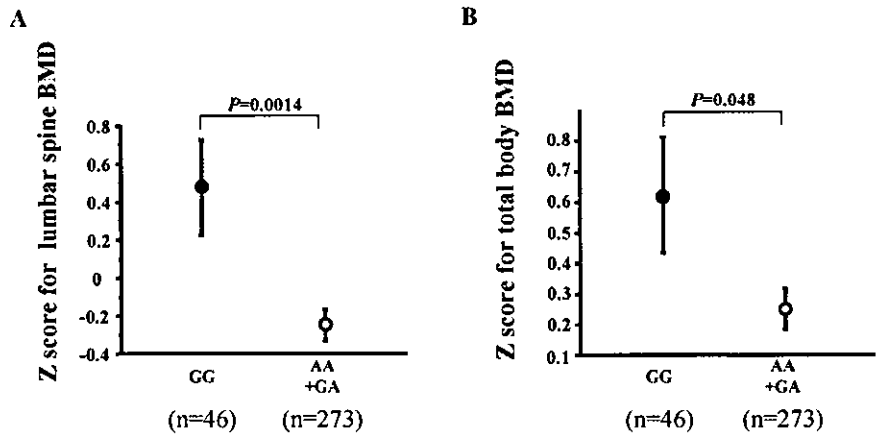
Fig. 1.


A
Z score for lumbar spine BMD
P=0.0014
0.8
0.6
0.4
0.2
0
-0.2
-0.4
GG
AA
+GA
(n=46)
(n=273)
B
Z score for total body BMD
P=0.048
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
GG
AA
+GA
(n=46)
(n=273)

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ORIGINAL ARTICLE

Association of a single nucleotide polymorphism in the secreted frizzled-related protein 4 (sFRP4) gene with bone mineral density

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Background: Wnt-β-catenin signaling pathway is involved in the regulation of bone mineral density (BMD). Secreted frizzled-related protein (sFRP) 4 that antagonize Wnt signals may modulate Wnt-β-catenin signaling pathway in the bone. Therefore, we analyzed expression of sFRP4 mRNA in primary osteoblasts and the association of a single nucleotide polymorphism (SNP) in the sFRP4 gene with BMD.

Methods: Expression levels of sFRP4 mRNA were analyzed during the culture course of rat primary osteoblasts. Association of a SNP in the sFRP4 gene at Arg262 (CGC to CGT) with BMD was examined in 372 healthy post-menopausal Japanese women.

Results: sFRP4 mRNA was detected and increased during the differentiation of rat primary osteoblasts. As an association study of the SNP in the sFRP4 gene, the subjects without the T allele (CC; $n = 129$) had significantly higher lumbar BMD than the subjects bearing at least one T allele (TT + CT; $n = 243$) (Z score; 0.054 *versus* −0.324; $P = 0.0188$).

Conclusion: sFRP4 mRNA was expressed and regulated in primary osteoblasts. A genetic variation at the sFRP4 gene locus is associated with BMD, suggesting an involvement of sFRP4 gene in the bone metabolism.

Keywords: bone mineral density, osteoblast, osteoporosis, secreted frizzled-related protein 4, single nucleotide polymorphism.

Introduction

Osteoporosis is a multifactorial disorder characterized by low bone mineral density (BMD), increased bone fragility, and increased risk of fracture.[1] Twin and sibling studies have shown that BMD is under genetic control with estimates of heritability ranging from 50% to 90%.[2–4] BMD is regulated by interplay between environmental factors and several genes, each with modest effects on bone mass and bone turnover.[5,6] Several candidate genes have been studied in relation to BMD.[7,8] These include vitamin D receptor (VDR) gene polymorphism,[9] estrogen receptor (ER) gene polymorphism,[10] collagen type Iα1 (COLIA1) gene polymorphism and parathyroid hormone (PTH) gene polymorphism.[11,12] Identification of candidate genes that affect bone mass will be useful for early detection of individuals who are at risk for osteoporosis and early institution of preventive measures.

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The Wnts represent a large group of secreted signaling proteins that are involved in cell proliferation and differentiation and play pivotal roles in morphogenesis.[13] It is also known that Wnt and bone morphogenetic protein (BMP) signals control apical ectodermal ridge (AER) formation and dorsal–ventral patterning during limb development.[14,15] Wnt proteins activate signal transduction through Frizzled which act as receptors for Wnt proteins and induce stabilization of cytoplasmic β-catenin protein.[16] Meanwhile, LDL receptor-related protein 5 and 6 (LRP5/6) were also found to be required for Wnt coreceptors.[17,18] Recent reports demonstrated that the Wnt-β-catenin signaling pathway regulates bone density through the LRP5.[19–22] It is also reported that activated β-catenin induced osteoblast differentiation of C3H10T1/2 cells.[23] These findings indicate that Wnt-β-catenin signaling pathway plays important roles in skeletal biology.

Recently, a secreted frizzled-related protein (sFRP) family was described.[24,25] Members of this family share the Wnt binding domain of the Frizzled proteins but lack their 7-transmembrane segments. It has been suggested that these sFRP proteins may control morphogenesis by binding Wnts extra-cellularly and antagonize their ability to signal through the plasma membrane-bound Frizzled receptors.[24,25] sFRP4, one of the sFRP family protein, is found to be expressed in the cartilage and osteoblasts.[26] Therefore, it is possible that sFRP4 modulates Wnt-β-catenin signaling pathway in the bone. In the present study, we examine the sFRP4 mRNA expression in primary osteoblasts and an association between a polymorphism in the sFRP4 gene and BMD in Japanese women to investigate possible contribution of the sFRP4 to bone metabolism.

Materials and methods

Cell culture

Rat primary osteoblasts were isolated from calvaria of 5-day-old-neonatal rats by enzymatic digestion as described previously with some modification.[27] Briefly, calvaria were minced and incubated at 37°C for 20 min in magnesium-free phosphate-buffered saline containing 0.1% collagenase and 0.2% dispase. The enzymatic digestion was repeated twice. The second digestion was performed for 70 min. Cells isolated at second digestion were cultured in α-MEM containing 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). Cells at the second passage were used for experiments.

Total RNA isolation and cDNA synthesis

Osteoblasts were cultured in 6-cm dishes with α-MEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate for 3, 5, 8, 11, 13, 15 or 18 days. Total RNAs were extracted from these cells using a Totally RNA Kit (Ambion, TX). cDNA was synthesized from 1 μg of total RNA of primary osteoblasts using first strand cDNA synthesis kit (Amersham, IL).

SYBR green real time PCR

Primers were designed using Primer Express 1.0 software (Applied Biosystems, CA). Definitive primers were: rat GAPDH forward 5′ -GGCACAGTCAAGGCTGA GAAT- 3′, reverse 5′ -TCGCGCTCCTGGAAGATG- 3′, rat alkaline phosphatase (ALP) forward 5′-TGACCACC ACTCGGGTGAA-3′, reverse 5′-GCATCTCAT-TGTCC GAGTACCA-3′ and rat sFRP4 forward 5′-GT TGAAGCCACCCTTACAGGATA-3′, reverse 5′-GGTC CTAAGGCAAGTGGTGTGT-3′. Polymerase chain reaction (PCR) products were 71 bp, 87 bp and 79 bp length for GAPDH, ALP and sFRP4, respectively. Quantitative PCR was carried out using a 2 × master mix composed from the SYBR Green PCR Core Reagents (Applied Biosystems) and 50 nmol/L primers. PCR reactions were performed using an ABI Prism 7000 system (Applied Biosystems) with the following sequence: 2 min at 50°C, 10 min at 95°C and 40 cycle of 15 s at 95°C and 1 min at 60°C. ALP or sFRP4 signal was normalized to GAPDH signal.

Subjects

Genotypes were analyzed in DNA samples obtained from 372 healthy post-menopausal Japanese women (mean age ± SD; 64.22 ± 9.83) living in Nagano prefecture, Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin sodium) or unusual gynecologic history. All were non-related volunteers and provided informed consent before this study.

Measurement of BMD and biochemical markers

The lumbar-spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI). We measured serum concentration of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact-osteocalcin (I-OC, ELISA; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin (CT), 1, 25(OH)2D3, total cholesterol (TC) and triglyceride (TG). We also measured the urinary pyridinoline (PD, HPLC method) and deoxypyridinoline (DPD, HPLC method). The BMD data were recorded as 'Z scores', that is deviation from the weight-adjusted average BMD for each age. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20 000 Japanese women.

Determination of the sFRP4 genotype

DNA was extracted from peripheral leukocytes by standard techniques. In sFRP4 gene, neither missense mutation nor polymorphism in the promoter region was found from JSNP-database (http://snp.ims.u-tokyo.ac.jp/index.html). Thus, silent mutation for Arg262 (CGC to CGT) of the sFRP4 gene was determined using the TaqMan (Applied Biosystems) PCR method.[28] To determine the sFRP4 SNP, we used Assays-on-Demand SNP Genotyping Products C_1328209_10 (Applied BioSystems), which contain sequence-specific forward and reverse primers and two TaqMan MGB probes for detecting alleles. During the PCR cycle, two TaqMan probes hybridize competitively to a specific sequence of the target DNA, and the reporter dye separate from the quencher dye, resulting in an increase in fluorescence of the reporter. The fluorescence levels of the PCR products were measured with the ABI PRISM 7000 (Applied Biosystems) resulting in clear identification of three genotypes of the SNP.

