Studies on the Effects of Estrogen Stimuli on Osteogenesis

Hyejin Kim

Department of Biological Science and Technology,
Faculty of Engineering,
The University of Tokushima

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CHAPTER I
General introduction

1. Bone development and remodeling

Two ossification processes occur during the fetal development of the mammalian skeletal system, intramembranous ossification and endochondral ossification. During intramembranous ossification, mesenchymal cells differentiate directly into bone and form the flat bones of the skull. In this process, mesenchymal cells first differentiate into osteoblasts, which are a type of bone-forming cells. Next, the osteoblasts begin to deposit unmineralized osteoid. Because calcium phosphate is deposited by osteoblasts, the osteoid forms new bone [Bradley *et al*., 2011].

During endochondral ossification, mesenchymal cells give first rise to a cartilaginous frame and are then ossified and replaced, leading to the formation of long bones such as limbs (Fig. 1). In particular, mesenchymal cells differentiate into chondrocytes to form a temporary cartilage model. The cartilage model grows, the chondrocytes mature and become hypertrophic, and the growing cartilage model then starts to calcify. Chondrocytes undergo apoptosis due to their distance to blood vessels and limited uptake of nutrients, etc. Osteoprogenitor cells and blood vessels from the periosteum invade this area; the osteoprogenitor cells proliferate and differentiate into osteoblasts, which then begin to lay down a bone matrix. Osteoclasts, formed from macrophages, assist in the removal of cartilage matrix. In the fetus, primary ossification develops first in the diaphysis and a secondary ossification center forms later in the epiphysis

[Gerver *et al*., 2000, Mackie *et al*., 2008, Gerver *et al*., 2000].

During fetal and postnatal life, continuous bone remodeling occurs in a coordinated action involving bone-forming osteoblasts and bone-resorbing osteoclasts to maintain the bone mass.

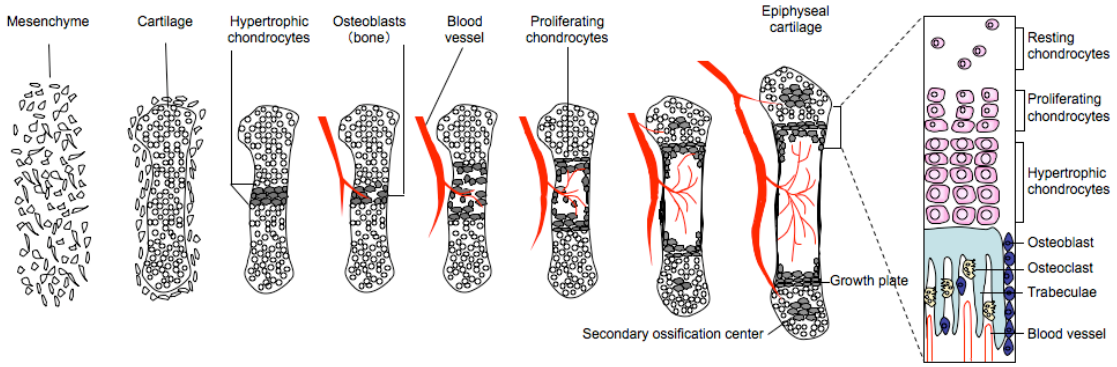

Fig.1 Endochondral ossification

2. Role of estrogen in osteoporosis

Osteoporosis is a disease caused by low bone mass due to increased osteoclast activity and/or decreased osteoblast activity. This disease is thought to be due to a multi-factorial condition such as low calcium intake, low vitamin D levels [Gennari, 2001], and low sex hormone levels [Albright *et al.,* 1940]. In particular, postmenopausal osteoporosis is caused primarily by estrogen deficiency.

With regard to its effects on bone resorption, estrogen has been shown to induce apoptosis in bone-resorbing osteoclasts [Kousteni *et al.,* 2002], and estrogen receptor (ER) knockout mice have an increased total number of osteoclasts due to the absence of

estrogen-induced osteoclast apoptosis [Parikka *et al*., 2005]. ERα expression in osteoclasts is required for apoptosis via estrogen and expression of the Fas ligand in bones leads to estrogen-induced apoptosis [Nakamura *et al*., 2007]. Moreover, estrogen affects osteoclast survival through upregulation of the Fas ligand in osteoblasts [Krum *et al*., 2008].

