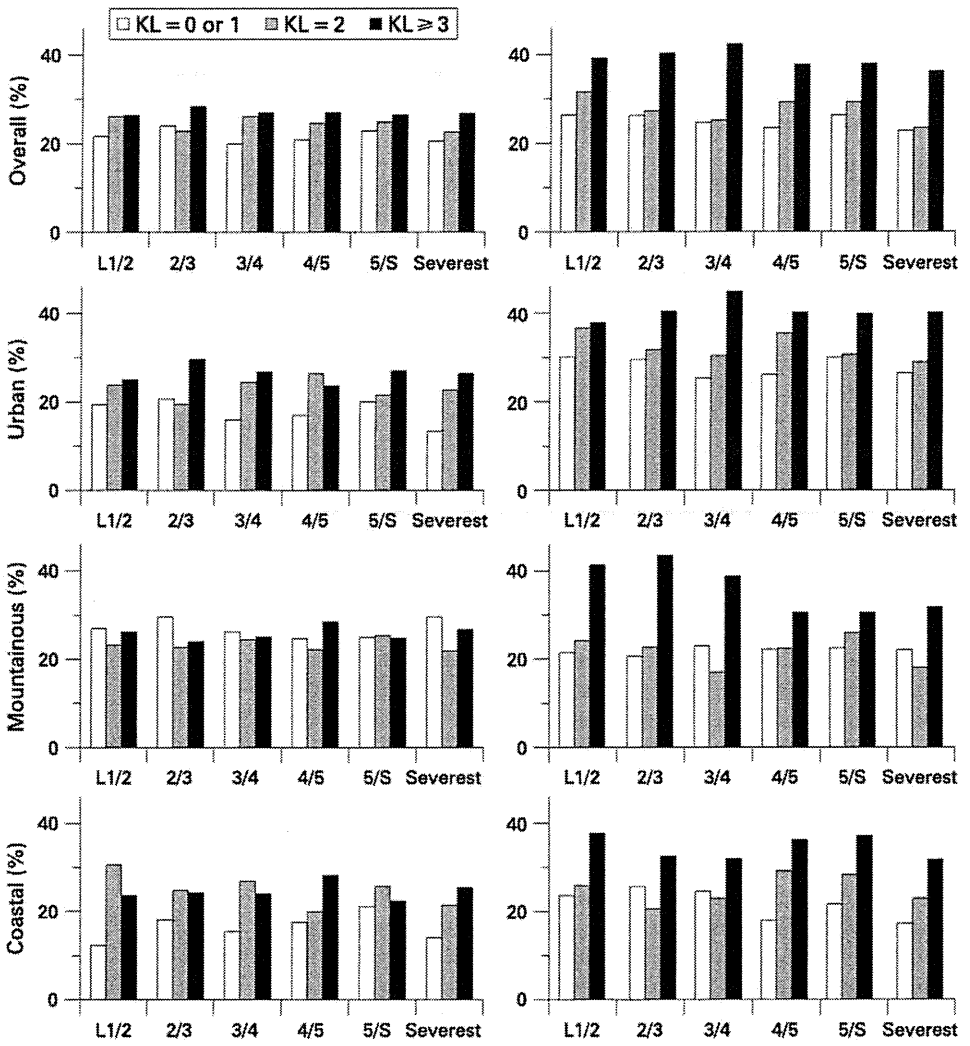

Figure 1 Percentage of subjects with low back pain according to the Kellgren/Lawrence (KL) grade in the overall population and in urban, mountainous and coastal communities.

analysis does not, of course, lead to the conclusion that individual lumbar spondylosis hardly progresses after 80 years. Since the ROAD study is a prospective cohort study of >10 years, the follow-up data will clarify the progression with ageing. Furthermore, there was a difference in prevalence between urban and mountainous communities. Considering that lumbar spondylosis is a common disease whose progression is governed by environmental and genetic factors, the regional difference is inevitable, as previously reported.[6] Although age and obesity are known to be representative risk factors for lumbar spondylosis,[2] the difference between communities in the present study was significant even after adjustment for age and BMI, indicating the involvement of other factors. Here again, a further longitudinal survey of the ROAD database including detailed environmental and genomic information will elucidate the underlying backgrounds.

Table 4 Number (%) of subjects with radiographic lumbar spondylosis at each intervertebral level in all cohorts

	KL≥2		KL≥3	
	Men	Women	Men	Women
L1/2	474 (57.9)	609 (41.4)	116 (14.2)	254 (17.3)
L2/3	541 (66.1)	749 (51.0)	164 (20.1)	355 (24.2)
L3/4	554 (67.7)	735 (50.0)	194 (23.7)	419 (28.5)
L4/5	523 (63.9)	736 (50.1)	306 (37.5)	605 (41.2)
L5/S	400 (48.9)	576 (39.2)	197 (24.2)	413 (28.1)

KL, Kellgren/Lawrence grading.

Interestingly, KL≥2 spondylosis was more prevalent in men than in women, while KL≥3 spondylosis was more prevalent in women. We and others also have reported that osteophytosis of the lumbar spine is more common in men than in women,[8 9] while disc space narrowing is more prevalent in women.[9] Based on the definition of the KL grading,[12] the discrepancy may be due to distinct aetiological mechanisms between osteophyte formation and disc space narrowing. A cross-sectional study which investigated the extent, prevalence and distribution of spinal spondylosis in women also showed that osteophytosis and disc space narrowing were significantly correlated, but each predicted only 19% of the variation in the other .[11] A previous prospective study in knee joints in the Chingford Study cohort found no association between osteophyte formation and joint space narrowing.[14] A recent study using quantitative magnetic resonance imaging (MRI) in knee joints also reported that osteophyte formation was unrelated to cartilage loss.[15] Furthermore, in an experimental mouse knee osteoarthritis model, we have identified a cartilage-specific molecule, carminerin, that induces only osteophyte formation without affecting cartilage degeneration during the progression of osteoarthritis.[16 17] Further clinical and basic research will disclose the distinct backgrounds of these two representative features of osteoarthritis.

Table 5 Association of Kellgren/Lawrence (KL) grade at each intervertebral level with low back pain

	L1/2	L2/3	L3/4	L4/5	L5/S	Severest
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Men						
KL = 2	1.30 (0.92 to 1.84)	0.94 (0.65 to 1.36)	1.43 (0.98 to 2.11)	1.24 (0.82 to 1.89)	1.12 (0.75 to 1.65)	1.15 (0.70 to 1.92)
KL≥3	1.30 (0.79 to 2.11)	1.25 (0.80 to 1.94)	1.49 (0.96 to 2.32)	1.42 (0.97 to 2.08)	1.22 (0.82 to 1.81)	1.44 (0.89 to 2.38)
Women						
KL = 2	1.20 (0.91 to 1.57)	0.99 (0.75 to 1.31)	0.96 (0.71 to 1.30)	1.25 (0.82 to 1.88)	1.07 (0.73 to 1.54)	0.99 (0.69 to 1.42)
KL≥3	1.66 (1.23 to 2.24)*	1.74 (1.32 to 2.30)*	2.10 (1.62 to 2.72)*	1.88 (1.48 to 2.38)*	1.60 (1.25 to 2.06)*	1.80 (1.38 to 2.37)*

The odds ratio was calculated by logistic regression analysis compared with subjects with KL grade 0 or 1 after adjustment for age and body mass index.
*$p<0.01$.
OR, odds ratio; CI, confidence interval.

Symptomatic low back pain was associated with KL≥3 spondylosis in women but not in men, but not with KL≥2 spondylosis in either gender. Considering the definition of KL grading, this may suggest that disc space narrowing but not osteophytosis of the lumbar spine contributes to low back pain, which is consistent with previous reports.[18] Differences in the association between genders might be dependent on muscle strength to compensate for spinal instability due to disc space narrowing, since men are known to have greater muscle strength than women at all ages.[19] However, approximately 30% of participants without definite radiographic lumbar spondylosis (KL = 0 or 1) had low back pain, and the odds ratio of KL≥3 spondylosis for pain was 1.44 in men and 1.80 in women, which is much lower than the previously reported odds ratio of 8.5 for KL≥3 osteoarthritis in the knee joint for knee pain.[20] This may be because low back pain arises from a number of disorders other than disc space narrowing such as nociceptive stimuli, inflammation, muscle weakness and abnormal load on muscle, ligament or capsular tissues.[21] Indeed, disc degeneration was detected by MRI in at least one lumbar level in all but one asymptomatic volunteers aged 60–80 years.[22] Furthermore, pain is also influenced by psychological factors such as depression, since a significant association between low back pain and depression has been confirmed in many longitudinal studies.[23–25] A recent psychophysical study has shown that anxiety was linked to self-reported and induced low back pain in men but not in women.[26] This might be an alternative reason for the lower association between radiographic spondylosis and low back pain in men.

This study has several limitations. First, prevalence figures using a large-scale population-based sample of elderly people may be generalisable to the Japanese population. However, this study investigated elderly participants who lived independently rather than those who lived in institutional settings, so the calculated prevalence may be underestimated. Second, the definition of low back pain in the present study did not determine the severity. The association of lumbar spondylosis with the severity of low back pain could not be examined in this study. Third, the analyses did not include facet joint osteoarthritis or vertebral fracture, which would probably be associated with low back pain. This is the next factor to be investigated in the ROAD study. Fourth, since the KL system emphasises osteophytosis, it is unclear how to handle lumbar spondylosis with disc space narrowing but no osteophytosis. Since quantitative MRI is still too laborious and expensive to perform in general clinical practice, we are now developing a computer-aided diagnostic program which enables the fully automatic measurement of major features of lumbar spondylosis including disc space narrowing and osteophytosis on plain radiographs.