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the T-allele and the group with only C-allele encoded at that locus were subjected to statistical analysis (Student's *t*-test; StatView-J 4.5). A *P*-value less than 0.05 was considered statistically significant.

Results

SFRP4 mRNA expression is regulated during the course of primary osteoblasts differentiation

At the inception of this study, we measured sFRP4 mRNA levels during the course of differentiation in rat primary osteoblasts. In the presence of ascorbic acid and β-glycerophosphate, primary osteoblasts proceed through differentiation normally with the deposition of a collagenous extracellular matrix that mineralizes.[29,30] The continual maturation of the osteoblasts was reflected by the increase in ALP mRNA levels (Fig. 1A). sFRP4 mRNA levels exhibited an increase that spanned day 11 to day 18, indicating that the up-regulation of sFRP4 mRNA paralleled with the increase of ALP mRNA expression (Fig. 1B).

Association of sFRP4 gene polymorphism in exon4 with BMD

We analyzed the genotypes for a sFRP4 polymorphism at Arg262 (CGC to CGT) in subjects, using TaqMan methods. Among 372 post-menopausal volunteers, 77 were TT homozygotes, 166 were CT heterozygotes, and 129 were CC homozygotes.

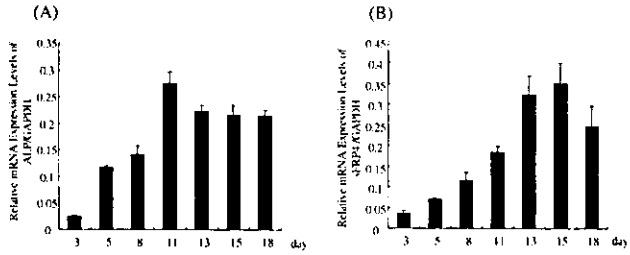

Figure 1 Expressions of ALP and sFRP4 mRNA during culture course of rat primary osteoblasts were analyzed by real time RT-PCR. Rat primary osteoblasts were cultured with α-MEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate up to 18 days. At the indicated times, RNA was extracted and the expression levels of ALP (A) and sFRP4 (B) were analyzed by real time PCR, normalized to GAPDH expression (n = 4 for each group). Values are means + SD.

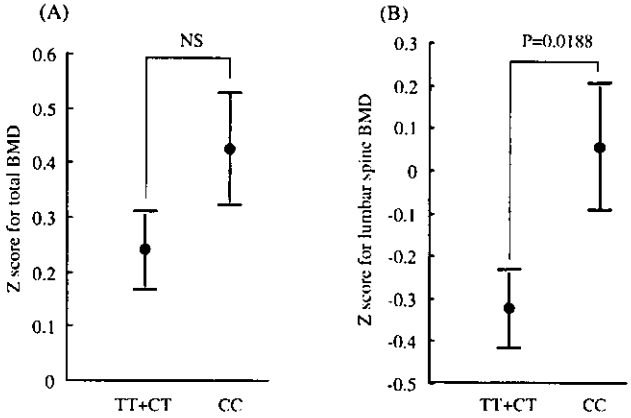

Figure 2 Z score values of total body and lumbar spine bone mineral density (BMD) in the groups with each genotype of sFRP4 polymorphism at Arg262 (CGC to CGT). (A) Z score values for total BMD are shown for genotype TT + CT and for genotype CC. Values are expressed as mean ± SE. Number of subjects are shown in parentheses. (B) Z scores for lumbar BMD as shown in the same manner as (A).

We compared Z scores for BMD of total body and lumbar spine between the subjects bearing at least one T allele (TT + CT) and subjects without the T allele (CC). Comparison of the Z scores of the total BMD between those with and without T allele showed a higher average value for the CC homozygote group, but the difference was not statistically significant (Fig. 2A). On the other hand, Z scores of the lumbar BMD in the CC homozygote group was significantly higher than in the other group (Fig. 2B). Among the biochemical markers examined, ALP was higher in the CC homozygote group than the other group (Table 1). Other than ALP, the background and biochemical data were not statistically different between these groups.

Table 1 Comparison of Z scores of lumbar spine (L2–L4) and total body bone mineral density, background and biochemical data of subjects between the two groups of genotypes

Items	Genotype (mean ± SD)		*P*-value
	TT + CT	CC	
Number of subjects	243	129	
Age (years)	65.18 (9.26)	65.90 (10.45)	NS
Body weight (kg)	50.40 (7.77)	49.93 (8.70)	NS
Body height (cm)	150.11 (6.36)	151.41 (6.21)	NS
Years after menopause	15.97 (9.81)	16.08 (11.06)	NS
Lumbar BMD (g/cm^2)	0.876 (0.183)	0.920 (0.209)	0.0366
Lumbar BMD (Z-score)	−0.324 (1.359)	0.054 (1.659)	0.0188
Total BMD (g/cm^2)	0.971 (0.114)	0.987 (0.112)	NS
Total BMD (Z-score)	0.240 (1.049)	0.426 (1.128)	NS
ALP (IU/L)	186.25 (62.16)	201.18 (71.43)	0.0385
Ca (mg/dL)	9.228 (0.448)	9.202 (0.424)	NS
P (mg/dL)	3.427 (0.473)	3.344 (0.462)	NS
I-OC (ng/mL)	8.012 (3.91)	7.782 (3.126)	NS
PD (pmol/umol of Cr)	35.66 (18.45)	33.25 (13.33)	NS
DPD (pmol/umol of Cr)	7.530 (3.920)	7.397 (2.795)	NS
PTH (pg/mL)	36.05 (16.31)	32.50 (12.64)	NS
CT (pg/mL)	22.67 (11.01)	16.35 (5.91)	NS
1,25-(OH)2D3 (pg/mL)	35.54 (11.48)	36.79 (8.91)	NS
TC (mg/dL)	199.19 (38.84)	198.10 (35.96)	NS
TG (mg/dL)	144.06 (87.13)	133.78 (73.22)	NS
%FAT	32.06 (7.37)	30.29 (8.39)	NS

Values are given as means ± SD.
NS, not significant; BMD, bone mineral density; Ca, calcium; P, phosphate; ALP, alkaline phosphatase; I-OC, intact osteocalcin; PD, pyridinoline; DPD, deoxypyridinoline; PTH, parathyroid hormone; CT, calcitonin; TC, total cholesterol; TG, triglyceride; %FAT, body fat percentage.

Discussion

During the course of primary osteoblast differentiation, the increase of sFRP4 expression was accompanied by the increase of ALP expression, which is a marker of osteoblast differentiation.[31] This result suggests some roles of sFRP4 in the osteoblasts. Recent reports demonstrated that the Wnt-β-catenin signaling pathway regulates bone density through the LRP5. Inactivating mutations in the LRP5 gene decrease bone mass and cause the autosomal-recessive disorder osteoporosis-pseudoglioma syndrome in humans and mice.[19,20] Conversely, activating mutations in the LRP5 gene are linked to autosomal-dominant high bone mass traits.[21,22] Thus, Wnt signaling plays a pivotal role in bone metabolism. Wnt signaling is initiated by binding of Wnts to the Frizzled, and antagonize glycogen synthase kinase 3β (GSK3β)/adenomatous polyposis coli (APC)/Axin complex, leading to the stabilization of β-catenin. Stabilized β-catenin accumulates in the nucleus, interact with lympoid enhancer factor (LEF)-1/T-cell factor (TCF) and activates Wnt target genes.[32] sFRP4, which is assumed to antagonize Wnt signaling, may also regulate Wnt target genes and be involved in bone metabolism.

To our knowledge, the present study is the first to investigate the influence of a polymorphism of the sFRP4 gene on BMD. We demonstrated that the Japanese post-menopausal women who had one or two allele(s) of a synonymous change of C-T transition showed significantly lower lumbar BMD. Total BMD was also lower in the subjects bearing at least one T allele, although the difference was not statistically significant. Lower BMD in post-menopausal women can be considered a result of abnormally rapid bone loss and/or lower peak bone mass. The SNP analyzed in this study would be useful as a genetic marker for low BMD and susceptibility to osteoporosis. Although the biological meanings of this polymorphism should be revealed by functional studies, three hypotheses could be proposed at present. First, silent polymorphism may be linked with other mutations in exons, which contributes to the change of the sFRP4 protein function. Second, SNP may be linked with a mutation in regulatory elements affecting the levels of expression through variable transcriptional regulation. Third, this SNP in the sFRP4 gene may be linked with a mutation of another undefined gene adjacent to the sFRP4 gene that causes low BMD directly or indirectly.

Bone resorption markers showed no significant differences between the genotypes of the sFRP4 gene. In contrast, the serum level of ALP was higher in the CC group, which had significantly higher lumbar BMD. These findings suggested that increased bone formation might be involved in higher BMD. However, the values of osteocalcin were not significantly increased in this group. Although both ALP and osteocalcin are bone formation markers, ALP is an early marker of osteoblast differentiation and osteocalcin is a marker of late osteoblast differentiation.[31] According to the *in vitro* study, β-catenin induced ALP expression but not osteocalcin expression in C3H10T1/2 suggesting that β-catenin might activate early osteoblast differentiation.[23] sFRP4 is thought to be an extracellular antagonist of Wnt signaling, therefore we could speculate that sFRP4 is involved in the regulation of early osteoblast differentiation by modulating the Wnt–β–catenin pathway. However, we measured total ALP in the present study, it is possible that this did not represent a useful parameter for bone formation.