During bone formation, estrogen increases osteoblast proliferation and decreases osteoblast and osteocyte apoptosis [Kousteni *et al*., 2002]. However, the detailed molecular mechanisms, e.g., identification of the peptide/protein factors or enzymes associated with osteoblast differentiation and the signal network—from the stimulation by estrogen to the expression of genes participating in bone differentiation—have not been clarified yet.

3. Characterization of subtilisin-like proprotein convertases (SPCs)

Many bioactive proteins such as peptide hormones and growth factors, including bone morphogenetic proteins (BMPs), are biosynthesized as inactive precursor proteins that convert into the mature form through partial cleavage by specific processing proteases at the C-terminal tail of basic amino acids with the recognition motif RXXR; the RXK/RR motifs provide an optimum processing site [Molloy *et al*., 1992] (Table 1). The partial cleavage process is required for the biosynthesis of bioactive proteins.

Homologues of kexin, which is a processing protease first isolated from yeast, were subsequently isolated in mammals in the following order: Furin [Fuller *et al.*, 1989], PC1/PC3 and PC2 [Smeekens *et al.*, 1990], PACE4 [Kiefer *et al*., 1991], PC4

[Nakayama *et al*., 1992], PC5/PC6 [Nakagawa *et al*., 1993], and PC7/PC8 [Bruzzaniti *et al*., 1996]. These proteases are regarded as subtilisin-like proprotein convertases (SPCs) because they are serine proteases with Ca^{2+} dependency and their catalytic region is similar to that of subtilisin (Fig. 2).

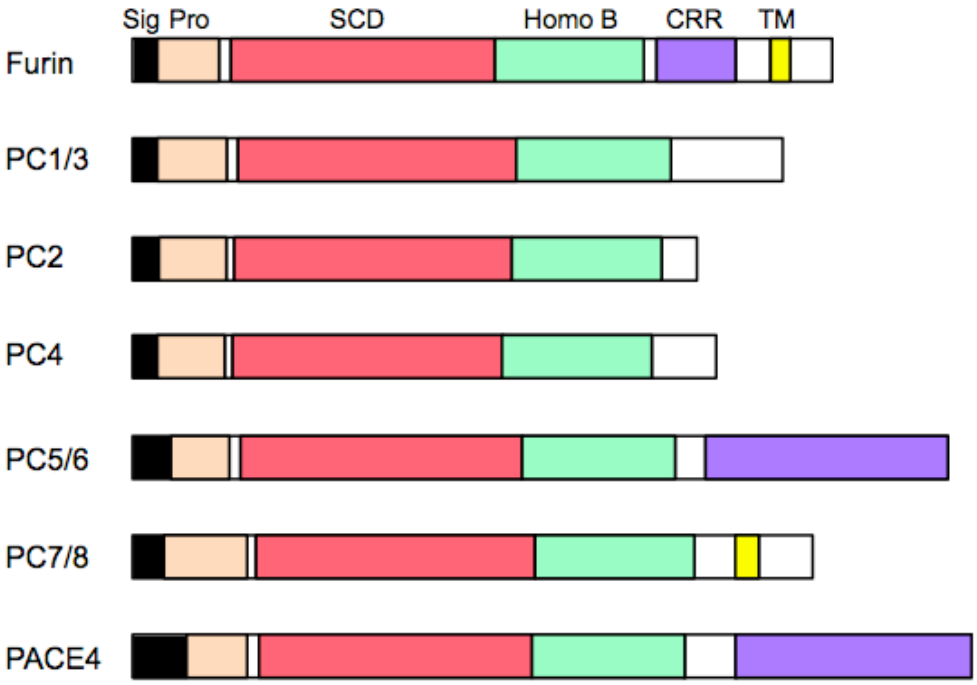

Fig.2 Domain structure of subtilisin-like proprotein convertases

Sig: signal peptide, Pro: propeptide, SCD: subtilisin-like catalytic domain
Homo B: homologous domain B, CRR: cysteine-rich region, TM: transmembrane domain

The N-terminal domain is conserved in all family members, whereas great diversity is found in the C-terminal domains of the different SPCs. These proteases become active through autocatalytic cleavage of an N-terminal propeptide. The propeptide domain functions as chaperone for the proenzyme [Nakayama, 1997], and the Homo B

domain, as well as the catalytic domain, are required for autocatalytic activation [Takahashi *et al*., 1995]. PACE4 and PC5/6 as extracellular matrix-bound enzymes have a heparin-binding region in the cysteine-rich region [Tsuji *et al*., 2003]. PACE4 is conserved in humans and mice, and the knockout mouse of this gene has been previously shown to have bone-morphogenesis defects [Constam *et al*., 2000] (Table 2).