In conclusion, this cross-sectional study using a large-scale population from the ROAD study revealed a high prevalence of radiographic lumbar spondylosis in elderly people. The prevalence differed to some extent by age, gender and community. Gender seems to be distinctly associated with KL≥2 and KL≥3 lumbar spondylosis, and disc space narrowing with or without osteophytosis in women may be a risk factor for low back pain. Further progress, along with continued longitudinal survey in the ROAD study, will elucidate the environmental and genetic backgrounds of lumbar spondylosis and its relation with low back pain.

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Competing interests: None.

Ethics approval: All participants provided written informed consent, and the study was conducted with approval of the ethical committees of the University of Tokyo and the Tokyo Metropolitan Institute of Gerontology.

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Original article

Association of low dietary vitamin K intake with radiographic knee osteoarthritis in the Japanese elderly population: dietary survey in a population-based cohort of the ROAD study

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Abstract

Background. The present study sought to identify dietary nutrients associated with the prevalence of radiographic knee osteoarthritis (OA) in the Japanese elderly of a population-based cohort of the Research on Osteoarthritis Against Disability (ROAD) study.

Methods. From the baseline survey of the ROAD study, 719 participants ≥60 years of age (270 men, 449 women) of a rural cohort were analyzed. Dietary nutrient intakes for the previous 1 month were assessed by a self-administered brief diet history questionnaire. The radiographic severity at both knees was determined by the Kellgren/Lawrence (KL) system.

Results. The prevalence of knee OA of KL ≥2 was 70.8%. Age, body mass index, and female sex were positively associated with the prevalence. Among the dietary factors, only vitamin K intake was shown to be inversely associated with the prevalence of radiographic knee OA by multivariate logistic regression analysis. The presence of joint space narrowing of the knee was also inversely associated with vitamin K intake. The prevalence of radiographic knee OA for each dietary vitamin K intake quartile decreased with the increased intake.

Conclusions. The present cross-sectional study using a population-based cohort supports the hypothesis that low dietary vitamin K intake is a risk factor for knee OA. Vitamin K may have a protective role against knee OA and might lead to a disease-modifying treatment.

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Introduction

Osteoarthritis (OA) is a major public health issue causing disability of the elderly in most developed countries.[1] There is an urgent need for safe, effective strategies for preventing and treating this disease. Such strategies could come from dietary nutrition as studies have indicated an association of nutritional factors with OA.[2–7] Diet and nutritional factors are important because they are modifiable. However, epidemiological data on the relation between nutritional factors and OA are insufficient. We thus set up a population-based prospective cohort study named Research on Osteoarthritis Against Disability (ROAD) in 2005. The present study investigated the association of the prevalence of radiographic knee OA with dietary nutritional factors assessed by a self-administered brief diet history questionnaire (BDHQ) in the Japanese elderly living in a rural community participating in the ROAD study.[8]

Participants and methods

Participants

The ROAD study is a population-based prospective cohort study designed to clarify the environmental and genetic risk factors for OA. The participants of the ROAD study were recruited from the residents of three communities that have different characteristics: an urban region in Itabashi, Tokyo; a mountainous region in Hidakagawa, Wakayama; and a coastal region in Taiji, Wakayama.[9] The inclusion criteria were as follows: The patient (1) had to be able to walk to the clinic at

which the survey was performed, (2) provide self-reported data, and (3) understand and sign an informed consent form. Residents of the urban, mountainous, and coastal regions were recruited from the resident registration list of the relevant region.

The age of the participants recruited from the urban region was ≥60 years, and that of the participants from the two other regions was ≥40 years. In the urban, mountainous, and coastal areas, 99.8%, 84.3%, and 54.7% of the participants, respectively, were >60 years of age. Two-thirds of the participants were women, and their mean age was 1 year less than that of the male participants. The baseline survey of the Hidakagawa cohort was conducted from November 2005 to February 2006. The community has a population of 11300/330 km^2 and residents ≥65 years constitute 30.5% of the population. All participants provided written informed consent, and the study was conducted with approval of ethics committees of the institution. From the baseline data of 723 participants who were ≥60 years in the cohort, we analyzed 719 participants (270 men, 449 women) after excluding four individuals who had undergone knee surgery.

Dietary assessment

For the dietary survey, we used the BDHQ and investigated dietary nutrient intakes for the previous 1 month. A questionnaire was given to each participant with detailed explanations to fill it out at home and was addressed by well-trained interviewers when the participant visited the clinic. The BDHQ is a 4-page, structured questionnaire that inquires about the consumption frequency of a total of 56 food and beverage items, with specified serving sizes described in terms of a natural portion or the standard weight and volume measurement of servings commonly consumed in general Japanese populations. The BDHQ was developed based on a comprehensive (16-page) version of a validated self-administered diet history questionnaire[8] and is now widely used for the dietary survey in Japan.[10–12] Estimates of dietary intake for the 56 food and beverage items, energy, and selected nutrients were calculated using an ad hoc computer algorithm for the BDHQ, which was based on the Standard Tables of Food Composition in Japan. Dietary intake levels of total energy and 16 nutrient factors (animal protein; vegetable protein; animal fat; vegetable fat; carbohydrate; vitamins B_1, B_2, B_6, and B_{12}; niacin; vitamins C, D, E, and K; dietary fiber; salt) were analyzed.

Radiographic assessment

All participants had plain radiographic examinations of both knees with an anteroposterior view with weight-bearing and foot map positioning. Knee radiographs were read, without knowledge of the participants' clinical status, by a single well-experienced orthopedist using the Kellgren/Lawrence (KL) radiographic atlas, and a KL grade (0–4) was determined.[13,14] The higher KL grade in both knees was designated as that of the participant. To evaluate intraobserver variability of the KL grading, 100 randomly selected radiographs of the knee were scored by the same observer more than 1 month after the first reading. Furthermore, 100 other radiographs were scored by two experienced orthopedic surgeons using the same atlas for interobserver variability. The intra- and interobserver variabilities were evaluated by kappa analysis and were confirmed to be sufficient for assessment (0.86 and 0.80, respectively).

Statistical analysis

Differences in crude mean values of dietary nutrient intakes were examined by a nonpaired *t*-test between the KL = 0 or 1 group and the KL ≥2 group for each sex, and those with significant differences were further evaluated by multivariate logistic regression analysis after adjustment for age, sex, body mass index (BMI), and total energy to estimate the odds ratio (OR) and its associated 95% confidence interval (95% CI). Association of the presence of joint space narrowing of the knee defined as KL ≥3 with nutrient intakes was also examined by logistic regression analysis. The Cochran-Mantel-Haenszel test was used to determine the association of the prevalence of knee OA for each dietary nutrient intake quartile for linear trend. Data analyses were performed using SAS version 9.0 (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered significant.

Results

Characteristics of the 719 participants are shown in Table 1. The prevalence of KL ≥2 knee OA was 70.8% (57.8% in men, 78.6% in women) and that of KL ≥ 3 was 25.9% (15.9% in men, 31.8% in women). Neither the age nor the BMI was significantly different between men and women in the overall population. Participants with KL ≥2 knee OA were older than those without it (KL = 0 or 1) in both sexes, and the BMI was higher in KL ≥2 than in KL = 0 or 1 in women.

We compared total energy and 16 dietary nutrient intakes between the groups with and without KL ≥2 knee OA (Table 2). Vegetable fat intake was significantly lower in the KL ≥2 group than in the KL = 0 or 1 group in women. Vitamin K intake was significantly lower in the KL ≥2 group than in the KL = 0 or 1 group in both sexes. Total energy and other nutrient intakes

Table 1. Characteristics of participants

	Men			Women		
	Overall	KL = 0 or 1	KL ≥2	Overall	KL = 0 or 1	KL ≥ 2
No. of participants	270	114	156	449	96	353
Age (years)	72.1 ± 6.3	70.4 ± 5.9	73.4 ± 6.3†	72.0 ± 7.0	68.8 ± 6.1*	72.8 ± 7.0†
Height (cm)	160.2 ± 6.2	160.1 ± 6.8	159.9 ± 5.8	146.9 ± 6.3*	148.5 ± 6.1*	146.8 ± 6.3*
Weight (kg)	58.9 ± 9.6	58.3 ± 9.6	59.2 ± 9.6	49.7 ± 8.5*	48.7 ± 6.7*	49.9 ± 8.9*
BMI (kg/m^2)	22.8 ± 2.9	22.5 ± 2.8	23.1 ± 3.0	22.9 ± 3.4	22.1 ± 2.6	23.2 ± 3.5†

Data are means ± SD
KL, Kellgren/Lawrence system; BMI, body mass index
* $P < 0.05$ vs. men in the corresponding group by nonpaired *t*-test
† $P < 0.05$ vs. KL = 0 or 1 in the corresponding group by nonpaired *t*-test

Table 2. Comparison of total energy and dietary nutrient intakes between participants with (KL ≥2) and without (KL = 0 or 1) radiographic knee OA according to sex