Recently, sFRP4 was shown to be a factor produced by tumors derived from subjects with tumor-induced osteomalacia (TIO).[33] This report suggests that sFRP4 is a circulating protein associated with renal phosphate wasting. Phosphate is a main constituent of the bone matrix and is required for proper bone mineralization. Thus, it is possible that sFRP4 may affect the bone metabolism not only by regulating osteoblastic Wnt-β-signaling, but also by regulating the phosphate metabolism. In the present study, the CC group of the SNP of the sFRP4 gene had significantly high bone density. However, the difference in serum phosphate was not significant between the CC group and the other group. In addition, whether this polymorphism is associated with circulating sFRP4 concentration remains unclear. Further studies will be required to clarify the sFRP4 function in skeletal homeostasis and the biological significance of this polymorphism.

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Association of a single-nucleotide polymorphism in low-density lipoprotein receptor-related protein 5 gene with bone mineral density

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Abstract Low-density lipoprotein receptor-related protein 5 (LRP5) is an important regulator of osteoblast growth and differentiation, affecting peak bone mass in vertebrates. Here, we analyzed whether the *LRP5* gene was involved in the etiology of postmenopausal osteoporosis, using association analysis between bone mineral density (BMD) and an *LRP5* gene single-nucleotide polymorphism (SNP). Association of an SNP in the *LRP5* gene at IVS17-1677C > A (intron 17) with BMD was examined in 308 postmenopausal Japanese women (65.2 ± 9.6 years; mean ± SD). The subjects bearing at least one variant A allele (CA + AA; n = 142) had significantly lower Z scores for total body and lumbar BMD than the subjects with no A allele (CC; n = 166) (total body, 0.08 ± 1.09 versus 0.50 ± 1.03; P = 0.0022; lumbar spine, −0.42 ± 1.43 versus −0.02 ± 1.42; P = 0.013). These findings suggest that the *LRP5* gene is a candidate for the genetic determinants of BMD in postmenopausal women, and this SNP could be useful as a genetic marker for predicting the risk of osteoporosis.

Key words wnt · LRP5 · osteoporosis · bone mineral density · polymorphism

Introduction

Osteoporotic fracture is a serious event in an increasingly aging population. Low bone mass is one of the most significant risk factors. Twin and sibling studies have revealed that the proportion of variance of bone mineral density (BMD) accounted for by genetic factors is around 50%–90% [1–6]. These studies have suggested that the variation in BMD among individuals is largely caused by genetic factors. Therefore, genetic markers that are correlated with BMD would be useful for predicting future bone loss and for clarifying the mechanism of bone loss in osteoporosis. After an association of BMD with vitamin D receptor (VDR) genotypes was reported [7], polymorphisms in several other genes were investigated [8]. These genes included those implicated in bone formation by the regulation of osteoblast growth and differentiation, such as transforming growth factor beta 1 (TGFβ1) [9], collagen type Ia1 (COLIA1) [10], parathyroid hormone (PTH) [11], and p57Kip2 (CDKN1C) [12]. Considering the polygenetic nature of BMD distribution and the multiplicity of endocrine factors known to regulate bone mass and bone turnover, it is important that the panel of candidate genes could be expanded to elucidate the whole genetic background of osteoporosis.

The Wnt signaling pathway plays a pivotal role in embryonic development and oncogenesis [13,14]. Studies using *Drosophila*, *Xenopus*, and mammalian cells have established a canonical signaling pathway [15–17]. Both genetic and biochemical results have provided solid evidence indicating that FZ proteins function as Wnt receptors. Wnt proteins bind Frizzled (FZ) and prevent glycogen synthase kinase 3 (GSK3)-dependent phosphonylation of β-catenin, leading to the stabilization of β-catenin. Meanwhile, the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) were found to be also required for the Wnt signaling pathway as Wnt co-receptors [18,19]. Recent reports have demonstrated that the Wnt-β-catenin signaling pathway regulates bone density through LRP5 [20–23]. Inactivating mutations in LRP5 decrease bone mass and cause the autosomal-recessive disorder osteoporosis-pseudoglioma syndrome in humans [20] and mice [21]. Conversely, activating mutations in LRP5 are linked to autosomal-dominant high-bone mass traits [22,23]. These data suggest that LRP5, which modulates

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Wnt signaling, controls bone metabolism in vivo in mammals. To examine the possible contribution of the *LRP5* gene to the etiology of involutional osteoporosis, we investigated an association between polymorphism in this gene and BMD in Japanese women.

Subjects and methods

Subjects

Genotypes were analyzed in DNA samples obtained from 308 healthy postmenopausal Japanese women (mean age ± SD; 65.2 ± 9.6 years) living in Nagano prefecture, Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g., corticosteroids, anticonvulsants, heparin), or unusual gynecologic history. All women were non-related volunteers and provided informed consent before this study.

Measurement of BMD and biochemical markers

The lumbar spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry, using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentrations of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact osteocalcin (I-OC; enzyme-linked immunosorbent assay [ELISA]; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin, 1, 25$(OH)_2D_3$, total cholesterol (TC), and triglyceride (TG). We also measured urinary pyridinoline (PD; HPLC method) and deoxypyridinoline (DPD; HPLC method). The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. The Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20000 Japanese women.

SNP Selection

A polymorphic variation of the *LRP5* gene was extracted from the JSNP-database (http://snp.ims.u-tokyo.ac.jp/index.html), and was denoted as IVS17-1677C > A according to its localization on the gene.

Genotyping procedure

Genotypes of IVS17-1677C > A were determined using the SNP-dependent (Sd)-polymerase chain reaction (PCR) method, a modified allele-specific PCR of polymorphic sequence as previously described [24,25]. Two allele-specific primers (AS-primers) and one reverse primer were prepared per single-nucleotide polymorphism (SNP). The AS-primers (long and short) have a five-base difference between them; each has a polymorphic nucleotide of the SNP sequence at the 3′ ends, and an additional artificial mismatch introduced near the 3′ end. Primer sequences used were as follows: IVS17-1677C > A FL-primer: 5′-TTTTTGGGCGGTAAAT ACACGTCTCTCGAG-3′; IVS17-1677C > A FS-primer: 5′-CCGCGGTAAATACACGTCTCTCGAT-3′; and IVS17-1677C > A reverse-primer: 5′-GTTTCC GTCAGAAC GCTGCACTA-3′.

This primer set allowed distinct discrimination of alleles. For the assay, a genomic DNA sample (10 ng) was amplified with 250 nM of each primer (two polymorphic forward, and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U Taq DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA). The Sd-PCR reaction was carried out in a thermal cycler (Gene-amp system 9600; Perkin-Elmer) with initial denaturalization at 94°C for 4 min, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles at 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system, using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, direct re-sequencing was carried out using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the minor A-allele and the group with only the major C-allele encoded at that locus were subjected to analysis. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group were normally distributed, we applied Student's *t*-test, using StatView-J4.5 software (SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

Association of LRP5 *gene polymorphism in intron 17 with BMD*

We analyzed the genotypes for the *LRP5* IVS17-1677C > A polymorphism (rs3781586 in the National Center for Biotechnology Information [NCBI] dbSNP data-

Table 1. Comparison of background and biochemical data between subjects bearing at least one A allele (AA + CA) and subjects with no A allele (CC) at IVS17-1677 (intron 17)

Items	Genotype (mean ± SD)		*P* value
	CC	CA + AA	
No. of subjects	166	142	
Age (years)	65.1 ± 9.6	65.4 ± 9.9	NS
Height (kg)	151.0 ± 6.2	150.3 ± 6.4	NS
Body weight (kg)	50.7 ± 8.4	50.3 ± 8.1	NS
Lumbar spine BMD (g/cm^2)	0.92 ± 0.20	0.87 ± 0.19	0.025
Lumbar spine BMD (Z score)	−0.02 ± 1.42	−0.42 ± 1.43	0.013
Total body BMD (g/cm^2)	1.00 ± 0.11	0.96 ± 0.12	0.015
Total body BMD (Z score)	0.50 ± 1.03	0.08 ± 1.09	0.0022
Ca (mg/dl)	9.2 ± 0.43	9.2 ± 0.45	NS
P (mg/dl)	3.4 ± 0.46	3.4 ± 0.48	NS
ALP (IU/l)	183.7 ± 62.6	195.4 ± 71.0	NS
I-OC (ng/ml)	7.6 ± 4.2	8.3 ± 3.7	NS
PD (pmol/μmol of Cr)	36.1 ± 24.7	34.8 ± 12.0	NS
DPD (pmol/μmol of Cr)	7.6 ± 5.2	7.4 ± 2.4	NS
Intact PTH (pg/ml)	35.1 ± 16.4	35.8 ± 16.6	NS
Calcitonin (pg/ml)	22.8 ± 11.1	23.4 ± 11.7	NS
1,25 $(OH)_2D_3$ (pg/ml)	37.5 ± 12.6	34.3 ± 10.4	NS
TC (mg/dl)	198.7 ± 37.5	195.7 ± 39.2	NS
TG (mg/dl)	141.5 ± 81.4	136.8 ± 71.4	NS
Percent fat	32.1 ± 7.9	31.6 ± 7.4	NS
BMI	22.2 ± 3.2	22.2 ± 2.9	NS