Table.1: Sequence around the cleavage site of potential precursor proteins in mouse bones

Precursor proteins	Sequences at the cleavage site
Osteocalcin	VNRLRR↓YL
ChM-1	IQRERR↓EV
PTHrP	SRRLKR↓AV
FGF23	PRRHTR↓SA
BMP family	
BMP1	RPRSRR↓AA
BMP2	HKREKR↓GA
BMP3	KKARRK↓GW
BMP4	RRRAKR↓SP
BMP5	LLRSVR↓AA
BMP6	HVRTTR↓SA
BMP7	HLRSIR↓ST
BMP8	PVRAPR↓AA
BMP9	LARRKR↓ST
BMP10	SARIRR↓NA
BMP15	LMRSVR↓GA

Arrows indicate the predicted cleavage sites.

Table.2: Comparison of human and mouse PACE4

Chromosomal localization		Identity	Gene length	Exon number	Tissue distribution	Knockout mouse
human	mouse	86%	>250 kb	22	nervous system, heart, liver, bone	75% viable phenotype with bone morphogenesis defects, cyclopia
15q26	7					

CHAPTER II

Estrogen stimuli promote osteoblastic differentiation via PACE4 in MC3T3-E1 cells

1. Introduction

Bone mass is constantly maintained through the so-called bone remodeling process, which consists of bone formation by osteoblasts and bone resorption by osteoclasts. However, bone mass decreases with age, especially in postmenopausal women with low blood estrogen levels [Albright *et al.,* 1940]. These women are predisposed to osteoporosis due to the collapse of the balance between bone formation and resorption, which results in net bone resorption [Jasani *et al*., 1965; Riggs *et al.,* 2002]. Because osteoporosis-related fractures are very problematic for elderly people [Kanis *et al.,* 2004], endochondral ossification is required for fracture healing and where a cartilage frame formed by chondrocytes is replaced with bone, estrogen replacement therapies for the treatment and prevention of osteoporosis are particularly important [Riggs *et al.,* 2002; Scammell *et al.,* 1996]. For example, the soybean isoflavone daidzein (Diz) is a phytoestrogen with estrogenic activity and its intake is effective for the maintenance of bone mass in postmenopausal women [Ma *et al.,* 2008]. Moreover, equol (Eq), which is a metabolite of Diz, has been shown to have greater estrogenic activity than Diz. [Setchell *et al.,* 2002].

It has been reported that estrogen induces apoptosis in osteoclasts [Kameda *et al.,* 1997], and the number of osteoclasts has been found to increase in ovariectomized mice [Kousteni *et al.,* 2002]. While estrogen suppresses bone resorption, it was also shown to attenuate apoptosis through activation of the Src/Shc/ERK signaling pathway in osteoblasts [Kousteni *et al.,* 2001]. Furthermore, estrogen stimuli, including phytoestrogens such as Diz, enhanced bone mineralization of a murine osteoblastic cell line [Kanno *et al.,* 2004]. Thus, understanding the mechanism of how estrogen stimuli facilitate bone formation is important to develop treatment and prevention therapies for osteoporosis. However, the detailed molecular mechanisms underlying bone formation triggered by estrogen stimuli have not been clarified yet. It is well known that BMPs are highly linked with ossification and bone formation via Smad signaling. It is thought that several factors related to ossification, including BMPs, have to undergo proteolytic processing to convert to the active form; furthermore, the SPC family, which are serine endoproteases, play key roles in activating the precursors of BMPs with a consensus cleavage motif for SPCs [Constam *et al.,* 1999]. Recently, it has been reported that an SPC regulates the hypertrophic conversion of murine chondrocytes through activation of BMP6 [Yuasa *et al.,* 2012]. Therefore, in the present study, I investigated the mechanism underlying the effect of estrogen stimuli on osteoblasts, with focus on the role of SPCs.