	Men		Women	
Parameter	KL = 0 or 1	KL ≥2	KL = 0 or 1	KL ≥2
Total energy (MJ/day)	9.77 ± 2.88	9.90 ± 2.73	7.07 ± 1.75	7.03 ± 1.78
Dietary nutrients				
Animal protein (g/day)	46.3 ± 20.7	48.4 ± 20.9	36.8 ± 12.9	37.4 ± 16.2
Vegetable protein (g/day)	34.1 ± 10.1	33.8 ± 9.4	27.2 ± 6.7	26.1 ± 6.8
Animal fat (g/day)	27.6 ± 13.3	28.7 ± 12.2	21.9 ± 7.8	22.1 ± 10.1
Vegetable fat (g/day)	21.2 ± 10.9	21.9 ± 10.4	19.7 ± 8.6	17.6 ± 8.1*
Carbohydrate, (g/day)	352 ± 116	356 ± 114	259 ± 72	261 ± 75
Vitamin D (μg/day)	22.0 ± 11.5	23.7 ± 13.0	16.7 ± 7.4	18.5 ± 9.9
Vitamin E (mgα-TE/day)	7.76 ± 3.43	7.89 ± 3.15	7.24 ± 2.51	6.84 ± 2.58
Vitamin K (μg/day)	266 ± 171	228 ± 131*	253 ± 125	213 ± 115*
Vitamin B_1 (mg/day)	0.81 ± 0.27	0.80 ± 0.24	0.71 ± 0.16	0.67 ± 0.19
Vitamin B_2 (mg/day)	1.09 ± 0.44	1.06 ± 0.37	0.97 ± 0.27	0.92 ± 0.33
Niacin (mgNE/day)	18.1 ± 6.9	18.0 ± 6.6	14.1 ± 4.1	13.6 ± 5.1
Vitamin B_6 (mg/day)	1.34 ± 0.49	1.32 ± 0.45	1.08 ± 0.29	1.05 ± 0.35
Vitamin B_{12} (μg/day)	12.1 ± 6.3	12.5 ± 6.5	9.2 ± 4.0	9.6 ± 4.9
Vitamin C (mg/day)	103 ± 43	96 ± 39	117 ± 45	113 ± 42
Dietary fiber (g/day)	11.7 ± 4.0	11.2 ± 3.3	11.0 ± 3.2	10.5 ± 3.0
Salt (g/day)	13.0 ± 4.1	12.5 ± 3.7	10.4 ± 2.6	10.4 ± 3.1

Data are the mean ± SD
TE, tocopherol equivalent; NE, niacin equivalent
* $P < 0.05$ vs. KL = 0 or 1 in each group by nonpaired *t*-test

were not significantly different between the groups in either sex. Logistic regression analysis was performed using the presence of KL ≥2 knee OA (1, yes vs. 0, no) as an objective variable and age, BMI, sex, total energy, vegetable fat, and vitamin K intakes (vs. +1 SD) as explanatory variables (Table 3). Age, BMI, and sex were associated with the presence of radiographic knee OA (KL ≥2). Although vegetable fat intake had no significant association, dietary vitamin K intake (OR = 0.75, 95% CI = 0.63–0.89 vs. +1 SD) was shown to be inversely associated with the presence of radiographic knee OA in the overall population.

Table 4 shows the association between KL grade and dietary vitamin K intake according to sex. Logistic

Table 3. Association of age, BMI, sex, and nutrient intakes with radiographic knee OA (KL ≥ 2) in the overall population

Parameter	OR	95% CI
Age (years)	1.11	1.07–1.14*
BMI (kg/m^2)	1.15	1.08–1.22*
Women (vs. men)	3.08	2.16–4.40*
Dietary nutrient intakes		
Vegetable fat[a] (SD)	0.93	0.78–1.10
Vitamin K[a] (SD)	0.75	0.63–0.89*

The odds ratios for KL ≥ 2 (vs. KL = 0 or 1) were calculated by logistic regression analysis
OR, odds ratio; CI, confidence interval
* $P < 0.01$
[a] Adjusted for age, sex, BMI, and total energy

Table 4. Association between KL grade and dietary vitamin K intake according to sex

Condition	Overall		Men		Women	
	OR	95% CI	OR	95% CI	OR	95% CI
KL ≥2 (vs. KL = 0 or 1)	0.75	0.63–0.89†	0.76	0.59–0.95*	0.74	0.58–0.96*
KL ≥3 (vs. KL ≤2)	0.67	0.53–0.84†	0.74	0.50–1.04	0.61	0.45–0.81†

Odds ratios were calculated by logistic regression analysis after adjustment for age, sex, BMI, and total energy
*$P < 0.05$
†$P < 0.01$

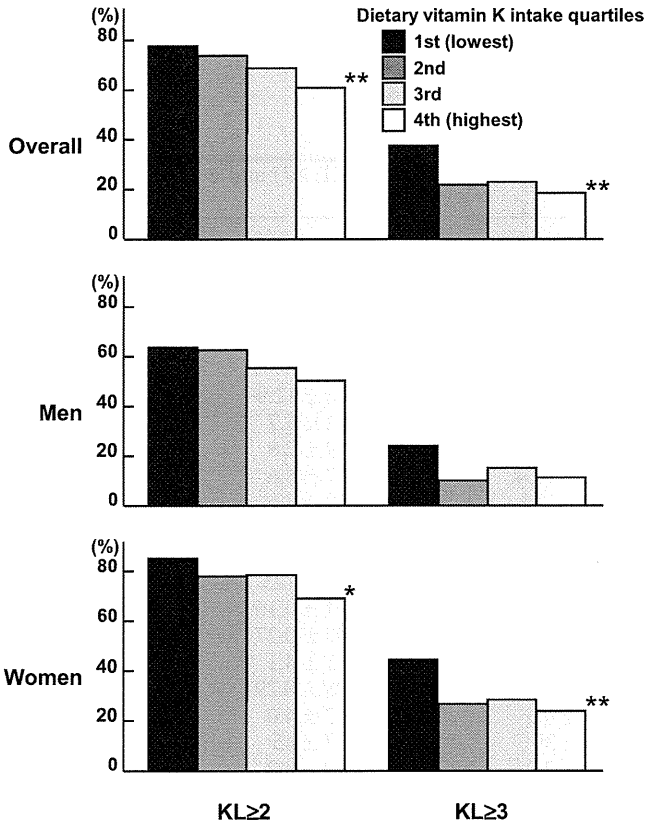

Fig. 1. Prevalence of KL ≥2 and KL ≥3 knee osteoarthritis per quartile of dietary vitamin K intake. The 25th, 50th, and 75th percentiles were, respectively, 141.4, 205.8, and 285.8 mg/day in the overall population; 145.0, 222.8, and 314.0 mg/day in men; and 137.4, 199.9, and 279.3 mg/day in women. *$P < 0.05$ and **$P < 0.01$ for linear trend

regression analysis using the presence of KL ≥2 knee OA as an objective variable showed that vitamin K intake was inversely associated with KL ≥2 knee OA in both sexes (OR = 0.76, 95% CI = 0.59–0.95 vs. +1 SD in men; OR = 0.74, 95% CI = 0.58–0.96 vs. +1 SD in women) as well as in the overall population. Furthermore, logistic regression analysis using the presence of KL ≥3 knees (vs. KL ≤2) as an objective variable revealed that KL ≥ 3 knees (vs. KL ≤2) were also inversely associated with vitamin K intake in the overall population (OR = 0.67, 95% CI = 0.53–0.84 vs. +1 SD) and in women (OR = 0.61, 95% CI = 0.45–0.81 vs. +1 SD), indicating that the presence of joint space narrowing of the knee was inversely associated with dietary vitamin K intake. Furthermore, we examined the prevalence of KL ≥2 and KL ≥3 knee OA for each dietary vitamin K intake quartile (Fig. 1), which decreased with ascending vitamin K intake. This tendency was significant in the overall population and in women.

Discussion

The present study investigated the association of radiographic knee OA with nutritional factors in a population-based cohort of the ROAD study. Total energy, protein, fat, and carbohydrate had no significant association with knee OA. Among dietary vitamin intakes, vitamin K was inversely associated with the prevalence of radiographic knee OA. Previous published epidemiological studies have suggested a relation between OA and vitamins.[2–7] Vitamin K includes vitamin K_1 or phylloquinone, which is contained in green leafy vegetables, and vitamin K_2 or menaquinone, which is synthesized by bacteria and abundantly contained in a traditional Japanese fermented soybean food called *natto*.[15,16] Vitamin K belongs to the fat-soluble vitamins, which may be the reason why vegetable fat intake was lower in the knee OA group in women, although it was not significant in the multivariate analysis. Plasma levels of phylloquinone has been reported to be inversely associated with the prevalence of OA in the hand and knee,[6] which is consistent with the results of the present study.