Statistical analysis was performed according to the method described in the text
BMD, bone mineral density; Ca, calcium; P, phosphate; ALP, alkaline phosphatase; I-OC, intact-osteocalcin; PD, pyridinoline; DPD, deoxypyridinoline; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; BMI, body mass index; NS, not significant

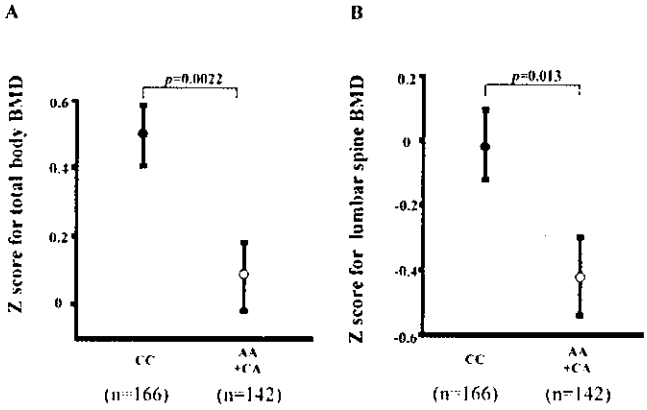

Fig. 1. Z Score values for total body and lumbar bone mineral density (*BMD*) in the groups with each genotype of the *LRP5* gene in intron 17 (IVS17-1677C > A). **A** Z Score values for total body BMD are shown as the *solid circle* for genotype CC at IVS17-1677 and as the *open circle* for genotype AA + CA at IVS17-1677. Values are expressed as means ± SE. Numbers of subjects are shown *in parentheses*. **B** Z Score values for lumbar spine BMD are shown in the same manner as in **A**

base) in 308 subjects, using Sd-PCR methods [25]. Among the 308 postmenopausal volunteers, 24 were AA homozygotes, 118 were CA heterozygotes, and 166 were CC homozygotes. Allelic frequencies were 0.731 for the C allele and 0.269 for the A allele in this population.

We compared Z scores for BMD of total body and lumbar spine between subjects bearing at least one chromosome with the A allele (genotype AA + CA; n = 142) and subjects with no A allele (CC; n = 166). The former subjects had significantly lower Z scores for total body BMD (0.08 ± 1.09 versus 0.50 ± 1.03; P = 0.0022, Fig. 1A) and lumbar BMD (−0.42 ± 1.43 versus −0.02 ± 1.42; P = 0.013; Fig. 1B). As shown in Table 1, the background data were not significantly different between these groups.

Discussion

We investigated the influence of a genetic variation of the *LRP5* gene on bone mineral properties. The allelic frequencies of an SNP in intron 17 (0.731 for IVS17-1677C and 0.269 for IVS17-1677A) in Japanese postmenopausal women were in Hardy-Weinberg equilibrium. The allelic frequencies of this SNP in the general Japanese population were reported in the JSNP database (IMS-JST137897). The database reported that the allelic frequencies were 0.726 for IVS17-1677C and 0.274 for IVS17-1677A, indicating that the allelic frequencies in the present study were in line with the JSNP database.

Recently, patients with homozygous *LRP5* gene disruption were reported [20]. There are many types of mutations affecting bone mass accrual during growth, causing the autosomal recessive disorder osteoporosis-pseudoglioma syndrome. Regarding the effect on the bone, these patients showed a marked decrease in their BMD. In addition, Kato et al. [21] created and characterized *LRP5* gene knockout mice. Interestingly, *LRP5* gene knockout mice showed lower bone mass density than wild-type mice because of decreasing osteoblast proliferation. In their report, Kato et al. [21] observed the presence of LRP5 protein in osteoblasts lining the endosteal and trabecular bone surfaces, but not in osteoclasts, by immunohistochemistry in wild-type mice. Recently, a gain-of-function mutation (G171V) in the *LRP5* gene was described in two kindreds with an enhanced bone density [22,23]. In vitro studies showed that the normal inhibition of Wnt signaling by another protein, Dickkopf-1 (Dkk1), was defective in the presence of this mutation, resulting in increased signaling due to unopposed Wnt activity. Thus, LRP5 may be one of the cellular mediators involved in bone formation, by regulating the proliferation and differentiation of osteoblasts.

In the present study, significant correlation was observed between BMD and a polymorphism in intron 17 (IVS17-1677C > A). To our knowledge, this is the first report that a common SNP in the *LRP5* gene affected BMD. However, it is still unclear how BMD is affected by this intronic polymorphism of the *LRP5* gene. For explaining this, three hypotheses could be proposed. (i) This intronic polymorphism may be linked with exon mutations and may contribute to changing LRP5 protein function. (ii) This polymorphism may be linked with mutations of regulatory elements and may affect the levels of expression through transcriptional regulation. (iii) The polymorphism in the *LRP5* gene may be linked with mutation of another unidentified gene adjacent to the *LRP5* gene which causes low BMD directly or indirectly.

In conclusion, our finding suggests that the *LRP5* gene may be a candidate for the genetic determinants of BMD in postmenopausal women. Examining *LRP5* gene variation will, it is hoped, enable us to understand one of the mechanisms of involutional osteoporosis. Wnt and LRP5 signaling have been implicated in other diseases, including cholesterol and glucose metabolism-related diseases [26]. The variant presented here may be involved in the risk of such diseases, as well as osteoporosis.

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Protein Phosphatase 5 Is a Negative Regulator of Estrogen Receptor-Mediated Transcription

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Estrogen receptors (ERs) are transcription factors that can be modulated by both estrogen-dependent and growth factor-dependent phosphorylation. A yeast two-hybrid screening identified a serine/threonine protein phosphatase (PP5) as an interactant of ERβ (1–481), a dominant negative ERβ mutant. Glutathione *S*-transferase pull-down assays, mammalian two-hybrid assays, and immunoprecipitation studies showed that PP5 directly binds to both ERα and ERβ via its tetratricopeptide repeat domain. E domains of ERα and ERβ, without containing activation domain core regions in transcription activation function 2, were required for the binding to PP5. In ERα-positive breast cancer MCF7 cells, estrogen- and epidermal growth factor-dependent phosphorylation of ERα on serine residue 118, a major phosphorylation site of the receptor, was reduced by expressing PP5 but enhanced by PP5 antisense oligonucleotide. Estrogen-induced transcriptional activities of both ERα and ERβ and mRNA expression of estrogen-responsive genes, including pS2, c-*myc*, and cyclin D1, were suppressed by PP5 but enhanced by PP5 antisense oligonucleotide. A truncated PP5 mutant consisting only of its tetratricopeptide repeat domain acted as a dominant negative PP5 that enhanced serine residue 118 phosphorylation of ERα and transactivations by ERα and ERβ. We present the first evidence that PP5 functions as an inhibitory regulator of ER phosphorylation and transcriptional activation *in vivo*. (*Molecular Endocrinology* 18: 1131–1143, 2004)

THE PHYSIOLOGICAL FUNCTIONS of estrogen are mediated by the two estrogen receptors, ERα and ERβ (1). ERs are transcription factors that regulate expressions of estrogen-targeted genes in response to hormone binding. Like other transcription factors, ERs are phosphoproteins (2). It has been observed that direct phosphorylation of ERα is induced upon addition of ligands (3) as well as mediated by protein kinases in a ligand-independent manner (4–7). Serine 118 (S118) is a major phosphorylation site within A/B domain, or N-terminal transcription activation function (AF)-1 of ERα, the mutation of which reduces transactivation by ERα (3, 8). Previous literature shows that S118 is phosphorylated by MAPK (4) or by Cdk-activating kinase, a cyclin-dependent kinase that phosphorylates the *Pol*II C-terminal tail domain (7). S118 phosphorylation is also induced by ligand binding to the receptor in a MAPK- or Cdk-activating kinase-independent manner (9).

The molecular mechanism(s) by which the phosphorylation of ERs is regulated remain(s) to be clarified. The reversible phosphorylation of proteins is catalyzed by protein kinases and phosphatases. Among the enzymes, serine/threonine protein phosphatases belong to the PPP family that specially targets phosphorylation of serine/threonine residues (10). The PPP family of phosphatases is comprised of several members including protein phosphatase 1 (PP1), PP2A, PP2B, PP2C, and PP4–7 (11). PP5 (serine/threonine protein phosphatase), another member of the PPP family, has a unique character in that it consists of a single polypeptide chain containing a phosphatase catalytic domain near its C terminus and four tetratricopeptide repeat (TPR) domains as a regulatory region in its N terminus (12). The TPR domain consists of a highly degenerate 34-amino acid repeat initially identified in several cell-cycle gene products and in proteins involved in the regulation of RNA synthesis (13, 14). TPR domains mediate protein-protein interactions

Abbreviations: AD core, activation domain core; AF, activation function; AS, antisense; E_2, 17β-estradiol; dccFCS, dextran-coated charcoal-treated fetal calf serum; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GR, glucocorticoid receptor; GST, glutathione *S*-transferase; PP5, serine/threonine protein phosphatase type 5; S118, serine 118; S118A, mutation of S118 to alanine; S118E, substitution of S118 to glutamic acid; Scr, scrambled; SDS, sodium dodecyl sulfate; SSC, sodium saline citrate; TPR, tetratricopeptide repeat; VP16, herpes simplex viral protein 16; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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(15), and there is evidence that the TPR domain of PP5 targets the phosphatase to other proteins, including heat shock protein 90-glucocorticoid receptor complex (16), apoptosis signal-regulating kinase 1 (17), the atrial natriuretic peptide receptor (18), the anaphase-promoting complex (19), and PP2A (20).