2. Materials and methods

2-1. Cell culture

ATDC5, a mouse chondrogenic cell line derived from embryonal carcinoma cells, was provided by Drs. Yuji Hiraki and Chisa Shukunami of Kyoto University [Yuasa *et al.,* 2012]. Cells were cultured in growth medium consisting of Dulbecco's modified Eagle's medium:Ham's F12 (1:1) containing 5% fetal bovine serum (FBS) (inactivated by treatment at 56°C), 10 μg/mL transferrin, 30 nM sodium selenite, 100 U/mL penicillin G potassium, and 100 μg (titer)/mL streptomycin sulfate. MC3T3-E1, an osteoblast cell line from C57BL/6 mouse calvaria, was obtained from the RIKEN Cell Bank (No. RCB1126). MC3T3-E1 cells were cultured in Minimum Essential Medium Alpha (Wako) containing 10% FBS, 100 U/mL penicillin G potassium, and 100 μg (titer)/mL streptomycin sulfate. Cell culture of both cell lines was carried out in a 5% CO_2-air incubator at 37°C.

2-2. Differentiation culture with estrogen stimuli

ATDC5 cells and MC3T3-E1 cells were seeded at 1×10^4 cells/cm^2 into wells of 24-well culture plates containing 0.5 mL/well or in 35-mm culture dishes containing 2 mL/dish of the respective growth medium. When the cells reached confluence, the

medium was replaced with the same volume of differentiation medium containing FBS or charcoal-dextran-treated FBS (CD-FBS). CD-FBS was prepared by treatment of FBS with dextran-coated charcoal (Sigma) to reduce the amount of endogenous estrogen, as described previously [Kanno *et al.,* 2004]. Briefly, 10 g of dextran-coated charcoal and 200 mL of FBS were mixed at 37°C for 1 h with constant stirring. The treated FBS was centrifuged at 2500 × *g* for 20 min, and the supernatant was sterilized by filtration through a 0.2-μm filter. Differentiation medium for ATDC5 cells was prepared by addition of 10 μg/mL insulin and that for MC3T3-E1 cells was prepared by addition of 5 mM β-glycerophosphate and 0.2 mM L-ascorbic acid to the growth medium. Estrogenic agents such as 17β-estradiol (E2) and the soybean isoflavones Diz and the S-form of Eq were dissolved in dimethyl sulfoxide and added to the differentiation medium containing CD-FBS. To evaluate the dependency of the effect of estrogenic agents on the estrogen receptor (ER), the ER inhibitor fulvestrant (Sigma) was added to the differentiation medium at 1 μM and incubated as described above.

2-3. Alcian blue staining and alizarin red S staining

After incubation in the differentiation medium for the designated time, the differentiation of ATDC5 cells and MC3T3-E1 cells was monitored by alcian blue staining and alizarin red S staining, respectively. ATDC5 samples were washed three times with phosphate-buffered saline and then fixed with 99% methanol at room temperature. After 20 min, the cells were stained with alcian blue for 1 day and then washed with dH_2O. MC3T3-E1 samples were washed three times with phosphate-buffered saline and then fixed with ice-cold 70% ethanol for 1 h on ice. Subsequently, the cells were washed with distilled H_2O and stained with alizarin red S for 20 min at room temperature. To quantify the relative differentiation, their absorbance at 520 nm was measured in a spectrometer (Tecan Infinite M200) after several washes with distilled H_2O.

2-4. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from MC3T3-E1 cells during the time course using the Qiagen RNeasy mini kit (Qiagen). Subsequently, cDNA was synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Both steps were performed according to the manufacturer's instructions. RT-PCR performed using 1 μL of template solution containing cDNA equivalent to 100 ng mRNA was performed for 15, 22, and 25 cycles for glyceraldehyde-3-phosphate dehydrogenase (for the data in Figs. 6 and 7) and all other genes, respectively, by using a gene-specific primer set: an initial step at 95°C for 2 min, followed by 95°C for 1 min, 50°C or 53°C for 1 min, and 72°C for 1 min (Table 3). Each primer set corresponding to the target genes was designed to amplify a partial region of the entire cDNA (Fig. 3). qRT-PCRs were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol with primer sets for glyceraldehyde-3-phosphate dehydrogenase, ERα, ERβ, and PACE4 (Table 3); those for furin [Hwang *et al.,* 2006] and PC7 [Marchesi *et al.,* 2011] were performed using the ABI Prism 7000 Sequence Detection System.