Vitamin K serves as an essential cofacotor of γ-glutamyl carboxylase, an enzyme for the γ-carboxylation of vitamin K-dependent proteins including matrix Gla protein (MGP).[17] MGP is an extracellular matrix protein of the mineral-binding Gla protein family that includes osteocalcin, the growth arrest-specific protein 6 (Gas6). Gas6 is up-regulated in growth-arrested cells,[18] suggesting a role in protection from certain cellular stresses, such as apoptosis. In fact, many studies demonstrated the ability of Gas6 to promote cell survival and proliferation.[19–22] MGP is expressed by proliferative and late hypertrophic chondrocytes,[23,24] and mutations in MGP

are responsible for Keutel syndrome in which patients are affected by aberrant cartilage calcification.[25] Studies of MGP-deficient mice suggest that MGP is an inhibitor of extracellular matrix calcification in the epiphyseal growth plate.[26] Warfarin, a vitamin K-antagonist anticoagulant, is known to cause warfarin embryopathy characterized by abnormal calcification and decreased growth of the cartilage.[27,28] These data demonstrate that vitamin K plays an important role in cartilage metabolism as a inhibitor of extracellular matrix calcification as well as a promotor of cell survival and proliferation. Habitual low dietary vitamin K intake may exert an inhibitory effect on the vitamin K-dependent MGP and Gas6 functions and modulate the pathogenesis of OA by influencing the process of osteophytosis and cartilage destruction.

The minimum amounts of vitamin K intake recommended by the Japanese Ministry of Health, Labor, and Welfare are 75 and 65 μg/day for men and women, respectively. The percentages of participants who did not meet the criteria in this study were 8.5% in men, 3.6% in women, and 5.4% in the overall population—all of whom belonged to the 1st quartile (lowest) in Fig. 1. However, even in the 2nd through 4th quartiles, the prevalence of radiographic OA decreased with ascending vitamin K intake, suggesting that the recommended amount of vitamin K intake may not be sufficient for the prevention of knee OA.

The management of knee OA is largely palliative, focusing on the alleviation of symptoms, although it is a major public health issue causing disabilities in the elderly. The Osteoarthritis Research Society International (OARSI) current recommendations include a combination of nonpharmacological interventions and pharmacological treatments.[29] Considering that nonsteroidal antiinflammatory drugs (NSAIDs) with serious adverse effects caused by their long-term use remain among the most widely prescribed drugs for OA,[30] there is a need for safe, effective alternative strategies for the prevention and treatment of this disease. Such strategies could come from dietary nutrition, and vitamin K might have a preventive role against OA.

There are limitations in the present study. This is a cross-sectional study of the baseline data, and a causal relation could not be determined. In addition, the dietary survey in this study investigated dietary habits only for the pevious month, which did not necessarily reflect a long habit of several years, despite the fact that OA is a slowly progressing chronic disease. This dietary survey also investigated whether participants had changed their dietary habits. Those who answered yes comprised 9.6%; and 90.4% of participants answered they had not changed their dietary habits. Although it is likely that dietary habits in middle-aged and elderly people are usually quite different from those in children and young adults, there is a possibility that most of participants in this study had not changed their dietary habits for several years or for a longer time, which may have affected the disease process of OA. Furthermore, the dietary survey in the present study was conducted from autumn to winter although there are four seasons in Japan and diets may vary with the season. Therefore, the present study could give some bias for the effect of season on the nutritional quality of diets. This is a limitation in this study because we could not follow participants during all seasons to get a measure of average diets during the year. We are planning a follow-up study during the same season to minimize the variation caused by seasonal differences. Longitudinal data are required to confirm the relation between vitamin K and OA.

Conclusion

The present cross-sectional study using a population-based cohort supports the hypothesis that low dietary vitamin K intake is a risk factor for knee OA. Vitamin K may have a protective role against knee OA and might lead to disease-modifying treatment.

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We declare that we have no conflict of interest regarding the present manuscript.

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COHORT PROFILE

Cohort Profile: Research on Osteoarthritis/ Osteoporosis Against Disability study

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How did the study come about?

Since the proportion of the ageing population in Japan is increasing, a comprehensive and evidence-based strategy is urgently required for the prevention of musculoskeletal diseases, including osteoarthritis (OA) and osteoporosis (OP), both of which affect the activities of daily living (ADL) and quality of life (QOL) and increase morbidity and mortality.[1–4] However, few prospective, longitudinal studies for the purpose of developing such a strategy have been conducted, and little information is available regarding the prevalence and incidence of musculoskeletal disorders, including OA and OP, as well as pain and disability in the Japanese population.[5–10] It is difficult to design rational clinical and public health approaches for the diagnosis, evaluation and prevention of OA and OP without such epidemiological data.

The Research on Osteoarthritis/osteoporosis Against Disability (ROAD) study was established in 2005 by N.Y., T.A., H.O., S.M., H.K. and K.N. (principal investigators). The principal investigators are affiliated with the 22nd Century Medical and Research Center, University of Tokyo.

What does the ROAD study cover?

The ROAD study is a multi-centre prospective observational study that aims to elucidate the environmental and genetic background of bone and joint diseases (with OA and OP as the representative bone and joint diseases). It is designed to examine the extent to which risk factors for these diseases are related to the clinical features of the diseases, laboratory and radiographic findings, bone mass, bone geometry, lifestyle, nutritional factors, anthropometric and neuromuscular measures and fall propensity. It also aims to determine how these diseases affect the ADL and QOL of Japanese men and women.

The study will provide the information required to develop clinical algorithms for the early identification of potential high-risk populations. It will also provide information required to develop policies for the detection and prevention of OA, OP and osteoporotic fractures. The immediate goal of this study is to establish a representative population of elderly people, principally for the study of bone and joint health. The establishment of this cohort will also facilitate the expansion of other studies in related areas of investigation. Moreover, the knowledge gained from the ROAD study will have major implications for understanding and managing several other common problems of ageing.

Who are in the sample?

The subjects were residents of any one of the three communities that have different characteristics: an urban region in Itabashi, Tokyo; a mountainous region in Hidakagawa, Wakayama; and a coastal region in Taiji, Wakayama (Figure 1). The inclusion criteria, apart from residence in the communities mentioned above, were the ability to (i) walk to the survey site, (ii) report data and (iii) understand and sign an informed consent form. The age of the participants recruited from the urban region was $\geqslant 60$ years, and that of the participants from the other

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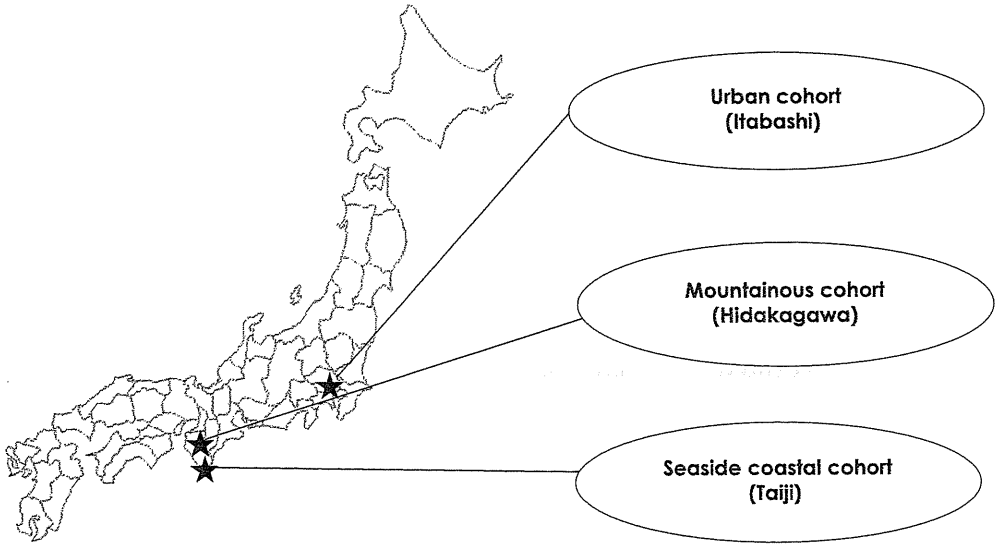

Figure 1 Locations of the three regions from which the study cohort was derived

Table 1 Age–sex distribution and mean values [standard deviation (SD)] of selected characteristics of the participants

Age strata (years)	Men				Women			
	Total	Urban	Mountainous	Coastal	Total	Urban	Mountainous	Coastal
⩽39	14	0	2	12	31	0	7	24
40–49	44	0	7	37	105	0	17	88
50–59	107	0	36	71	211	2	67	142
60–69	168	11	93	64	385	60	183	142
70–79	535	315	150	70	913	594	196	123
⩾80	193	139	31	23	334	229	75	30
Total	1061	465	319	277	1979	885	545	549
Age (years)	71.0 (10.7)	77.2 (4.3)	69.5 (9.1)	62.6 (13.2)	69.9 (11.2)	76.3 (5.0)	68.6 (10.4)	60.8 (12.5)
Height (cm)	162.5 (6.7)	161.3 (5.9)	161.4 (6.9)	165.8 (6.8)	149.8 (6.5)	148.5 (5.6)	148.2 (6.7)	153.2 (6.2)
Weight (kg)	61.3 (10.0)	60.0 (8.5)	60.0 (10.2)	64.8 (11.0)	51.5 (8.6)	50.8 (8.3)	50.5 (8.6)	53.5 (8.8)
BMI (kg/m^2)	23.1 (3.0)	23.0 (2.8)	23.0 (3.0)	23.5 (3.4)	22.9 (3.5)	23.0 (3.4)	23.0 (3.4)	22.8 (3.6)
Current smoker (%)	25.9	19.0	28.9	31.1	3.5	2.9	4.7	2.9
Current drinker (%)	64.4	60.5	69.8	63.2	25.9	27.4	26.1	24.2

BMI = body mass index.

two regions was ⩾40 years. In the urban region, invitation letters were distributed only to the inhabitants whose name was on a list of community-dwelling people that was prepared in 2002.[11]

Subjects from each area who were willing to attend the study were invited to participate. Despite being younger (58 years) than the age limit defined in the inclusion criteria, 2 inhabitants from the urban area, 9 from the mountainous area and 36 from the coastal area were included in the study because they were very keen to participate. Over the 1.5-year period from October 2005 to March 2007, 3040 of 5785 candidates were enrolled from the three regions (participation rate, 52.5%).

