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# The Fracture and Immobilization Score (FRISC) for risk assessment of osteoporotic fracture and immobilization in postmenopausal women—A joint analysis of the Nagano, Miyama, and Taiji Cohorts

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ABSTRACT

*Introduction:* We aimed to (i) explore risk factors for major osteoporotic fracture or immobilization; (ii) develop a prediction model that can be used to assess the risk of fracture and immobilization; and (iii) assess external validity of the final model.

*Methods:* A total of 1787 postmenopausal Japanese women were followed in a hospital-based cohort study. Endpoints included the annual incidence of major osteoporotic fracture and immobilization. For each endpoint, multivariate Poisson regression models were fitted separately and risk factors were screened through backward variable selection. The predictive accuracy of the final model (FRISC) was evaluated in two independent community-based cohorts.

*Results:* Over a median follow-up of 5.3 years, a total of 383 major osteoporotic fractures (279 clinical vertebral, 44 hip, 60 distal forearm) and 83 immobilizations occurred in the developmental dataset. Backward variable selection confirmed that the following are risk factors for major osteoporotic fracture: age, weight, prior fracture, back pain, and lumbar bone mineral density (BMD). Age, prior fracture and dementia were significant risk factors for immobilization. Hosmer–Lemeshow tests did not indicate any significant deviation between the observed fracture frequency and prediction from the FRISC in the independent validation dataset. The C statistic for the FRISC was 0.727 (95% confidence interval: 0.660 to 0.794) and was higher than that for BMD alone significantly ($p = 0.03$).

*Conclusions:* We developed a novel prediction model for fracture and immobilization, FRISC, and the clinical risk factors in the FRISC allows better identification of populations at high risk of fracture than BMD alone. A web application is available at http://www.biostatistics.jp/prediction/frisc.



## Introduction

Fracture due to osteoporosis results in increased mortality, morbidity and medical expense in the US and Japan [1–3]. As an adjunct to the development of effective treatments, early identification of populations at high risk of fracture is regarded as an effective strategy for decreasing both the burden of illness and the associated cost in countries with aging populations [4]. The US and Japanese guidelines both recommend that bone mineral density (BMD) should be used to determine when to intervene in patients with osteoporosis [5,6]. However, epidemiological studies have demonstrated that many major osteoporotic fractures occur among individuals with a BMD T score value above the intervention threshold value, although the incidence of fracture certainly increases with decreasing BMD [7,8]. As a solution to this problem, a WHO scientific group proposed the use of 10-year probabilities of osteoporotic or hip fracture, calculated using multiple risk factors. The result was the WHO fracture risk assessment tool (FRAX) [9]. Several other prediction models/risk assessment tools were also developed based on data from cohort studies and clinical trials [10–13].

*Abbreviations:* BMD, bone mineral density; FRAX, fracture risk assessment tool; FRISC, Fracture and Immobilization Score; CHD, coronary heart disease; CVD, cerebrovascular disease; ROC, receiver operating characteristic; CI, confidence interval.

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Although case-finding strategies optimized by risk assessment tool for fracture appear to be promising, there are several problems which must be addressed before clinical application. First, whether combination of BMD and clinical risk factors allows better discrimination of fracture than BMD alone is still controversial; the discriminatory power of the FRAX was excellent in eleven population-based cohorts [14] but was similar to BMD alone in a cohort study [15]. Second, since the Japanese version of FRAX was validated only in the community dwelling cohorts in Japan [9], there has been no available data whether the FRAX probability performs well in hospital-based population. As Justice et al. pointed out, the effectiveness of prediction models in clinical practice depends on the extent to which they can be generalized to the population in question [16]. In general practice, the FRAX was often adapted to assess fracture risk in a patient who has a more complicated risk for fracture, such as atherosclerosis, diabetes or other potential risks to deteriorate bone strength. In this regard, it is necessary to evaluate prediction models for fracture not only in community but also hospital-based populations. Third, outcomes other than fracture, such as immobilization, appear to be also important for optimizing osteoporotic treatment from the health economic perspective. In fact, we have found that osteoporotic fracture was an independent risk for immobilization and this morbid state would require a large amount of resources of care [17].

The present study was therefore performed with three main aims. The first was to explore risk factors for the incidence of major osteoporotic fracture or immobilization. This was done using data from the Nagano Cohort, a hospital-based cohort study of postmenopausal Japanese women. The second aim of this study was to develop a prediction model named as the Fracture and Immobilization Score (FRISC), to assess the risk of major osteoporotic fracture and immobilization, based on the risk factors confirmed in the first part of the study. Finally, we assess the external validity of the FRISC and investigate whether the predictive accuracy was improved from BMD alone using pooled data from the Miyama and Taiji Cohorts, which followed 400 Japanese women from communities over a 10-year period.

## Methods

### *Development and validation datasets*

We used two independent datasets in the current analysis; a developmental dataset from the Nagano Cohort and a validation dataset from the Miyama and Taiji Cohorts. Profiles of these three cohorts have been detailed previously [17–28]. The Nagano Cohort recruited and followed up postmenopausal women who were receiving medical care as outpatients or visitors at a medical institute in Nagano Prefecture, Japan since April 1993 [16–20]. A total of 1787 participants were included in the developmental dataset; exclusion criteria were (i) metabolic bone disease and (ii) secondary osteoporosis (e.g. hyperparathyroidism, hyperthyroidism other than patients on T4 replacement and with euthyroid for more than one year, chronic renal failure or osteomalacia). We excluded those who met the exclusion criteria regardless of BMD.

However, steroid users were enrolled to the present study because the history of steroid use was required in the FRAX. The protocol was approved by the ethics committee at the Research Institute and Practice for Involutional Diseases and we obtained written informed consent from all participants. The Miyama Cohort was set up in 1988 as subsets of nationwide community-based cohort studies sponsored by the Ministry of Education or Ministry of Health and Welfare [21–23]. A total of 1453 inhabitants aged 40–79 years in Miyama Village were listed from the resident registration in December 1988. Then, 200 men and 200 women were recruited and followed up between 1990 and 2000. The Taiji Cohort is a community-based cohort study in Taiji Town, Wakayama Prefecture, Japan [25–27]. From a list of 2261 inhabitants aged 40–79 years obtained from the resident registration in June 1992, 50 men and 50 women in each decade age group between 40 and 79 years (a total of 400 participants) were recruited randomly and followed up between 1993 and 2003. All the sampled participants were contracted and agreed to participate. The validation dataset included all the women in the Miyama and Taiji Cohorts.

### *Data collection in the Nagano Cohort*

At baseline, anthropometric indices, including body weight and body height, were obtained for all patients. Subjects were also interviewed to obtain data about age at menopause, smoking habit, alcohol consumption, past and present occupation, presence of pain, medical history (including rheumatoid arthritis, diabetes mellitus, hypertension, dyslipidemia, cancer, dementia, coronary heart disease [CHD] and cerebrovascular disease [CVD]). Pain was defined as any symptom of pain in the back, hip muscles, ribs, legs, knees, neck, shoulders, wrists, or other joints. Back pain was defined as any symptom of pain in the back trunk area, regardless of the degree or consistency of the pain [18]. Rheumatoid arthritis was diagnosed according to the diagnostic criteria proposed by the American Rheumatism Association [28]. The Japanese version of the Mini-mental State Examination (MMSE) was performed in the subjects who were suspicious dementia and a subject with the total score of MMSE less than 20 points was considered to have obvious cognitive dysfunction [29]. A history of CHD was defined as any previous acute coronary symptoms or events requiring coronary intervention, and was confirmed by coronary angiography, echocardiogram, or MD-CT imaging study. A history of CVD was diagnosed based on evidence of apparent brain attack or an existing brain lesion as observed by magnetic resonance imaging or computer-assisted X-ray tomography. Self-reports of a history of malignancy were confirmed by referring to the patient's medical records [19]. The BMD of the lumbar spine was measured at baseline using dual-energy X-ray absorptiometry (Lunar DPX-L or DPX-IQ; Lunar Corporation, Madison, WI) and a quality assurance test was carried out for every measurement to detect machine drift. The inter-assay variance of the lumbar BMD measurements in our laboratory was $0.5 \pm 0.5\%$ (coefficient of variation ± standard deviation) [20]. T score was calculated by using Japanese standard values [30].

