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中村耕三	特集　ロコモティブシンドロームを知っていますか	健康づくり	9	7	2010
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大森豪	小児骨折の疫学	Clinical Calcium	20	59-64	2010
大森豪	疫学調査から見た危険因子と生活指導	関節外科	29	24-30	2010
織田広司、林豊彦、大森豪、渡辺聡、谷藤理、佐藤卓、古賀良生	関節鏡視下手術支援システムを用いた大腿骨孔位置の決定	臨床バイオメカニクス	31	327-332	2010

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小田川健一、豊田貴嗣、笹川圭右、小林公一、坂本信、田邊祐治、谷藤理、佐藤卓、古賀良性、大森豪	イメージレジストレーション法による膝関節の接触動態解析	臨床バイオメカニクス	31	349-356	2010
木村太郎、松尾智史、西野勝敏、田邊祐治、古賀良生、大森豪	内側型変形性膝関節症における歩行時スラスト現象と3次元下肢荷重線の関連性	臨床バイオメカニクス	31	401-408	2010
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川本雅渉、 辻井雅也、飯田竜、長谷川正裕、植村和司、須藤啓広	烏口突起下転位を認めた鎖骨遠位部骨折の1例	中部日本整形外科災害外科学会雑誌	53	313-314	2010
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長谷川正裕、須藤啓広	フルポーラスロングステムを用いた人工股関節再置換術の中期成績	Hip Joint	36	290-293	2010
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西村誠、倉田竜也、藤原達彦、近藤哲士、塩川靖夫、井阪直樹、須藤啓広	人工股・膝関節置換術前後における下肢深部静脈血栓の発生率	東海関節	2	13-16	2010
山口敏郎、長谷川正裕、新美塁、須藤啓広	フォンダパリヌクス投与による下肢人工関節置換術後の深部静脈血栓症の予防効果について	東海関節	2	21-25	2010
里中東彦、辻井雅也、長谷川正裕、若林弘樹、平田仁、須藤啓広	半拘束型人工肘関節置換術の治療成績	中部日本整形外科災害外科学会雑誌	53	1279-1280	2010
辻井雅也、植村和司、里中東彦、堀和一郎、平田仁、須藤啓広	手根中央関節鏡視下手術が有用であった手根骨障害	中部日本整形外科災害外科学会雑誌	53	1391-1392	2010
堀和一郎、新美塁、内田淳正、松峯昭彦、須藤啓広	脳神経膠腫を合併したOllier病の1例	中部日本整形外科災害外科学会雑誌	53	1397-1398	2010
中川太郎、松峯昭彦、中村知樹、三浦良浩、新美塁、須藤啓広	骨肉腫治療後に発生したEwing肉腫の1例	中部日本整形外科災害外科学会雑誌	53	1399-1400	2010
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下方浩史、安藤富士子	疾病予防のための理想的生活．生活習慣改善による疾病予防－エビデンスを求めて	成人病と生活習慣病	40	1026-1031	2010
下方浩史、安藤富士子	運動器疾患の長期縦断疫学研究．ロコモティブシンドロームと生活習慣病	Progress in Medicine	30	3021-3024	2010
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安藤富士子、北村伊都子，金興烈，李成喆，下方浩史	潜在性慢性炎症と中高年者のサルコペニアに関する縦断的検討	日本未病システム学会誌			in press
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Ⅳ. 研究成果の刊行物・別刷