Selected characteristics of the study population, including age, height, weight, BMI and proportions of participants who smoked and consumed alcohol, are shown in Table 1. In the urban, mountainous and coastal areas, 99.8, 84.3 and 54.7% of the participants, respectively, were >60 years of age. Two-thirds of the participants were women, and their mean age was 1 year less than that of the male

participants. No significant differences were observed in BMI values between the genders, but the proportions of both current smokers and alcohol consumers were significantly higher among men than among women.

All participants provided written informed consent, and the study was conducted with the approval of the ethics committees of the University of Tokyo (nos 1264 and 1326) and the Tokyo Metropolitan Institute of Gerontology (no. 5). Careful consideration was given to ensure a safe experience for the participants during the examination and during any other study procedures.

How often have they been followed up?

We intend to follow-up the three population-based cohorts of the ROAD study for at least 10 years. In October 2008, after a follow-up period of 3 years, a second comprehensive clinical examination was started and is ongoing. We will repeat the baseline measurements during the second examination. A third and fourth examination will be performed at 6 and 10 years, respectively, after the baseline examination.

What has been measured?

The baseline examination of the ROAD study consisted of the following: interviewer-administered questionnaire, dietary assessment, anthropometric measurements, visual and neuromuscular function assessment, biochemical measurements, medical history taking, radiographic assessment and bone mineral density (BMD) measurement (Table 2).

Interviewer-administered questionnaire

A questionnaire was prepared by modifying the questionnaire used in the Osteoporotic Fractures in Men Study (MrOS),[12] and adding some new items to the modified questionnaire. Knee symptoms were

Table 2 Summary of data collected in the ROAD study

Interviewer-administrated questionnaire	
Cigarette smoking, alcohol consumption	
Medical history, medications	
Reproductive variables, lactation	
Dietary history, history of falls and fractures	
Physical activity using PASE	
Family history	
Evaluation of knee symptoms using WOMAC	
Health-related QOL (EQ5D, SF-8)	
Dietary assessment	
Nutrient intake calculated using BDHQ	
Anthropometric measurements	
Height, weight, arm span, grip strengths	
Circumference of both wrists, circumference of waist	
Heart rate, systolic and diastolic blood pressure	
Visual and neuromuscular function	
Visual acuity	
Walking speed with tandem walking 6 m x 20 cm	
Rise from a chair	
Biochemical measurements	
Blood samples	Blood counts, haemoglobin, haemoglobin A1C, blood sugar
Sera	Total protein, AST, ALT, GGT, total cholesterol, HDL-cholesterol, triglyceride
	BUN, uric acid, creatinine
DNA samples extracted	
Urine samples	Urinary protein, occult blood, sugar, urobilinogen
Medical information	
Pain in back, lumbar, knee and hip	
Swelling and range of motion of the joints	
Tendon reflexes	
Cognigitve function used by Mini-Mental Status Examination	
Radiographic assessment	
Anteroposterior and lateral views of lumbar spine	
Anteroposterior view of both knees	
Anteroposterior view of both hips	
BMD measurements	
Lumbar spine and proximal femur (mountainous and coastal areas)	

AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = γ-glutamyltranspeptidase; HDL = high-density lipoprotein; BUN = blood urea nitrogen; BDHQ = Brief Diet History Questionnaire; PASE = Physical Activity Scale for the Elderly; WOMAC = Western Ontario and McMaster University Osteoarthritis Index; EQ5D = European QOL-5 dimensions instrument; SF-8 = Medical Outcomes Study 8-item Short Form.

evaluated using the WOMAC.[13] The health-related QOL was evaluated using the EuroQOL, EQ5D[14] and the SF-8.[15] The study staff recorded all the medications administered and their doses. Physical activity was quantified using the PASE.[16]

Dietary assessment

Dietary assessment was made using a BDHQ, and the dietary intakes of nutrients during the previous month were determined. Each participant received a questionnaire that included detailed explanations. Well-trained interviewers clarified any unclear sections in the questionnaire, which was to be completed by the participants at their leisure. The BDHQ is a four-page structured questionnaire that includes questions about the frequency of consumption of 80 principal foods. The serving sizes of the foods are described as normal portions, i.e. the standard weight and volume of servings commonly consumed by the general Japanese population. The BDHQ was modified from a comprehensive, 16-page version of a validated self-administered diet history questionnaire.[17] A total of 141 components, including dietary energy and nutrient intakes, were calculated using an *ad hoc* computer algorithm for the BDHQ.

Anthropometric measurements

Anthropometric factors were measured by well-trained medical nurses. The height and weight of the participants at age 25 years were also noted. BMI [weight in kilograms/(height in metres)^2] was calculated on the basis of the current height and weight.

Visual and neuromuscular function

Visual acuity was assessed by the Landolt ring test. Walking speed was determined by recording the time taken by a subject to walk 6 m at the fastest possible speed. The time required for tandem walking across a 6-m long and 20-cm wide path was used to determine balance. The ability to rise from a chair without using the arms (chair stand) and the ability to perform five chair stands was evaluated; the time required to complete the tasks was noted.

Biochemical measurements

Blood and urine samples were obtained from each participant for biochemical and genomic examinations. Urinary protein, occult blood, sugar and urobilinogen were tested using disposable reagent strips (uro-hema-combi sticks; Siemens Medical Solutions Diagnostics, Tokyo, Japan). Residual blood, plasma, serum and urine specimens were processed and stored in a deep freezer (−80°C). DNA was extracted from stored whole-blood specimens, and biochemical markers of bone turnover and cartilage will be measured using these stored serum and urine samples.

Medical history

Medical history was obtained by experienced orthopaedic surgeons (S.M. and H.O.). To quantify cognitive function, the participants were instructed to complete the modified Mini-Mental Status Examination—Japanese version.[18] Physicians explained any unclear sections of this questionnaire to the participants and assessed the participants' cognitive status on the basis of the completed questionnaire.

Radiographic assessment

The severity of OA was radiographically determined according to the Kellgren–Lawrence (KL) grading system as follows[19]: KL0—normal joint; KL1—slight osteophytes; KL2—definite osteophytes; KL3—disc-space narrowing and large osteophytes; and KL4—bone sclerosis, disc-space narrowing and large osteophytes. In the ROAD study, joints that exhibited only disc-space narrowing and no large osteophytes were graded as KL3. The radiographs were examined by a single, experienced orthopaedic surgeon (S.M.), who was blinded to the clinical status of the participants. If at least one knee joint was graded as KL2 or higher, the participant was diagnosed with radiographic knee OA. Similarly, if at least one intervertebral joint of the lumbar spine was graded as KL2 or higher, the participant was diagnosed with radiographic lumbar spondylosis.

BMD measurement

In the mountainous and coastal areas, the BMD of the lumbar spine and proximal femur was measured using dual energy X-ray absorptiometry (DXA) (Hologic Discovery; Hologic, Waltham, MA, USA) during the baseline examination. Another BMD measurement was scheduled for the second examination.

To maintain the quality of measurement, the same DXA equipment was used, and the same spine phantom was scanned daily to monitor the machine's performance in study populations from different regions. The BMD of the phantom was adjusted to 1.032 ± 0.016 g/cm^2 ($\pm 1.5\%$) during all examinations. In addition, to exclude inter-observer variability, the same physician (N.Y.) examined all participants. In another study, N.Y. had measured the intra-observer variability in both *in vitro* and *in vivo* experiments using Lunar DPX.[20] In the case of the *in vitro* experiment, the coefficient of variance (CV) for the BMD of the L2–L4 vertebrae was 0.35%. In the case of the *in vivo* experiments, which were performed on five male volunteers, the CVs for the BMDs of the L2–L4 vertebrae, the proximal femur, Ward's triangle and the trochanter were 0.61–0.90, 1.02–2.57, 1.97–5.45 and 1.77–4.17%, respectively.

OP was defined on the basis of the World Health Organization (WHO) criteria; specifically, it was diagnosed when the BMD T-scores were lower than the mean lumbar peak bone mass minus 2.5 SDs.[21]

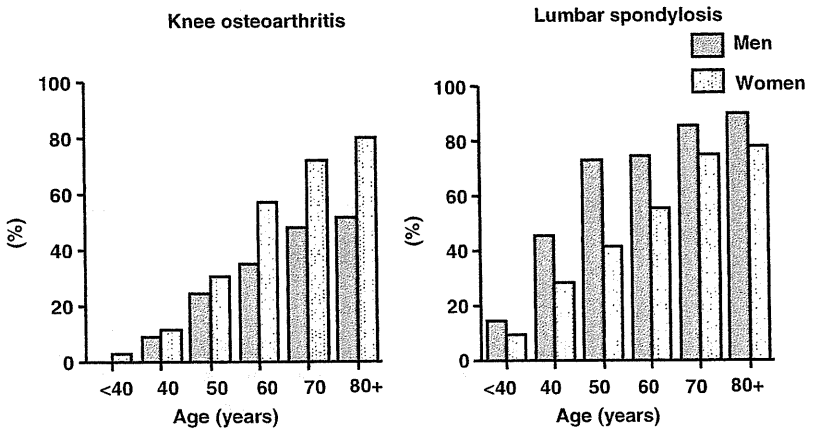

Figure 2 Prevalence of radiographic knee osteoarthritis and lumbar spondylosis, classified by age and gender

In Japan, the mean BMD of the L2–L4 vertebrae among both young male and female adults has been measured using Hologic DXA.[22] These indices were used in the present study; lumbar spine BMD $<0.714\,g/cm^2$ (in case of both men and women), and femoral neck BMD $<0.546\,g/cm^2$ (men) or $0.515\,g/cm^2$ (women) were considered to indicate OP.