### *Data collection in the Miyama and Taiji Cohorts*

A self-administered questionnaire was used for baseline data collection in the Miyama Cohort, while both self-administered and interviewer-administered questionnaires were used in the Taiji Cohort [22–27]. The items in these questionnaires included birth date, body weight, body height, current smoking status, current alcohol intake, presence of back pain, use of steroids, and medical history such as rheumatoid arthritis. Parental history of fracture was asked only in the Taiji Cohort. The BMD of L2-4 and BMD at femoral neck, Ward's triangle and the trochanteric region were measured by dual-energy X-ray absorptiometry (Lunar DPX; Lunar Corporation, Madison, WI in the Miyama Cohort, Hologic QDR-1000; Hologic Inc., Crosby Drive Bedford, MA in the Taiji Cohort) and treated as T scores. The incidence of clinical fracture was evaluated in both the cohorts. However, radiographs for morphometrical vertebral fracture were available only in the Miyama Cohort. Consequently, parental history or morphometrical vertebral fracture was missing data in either cohort systematically and thus we assumed that participants with these missing data did not have parental history or prior fracture. We calculated the 10-year probability of major osteoporotic fracture by entering the following data into online version of the FRAX; age, sex, weight, height, previous fracture, parental history of hip fracture, current smoking status, glucocorticoid use, rheumatoid arthritis, alcohol intake and femoral neck BMD.

### *Endpoints*

Endpoints included the annual incidence of major osteoporotic fracture and immobilization. Major osteoporotic fracture was defined

as first occurrence of any clinical fracture (hip fracture, surgical neck fracture of the humerus, distal forearm fracture, or clinical vertebral fracture). We also evaluated a radiographical vertebral fracture by the semi-quantitative visual method [31], but major osteoporotic fracture does not include morphometrical fracture by definition. A validation analysis of our semi-quantitative method for analyzing incident vertebral fracture has been reported elsewhere [32]. Prior vertebral fractures were defined as those fractures for which the ratio of the height of the central or anterior vertebral body to that of the posterior vertebral body was less than 0.8, or when any of these three vertebral body heights was less than 80% of the height of the adjacent vertebral body [6]. Immobility was defined in accordance with the subject's locomotive ability. Subjects bed-bound at home (lying in bed almost all day) for more than 6 months or institutionalized in nursing homes (lying in bed or using a wheelchair for locomotion), were defined as immobile [17]. Some immobile subjects could be sitting on a bed and could be going to a portable toilet which located besides the bed. Participants who died from any cause, moved to the home of a relative because they were not able to perform the activities of daily living independently, were lost to follow-up, or were followed up until June 1, 2009 were treated as censored. For each endpoint, the accumulation of person-years at risk started from registration of each patient.

### Statistical considerations

For each endpoint, we fitted multivariate Poisson regression models separately and rate ratios for risk factors estimated by the Poisson regression models were reported with 95% confidence intervals (CI) and p values. The following variables were initially identified from the literature as the traditional risk factors for osteoporotic fracture: covariates included in the FRAX other than femoral neck BMD (age, height, weight, prior fracture, parental history of fracture, current smoking status, use of steroids, rheumatoid arthritis, alcohol intake), lumbar BMD, presence of back pain, presence of any pain, and drug treatment for osteoporosis [9,18,22]. These covariates were screened via backward variable selection with a significance level of $p=0.2$. We constructed a prediction model for immobilization using the same procedure, except that three covariates (dementia, history of CVD, and history of malignancy) were used in addition to those used in the osteoporotic fracture analysis. Finally, the FRISC was developed using the following formula:

$$\text{Prob}(t) = \int_0^t \lambda \exp(X\beta) \exp\left[-\int_0^u \{\lambda \exp(X\beta) + m(v)\} dv\right] du$$

Here, $t$ is a time point for prediction (i.e. the formula calculates 10-year probability if $t=10$), $\beta$ is a vector of log-rate ratios for covariates $X$, $\lambda$ denotes baseline incidence rate, and $m(v)$ is mortality at time $v$ obtained from sex- and age-specific mortality in Vital Statistics of Japan in 2008 [33].

We assessed the predictive accuracy of the FRISC in terms of calibration and discrimination [34] using occurrence of major osteoporotic fracture within a 10-year period, which was treated as a binary event, in the validation dataset. Calibration, namely how closely the prediction reflects actual events, was assessed using ratio of observed and predicted events and the Hosmer–Lemehshow test. Discrimination, the ability to distinguish between those who experience the event and those who do not, was evaluated using receiver operating characteristic (ROC) curves and Harrell's C statistic. Improvement in the C statistics of the two models from BMD alone was assessed by using contrast tests.

All reported p values for statistical tests are two-tailed, and $p<0.05$ was taken to indicate statistical significance. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

## Results

### Characteristics of participants and follow-up

Baseline characteristics of participants in the Nagano, Miyama and Taiji Cohorts are summarized in Table 1. The Nagano Cohort included older participants and mean lumbar BMD in this cohort was lower than the other cohorts. Prior fracture, back pain and parental history were observed more frequently in the Miyama and Taiji Cohorts. In the Nagano Cohort, 37.4% of participants were being treated with bone resorption inhibitors (bisphosphonates or a selective estrogen receptor modulator), and 16.7% were receiving 1-alpha-OH vitamin $D_3$ or vitamin $K_2$ at baseline. Table 2 describes the incidence of fractures and immobilization. In the Nagano Cohort, over a median follow-up time of 5.3 years (range, 0.03–16.5 years), a total of 383 major osteoporotic fractures occurred (279 clinical vertebral fractures, 44 hip fractures, 60 distal forearm fracture, Table 2). In the Miyama and Taiji Cohorts, 337 of 400 participants completed the planned follow-up of the 10-year period (84%) and a total of 60 major osteoporotic fractures occurred (44 clinical vertebral fractures, 8 hip fractures, 8 distal forearm fracture, Table 2). Incidence rates of fractures in the two cohorts were much lower than the Nagano Cohort possibly due to the difference in average age and lumbar BMD at baseline (Tables 1 and 2). Immobilization occurred in 83 participants in the Nagano Cohort.

