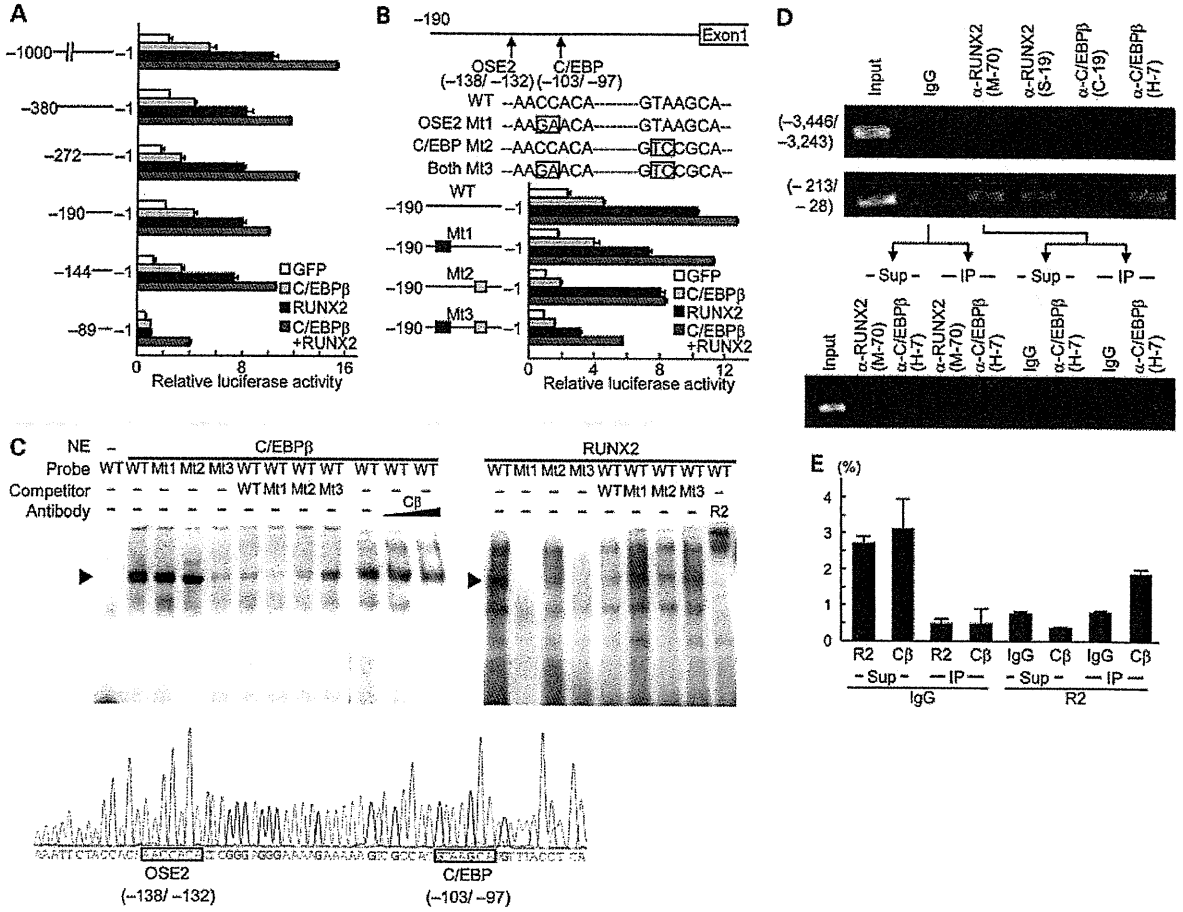

Figure 5. Transcriptional regulation of MMP13 by C/EBPβ and RUNX2. (**A**) Deletion analysis using luciferase-reporter constructs containing the 5′-flanking sequence from −1000 to −1 bp of the *MMP13* gene and a series of deletion fragments in SW1353 cells transfected with *CEBPB, RUNX2*, their combination 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

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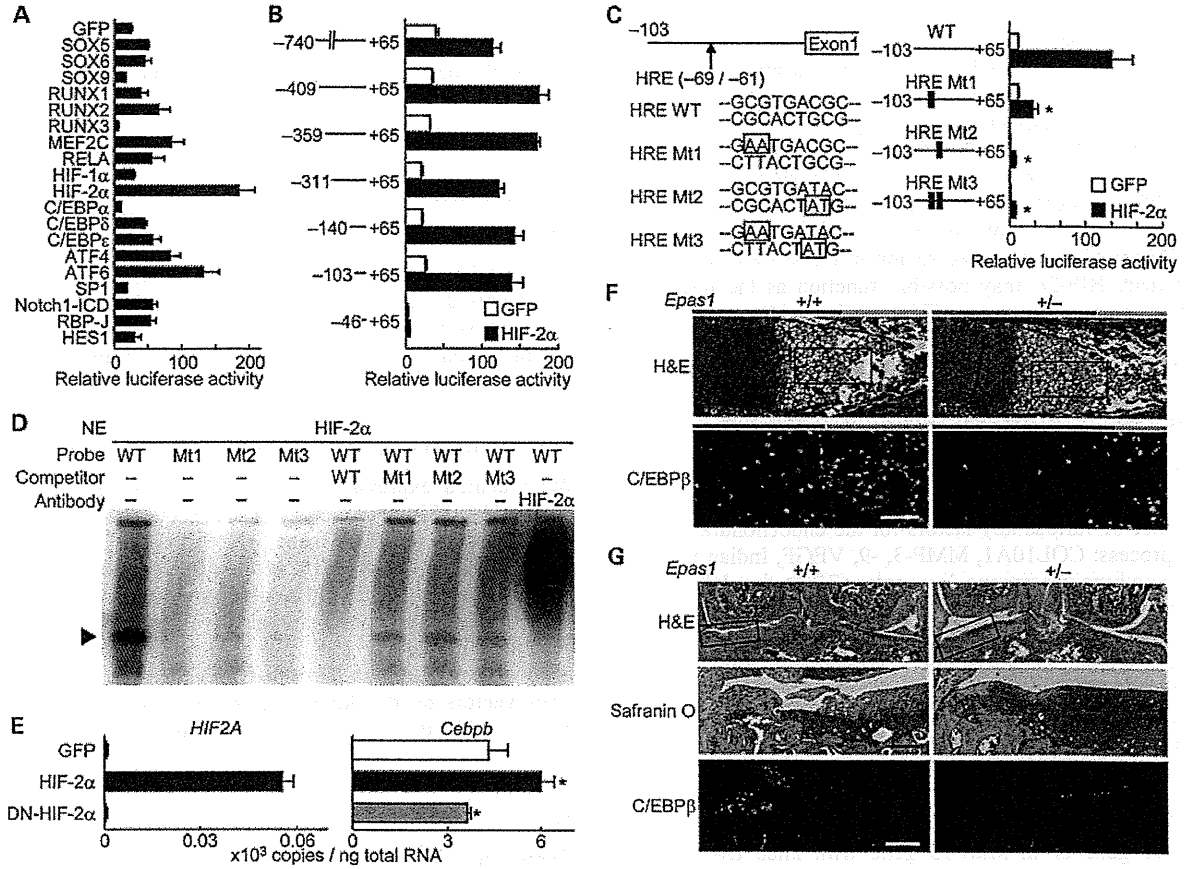

Figure 6. Upstream mechanism that regulates C/EBPβ. (**A**) Luciferase activities after transfection of putative chondrocyte-related transcription factors into SW1353 cells with a reporter construct containing a fragment (−740 to +65 bp) of the *CEBPB* gene. Experiments were done in triplicate with data 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;

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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.

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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

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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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