All assessments performed in the baseline study will be repeated at the first, second and third follow-ups.

What is attrition like?

The first follow-up (second examination) commenced on October 2008, 3 years from baseline assessment. By the end of 2008, follow-up was completed in Hidakagawa, the mountainous region. Of the 864 participants (319 men and 545 women) in the baseline study, 635 subjects (224 men and 411 women) attended the second examination. The response rate for the second examination in the mountainous area was 73.5%. The most common reasons for non-participation were illness and difficulty in visiting the clinic (43% of the dropouts). Further, 26 people (12% of the dropouts) who participated in the baseline study died during the 3-year period following the initial assessment. In other two areas, the follow-ups are on going. The total attrition will be determined at the end of March 2010.

What has the ROAD study found?

By analysing the data from the baseline study, we have determined the prevalence of OA and OP.

OA

The age–sex distribution of radiographic knee OA and lumbar spondylosis was calculated (Figure 2); both conditions were diagnosed at KL grades of $\geqslant 2$. In the overall population, the prevalence of radiographic knee OA and lumbar spondylosis was 54.6% (42.0% in men and 61.5% in women) and 70.2% (80.6% in men and 64.6% in women), respectively. Thus, both the overall and sex-specific prevalence of lumbar spondylosis were higher than those of knee OA.[23]

OP

The prevalence of OP was calculated for the participants from mountainous and coastal regions in the ROAD study (Figure 3). The prevalence of OP of the lumbar spine and femoral neck in women was 6- and 5-fold, respectively, than in men. The differences were significant ($P < 0.001$).[23]

What are the main strengths and weaknesses of the ROAD study?

Strengths

In Japan, little epidemiological information is available of musculoskeletal diseases such as OA and OP. The ROAD study is the first large population-based prospective study conducted on the Japanese population and is designed to supply essential information, chiefly of OA and OP.

We confirmed the high prevalence of OA and OP among the ROAD study participants, and we will conduct follow-up examinations for at least 10 years in order to clarify the relationships of OA, OP and osteoporotic fractures with the following parameters: lifestyle, anthropometric and neuromuscular measurements, bone mass, bone geometry and fall propensity. Further, we will determine how these impairments affect QOL and mortality. We also expect to assess the similarities and differences in the risk factors of OA and OP. In addition, we will clarify the incident morbidity of other lifestyle-related disorders,

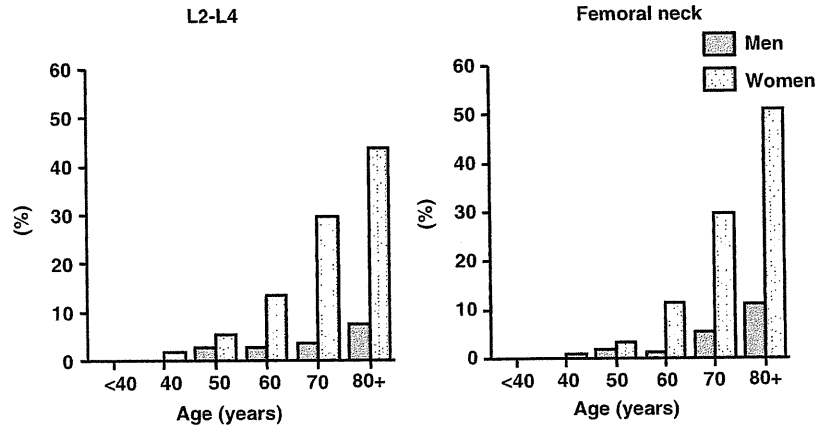

Figure 3 Prevalence of osteoporosis of the lumbar spine and femoral neck

such as obesity, hypertension, diabetes mellitus, cardiovascular and metabolic diseases and dementia.

The ROAD study data will facilitate the development of clinical guidelines for the detection and prevention of osteoporotic fractures in other countries. This study was designed such that it would be similar to the Study of Osteoporotic Fractures, a large observational study on the determinants of fractures in older women,[24] and to MrOS, a large observational study on the determinants of fractures in older men[25] in the USA.

Finally, the completion of the ROAD study will provide unique opportunities for the study of other conditions that are common among older men and women, such as obesity, diabetes, cardiovascular disease, cognitive disorders and frailty. The blood, plasma, serum and urine specimens stored during the ROAD study will enable the clarification of a variety of new biochemical and genetic factors associated with musculoskeletal disorders and the aforementioned diseases.

Weaknesses

Although the ROAD study includes a large number of subjects (more than 3000), these subjects are voluntary participants and have been recruited from only three areas; hence, they do not truly represent the general population. The 'healthy' and 'regional' selection biases should be confirmed.[26] We could not directly compare the baseline characteristics between the responders and non-responders owing to lack of data regarding the non-responders. Hence, to determine whether a selection bias existed in the ROAD study, we compared the anthropometric measurements and frequencies of smoking and alcohol drinking between the participants and the general Japanese population. The values for the general population were obtained from the 2005 National Health and Nutrition Survey conducted by the Ministry of Health, Labour and Welfare, Japan, which is an annual survey to clarify the health status of the Japanese population and is conducted on approximately 18 000 inhabitants from 6000 randomly selected families.[27]

The BMIs of ROAD study participants and the Japanese population were compared (Table 3). No significant differences were identified, except that the male participants aged 70–74 years were significantly smaller in build than men of this age group in the overall Japanese population ($P < 0.05$).

The proportion of current smokers and current drinkers (those who regularly smoked or drank more than once a month) in the general Japanese population was compared with that in the study population (Figure 4). Both proportions were significantly higher in the general Japanese population than in the study population (smokers: men, $P < 0.001$ and women, $P < 0.001$; drinkers: men, $P < 0.01$ and women, $P < 0.001$), suggesting that participants of the ROAD study had healthier lifestyles than the general Japanese population. This bias due to the selection of 'healthy' individuals should be taken into consideration while generalizing the results of the ROAD study.

Further, BMD was measured only in the participants from the mountainous and coastal areas. The total number of participants from these two areas (1690) may be large enough to accurately estimate the incidence and evaluate risk factors. Nevertheless, regional bias should be taken into account while generalizing the results.

Can I get hold of the data? Where can I find out more?

The ROAD study group welcomes specific and detailed proposals for new collaborations. Initial enquiries should be addressed to N.Y. Some information about the ROAD study is available on the website of the Department of Joint Disease Research, 22nd Century Medical and Research Centre,

Table 3 Comparison of BMI (SD) (kg/m^2) of the participants with general Japanese population

	Men		Women	
Age strata (years)	ROAD	Japanese	ROAD	Japanese
40–49	24.5 (4.4)	24.0 (3.3)	21.9 (4.1)	22.4 (3.5)
50–59	23.6 (2.9)	23.7 (3.1)	23.0 (3.3)	23.1 (3.4)
60–69	23.8 (3.2)	23.8 (2.9)	23.3 (3.2)	23.5 (3.7)
70–74	23.1 (2.8)	23.7 (3.2)	23.4 (3.5)	23.2 (3.4)
75–79	22.8(2.9)	23.3 (3.0)	23.0 (3.7)	23.4 (3.5)
⩾80	22.6 (2.9)	22.3 (2.6)	22.2 (3.2)	22.5 (4.0)

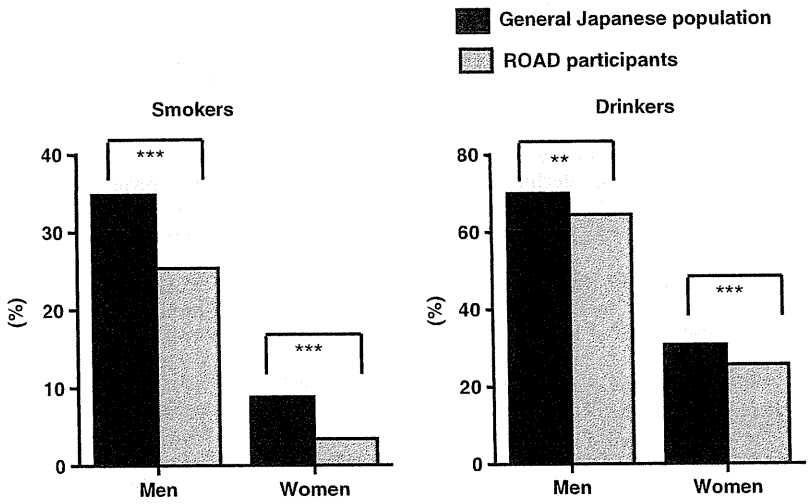

Figure 4 Comparison of the proportion of current smokers and drinkers between the participants of the ROAD study and the general Japanese population. $^{**}P < 0.01$, $^{***}P < 0.001$

University of Tokyo Hospital (http://www.h.u-tokyo.ac.jp/center22/kansetu.html).