### Risk factors for fracture and immobilization

We fitted multivariate Poisson regression models to the validation dataset of 1787 participants. Backward variable selection identified the following six risk factors for major osteoporotic fracture: age, weight, lumbar BMD, prior fracture and presence of back pain (Table 3). That is, parental history of fracture, smoking status, alcohol consumption, rheumatoid arthritis, and use of steroids, which are all

**Table 1**
Characteristics of participants in the three cohorts.

| | | The Nagano Cohort (N = 1787) | | | The Miyama and Taiji Cohorts (N = 400) | | |
|---|---|---|---|---|---|---|---|
| | | Mean | SD | 5–95 percentile | Mean | SD | 5–95 percentile |
| Age (years) | | 63.4 | 11.1 | 45–81 | 59.5 | 11.3 | 41–77 |
| Height (cm) | | 150.9 | 6.6 | 140–161 | 150.2 | 6.2 | 140–159 |
| Weight (kg) | | 51.1 | 8.5 | 38–65 | 51.2 | 9.3 | 37–66.5 |
| Lumbar BMD (T score) | | −1.55 | 1.22 | −3.5–0.5 | −1.36 | 1.19 | −3.85–1.57 |
| Femoral neck BMD (T score) | | * | | | −1.61 | 1.84 | −3.29–0.53 |
| | | | Frequency | % | Frequency | | % |
| Prior fracture | | | 403 | 22.6 | 49† | | 25.0 |
| Presence of pain | Back | | 572 | 32.0 | 251 | | 63.0 |
| | Other sites | | 449 | 25.1 | †‡ | | |
| Parental history | | | 22 | 1.2 | 20‡ | | 10.0 |
| Current smoker | | | 38 | 2.1 | 16 | | 4.0 |
| Current alcohol drinker | | | 137 | 7.7 | 46 | | 11.5 |
| Medication | Bone resorption inhibitors | | 369 | 37.4 | †‡ | | |
| | Active vitamin $D_3$ or vitamin $K_2$ | | 299 | 16.7 | †‡ | | |
| | Steroids | | 27 | 1.5 | 0‡ | | 0.0 |
| Rheumatoid arthritis | | | 224 | 12.5 | 0 | | 0.0 |
| Dementia | | | 97 | 5.4 | †‡ | | |

SD: standard deviation; BMD: bone mineral density.
*Not measured in the Nagano Cohort. †Not measured in the Miyama Cohort (N = 200).
‡Not measured in the Taiji Cohort (N = 200).

**Table 2**
Frequencies and incidence rates of fracture and immobilization in participants in the three cohorts.

| | The Nagano Cohort (N = 1787) | | | | The Miyama and Taiji Cohorts (N = 400) | | | |
|---|---|---|---|---|---|---|---|---|
| | Frequency | IR | 95% CI | | Frequency | IR | 95% CI | |
| Major osteoporotic fracture | 383 | 34.1 | 30.9 | 37.7 | 60 | 16.1 | 12.5 | 20.7 |
| Clinical vertebral facture | 279 | 24.9 | 22.1 | 28.0 | 44 | 11.8 | 08.8 | 15.9 |
| Hip fracture | 44 | 3.9 | 2.9 | 5.3 | 8 | 2.2 | 1.1 | 4.3 |
| Immobilization | 83 | 7.4 | 6.0 | 9.2 | - | | | |

IR, incidence rate per 1,000 person-years; CI, confidence interval.

included in the FRAX, were excluded based on having p values less than 0.2. Importantly, incidence rate of major osteoporotic fracture increased as weight elevated and this direction is opposite to the FRAX and this trend remains significant even when all the other risk factors listed initially in the variable selection procedure are adjusted for (rate ratio for 10 kg increase in weight: 1.22, 95% CI: 1.07 to 1.40, $p<0.01$). Multivariate analysis for immobilization, using the same variable selection procedure, showed that age, prior fracture and dementia were associated with the incidence of immobilization (Table 3).

### *Input and output of the FRISC*

All the risk factors that were retained through the variable selection procedure were incorporated into the final prediction model named as the FRISC. Interface of web application of the FRISC is displayed in Fig. 1. The input comprises the sex risk factors and, menopausal status and secondary osteoporosis which were used only for assessment of the applicability. The output comprises the 1, 3, 5 and 10-year probabilities of major osteoporotic fracture and those of immobilization and is calculated by using the algorithm described in Supplementary Data.

### *External validation of the FRISC*

Fig. 2 displays histograms of the calculated 10-year probabilities of major osteoporotic fracture for the 400 participants in the validation dataset (upper: the FRISC, lower: the FRAX). An apparent difference was observed in the left tail of the two histograms; in the upper figure participants with fracture probability less than 0.05 were very few, while the FRAX gave the fracture probability less than 0.05 to a substantial portion of the participants. As a result, the fracture probabilities from the FRISC were much higher on average. Table 4 compares the predictive accuracy of the two prediction models and prediction from BMD alone. Over the 10-year follow-up, major osteoporotic fracture developed in 60 of 400 participants in the validation dataset. The predicted event frequency calculated from the FRISC was slightly higher than the observation (observed/predicted ratio: 0.74), while the FRAX tended to underestimate (observed/predicted ratio: 1.59). The Hosmer–Lemeshow test did not indicate any significant deviation between the observed event frequency and prediction from the FRISC. The C statistics for the FRISC was 0.727, indicating that the discriminatory power of the FRISC is moderate, while that for prediction from BMD alone was 0.651. That is, the discriminatory power of the FRISC, which combines BMD and additional clinical risk factors, was better than BMD alone significantly even in independent community-based cohort studies ($p=0.03$, Table 4). Fig. 3 shows ROC curves for major osteoporotic fracture probability from the FRISC (solid curve), the FRAX (dashed curve) and BMD alone (dotted curve). Both the ROC curves of the prediction models increased almost identically at first, but the curve for the FRISC was slightly above the curve for the FRAX where sensitivity is higher than 0.7 and where lower probability is used as a cutoff point (i.e. 16% or lower in the FRISC, 14% or lower in the FRAX), indicating that the FRISC is advantageous over the FRAX for screening of low-risk osteoporotic patients.

**Table 3**
Multivariate Poisson regression analysis of risk factors for major osteoporotic fracture and immobilization in the development dataset of 1,787 participants.

| | Major osteoporotic fracture | | | | Immobilization | | | |
|---|---|---|---|---|---|---|---|---|
| | Rate ratio | 95% CI | | p | Rate ratio | 95% CI | | p |
| Age, + 10 years | 1.62 | 1.43 | 1.83 | <0.01 | 2.80 | 2.09 | 3.73 | <0.01 |
| Weight, + 10 kg | 1.25 | 1.10 | 1.42 | <0.01 | - | - | - | - |
| Lumbar BMD, + 1 T score point | 0.85 | 0.76 | 0.94 | <0.01 | - | - | - | - |
| Prior fracture, yes/no | 2.00 | 1.57 | 2.54 | <0.01 | 2.04 | 1.21 | 3.44 | 0.01 |
| Back pain, yes/no | 1.58 | 1.27 | 1.96 | <0.01 | - | - | - | - |
| Dementia, yes/no | - | - | - | - | 2.09 | 1.32 | 3.29 | <0.01 |

BMD: bone mineral density; CI: confidence interval.

## Discussion

In the current study, we explored clinical risk factors for major osteoporotic fracture and immobilization and developed a novel prediction model, the FRISC. Importantly, the assessment of external validity showed that the FRISC allows accurate prediction of major osteoporotic fracture even in the community-based setting and after a long-term follow-up of ten years, although it was developed in a hospital-based cohort study (i.e. for outpatients and visitors to a clinic). Therefore, the FRISC is useful both not only for patients who have a more complicated risk for fracture, such as atherosclerosis, diabetes or other potential risks to deteriorate bone strength, but also general postmenopausal women. Further the discriminatory power of the FRISC was shown to be better than BMD alone. We have previously noted that there is a close relationship between bone fractures and subsequent immobilization in postmenopausal Japanese women, and that these two conditions are morbid states that require a large amount of health resources [17]. Therefore, an accurate measure to predict these two conditions is particularly valuable in the context of an aging society. A web application of the FRISC is available at http://www.biostatistics.jp/prediction/frisc (Fig. 1).