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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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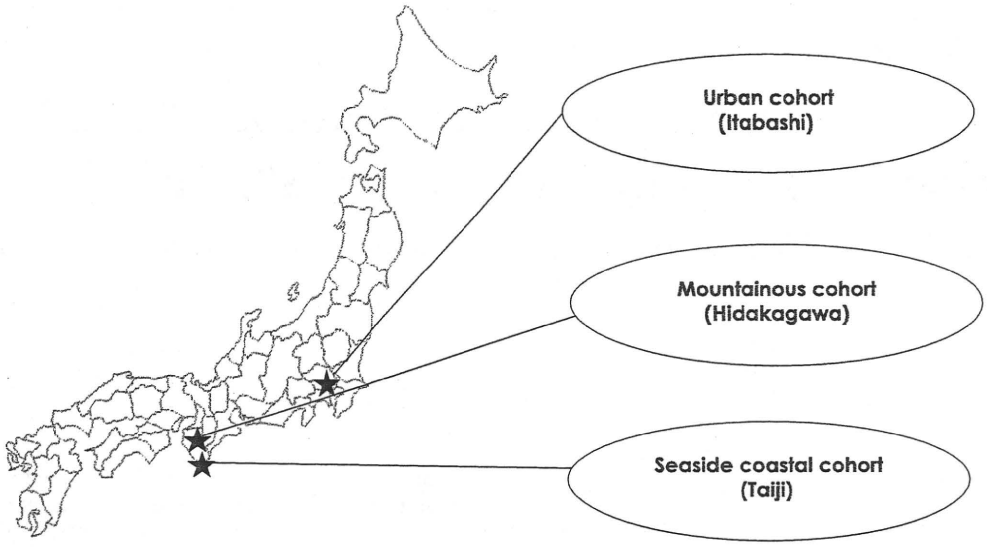

Figure 1 Locations of the three regions from which the study cohort was derived

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]

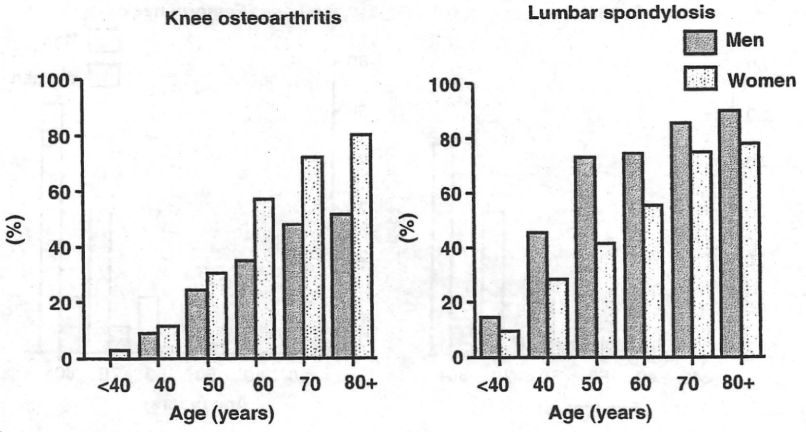

Figure 2 Prevalence of radiographic knee osteoarthritis and lumbar spondylosis, classified by age and gender

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,

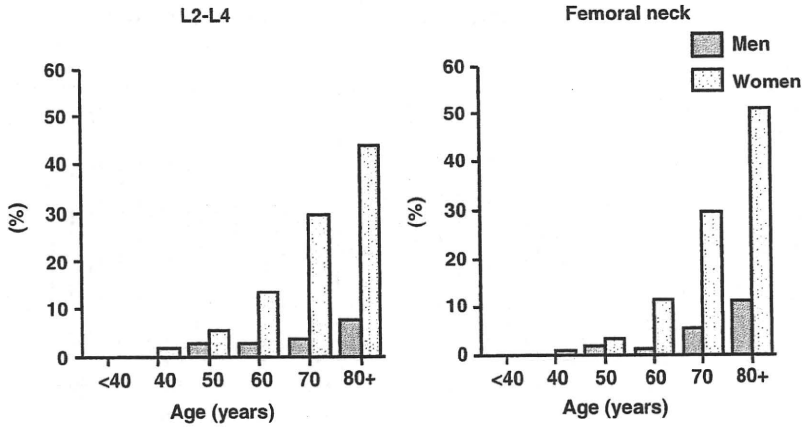

Figure 3 Prevalence of osteoporosis of the lumbar spine and femoral neck

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

Age strata (years)	Men		Women	
	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)