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Conflict of interest: None declared.

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nature medicine

Transcriptional regulation of endochondral ossification by HIF-2α during skeletal growth and osteoarthritis development

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Chondrocyte hypertrophy followed by cartilage matrix degradation and vascular invasion, characterized by expression of type X collagen (COL10A1), matrix metalloproteinase-13 (MMP-13) and vascular endothelial growth factor (VEGF), respectively, are central steps of endochondral ossification during normal skeletal growth and osteoarthritis development. A *COL10A1* promoter assay identified hypoxia-inducible factor-2α (HIF-2α, encoded by *EPAS1*) as the most potent transactivator of *COL10A1*. HIF-2α enhanced promoter activities of *COL10A1*, *MMP13* and *VEGFA* through specific binding to the respective hypoxia-responsive elements. HIF-2α, independently of oxygen-dependent hydroxylation, was essential for endochondral ossification of cultured chondrocytes and embryonic skeletal growth in mice. HIF-2α expression was higher in osteoarthritic cartilages versus nondiseased cartilages of mice and humans. *Epas1*-heterozygous deficient mice showed resistance to osteoarthritis development, and a functional single nucleotide polymorphism (SNP) in the human *EPAS1* gene was associated with knee osteoarthritis in a Japanese population. The *EPAS1* promoter assay identified RELA, a nuclear factor-κB (NF-κB) family member, as a potent inducer of HIF-2α expression. Hence, HIF-2α is a central transactivator that targets several crucial genes for endochondral ossification and may represent a therapeutic target for osteoarthritis.

Endochondral ossification is an essential process not only for physiological skeletal growth[1], but also for development of osteoarthritis, which is the most common joint disorder and is characterized by cartilage degradation and osteophyte formation[2–7]. The process of endochondral ossification requires both the hypertrophic differentiation of chondrocytes, which is characterized by secretion of COL10A1, and the conversion of avascular cartilage tissue into highly vascularized bone tissue via degradation of the cartilage matrix and vascular invasion[1,8]. The matrix degradation requires proteinases, among which MMP-13 has a major role[8,9], and the vascular invasion depends on an angiogenic switch by VEGF[8,10]. These steps of chondrocyte hypertrophy, cartilage degradation and vascular invasion are well coordinated; however, the molecular mechanism that extensively controls the sequential steps remains an enigma. Here we initially performed a screen of transcription factors that potentiate the expression of *COL10A1* and identify HIF-2α, an α-subunit member of the HIF family, as the most potent transactivator.

The HIF protein family consists of α- and β-subunit members that function by forming heterodimers[11]. Under normoxic conditions, the α-subunit members HIF-1α, HIF-2α and HIF-3α undergo oxygen-dependent hydroxylation, resulting in ubiquitination and degradation by the proteasome[12,13]. In contrast, under hypoxic conditions, they are neither hydroxylated nor degraded, and they heterodimerize with the constitutive β-subunit members known as aryl hydrocarbon receptor nuclear translocator (ARNT), ARNT2, ARNT-like (ARNTL) and ARNTL2. The heterodimers activate transcription of the target genes by binding the consensus sequence called hypoxia-responsive element (HRE) in the promoters[11]. As cartilage is an avascular and hypoxic tissue, HIF proteins may have a crucial role in the functions of chondrocytes, and, in fact, HIF-1α is known be a potent regulator of cartilage homeostasis[14–16]. However, HIF-2α and HIF-1α are not functionally redundant[17–21], and little is known about the function of HIF-2α in chondrocytes. Here we examined the role of HIF-2α in endochondral ossification during skeletal growth and osteoarthritis development and investigate the underlying mechanism.

RESULTS

Identification of HIF-2α as a transactivator of *COL10A1*

We initially performed a screen of transcription factors that induce hypertrophic differentiation using mouse chondrogenic ATDC5 cells and human nonchondrogenic HeLa cells transfected with a proximal promoter fragment of the *COL10A1* gene. For candidate molecules, we

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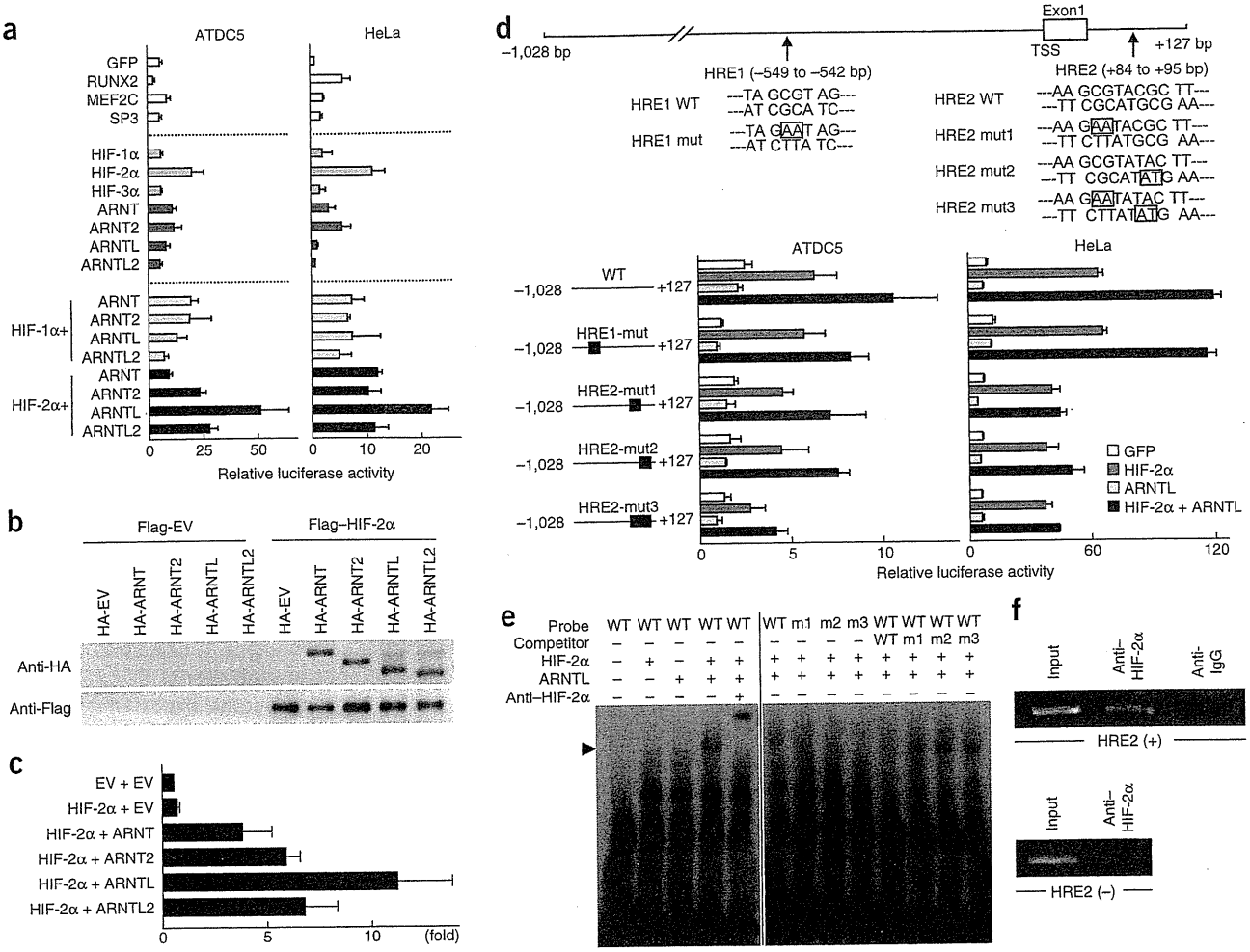

Figure 1 Transcriptional regulation of *COL10A1* by HIF-2α. (**a**) Luciferase assay for screening transcription factors that activate the *COL10A1* promoter by the transfections of candidate genes into ATDC5 and HeLa cells with a reporter construct containing a fragment (−1,028 to +127 bp) of the *COL10A1* gene. Data are shown as means ± s.d. (**b**) Immunoprecipitation and immunoblotting analysis by co-transfections of Flag-tagged HIF-2α or the control empty vector (EV) and hemagglutinin (HA)-tagged β-subunit members or the EV in ATDC5 cells. (**c**) Mammalian two-hybrid assay by transfections of vectors expressing GAL4–HIF-2α and VP16–β-subunit fusion proteins with the luciferase reporter vector with GAL4 binding sites into HeLa cells. Data are shown as means ± s.d. of relative fold increase in luciferase activity as compared to EV + EV (which is arbitrarily set to 1). (**d**) Site-directed mutagenesis analyses of the luciferase assay; one in HRE1 and three in HRE2 (+87 and +88 for mut1, +91 and +92 for mut2, and both for mut3), in the two cell lines transfected with GFP, HIF-2α, ARNTL or both HIF-2α and ARNTL. Data are shown as means ± s.d. (**e**) EMSA for specific binding (arrowhead) of the wild-type (WT) oligonucleotide probe containing HRE2 or the mutated probes described in **d** (m1, m2 and m3) with *in vitro*–translated HIF-2α, ARNT or both. Supershift by an antibody to HIF-2α (anti–HIF-2α) and cold competition with a 50-fold excess of unlabeled WT or the mutated probe are presented. (**f**) ChIP assay with cell lysates of human chondrogenic SW1353 cells that were amplified by a primer set spanning the HRE2 (+, +32 to +249 bp) or not spanning the HRE2 (−, −2,131 to −1,900 bp) before (input) and after immunoprecipitation with anti-HIF-2α or nonimmune IgG (anti-IgG).

prepared expression vectors of more than 100 transcription factors that are known to be expressed in chondrocytes, including HIF proteins, runt-related transcription factor-2 (RUNX2)[1,22], myocyte enhancer factor-2C (MEF2C)[23] and specificity protein-3 (SP3)[24] (**Fig. 1a**). Among them, HIF-2α showed the strongest activation in both cell lines. Although all β-subunit members were physically associated with HIF-2α in ATDC5 cells (**Fig. 1b**), ARNTL showed the strongest binding affinity to HIF-2α (**Fig. 1c**), and HIF-2α–ARNTL was the most potent combination for *COL10A1* transactivation (**Fig. 1a**).