Here we show that PP5 directly binds to ERα and ERβ and inhibits transcriptional activities of the receptors. ERα and ERβ interact with the TPR domain of PP5 through their E domains without including activation domain (AD) core regions of helix 12 within C-terminal transcription AF-2, which was previously shown to be important for binding to coactivators (21–23). 17β-Estradiol (E_2)-dependent or epidermal growth factor (EGF)-dependent phosphorylation of ERα on S118 is inhibited by PP5 but enhanced by a truncated mutant of PP5 consisting of only the TPR domain. PP5 suppresses the transactivations by ERα and ERβ but not those by ERα mutants with one amino acid substitution, S118A (mutation of S118 to alanine) or S118E (substitution of S118 to glutamic acid (3). PP5 inhibits E_2-induced mRNA expression of estrogen-targeted genes including pS2, c-*myc*, and cyclin D1. Furthermore, an antisense (AS) oligonucleotide against PP5 that could reduce endogenous PP5 expression enhanced both E_2-dependent and EGF-dependent phosphorylation of ERα, transactivation by ERα, and E_2-induced mRNA expression of estrogen-targeted genes. Our results may present a novel molecular mechanism that PP5 is a key regulator of the signaling pathways of ERα and ERβ in a negative manner.

RESULTS

Identification of PP5 that Interacts with a Dominant Negative ERβ

To identify novel binding partners of ERs, we used the yeast two-hybrid system based on a Lex A-ERβ (1–481) fusion protein to screen a cDNA library derived from estrogen-depleted MCF7 breast cancer cells. ERβ (1–481) mutant is a dominant negative form of ER that has potential to repress both ERα- and ERβ-mediated transactivation (24). Among positive clones, three independent clones encoding PP5 were obtained. The interactions of the PP5 clones between ERβ (1–481) were confirmed by the galactose-dependent growth of yeast strain EGY48, which was cotransformed with pSH18–34 LacZ reporter plasmid, pEG202NLS-ERβ (1–481), and the PP5 clones in galactose-inducible pJG4–5 (Fig. 1).

ERs Directly Bind to and Are Substrates for PP5

To characterize whether PP5 directly interacts with ERs *in vitro*, we performed glutathione *S*-transferase (GST) pull-down assays using bacterially expressed GST or GST-fused PP5 mutants and *in vitro* translated

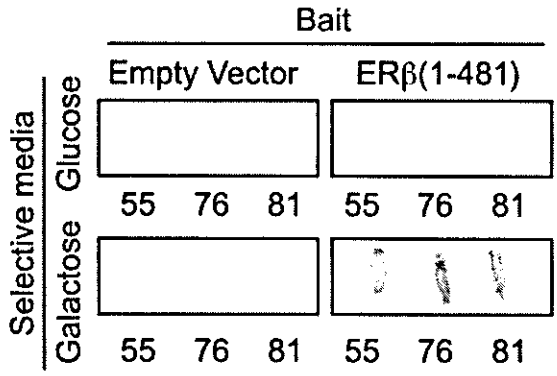

Fig. 1. Specific Interaction between PP5 and ERβ (1–481) in Yeast

Galactose-inducible plasmids pJG4–5 including PP5 clones (nos. 55, 76, and 81) obtained from yeast two-hybrid screening were transformed into yeast EGY48 along with LacZ reporter plasmid and pEG202NLS containing ERβ (1–481) or its empty vector. The specific interaction between PP5 clones and ERβ (1–481) construct was observed in galactose-containing medium through β-galactosidase staining.

ERα and ERβ (Fig. 2). Both ERα and ERβ were pulled down by full-length PP5, but not by GST alone. GST-PP5 (28–165), GST-PP5 (2–181), and GST-PP5 (2–312) can bind both ERs, whereas neither GST-PP5 (2–71) nor GST-PP5 (181–499) can bind the receptors (Fig. 2, B and C). The data suggest that both ERα and ERβ directly bind to PP5, and PP5 interacts with ERs through its TPR domains.

We next examined whether the interaction between PP5 and ERs has ligand dependency (Fig. 2, D and E). The amounts of pulled-down ERα or ERβ did not change for GST-PP5 or GST-PP5 (28–165) in the presence or absence of E_2, indicating that the association of PP5 with ERs does not require ligand activation.

We further investigated which domain(s) of ERs is/are responsible for the association with PP5 *in vivo* by performing mammalian two-hybrid assays. Cotransfection of expression constructs for the herpes simplex viral protein 16 (VP16) transactivation domain fused to PP5 and the GAL4 DNA binding domain fused to full-length or fragments of ERα/ERβ was performed into 293T cells, and the binding ability of PP5 with ER fragments was assessed by measuring luciferase activity that is derived from a GAL4-driven luciferase reporter (Fig. 3). The most significant luciferase activity was observed when PP5 interacted with E domains of ERs without containing the activation domain (AD) core regions within AF-2 [*i.e.* ERα (302–530) and ERβ (248–481)] (Fig. 3B). ER fragments including ABCD domains, ABC domains, and the AD core regions within E/F domains showed no binding activity to PP5.

The finding that TPR domains in PP5 are requisite sites for interaction with ERs was further confirmed by coimmunoprecipitation experiments, using 293T cells cotransfected with green fluorescent protein (GFP)-tagged ERα along with Flag-tagged full-length PP5 or

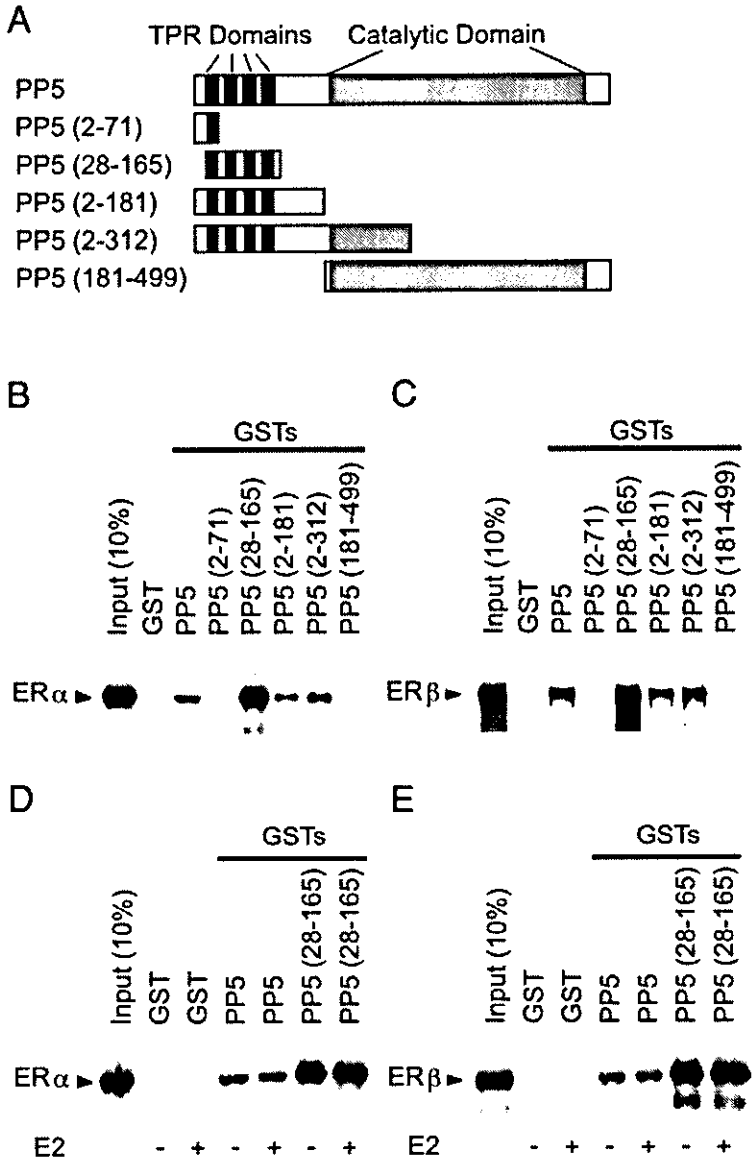

Fig. 2. ERα and ERβ Proteins Are Pulled Down by GST Fusion Proteins Containing PP5 or Its TPR Domains

A, Schematic diagrams of human PP5 constructs. PP5 cDNAs were inserted into pGEX4T-1 downstream of and in frame with the GST tag and GST or GST fusion proteins were expressed in *Escherichia coli*. *Solid* and *shaded boxes* indicate TPR domains and catalytic domain, respectively. B and C, *In vitro* translated ERα (B) and ERβ (C) proteins labeled with ^{35}S-methionine were incubated with GST or GST fusion proteins containing full-length PP5 or PP5 mutants. Labeled proteins corresponding to 10% of input and materials bound to glutathione-Sepharose were separated by 10% SDS-PAGE and detected by radioautography. D and E, Estrogen stimulation does not affect the *in vitro* interaction of full-length PP5 or its TPR domains [PP5 (28–165)] with ERα (D) and ERβ (E). GST pull-down assays were performed as described above except for the addition of 10 nM E_2 in the binding solution.

a PP5 mutant with only its TPR domains (Fig. 4B). The *in vivo* interaction between endogenous PP5 and ERα in human breast cancer MCF7 cells was also shown by coimmunoprecipitation study (Fig. 4C).