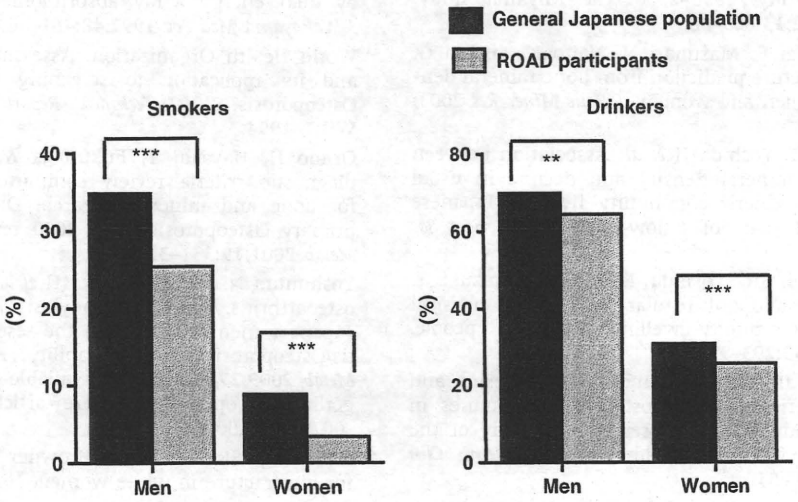

Figure 4 Comparison of the proportion of current smokers and drinkers between the participants of the ROAD study and the general Japanese population. $^{**}P < 0.01$, $^{***}P < 0.001$

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

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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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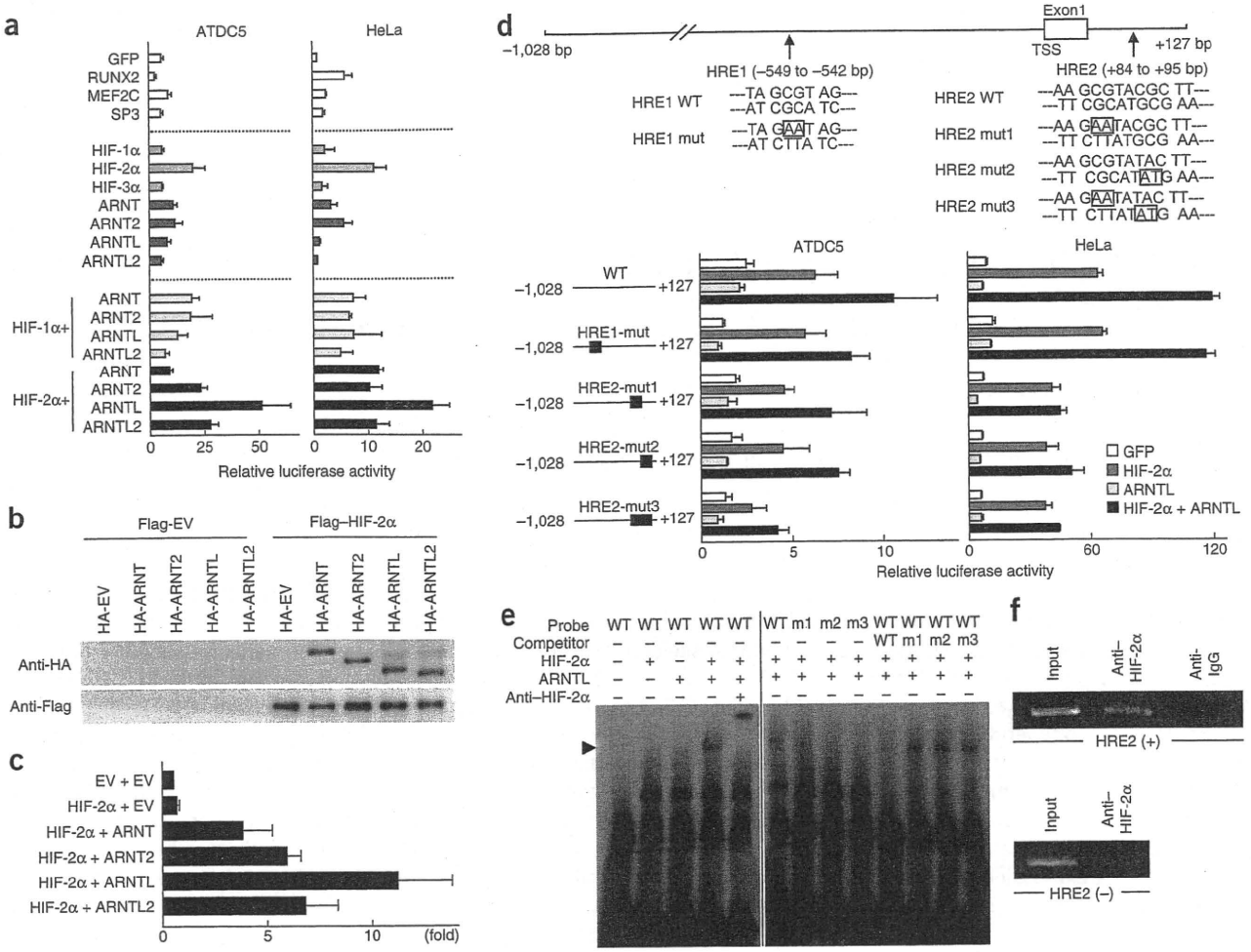