The major finding of the current study is that inclusion of the four clinical risk factors, namely age, weight, prior fracture and back pain, in addition to BMD significantly improved the accuracy of the prediction model for major osteoporotic fracture. In contrast, parental history of fracture, smoking status, alcohol consumption, rheumatoid arthritis and use of steroids, which are all included in the FRAX, were not associated with incidence of fracture in the present analysis. The reason for this observation does not appear to be a lack of power given the number of observed events in the Nagano Cohort. Diet and other lifestyle factors, which were Westernized among smokers in this cohort, may have contributed to this unexpected result. One implication of these findings is that the association between lifestyle factors and fracture risk is possibly biased due to confounding factors, and it is necessary for prediction models to reflect the multidimensional nature of lifestyles. Although there were smokers and drinkers in the present population, the extent of their smoking and drinking was very mild, and smaller percentages of patients had these habits than in comparable Caucasian populations. In the practical point of view, a more parsimonious model is desirable and the FRISC would therefore provide a simple but sufficiently accurate measure for prediction of major osteoporotic fracture.

The present results indicated that incidence of fracture increases with heavier body weight, although low BMI has been considered as a significant risk factor of fracture as proposed in the FRAX. This trend remained even after the adjustment for the other risk factors

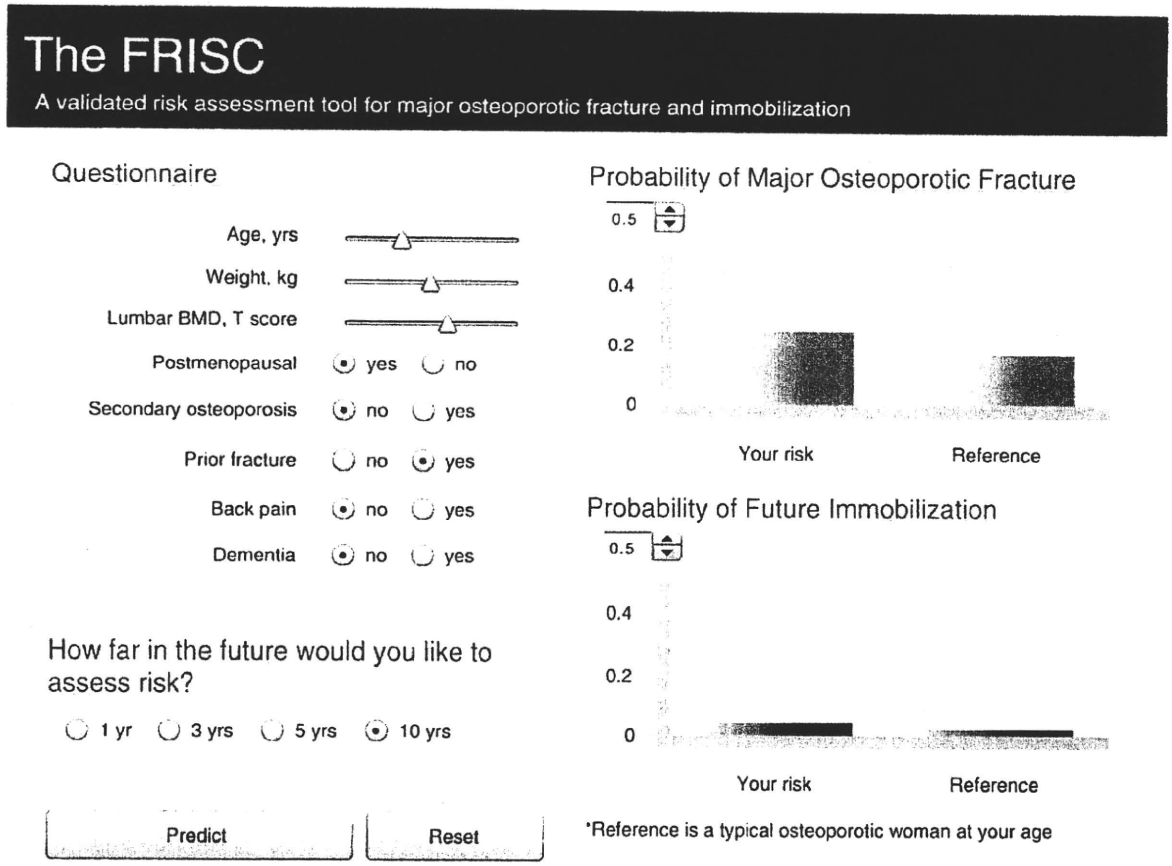


**Fig. 1.** Input and output of the web application of the FRISC.

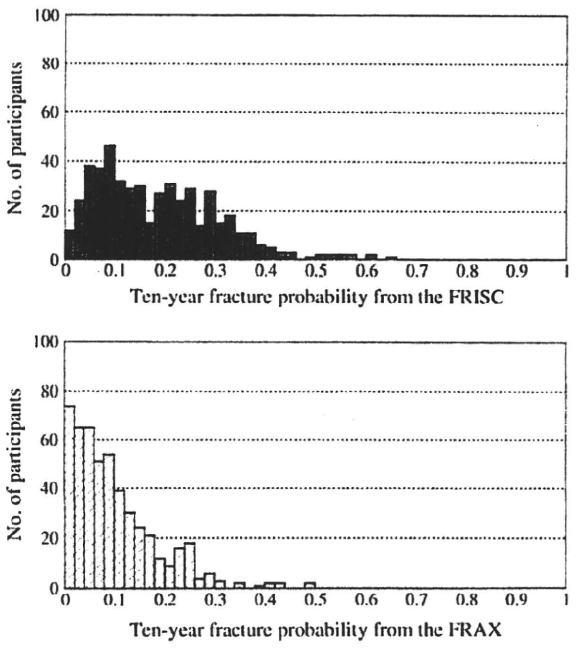


**Fig. 2.** Histogram of 10-year probabilities of major osteoporotic fracture from the FRISC (upper) and the FRAX (lower) in the Miyama and Taiji Cohorts.

(p<0.01) and therefore seemed to be attributable to confounders. This may be one of the causes of discrepancy in 10-year probability between the FRAX and the actual fracture rate in the three cohorts. Recent report indicated that morbid obesity had a higher susceptibility of fractures comparing to the postmenopausal women with normal weight although the BMD of the obesity was higher than the controls [35]. As it is well known that obesity will connect to have diabetes mellitus or at least to have glucose intolerance and diabetes may deteriorate bone quality due to an increase in non-enzymatic glycation induced cross-links of collagen, which increased collagen

**Table 4**
Predictive accuracy of major osteoporotic fracture probability from the FRISC compared the FRAX evaluated in the validation dataset from general population.

| | Calibration | | | Discrimination | | | |
|---|---|---|---|---|---|---|---|
| | Predicted no. of cases | Observed/ predicted ratio | $p^*$ | C statistics[†] | 95% CI | | $p^‡$ |
| BMD alone | – | – | – | 0.651 | 0.575 | 0.728 | – |
| The FRAX | 37.8 | 1.59 | <0.01 | 0.699 | 0.629 | 0.768 | 0.23 |
| The FRISC | 81.2 | 0.74 | 0.17 | 0.727 | 0.660 | 0.794 | 0.03 |

CI: confidence interval.

* Hosmer–Lemeshow test, $p$ value less than 0.05 indicates a significant deviation between the observed and predicted event frequencies. Number of strata and degree of freedom are 10 and 8, respectively.

† The proportion of all patient pairs in which prediction and observed occurrence of event are concordant.

‡ Contrast test comparing C statistics of the FRAX and FRISC from that of BMD alone, $p$ value less than 0.05 indicates a significant improvement from BMD alone.