In the *COL10A1* promoter, we identified two HREs by the consensus sequence [A/G]CGT (ref. 25), one in the 5′-end flanking region (HRE1) and the other in intron 1 (HRE2) (**Fig. 1d**). We introduced mutations in HRE1 and HRE2, but only the latter mutation resulted in suppression of transactivation by HIF-2α and the HIF-2α–ARNTL combination (**Fig. 1d**). We then confirmed the specific binding of the HIF-2α protein to HRE2 by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay (**Fig. 1e,f**).

HIF protein expression during chondrocyte differentiation

Although the HIF α- and β-subunit members were widely expressed in major tissues of adult mice, *Epas1* was most predominantly expressed in the tracheal cartilage (**Supplementary Fig. 1a**). During differentiation of ATDC5 cells, *Epas1* expression increased in accordance with the three representative factors for central steps of endochondral ossification: *Col10a1*, *Mmp13* and *Vegfa*, whereas *Hif1a* expression was strong at the early stage and decreased thereafter (**Fig. 2a**). *Hif3a* expression was very low, and the β-subunit members were extensively expressed in all differentiation stages (**Fig. 2a**).

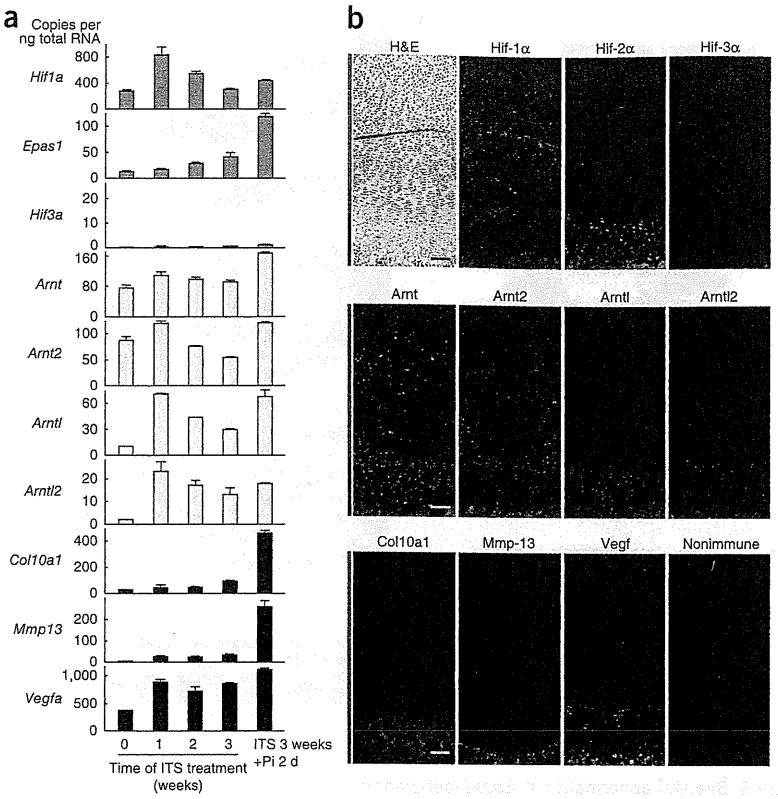

Figure 2 *In vitro* and *in vivo* expression patterns of the HIF α- and β-subunit members and Col10a1, Mmp-13 and Vegf during chondrocyte differentiation. (**a**) Time course of mRNA levels of the indicated genes during differentiation of mouse chondrogenic ATDC5 cells cultured with ITS (insulin, transferrin and sodium selenite) for 3 weeks and for 2 d more with inorganic phosphate (Pi). Data are expressed as means ± s.d. (**b**) H&E staining and immunofluorescence with antibodies to the indicated proteins, as well as a nonimmune control, in the proximal tibias of mouse embryos (embryonic day 18.5 (E18.5)). Scale bars, 100 μm. Red and blue bars to the left of each row indicate layers of proliferative and hypertrophic zones, respectively.

In tibial limb cartilage of mouse embryos, Hif-2α was localized primarily in the hypertrophic zone, similarly to Col10a1, Mmp-13 and Vegf (**Fig. 2b**). In contrast, Hif-1α was predominantly localized in chondrocytes at earlier differentiation stages in the proliferative zone, and Hif-3α was hardly detectable (**Fig. 2b**). The localizations of Arntl and Arntl2 were similar to Hif-2α, whereas those of Arnt and Arnt2 were similar to Hif-1α (**Fig. 2b**).

Physiological role of HIF-2α in endochondral ossification

To determine the involvement of HIF-2α in skeletal growth, we investigated the skeletal phenotype of *Epas1*-deficient mice. The homozygous deficient mutants (*Epas1*$^{-/-}$) were extraordinarily small and died at the early embryonic stage, as reported previously[20,21] (**Fig. 3a**). Although the heterozygous deficient mutants (*Epas1*$^{+/-}$) developed and grew without abnormalities of major organs, they showed mild but proportional dwarfism compared to wild-type littermates from embryonic stages up to 1 week after birth (**Fig. 3a,b** and **Supplementary Fig. 1b**). In the embryos, the limbs and vertebrae were 7–16% shorter in *Epas1*$^{+/-}$ mice than in the wild-type littermates (**Fig. 3c**). Although the actual length of the proliferative zone of the *Epas1*$^{+/-}$ limb was comparable to that of wild-type, the percentage of the proliferative zone relative to the total limb length was moderately increased (**Fig. 3d,e**) with normal BrdU-positive proliferative cells but suppressed Col10a1 expression (**Fig. 3f,g**), indicating impaired hypertrophic differentiation without an effect on proliferation caused by Hif-2α insufficiency. The percentage of the hypertrophic zone relative to the total limb length was also increased and that of the bone area was considerably decreased in the *Epas1*$^{+/-}$ limbs (**Fig. 3d,e**), indicating that Hif-2α insufficiency impaired not only chondrocyte hypertrophy but also subsequent steps such as matrix degradation and vascularization. This difference was gradually decreased with developmental compression of the hypertrophic zone after birth (**Supplementary Fig. 1c**). Immunohistochemistry confirmed that Mmp-13 and Vegf, as well as Col10a1, were suppressed by the Hif-2α insufficiency, which may cause the decrease in cartilage calcification shown by von Kossa staining (**Fig. 3f**).

Function of HIF-2α in cultured chondrocytes

In cultured ATDC5 cells, *Col10a1*, *Mmp13* and *Vegfa* amounts, as well as the activity of alkaline phosphatase and Alizarin red staining (both indicators of differentiation), were increased by overexpression of HIF-2α or the HIF-2α–ARNTL combination, whereas none of the expression levels or staining was affected by ARNTL alone (**Fig. 4a**). To examine the regulation of HIF-2α function by oxygen-dependent hydroxylation, we created ATDC5 lines overexpressing four kinds of HIF-2α mutants bearing mutations at the oxygen-dependent hydroxylation residues, including N847A and P531A (or both), which result in enhancement of the transactivation activity of the protein even under normoxic conditions, as well as P849A, which abrogates transactivation activity even under hypoxic conditions[13]. We found that none of these mutations affected the HIF-2α action on endochondral ossification parameters (**Fig. 4b**). All parameters were decreased, however, by loss of function of HIF-2α in ATDC5 cells achieved through overexpression of a dominant-negative mutant or expression of an siRNA specific for HIF-2α (**Fig. 4c**). In addition to ATDC cells, primary chondrocytes derived from *Epas1*$^{+/-}$ mice showed suppressed expression of the three factors, and the suppression of each factor was restored to wild-type levels by adenoviral overexpression of HIF-2α (**Fig. 4d**).

We then examined the transcriptional regulation of *MMP13* and *VEGFA* by HIF-2α. Among the α- and β-subunit members of the HIF proteins, HIF-2α most notably transactivated both *MMP13* and *VEGFA*, and the transactivation was further enhanced by ARNTL (**Supplementary Fig. 2a,b**), as is true for *COL10A1* (**Fig. 1a**). Deletion and site-directed mutagenesis analyses of the luciferase assay identified the core responsive elements to HIF-2α and the HIF-2α–ARNTL combination at HRE3 (−106 to −101) and HRE4 (−982 to −977) in the promoters of *MMP13* and *VEGFA*, respectively (**Supplementary Fig. 2c,d**). Further EMSA and ChIP assays confirmed the specific binding of the HIF-2α protein to HRE3 and HRE4 (**Supplementary Fig. 2e–h**).