Figure 1 Transcriptional regulation of *COL10A1* by HIF-2α. (**a**) Luciferase assay for screening transcription factors that activate the *COL10A1* promoter by the transfections of candidate genes into ATDC5 and HeLa cells with a reporter construct containing a fragment (−1,028 to +127 bp) of the *COL10A1* 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**).

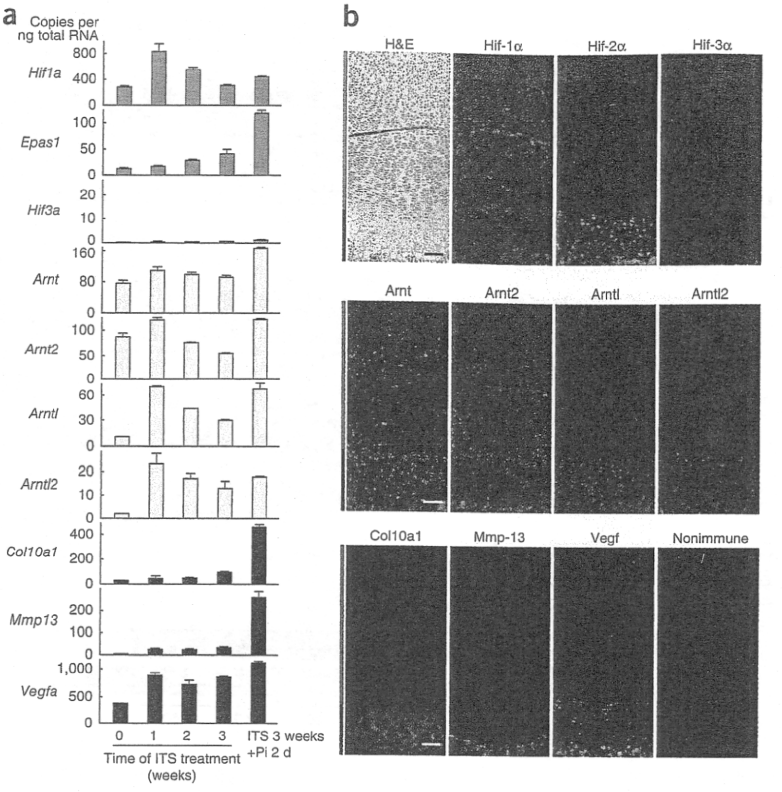

Figure 2 *In vitro* and *in vivo* expression patterns of the HIF α- and β-subunit members and Col10a1, Mmp-13 and Vegf during chondrocyte differentiation. (**a**) Time course of mRNA levels of the indicated genes during differentiation of mouse chondrogenic ATDC5 cells cultured with ITS (insulin, transferrin 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**).