PP5 Inhibits Phosphorylation of ERα on S118

To assess the phosphatase activity of PP5 on ERs, we next examined whether PP5 modulates the phosphorylation of ERα on S118, which is a major phosphorylation site for ERα. In MCF7 cells, maximal phosphorylation levels of ERα were observed 60 min and 5 min after E_2 stimulation and EGF stimulation, respectively, analyzed by immunoblotting using a specific antibody against ERα phosphorylated at S118 (ERα-P-S118). In cells transfected with PP5, the phosphorylation level of ERα 60 min after E_2 stimulation was 29% reduced compared with control cells transfected with a control

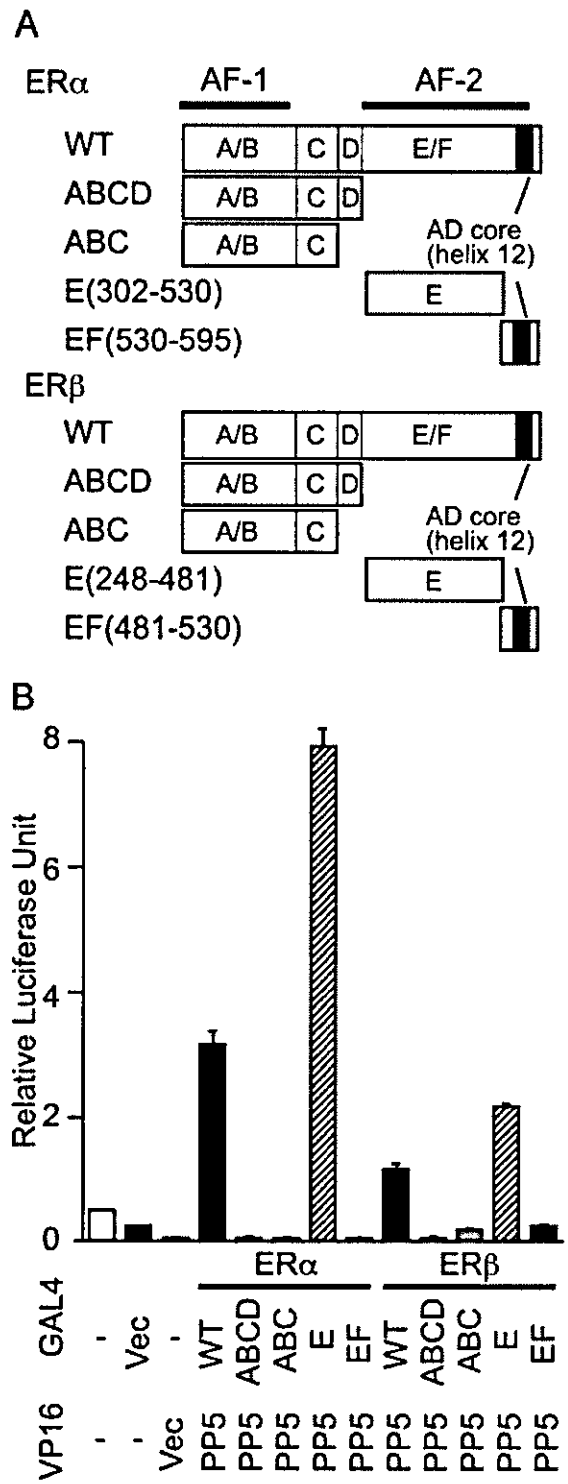

Fig. 3. Interaction of PP5 with ERα and ERβ in Mammalian Two-Hybrid Assays

A, Structure of full-length and truncated mutants of human ERα and ERβ. The cDNAs encoding wild-type (WT) ERα and ERβ or cDNA fragments encoding structural ABCD domains (amino acids 1–302), ABC domains (amino acids 1–263), E domain (amino acids 302–530), and a part of EF domains (amino acids 530–595) of ERα, and ABCD domains (amino acids 1–248), ABC domains (amino acids 1–213), E domain (amino acids 248–481), and a part of EF domains (amino acids 481–530) of ERβ were subcloned into an expression vector pCMX-GAL4 downstream of and in frame with GAL4 DNA binding domain. B, Mammalian two-hybrid assays were carried out using expression vectors of the pCMX-GAL4 containing full-length or mutants of ERα and ERβ (GAL4-ER), and pCMX-VP16 containing PP5 (VP16-PP5), in which PP5 was inserted into pCMX-VP16 downstream of and in frame to the VP16 transactivation domain in pCMX-VP16. 293T cells were cotransfected with 0.7 μg pRL-CMV vector for internal control, 0.8 μg TK-MH100 × 4Luc, 0.2 μg VP16-PP5, and 0.1 μg each pCMX-GAL4 constructs of ERα or ERβ in a well of 24-well plates. The cells were cultured for 24 h and the luciferase assay was performed. Data are the mean ± SD of three independent experiments performed in triplicate.

vector (Fig. 5A), and the phosphorylation level 5 min after EGF stimulation was 29% reduced relative to control cells (Fig. 5B). The maximal S118 phosphorylation levels induced by either E_2 (Fig. 5A) or EGF (Fig. 5B) were approximately 65% and 80% enhanced, respectively, in MCF7 cells transfected with the TPR domains of PP5 compared with control cells. To confirm the inhibitory activity of PP5 on ERα phosphorylation at S118, we investigated the effect of PP5 AS oligonucleotide on the phosphorylation levels of ERα in MCF7 cells. Either E_2-dependent (Fig. 5C) or EGF-dependent (Fig. 5D) phosphorylation of ERα was enhanced by treatment with PP5 AS compared with control mismatch scrambled (Scr) or PP5 sense (Sense) oligonucleotides (~150% increase by 60-min E_2 incubation in AS *vs.* Scr, ~80% increase by 5-min EGF stimulation in AS *vs.* Scr). The results indicate that PP5 has an inhibitory activity on ERα phosphorylation at S118, and the disruption of PP5 activity either by TPR alone or PP5 AS oligonucleotide causes an enhancement of S118 phosphorylation levels.

Suppression of ER Transcription Activity by PP5

To determine whether PP5 affects estrogen-regulated transcription, we next examined transcriptional activity of ERs and ER mutants using an estrogen response element (ERE)-driven luciferase reporter cotransfected with full-length PP5 or truncated PP5 mutants (Fig. 6). PP5 attenuated the ER transcriptional activity in a concentration-dependent manner, as E_2-dependent transactivations by both ERα and ERβ in 293T cells transfected with 50 ng of PP5 were approximately 50% decreased compared with control cells without PP5 transfection (Fig. 6A). Transfection with equal amounts of TPR domain cDNA (5 ng) relative to ER expression vector did not affect E_2-dependent transactivation, whereas 10-fold excess dose of TPR cDNA (50 ng) relative to ER cDNAs elicited a 30–40% increase in E_2-dependent transactivations by both ERα and ERβ compared with control cells. Another PP5 mutant with its catalytic domain alone did not affect ERα and ERβ transactivations. Because levels of S118 phosphorylation of ERα were modulated by PP5 ex-