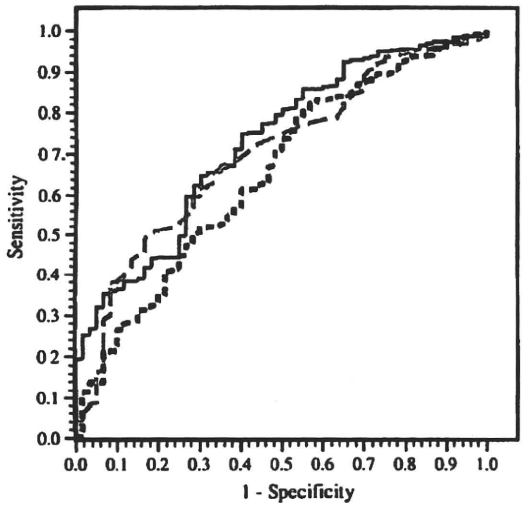


**Fig. 3.** Receiver operating characteristic curve for major osteoporotic fracture probability from the FRISC (solid curve), the FRAX (dashed curve) and BMD alone (dotted curve) in the Miyama and Taiji Cohorts.

brittleness [36,37]. Lifestyle factors such as diet and exercise may also be other explanations for this observation.

Although the intrinsic properties of the bone are important components of fracture risk, assessment of these factors alone does not adequately reflect the full range of factors associated with the occurrence of fracture [38]. Loss of bone mass and impaired bone quality are commonly held to be the two major causes of increased bone fragility in osteoporosis [2]; however, existing prediction models do not directly take bone quality into consideration. Despite recent progress in understanding the composition and structure of the bone, there are currently no standard assessments of bone quality. Novel bone quality-related markers such as homocysteine [39,40] and pentosidine [41] appear to improve predictive accuracy, but further research is required to determine whether they will be useful in the context of predicting osteoporotic fracture.

The incidence of clinical vertebral fracture in the Japanese population is substantially high (Table 2). As a result, the 10-year fracture probabilities generated using the FRISC is much higher than the FRAX (Fig. 2). The major underlying cause of the discrepancy in the 10-year probabilities is likely to be the difference in population. The FRISC was developed in a cohort study conducted at one medical institute and included subjects who were receiving treatment for osteoporosis, whereas the FRAX was developed using data from a community-based population. Although the effectiveness of bisphosphonate and selective estrogen receptor modulators in reducing fracture risk has been demonstrated, in the current analysis, drug treatment for osteoporosis was not a significant factor at the site of major osteoporotic fracture after adjustment for other risk factors, suggesting that its influence on risk is smaller than that of the risk factors. People who visit a hospital or clinic possibly have a higher prevalence of co-morbid conditions than people in the general population, yielding an increased incidence of fracture because of deterioration in both bone quality and quantity.

Given the large difference in incidence rates of fracture between the Nagano Cohort and the Miyama and Taiji Cohorts (Table 2), it may not seem to be sensible to choose the Miyama and Taiji Cohorts as validation cohorts since a good fit is unexpected. However, as shown in Table 1, the Nagano cohort included older participants and the mean lumbar BMD in this cohort was lower than the other cohorts. Therefore the difference in participants' characteristics may be attributable to the higher incidence rate in the Nagano cohort relative to the other cohorts. Further, the Miyama and Taiji Cohorts followed participants over a 10-year period and are more suitable for the validation analysis. A limitation of our validation analysis was that parental history or morphometrical vertebral fracture was missing data in either of the validation cohorts systematically. We assumed that participants with these missing data did not have parental history or prior fracture, yielding a somewhat lower 10-year probability of major osteoporotic fracture. Given that we did not find any evidence of deviation between the observed fracture frequency and prediction from the FRISC even in independent community-based cohort studies, the FRISC appears to allow accurate prediction of major osteoporotic fracture both in community-based and hospital-based settings.

Supplementary materials related to this article can be found online at doi:10.1016/j.bone.2010.08.019.

## Conclusion

We developed a novel prediction model for fracture and immobilization, FRISC, and the clinical risk factors in the FRISC allows better identification of populations at high risk of fracture than BMD alone.

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# Plasminogen/Plasmin modulates bone metabolism by regulating the osteoblast and osteoclast function.

Running Title Plasminogen/Plasmin modulates bone metabolism

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**The contribution of plasminogen (Plg)/plasmin which have been claimed to be the main fibrinolytic regulators in the bone metabolism remains unclear. This study evaluated how the absence of Plg affects the function of osteoblast (OB) and osteoclast (OC). There was a larger population of pre-OCs in bone marrow-derived cells from the $Plg^{-/-}$ mice than the population of that from the WT mice. In addition, the absent of Plg suppressed the expression of osteoprotegerin (OPG) in OBs. Moreover, an exogenous plasmin clearly induced the OPG expression in $Plg^{-/-}$ OBs. On the other hand, the osteoclastogenesis of RAW264.7 mouse monocyte/macrophage lineage cells in coculture with OBs from the $Plg^{-/-}$ mice was significantly accelerated in comparison to that in co-culture with OBs from the WT mice. Intriguingly, the accelerated OC differentiation of RAW264.7 cells co-cultured with $Plg^{-/-}$ OBs was clearly suppressed by the treatment of an exogenous plasmin. Consequently, $Plg^{-/-}$ mice display decreased bone mineral density. These findings could eventually lead to the development of new clinical therapies for bone disease caused by a disorder of the fibrinolytic system.**

The fibrinolytic system contains plasminogen (Plg) a proenzyme, which is converted to the active serine protease plasmin, a main component of the fibrinolytic system, through the action of a tissue-type plasminogen activator (tPA) or urokinase-type PA (uPA). The inhibition of the system may occur through the neutralization of the plasminogen activators or plasmin, and this neutralization is achieved mainly by the plasminogen activator inhibitor-1 (PAI-1) or α2-antiplasmin (α2AP), respectively. PAI-1, the primary endogenous inhibitor of tPA or uPA, plays an important role in inhibiting arterial clot lysis (1). On the other hand, α2AP rapidly inactivates plasmin, resulting in the formation of a stable inactive complex, plasmin-α2AP (2). Apart from the removal of fibrin, the fibrinolytic system also plays a pivotal role in such phenomena as embryogenesis, proliferation, migration, wound healing, fibrosis, and tumorigenesis (3-9).

It is suggested that fibrinolytic factors such as tPA, uPA, uPA receptor (uPAR) and PAI-1 are involved in the bone metabolism as follows: The absence of tPA and uPA enhanced OB differentiation and formation of a mineralized bone matrix, and increased bone formation and bone mass (10). On the other hand, the absence of PAI-1 protects against trabecular bone loss induced by estrogen deficiency, suggesting a site-specific

role for PAI-1 in bone turnover (11). In addition, uPAR-lacking mice displayed increased bone mineral density (BMD), increased osteogenic potential of OBs, decreased OCs formation, and cytostkeletal reorganization in mature OCs (12). However, the physiological roles of fibrinolytic main regulators such as Plg/plasmin in bone metabolism are not precisely understood.

The receptor activator of NF-κB (RANK), its ligand RANKL and osteoprotegerin (OPG) control OCs function (13,14). RANK activated by RANKL has proven to be absolutely required for OCs development (15). RANKL is neutralized by OPG that specifically binds to RANKL. OPG is expressed in many tissues apart from OBs, including heart, kidney, liver, spleen, and bone marrow (13). However, molecular mechanisms of OPG expression remain to be elucidated.