Table 3: Primer sets for RT-PCR/qRT-PCR

Gene	Primer Sequence	Gene	Primer Sequence
ER α	Fw: 5'-cagctcaacagcgtgtcgccta-3'	BMP1	Fw:5'-gccgaggaaggctatggcgtgga-3'
	Rev: 5'-gtttcctttctcgttactgctgg-3'		Rev:5'-gcttgtgtaccgcaggtggaagc-3'
ER β	Fw: 5'-atggccaacttctggacacctc-3'	BMP2	Fw:5'-ccaggagcgcccgccccagacc-3'
	Rev:5'-cttggcgcttggactagtaa-3'		Rev:5'-tggttggtgtgtccctgtgt-3'
GR	Fw:5'-acctcaataggtcgaccagc-3'	BMP3	Fw:5'-ggtcatctgtctgtagatgtgg-3'
	Rev:5'-cccgccaaaggagaaagcaag-3'		Rev:5'-caagggcagcaagatcccagtag-3'
SOX5	Fw:5'-cttggtgctgccgtgtctcctacc-3'	BMP4	Fw:5'-cgctggacccgggaaaagcaac-3'
	Rev:5'-atagcctattgtgctaactcttgc-3'		Rev:5'-gcgacggcagttcttattcttctt-3'
SOX9	Fw: 5'-gcaaagttgatctgaagcgagagg-3'	BMP5	Fw:5'-ctcatcagaggaggcattacaaaga-3'
	Rev:5'-ccagtgtaggtgacctggccgt-3'		Rev:5'-ctgcacagagctgtaagcccaaa -3'
RUNX2	Fw:5'-tcgcctcagtgatttagggcgca-3'	BMP6	Fw:5'-ggcggtgcgtccccactgacta-3'
	Rev:5'-gtggcagtgtcatcatctgaaatac-3'		Rev:5'-gccccatgttgtgctgcggtgt-3'
Osterix	Fw:5'-tccctacccagcgccccacctct-3'	BMP15	Fw:5'-catcaaaccaggtagcatacg-3'
	Rev:5'-ctgtgaatgggcttcttcctcagc-3'		Rev:5'-gagtagcaagaaggcaacatccaag-3'
Osteopontin	Fw:5'-gatgaatctgacgaatctcacc-3'	ChM-1	Fw:5'-gaactcgctgatttgggtggccgt-3'
	Rev:5'-ctcagaagctgggcaacagggat-3'		Rev:5'-ctggtggtaaggattgtcagggt-3'
Osteocalcin	Fw:5'-ccctgagtctgacaaagccttca-3'	PTHrP	Fw:5'-gatcgcggagatccacacagcc-3'
	Rev:5'-tactggtctgatagctcgtc-3'		Rev:5'-cgcttctttttctcctgttctc-3'
FGF23	Fw:5'-gagaatggctatgacgtctacttg-3'	furin	Fw:5'-gagaatgatgtggagatcatccgtg-3'
	Rev:5'-gctcgcgagagcaggataca-3'		Rev:5'-tctgagtccgatgggcactcct-3'
FGFR1	Fw:5'-gttcaagcagttggtggaagtcc-3'	PC1	Fw:5'-cctcggaggtcccgaagaagc-3'
	Rev:5'-cagcgccgtttgagtccactg-3'		Rev:5'-gtgtgattccactccaagccatca-3'
FGFR2	Fw:5'-gaggaatacttggatctcacccag-3'	PC2	Fw:5'-acctggagcacgtccaagctgtc-3'
	Rev:5'-aacactgccgtttatgtgtggatac-3'		Rev:5'-ccgtgaagcatcagggtccattc-3'
FGFR3	Fw:5'-cgcatcctcactgtgacatcaacc-3'	PC4	Fw:5'-cactcactactgggatgagga-3'
	Rev:5'-cgttactgggtggacctgggg-3'		Rev:5'-cagctggcctgtccttcggt-3'
Col-I	Fw:5'-gagaagtctcaagatggtggc-3'	PC6	Fw:5'-gtcttcagggatcccgctgttcg-3'
	Rev:5'-gcggggtcggagccctcgctt-3'		Rev:5'-gaatcctggcccattgcatgtc-3'
Col-II	Fw:5'-ccaccccgagtggaagagcggaga-3'	PC7	Fw:5'-ctggatggagtggattcagagc -3'
	Rev:5'-cagccatccttcagggcagtgtat-3'		Rev:5'-agcaaatctgcccgctcttccc -3'
Col-X	Fw:5'-ggcagcagcattacgacccaag-3'	PACE4	Fw:5'-accgggtacctacttcgattca-3'
	Rev:5'-gcattgggcaattggagccatacc-3'		Rev:5'-tcgcagctcaggcagttctc-3'
ALP	Fw:5'-cactcgggtgaaccacgccaca-3'	GAPDH	Fw:5'-gggtggagccaaacgggtc-3'
	Rev:5'-ctgatgagatccagaccatctagcc-3'		Rev:5'-ggagttgctgttgaagtcgca-3'