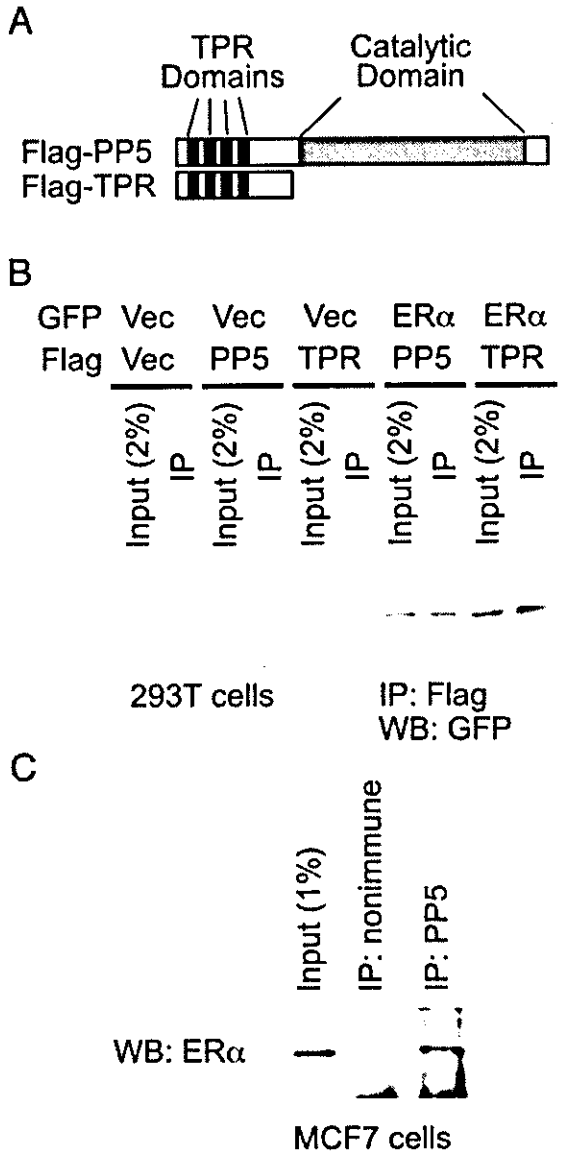

Fig. 4. Interaction and Phosphorylation Inhibition of ERα with PP5

A, Schematic representation of the full-length and TPR domain of PP5. B, Coimmunoprecipitation experiments of PP5 with ERα in 293T cells. Flag-tagged full-length and TPR domain of PP5 or control pcDNA3 vector containing Flag tag (Vec) and GFP-tagged ERα or control pEGFP-C2 vector (Vec) were transfected for 24 h in 293T cells. Cell extracts were obtained after a chemical cross-linking with 200 μg/ml of dithiobis[succinimidyl propionate] (DSP). Extract (1 mg) was incubated with an anti-Flag antibody M2 for 3 h at 4 C and then incubated with protein G-Sepharose beads for 1.5 h at 4 C. Proteins in the immune complexes or 20 μg of the cell extracts (corresponding to 2% of input) were resolved by SDS-PAGE, and the Western blot (WB) was probed with an antibody against GFP. Signals were visualized by an enhanced chemiluminescence system. C, Coimmunoprecipitaion of PP5 with ERα in MCF7 cells. Cell extract (1 mg) from MCF7 cells that were chemically cross-linked with DSP was incubated with either anti-PP5 antibody (PP5/PPT) or nonimmune serum for 3 h at 4 C and then incubated with protein G-Sepharose beads for 1.5 h at 4 C. Proteins in the immune complexes or 10 μg of the cell extracts (corresponding to 1% of input) were resolved by SDS-PAGE, and the Western blot was probed with an antibody against ERα (H-184). IP, Immunoprecipitation.

pression in Fig. 5, we next investigated whether PP5 affects transactivations by ERα mutants on S118. Mutation of S118 to alanine (S118A) causes a reduction in E_2-dependent transcriptional activation in a number of cell types, whereas substitution of S118 to glutamic acid (S118E) resulting in a constitutive negative charge leads to transactivation at levels higher than wild-type ERα (3). E_2-dependent transactivation by S118A was 30% reduced compared with wild-type ERα, whereas that by S118E was approximately 30% enhanced compared with wild-type ERα (Fig. 6B). PP5 expression did not further alter E_2-dependent transactivation by either S118A or S118E, suggesting that PP5 exerts its phosphatase activity on S118. To confirm the inhibitory activity of PP5 on ER-dependent transactivation, we examined the effect of PP5 AS oligonucleotide on the transcriptional activity by ERα (Fig. 6C). The E_2-stimulated ERα transactivation was 2-fold increased by PP5 AS transfection compared with Scr or PP5 sense oligonucleotides transfection, indicating that PP5 negatively regulate ligand-dependent transactivation by ERα.

PP5 Attenuates Induction of Estrogen-Responsive Genes

The ligand-induced ER activation leads to transcriptional induction of estrogen-responsive genes. We next investigated whether PP5 affects E_2-induced expression of the estrogen-responsive genes including pS2 (25), c-*myc* (26), and cyclin D1 (27, 28) by Northern blot analysis (Fig. 7A). Adenoviral expression of PP5 in MCF7 cells markedly suppressed the E_2 (10 nM)-induced mRNA expression of pS2, c-*myc*, and cyclin D1 under the serum-depleted condition, compared with the results of a control adenovirus (Ad-GFP) or vehicle alone. Each mRNA level was normalized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the same sample (Fig. 7, B–D). Approximately 40% reduction of pS2 expression was observed in adenoviral PP5-transduced MCF7 cells 24 h after E_2 stimulation, compared with the gene expression at the same period in control cells including adenoviral GFP-transduced cells and vehicle-transfected cells (Fig. 7B). Regarding c-*myc*, the maximal mRNA expression in Ad-PP5 cells was observed at 2 h after E_2 treatment, and it was only 30% of the maximal expression levels in the control cells (Fig. 7C). The maximal expression of cyclin D1 mRNA in Ad-PP5 cells was delayed compared with that in control cells: the former was at 12 h whereas the latter was at 2 h after E_2 treatment (Fig. 7D). The maximal level of cyclin D1 in Ad-PP5 cells was approximately half of that in vehicle-transfected cells.

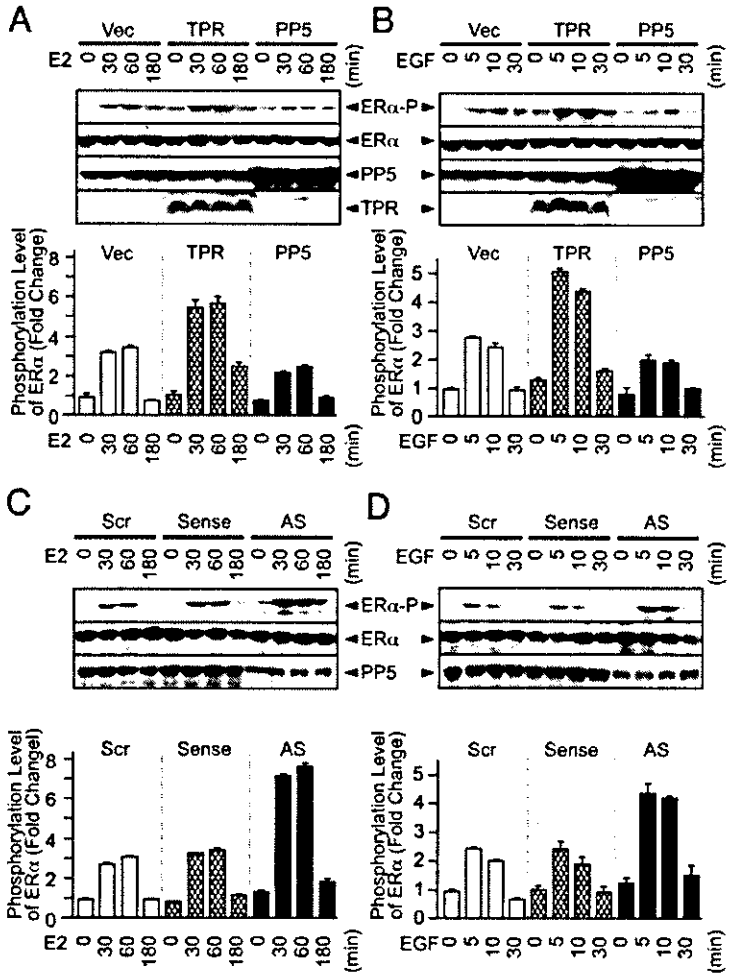

Fig. 5. PP5 Inhibits Phosphorylation of ERα on S118 in MCF7 Cells

MCF7 cells were plated at a density of 1.4×10^5 cells per well on six-well plates overnight, transfected with 1 μg expression plasmids (A and B) or with 1 μg oligonucleotides (C and D) for 12 h, serum starved for 24 h, and subsequently treated with E_2 (10 nM)(A and C) or EGF (100 ng/ml)(B and D) for indicated times. Cell extracts were collected using 80 μl of sample buffer for SDS-PAGE. Extracts (25 μl) were separated by SDS-PAGE and detected by antibody against phosphorylated human ERα on S118 (ERα-P), antibody against whole ERα (H-184), and anti-PP5 antibody (PP5/PPT). A–D, *Upper panels* show representative data from three independent experiments of Western blots after chemiluminescent detection. A–D, *Lower panels* show phosphorylation levels at ERα S118 normalized by total protein amounts of ERα. Quantification of signal intensities was performed using LAS 1000 image analyzer, and data are expressed as the mean ± SD of three independent experiments.

Because S118 phosphorylation levels and ERα transactivation were augmented by treatment with PP5 AS oligonucleotide, we further investigated whether PP5 AS enhances mRNA expression of estrogen-responsive genes (Fig. 8). Transfection with PP5 AS increased basal levels of pS2 (Fig. 8B), c-*myc* (Fig. 8C), and cyclin D1 (Fig. 8D) mRNAs in serum-starved condition (~170%, ~100%, and ~80% increase for pS2, c-Myc, and CycD1, respectively; AS *vs.* Scr). E_2 (10 nM)-induced mRNA expression of those three genes was 25–50% enhanced by PP5 AS compared with PP5 sense or Scr oligonucleotides, without affecting the time course of each gene. The maximal mRNA expression of pS2, c-*myc*, and cyclin D1 detected in 24 h after E_2 stimulation was 25%, 48%, and 36% increased by treatment with PP5 AS, respectively, when compared with Scr oligonucleotide (at 24 h, 2 h, and 6 h after E_2 stimulation for pS2, c-Myc, and CycD1, respectively. $P < 0.05$ for pS2 at 24 h, Scr *vs.* AS; $P < 0.001$ for c-Myc at 2 h, Scr *vs.* AS; $P < 0.05$ for CycD1 at 6 h, Scr *vs.* AS).

Taken together, the data demonstrate that PP5 negatively regulates ER phosphorylation levels, transcriptional activity, and transcriptional induction of estrogen-responsive genes.