We herein report the crucial role of fibrinolytic main regulators Plg/plasmin in bone metabolism especially on the point of view that how the regulators affects the ability of pre-OCs in bone marrow to differentiate into OCs, that of OBs to induce OC differentiation, and that of OBs to mineralize extra-cellular matrix (ECM).

## Materials and Methods

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### Animals

The Plg deficient ($Plg^{-/-}$) mice (16) were kindly provided by Prof. D Collen (University of Leuven, Belgium).

Wild type (WT) and $Plg^{-/-}$ mice littermates were housed in groups of two to five in filter-top cages with a fixed 12 hours light, 12 hours dark cycle. The body weights of mice were measured weekly.

### Reagents

Plasmin, aprotinin, α2AP, epsilon amino caproic acid (EACA) and other chemical substances were obtained from Sigma Chemical (St Louis, MO USA).

### Cell culture

Bone marrow cells, RAW264.7 mouse monocyte/macrophage lineage cells (American Type Culture Collection) and primary OBs were maintained in α-MEM (Invitrogen, Carlsbad, CA USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA USA) at 37 ℃ in a humidified

atmosphere of 5 % $CO_2$ / 95 % air.

Primary OBs derived from mice calvaria were obtained as previously described (17).

OC differentiation assay

Bone marrow-derived cells that include a population of pre-OCs were obtained from tibia of 5- to 7-weeks old adult mice. Mouse bone marrow cells were cultured for 3 days with RANKL (100 ng/ml) and M-CSF (100 ng/ml) in 48-well plates. In other experiments, Raw 264.7 cells were co-cultured with OBs from the $Plg^{+/+}$ and $Plg^{-/-}$ mice for 3 days in the absence or presence of interleukin-1β (IL-1β) (5 ng/ml) or prostaglandin $E_2$ ($PGE_2$) (1 μM) in 48-well plates. Cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of OCs) as described (17). TRAP-positive multinucleated cells containing three or more nuclei were counted as OCs, under microscopic examination.

Bone resorption assay

To estimate bone resorption activity of differentiated OCs from bone marrow cells of the $Plg^{+/+}$ and $Plg^{-/-}$ mice, the cells were stimulated with RANKL (100 ng/ml) and M-CSF (100 ng/ml) for 7 days on the BioCoat™ Osteologic™ multi-test slides, which consisted of sub-micron synthetic calcium phosphate thin film coated onto various culture vessels (Becton Dickinson and Company, Bedford, MA USA). Then, the non-resorbed area of calcium phosphate film was visualized by using a method of von Kossa staining, as follows: After fixation of the cells in the culture with 5 % glutaraldehyde, the calcium phosphate film was treated with 5 % silver nitrate for 30 min. Then, the staining was developed with 5 % sodium carbonate in 25 % formalin. The stained film in each well was photographed under a light microscopy, then, the image was inverted to yield the negative image: black image represents the resorbed area in the calcium phosphate film.

Bone histology

Bone histomorphometry of tibia in 5-weeks old male $Plg^{+/+}$ and $Plg^{-/-}$ mice were performed. Each tibia was removed and fixed in 4 % paraformaldehyde for 2 days, and then demineralized with 10% EDTA for 14 days before embedding in paraffin. Paraffin-embedded tissue was serially sectioned at 4-7-μm distances. Then, the sections were stained with Hematoxylin-Eosin (H-E) and TRAP by using TRAP kit (Sigma- Aldrich, St Louis, MO USA).

For the quantitative evaluation of the intensity of TRAP-staining of bone marrow tissue in decalcified sections of tibia from the $Plg^{+/+}$ and $Plg^{-/-}$ mice, the TRAP-stained images obtained from separate fields on the

specimens (n=6) were analyzed by using ImageJ.

Measurement of bone mineral density

Bone mineral density (BMD) was measured as described by Kanazawa et al and Nishiwaki T et al (18,19). BMD of the proximal tibia of the $Plg^{+/+}$ and $Plg^{-/-}$ mice at the indicated time was evaluated by using peripheral quantitative computed tomography with a fixed x-ray fan beam of 50-μm spot size, at 1 mA and 50 kVp (LaTheta LCT-100S; Aloka, Tokyo, Japan).

RNA isolation and quantitative RT-PCR

Total RNA was extracted as previously described (6). First-strand cDNA was synthesized from total RNA by using the PrimeScript RT reagent Kit (Takara). Quantitative RT-PCR (qRT-PCR) was performed on the IQ5 real-time PCR detection system (Bio-Rad, CA, USA) with SYBR Green technology on cDNA generated from the reverse transcription of purified RNA. The 2 step PCR reactions were performed as 92℃ for 1s and 60℃ for 10 sec. OPG mRNA expression was normalized against GAPDH mRNA expression using the comparative cycle threshold method. We used the following primer sequence: OPG, 5'-CAATGGCTGGCTTGGTTTCATAG-3' and 5'-CTGAACCAGACATGACAGCTGGA-3'; GAPDH, 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-TTGCTGTTGAAGTCGCAGGAG -3'

Western blot analysis

We performed a Western blot analysis for detection of OPG, phospho-ERK1/2, phospho-p38 MAPK, ERK1/2, and p38 MAPK as previously described (20). We detected OPG, phospho-ERK1/2, phospho-p38 MAPK, ERK1/2, and p38 MAPK by incubation with a polyclonal OPG antibody (rabbit IgG, from Gene Tex, Inc. CA, USA), anti-phospho-ERK1/2 antibody (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-p38 MAPK antibody (Cell Signaling Technology, Danvers, MA, USA), anti-ERK1/2 antibody (Cell Signaling Technology, Danvers, MA, USA), and anti-p38 MAPK antibody (Cell Signaling Technology, Danvers, MA, USA).

Measurement of alkaline phosphatase activity

We measured alkaline phosphatase (ALP) activity as previously described (20). Primarily cultured OBs were cultured for 14 day with differentiation media (media supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone and 50 μg/ml ascorbic acid) in 6-well plates. After 14 days, cells were then washed, and proteins in cells were extracted with a lysis

buffer (10 mM Tris-HCl, pH 7.5, 0.1 % Triton X-100). ALP activity was determined using *p*-nitro phenyl phosphate (Sigma-Aldrich Stein-heim, Germany) as a substrate.

Statistical analysis

All data are expressed as mean±SEM. The significance of the effect of each treatment ($P$ <0.05) was determined by analysis of variance (ANOVA) followed by the Student Newman-Keuls test.

## Results

### *Histological and radiological evaluation of the status of endochondral ossification in Plg-deficient mice*

The bone mineral density (BMD) in the $Plg^{+/+}$ and $Plg^{-/-}$ mice at 4 to 20 weeks were radiologically assessed using peripheral quantitative computed tomography. Intriguingly, the trabecular BMD in tibia from the $Plg^{-/-}$ mice was significantly lower than that from the $Plg^{+/+}$ mice at 4 to 6 weeks after birth (Fig. 1A). In addition, the cortical BMD in tibia from the $Plg^{-/-}$ mice was significantly lower than that from the $Plg^{+/+}$ mice at 4 to 18 weeks after birth (Fig. 1B). The decrease of cortical BMD seemed to parallel that of the body weight-decrease in the $Plg^{-/-}$ mice at 4 to 18 weeks after birth (Fig. 1B and 1C). Next, the status of endochondral ossification in tibia from the $Plg^{+/+}$ and $Plg^{-/-}$ was histologically compared to clarify the effect of the fibrinolytic system in bone metabolism. As shown in Figure 1D, H-E staining of decalcified section of tibia from the 5-week old mice showed that the layer of chondrocytes and trabecular bone formation in the medullary cavities were observed in both $Plg^{+/+}$ and $Plg^{-/-}$ mice. The TRAP-staining of the decalcified section of the tibias from the 5-week old mice revealed that the area of TRAP-positive bone marrow tissue in the tibias from the $Plg^{-/-}$ mice was significantly larger than that of the tissue from the $Plg^{+/+}$ mice (Fig. 1E). In addition, the intensity of the TRAP-staining on the decalcified sections of bone marrow tissue in the $Plg^{+/+}$ and $Plg^{-/-}$ mice was quantitatively evaluated as described in the Materials and Methods. As shown in Fig. 1F, the intensity of TRAP-staining on decalcified sections of bone marrow tissue in tibias from the $Plg^{-/-}$ mice was much stronger than in those from the $Plg^{+/+}$ mice.