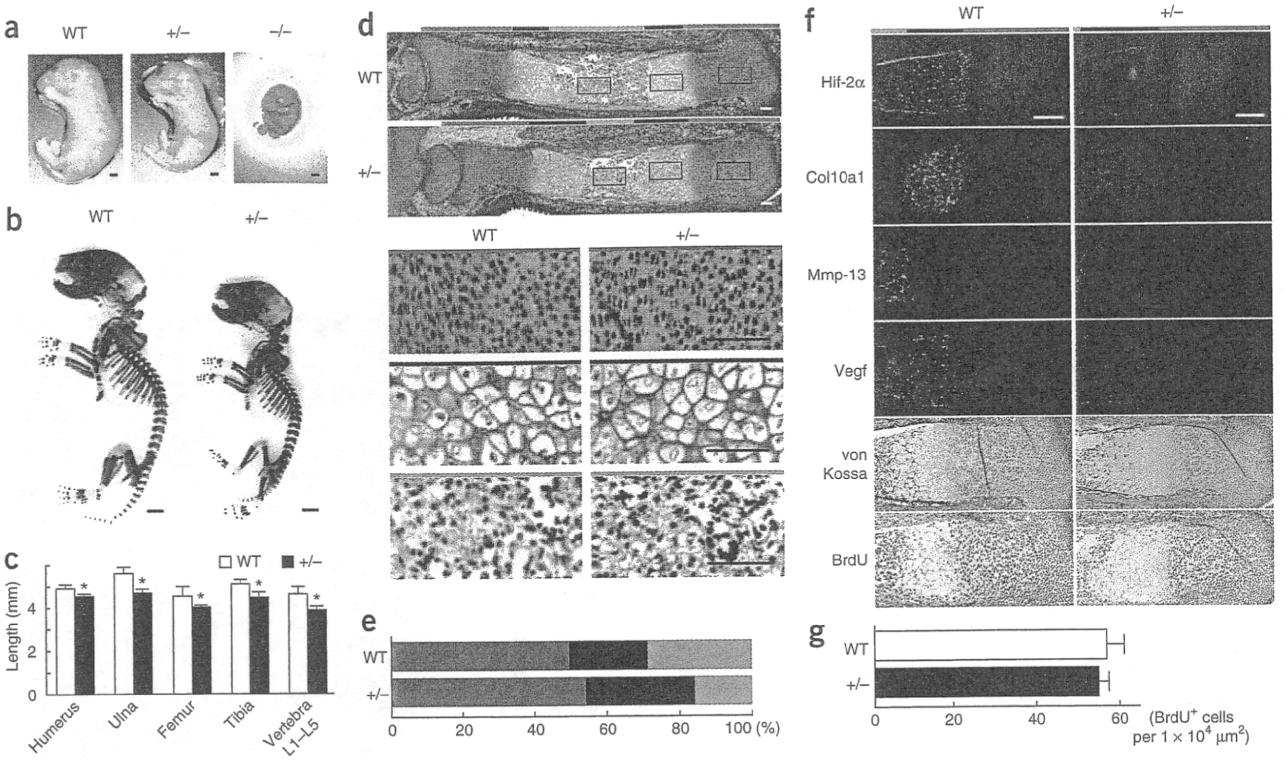

Figure 3 Skeletal abnormality in *Epas1*-deficient mice. (**a**) Wild-type (WT), heterozygous-deficient (*Epas1*$^{+/-}$) and homozygous-deficient (*Epas1*$^{-/-}$) littermate embryos (E17.5). All *Epas1*$^{-/-}$ embryos died at mid-gestation. Scale bars, 1 mm. (**b**) Double staining with Alizarin red and Alcian blue of the whole skeleton of WT and *Epas1*$^{+/-}$ littermate embryos (E17.5). Scale bars, 1 mm. (**c**) Length of long bones and vertebra (first to fifth lumbar spines) of WT and *Epas1*$^{+/-}$ littermate embryos. Data are expressed as means ± s.d. *$P < 0.05$ versus WT. (**d**) H&E staining of whole tibias of the WT and *Epas1*$^{+/-}$ littermate embryos. Inset boxes indicate the regions of the bottom three rows representing proliferative zone, hypertrophic zone and bone area, shown by red, blue and green bars, respectively. Scale bars, 100 μm. (**e**) Percentage of the length of proliferative zone (red), hypertrophic zone (blue) and bone area (green) over the total tibial length of the WT and *Epas1*$^{+/-}$ littermate embryos. (**f**) Immunofluorescence with antibodies to Hif-2α, Col10a1, Mmp-13 and Vegf, as well as bromodeoxyuridine (BrdU) labeling and von Kossa staining of the proximal tibias of WT and *Epas1*$^{+/-}$ littermate embryos (E17.5). Color bars indicate layers as indicated in **d**. Scale bars, 200 μm. (**g**) The number of BrdU-positive cells in 1×10^4 μm^2 of the proximal tibia of WT and *Epas1*$^{+/-}$ littermate embryos. Data are expressed as means ± s.d.

Contribution of HIF-2α to osteoarthritis in mice and humans

We next compared osteoarthritis development between adult littermates of wild-type and *Epas1*$^{+/-}$ mice that had undergone comparable skeletal growth after birth (**Supplementary Fig. 1b**) by creating a surgical osteoarthritis model through induction of instability to the knee joints[4,5]. The expression of Hif-2α, as well as of Col10a1, Mmp-13 and Vegf, increased in the joint cartilage with osteoarthritis development for 8 weeks after surgery in the wild-type mice; however, in the *Epas1*$^{+/-}$ littermates, the cartilage degradation and the expression of the three factors were notably suppressed (**Fig. 5a**). Quantification by grading systems[4,26] confirmed that the Hif-2α insufficiency