DISCUSSION

In the present study, we isolated a serine/threonine phosphatase PP5 as a direct interactant with ERβ through yeast two-hybrid studies. GST pull-down as-

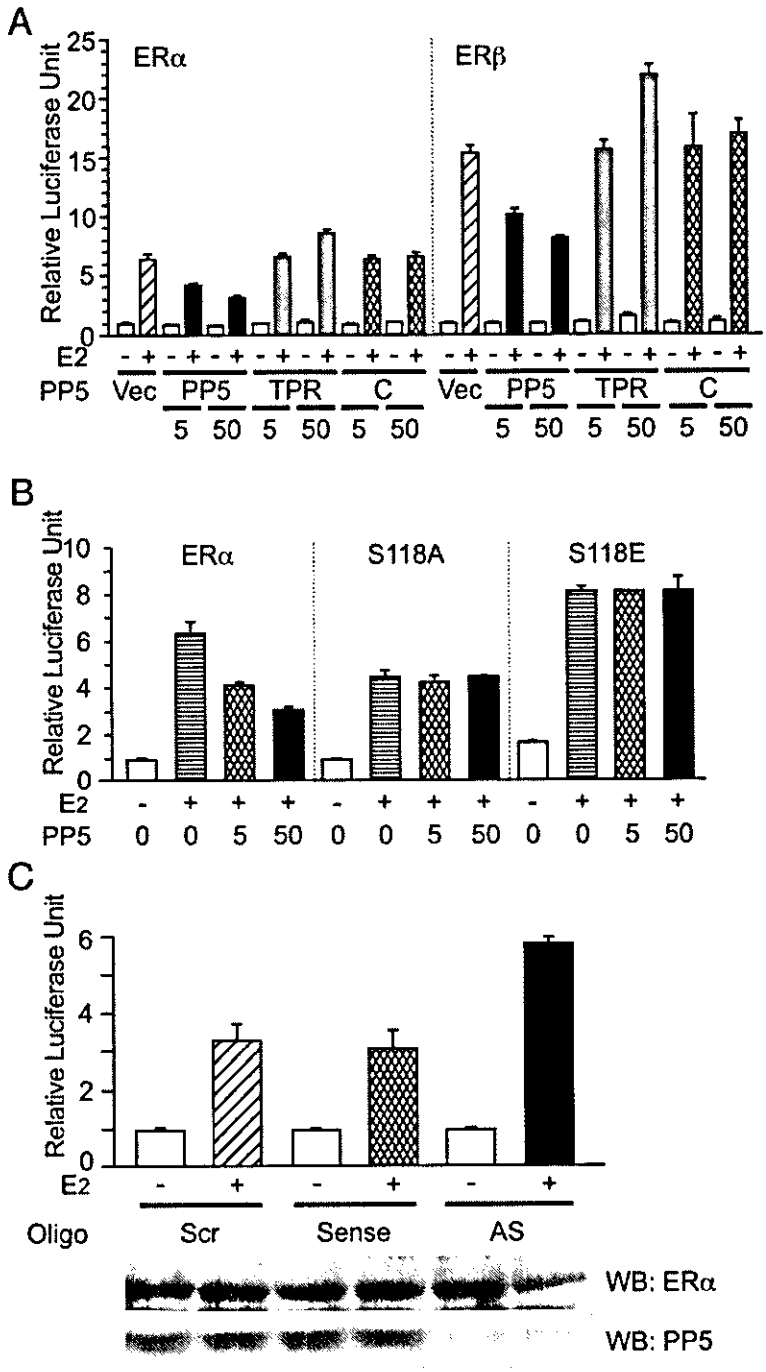

Fig. 6. Inhibition of ER-Mediated Transcription by PP5

A, 293T cells were plated at a density of 1 × 10^4 cells per well on 24-well plates and transfected with 0.8 μg ERE-tk-Luc, 0.7 μg pRL-CMV, 0–50 ng expression vectors for full-length (PP5), TPR domain (TPR), or catalytic domain (C) of PP5, and 5 ng expression vectors for ERα and ERβ. Cells were treated with or without E_2 (10 nM) for 24 h, and luciferase assay were performed. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. B, 293T cells were transfected with 0.8 μg ERE-tk-Luc, 0.7 μg pRL-CMV, 0–50 ng expression vectors for full-length PP5, TPR domain, or catalytic domain (C), and 5 ng expression vestors for wild-type ERα or ERα mutants S118A and S118E. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. C, Effect of PP5 AS oligonucleotide on ER-mediated transcription. Luciferase assays were performed as in panel B except transfection with 0.8 μg ERE-tk-Luc, 0.7 μg pRL-CMV, and 200 ng oligonucleotides into MCF7 cells. *Upper panel* shows the data of three independent experiments performed in triplicate. *Lower panels* show representative data from three independent experiments of Western blots (WB) using the identical cell extracts for luciferase assays. Signals were detected by antibodies against ERα and PP5.

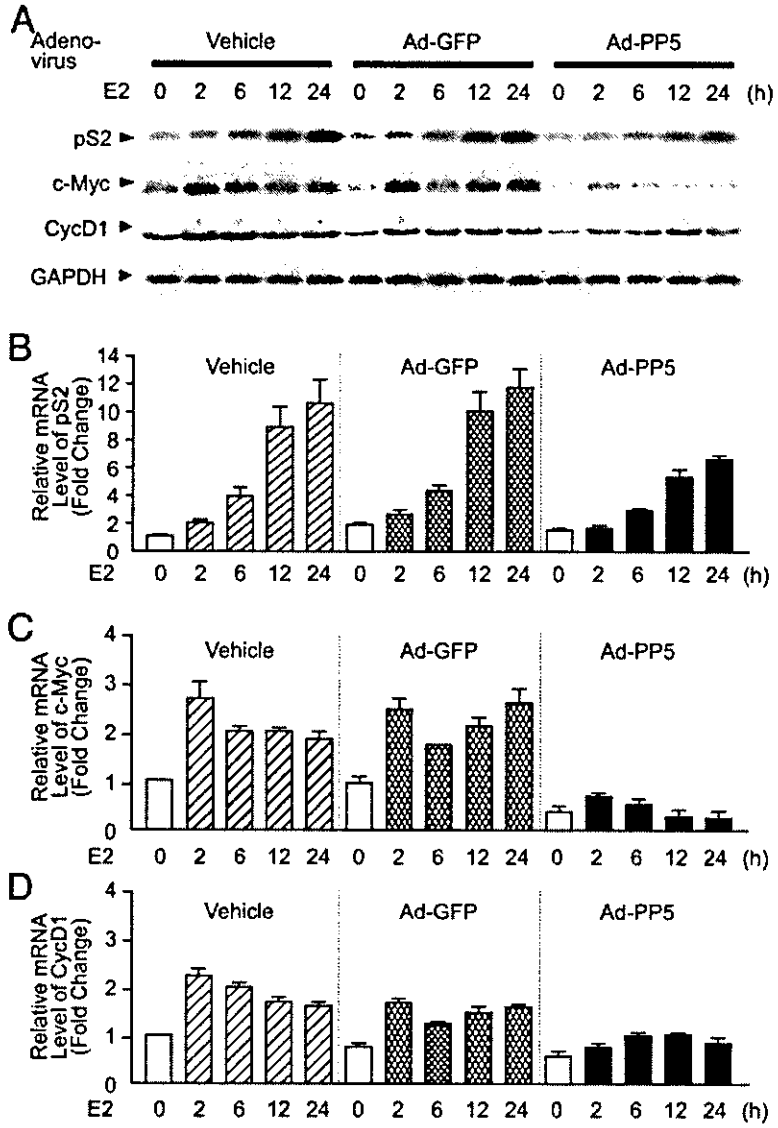

Fig. 7. PP5 Attenuates E_2-Induced Transcription of Estrogen-Responsive Genes in MCF7 Cells

MCF7 cells were plated at a density of 1×10^6 cells per well on 10-cm plates and infected with recombinant adenoviruses expressing PP5 (Ad-PP5) and GFP (Ad-GFP), or transfected with vehicle (Vehicle) for 12 h at a multiplicity of infection of 10. Cells were serum starved for 24 h after the infection and treated with E_2 (10 nM) for indicated times. Northern blot analysis was carried out using 20 μg total RNA and hybridized with ^{32}P-labeled cDNAs for pS2, c-*myc*, cyclin D1 (CycD1), and GAPDH. A, The representative result of three independent experiments of Northern blotting. B, C, and D, Quantification of mRNA levels of estrogen-responsive genes including pS2 (B), c-*myc* (C), and CycD1 (D) after normalization to GAPDH mRNA levels in MCF7 cells after E_2 treatment. Data are expressed as the mean ± SD values of fold change over control from three independent experiments. Data for cells treated with vehicle and harvested at the starting point of E_2 treatments are used as control mRNA levels.

says, mammalian two-hybrid assays, and immunoprecipitation studies show that PP5 interacts with both ERα and ERβ through its TPR domains. We demonstrated that PP5 negatively regulates the degree of both estrogen-dependent and EGF-dependent ERα phosphorylation on S118, leading to the suppression of ER transcriptional activity and the reduction of E_2-induced expression of estrogen-responsive genes including pS2, c-*myc*, and cyclin D1. We thus present the first evidence that PP5 acts as an inhibitory regulator of ER signaling pathway in a negative direction.

The status of ER phosphorylation can be regulated by phosphatases, as okadaic acid treatment enhances ERα phosphorylation and ERα transactivation (29). While preparing this manuscript, another member of the PPP family PP2A has been found to interact with ERα and dephosphorylate S118 of the receptor (30). Here we have shown the evidence that ER phosphorylation and function can be modulated by PP5. There are several differences between PP5 and PP2A regarding the interactions with ERs. In our results, the TPR domains of PP5 are the sites responsible for