### *The effect of the Plg-deficiency on the osteoclastogenesis of bone marrow-derived cells*

We evaluated how the fibronolytic system affects OC differentiation and function. The pre-OCs population in bone marrow-derived cells from the $Plg^{+/+}$ and $Plg^{-}$

$^{-/-}$ mice were respectively evaluated after stimulation with RANKL and macrophage colony-stimulating factor (M-CSF). As shown in Figure 2A, many TRAP-positive multinucleated OCs were observed in bone marrow cell cultures derived from the $Plg^{-/-}$ mice tibia. Therefore, an up-regulation of the TRAP-positive cell number in the $Plg^{-/-}$ mice-derived bone marrow cell was observed (Fig. 2B). In addition, the bone resorption activity of OCs differentiated from bone marrow-derived cells was compared in the $Plg^{+/+}$ and $Plg^{-/-}$ mice. There was an up-regulation of the bone resorption activity of $Plg^{-/-}$ mice-derived bone marrow cells (Fig. 2C). Intriguingly, plasmin significantly inhibited the M-CSF- and RANKL-induced OC differentiation of bone marrow cells derived from the $Plg^{-/-}$ and $Plg^{+/+}$ mice (Fig. 2D).

***Plasmin induced the OPG expression in OBs.***

In order to clarify how plasmin suppresses osreoclastgenesis *in vivo*, we examined whether plasmin up-regulates the expression of OPG in OBs from the WT mice *in vitro* by qRT-PCR and a Western blot analysis. Plasmin clearly induced OPG expression in OBs from the WT mice (Fig. 3A, B). In addition, the effect of various plasmin inhibitors (α2AP; serine protease inhibitor, aprotinin; lysine analogue, epsilon amino caproic acid (EACA)) on plasmin-induced OPG expression was investigated. These plasmin inhibitors clearly abrogated the plasmin-induced OPG expression (Fig. 3C, D).

In addition, we examined the plasmin-stimulated phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) in order to determine whether plasmin activates ERK1/2 and p38 MAPK in OBs. Plasmin activated ERK1/2 and p38 MAPK in OBs (Fig. 3E). We also examined whether the ERK1/2 and p38 MAPK pathways are associated with the plasmin-induced expression of OPG in OBs by using the inhibitor of mitogen activated protein kinase kinase (MEK) and p38 MAPK (PD98059, SB203580). PD98059 and SB203580 attenuated plasmin-induced expression of OPG in OBs (Fig. 3F). These data suggest the plasmin induces OPG expression through the ERK1/2 and p38 MAPK pathways.

Moreover, qRT-PCR and a Western blot analysis revealed that the expression of OPG was suppressed in OBs from the $Plg^{-/-}$ mice (Fig. 3G, H), thus suggesting that the absence of plasmin may result in the acceleration of osteoclastogenesis of pre-OCs in accordance with the depletion of OPG-synthesis in OBs. On the other hand, there was no difference in the status of RANKL

mRNA expression in OBs from the $Plg^{+/+}$ and $Plg^{-/-}$ mice (data not shown). Moreover, plasmin induced OPG expression in $Plg^{-/-}$ OBs (Fig. 3I, J).

***Effects of Plg-deficiency on the ability of OBs to induce osteoclastogenesis of RAW264.7 mouse monocyte/macrophage lineage cells***

The status of OC differentiation of RAW264.7 mouse monocyte/macrophage lineage cells in co-culture with $Plg^{-/-}$ OBs was examined to clarify how Plg deficiency affects OBs function for osteoclastogenesis. The ability of $Plg^{-/-}$ OBs to induce OCs-differentiation of pre-OCs RAW264.7 cells was compared with $Plg^{+/+}$ OBs. The OBs were co-cultured with RAW264.7 cells under stimulation with the inflammatory mediators interleukin 1-β (IL-1β) or prostaglandin $E_2$ ($PGE_2$). Inflammatory mediators induce RANKL expression on OBs (21). The inflammatory mediators-induced RANKL expression on OBs was expected to induce the osteoclastogenesis of the co-cultured RAW264.7 cells. As shown in Figure 4A, IL-1β or $PGE_2$ increased the number of TRAP-positive multinucleated cells co-cultured with OBs. Intriguingly, the number of TRAP-positive multinucleated cells co-cultured with $Plg^{-/-}$ OBs lacking OPG expression was significantly higher than that co-cultured with $Plg^{+/+}$ OBs with or without IL-1β or $PGE_2$. In addition, the number of TRAP-positive multinucleated cells co-cultured with $Plg^{-/-}$ OBs was decreased by plasmin (Fig. 4B).

***Effect of Plg-deficiency on the ALP activity in OBs***

The ALP activity in $Plg^{-/-}$ OBs was compared with $Plg^{+/+}$ OBs under stimulation with OB-differentiation media as described in Materials and Methods. The absence of Plg did not affect the ALP activity in undifferentiated- and differentiated OBs (Fig. 5).

***Rescue of the down-regulated BMD in Plg-deficient mice by the injection of plasmin***

To clarify the effect of exogenous plasmin on bone formation *in vivo*, we evaluated the status of the BMD in the $Plg^{-/-}$ mice with or without plasmin injection. The plasmin injection clearly increased the trabecular BMD in the $Plg^{-/-}$ mice (Fig. 6A). However, the plasmin injection did not affect the cortical BMD and the weight in the $Plg^{-/-}$ mice (Fig. 6B and C).

## Discussion

Fibrinolytic factors have been suggested to play an important role in bone

metabolism. PAs and PAI-1 are involved in bone resorption by OCs (22,23). However, the role of Plg/plasmin in bone metabolism was not precisely understood. This study showed that Plg/plasmin plays an important role in bone metabolism by regulating the function of both OBs and OCs.

The trabecular BMD in the tibias from the $Plg^{-/-}$ mice was significantly lower than that from the $Plg^{+/+}$ mice at 4 to 6 weeks after birth (Fig. 1A). In contrast, the cortical BMD in the tibias from the $Plg^{-/-}$ mice was significantly lower than that from the $Plg^{+/+}$ mice at 4 to 18 weeks after birth (Fig. 1B). Therefore, the decrease in the trabecular BMD in $Plg^{-/-}$ mice seemed to be transient, however, the decrease in the cortical BMD in the mice was consistently observed from the juvenile growth period to adulthood. In addition, the TRAP-staining of decalcified sections of tibias from the 5-week old mice revealed that the intensity of TRAP-staining of bone marrow tissue in the tibias from the $Plg^{-/-}$ mice was significantly stronger than that from the $Plg^{+/+}$ mice (Fig. 1E and F). Thus, the histoenzymatic assessment indicated that the OC-differentiation in bone marrow tissue of the $Plg^{-/-}$ mice might be more vigorously induced than that in the $Plg^{+/+}$ mice.