caused significant resistance to cartilage degradation and osteophyte formation (**Fig. 5b**). There was no difference in the subchondral bones between the two genotypes under the sham operation, suggesting that the *Epas1* deficiency does not affect physiological bone homeostasis. However, after surgical induction, subchondral bone sclerosis, an osteoarthritic disorder secondary to cartilage destruction, was apparent in the wild-type joints, whereas it was suppressed in the *Epas1*$^{+/-}$ joints (**Supplementary Table 1**).

In human knee joint samples, as well, the HIF-2α expression increased with osteoarthritis development, reached a maximum at the initial and progressive stages and decreased thereafter at the terminal stage, although it was hardly detected in subchondral bone or synovium (**Fig. 5c**). To further investigate a possible association of the human *EPAS1* gene with knee osteoarthritis of humans, we searched a Japanese population–based cohort of the ROAD study[27] for sequence variations in exons and the 5′-end flanking region up to −1,000 bp from the transcription start site (TSS) of the human *EPAS1* gene and identified only one common SNP with a minor allele frequency >0.1, rs17039192 (+18C and +18T for major and minor alleles, respectively, relative to the TSS; minor allele frequency = 0.132) (**Fig. 5d**). A comparison of allelic frequencies between 397 individuals with knee osteoarthritis and 437 controls showed significant association of the rs17039192 SNP with knee osteoarthritis ($P = 0.013$, odds ratio = 1.44) (**Fig. 5d**). Because this SNP was located close to the TSS, we further examined the effects of the allelic difference (+18C/T) on *EPAS1* promoter activity in chondrogenic and nonchondrogenic cells transfected with a luciferase reporter gene and the *EPAS1* promoter fragment (−1,000 bp to 488 bp) containing +18C or +18T. The susceptibility allele (18C) showed higher promoter activity in chondrogenic cells, but not in nonchondrogenic cells (**Fig. 5e**), confirming that enhanced transactivation of *EPAS1* in chondrocytes is associated with osteoarthritis in humans.

Molecular network around HIF-2α in endochondral ossification

Regarding downstream molecules of HIF-2α, we have focused on COL10A1, MMP-13 and VEGF as representative factors for the