The binding of RANKL to its receptor RANK triggers intricate and distinct signaling cascades that control lineage commitment and osteoclasts activation (13). OPG inhibits osteoclasts formation and bone resorption by blocking RANKL/RANK interactions (14). The current study showed that plasmin increased the OPG expression in WT OBs (Fig. 3A-D). Moreover, the expression level of OPG was decreased in $Plg^{-/-}$ OBs compared to $Plg^{+/+}$ OBs (Fig. 3G, H), suggesting that absence of plasmin may result in an acceleration of OB-mediated osteoclastogenesis of pre-OCs in accordance with the depletion of OPG expression in OBs. In fact, the number of TRAP-positive multinucleated RAW264.7 cells co-cultured with $Plg^{-/-}$ OBs was significantly higher than that of the cells co-cultured with $Plg^{+/+}$ OBs (Fig. 4A). Intriguingly, plasmin significantly inhibited the M-CSF- and RANKL-induced OC-differentiation of bone marrow cells derived from the $Plg^{+/+}$ and $Plg^{-/-}$ (Fig. 2D), suggesting that plasmin might attenuate osteoclastogenesis by its some direct effects on pre-OCs. In addition, there was a larger population of pre-OC in bone marrow-derived cells from the $Plg^{-/-}$ mice in comparison to the $Plg^{+/+}$ mice (Fig. 2A, B and C). On the other hand, the level of ALP activity in $Plg^{-/-}$ OBs was similar to that in $Plg^{+/+}$ OBs (Fig. 5), thus suggesting that the bone-mineralizing activity of OBs in the $Plg^{-/-}$ mice might be comparable with that in the $Plg^{+/+}$ mice. Consequently, the $Plg^{-/-}$ mice display decreased bone mineral density in

accordance with the enhanced ability of OBs to induce osteoclastogenesis of pre-OCs, the loss of the direct and suppressive effect of plasmin on pre-OCs differentiating into mature OCs and the increased pre-OCs population in bone marrow cells. In fact, the injection of plasmin into the $Plg^{-/-}$ mice clearly rescued the diminished trabecular BMD during the juvenile growth period (Fig. 6).

Plasmin activates a latent transforming growth factor β (TGF-β) (24,25) trapped in extra cellular matrix (ECM) to induce an OPG expression in ECM-harbored OBs. The accelerated expression of OPG on OBs might result in the suppression of the OB-mediated osteoclastogenesis. It is under the investigation by us whether deficiency of activated TGF-β causes decreased bone mineral density and decreased body weight in $Plg^{-/-}$ mice. On the other hand, plasmin directly activates various intracellular signaling through annexin A2 in macrophage (26). Plasmin activates macrophages via the annexin A2 heterotetramer composed of annexin A2 and S100A10 with subsequent stimulation of Janus kinase JAK1/TYK2 signaling. JAK1/TYK2 leads to STAT3 activation, Akt-dependent nuclear factor kappaB (NF-κB) activation, and phosphorylation of ERK1/2 and p38 MAPK. Furthermore, inhibitors of JAK, p38 MAPK, and NF-κB revealed that these signaling pathways are indispensable for the plasmin-mediated tumor necrosis factor-a and IL-6 induction in the cells. In addition, angiostatin, a fragment of plasmin(ogen), is a ligand and an antagonist for integrin α9β1 (27). Angiostatin, representing the kringle domains of plasmin, alone did not induce the migration of chinese hamster ovary (CHO) cells, but simultaneous activation of the G protein-coupled protease-activated receptor (PAR)-1 with an agonist peptide induced the migration on angiostatin. These facts suggest that plasmin directly stimulates various cell-lineages without an indirect cell-stimulation through an activation of some growth factors such as TGF-β. We showed that plasmin activated ERK1/2 and p38 MAPK, and the inhibition of ERK1/2 and p38 MAPK attenuated plasmin-induced OPG expression (Fig. 3E, F). In addition, plasmin activated c-jun N-terminal kinase (JNK), but the inhibition of JNK did not attenuate plasmin-induced OPG expression (data not shown). These data suggest that plasmin induces OPG expression through ERK1/2 and p38 MAPK pathway. On the other hand, the time lag between the activation of p38 MAPK and ERK1/2 after the plasmin stimulation in OBs might depend on the hierarchy of ERK1/2 and p38 MAPK in the plasmin-induced signal transduction: The ERK1/2 might be the downstream target of p38 MAPK directly activated by plasmin

in OBs. Further investigations would be required to clarify the details.

The current results strongly suggest that the plasmin activity regulates both OBs and OCs function, and then plays an important role in the bone metabolism. These findings may provide new insights into the development of clinical therapies for the prevention of bone loss related disorders.

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**Legends to Figures**

**Figure 1.** ***The bone histomorphometry and bone mineral density in Plg deficient mice***

(A) Trabecular BMD in the proximal tibia of male $Plg^{+/+}$ and $Plg^{-/-}$ mice was obtained from pQCT measurement (n=13). (B) Cortical BMD in the proximal tibia of the $Plg^{+/+}$ and $Plg^{-/-}$ mice was obtained from pQCT measurement (n=13). (C) The growth curves of the $Plg^{+/+}$ and $Plg^{-/-}$ mice (n=13). (D and E) Bone histomorphometry of tibia in 5-week-old male $Plg^{+/+}$ and $Plg^{-/-}$ mice (D: hematoxylin and eosin, E: TRAP). In D, the layer of chondrocytes and trabecular bone formation in the medullary cavities were observed in both $Plg^{+/+}$ and $Plg^{-/-}$ mice. In E, the TRAP-positive area in the bone marrow tissue of the tibias from $Plg^{-/-}$ mice was much larger than that in the tissue specimens obtained from $Plg^{+/+}$ mice. (F) The intensity of TRAP-staining on the decalcified sections of bone marrow tissue in the $Plg^{+/+}$ and $Plg^{-/-}$ mice was quantitatively evaluated as described in the Materials and Methods (n=6). The intensity of TRAP-staining on the sections from the $Plg^{-/-}$ mice was much stronger than that of sections from $Plg^{+/+}$ mice. The data represent the mean ± SEM. *, $P<0.01$; #, $P<0.05$.

**Figure 2.** ***The effect of the Plg deficiency on osteoclastogenesis and the OCs-function***

(A) Bone marrow cells from the $Plg^{+/+}$ and $Plg^{-/-}$ mice were cultured for 3 days in the absence or presence of RANKL (100 ng/ml) and M-CSF (100 ng/ml). Mature OCs were identified as TRAP positive multinucleated cells. The magnified image of boxed area was showed on the right of the original image. The arrowheads indicate osteoclasts. (B) The number of TRAP-positive multinucleated cells in (A) was determined from three different cultures. (C) The bone resorption activity of OCs differentiated from bone marrow-derived cells obtained from the $Plg^{+/+}$ and $Plg^{-/-}$ mice was compared. Bone marrow-derived cells from the $Plg^{+/+}$ and $Plg^{-/-}$ mice were cultured on the BioCoat™ Osteologic™ multi-test slides, which consisted of sub-micron synthetic calcium phosphate thin film coated onto various culture vessels, for 7 days in the presence of RANKL (100 ng/ml) and M-CSF (100 ng/ml) (n=4). Next, the resorbed areas of the calcium phosphate film were visualized as described in the Materials and Methods. The histogram on the right panels shows quantitative representations of bone resorption obtained from densitometry analysis. The densitometry results were expressed as the mean density. (D) Bone marrow cells from the $Plg^{+/+}$ and $Plg^{-/-}$ mice were cultured for 3 days in the presence of M-CSF (100 ng/ml). Some cells were cultured in the presence or absence of RANKL (100