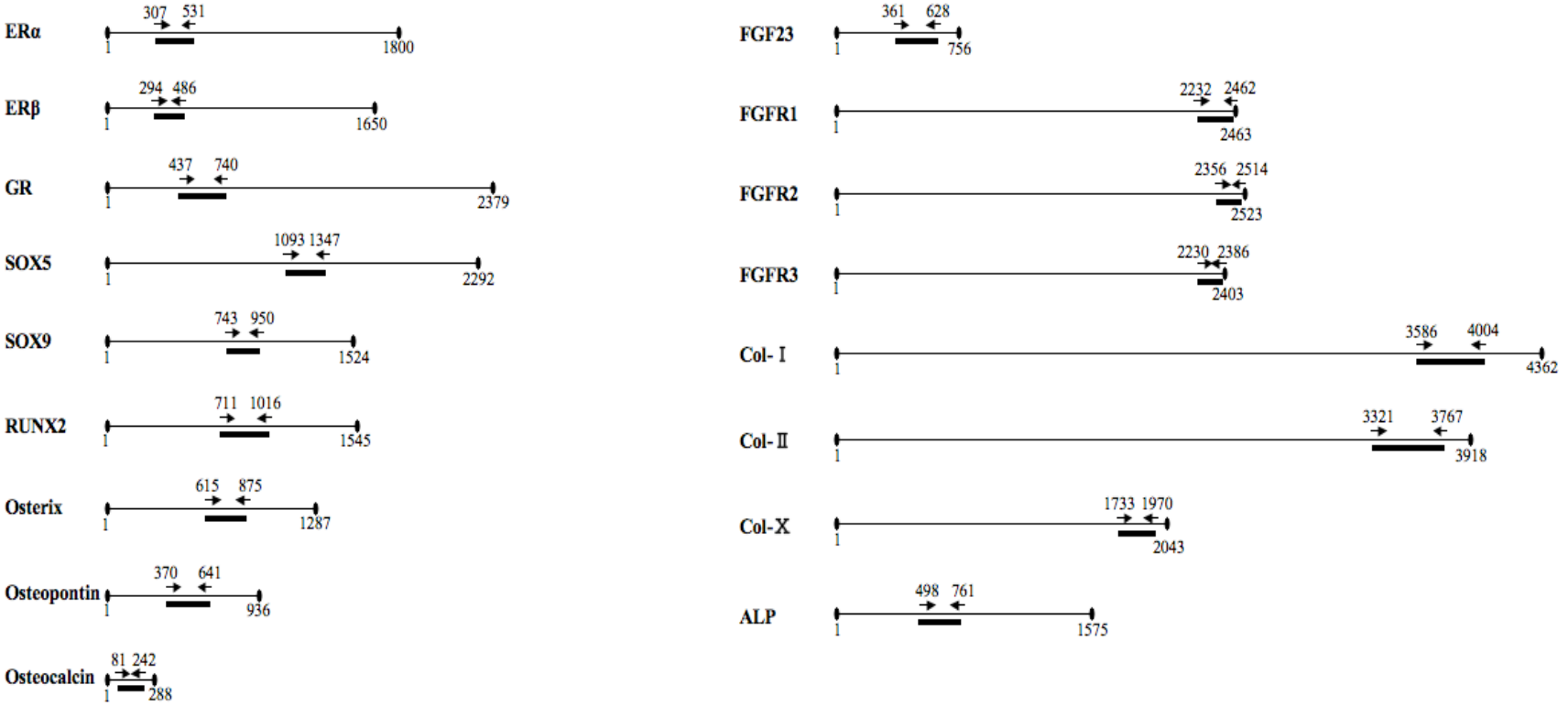

Fig 3. Location of the primer sets in cDNA of the target genes

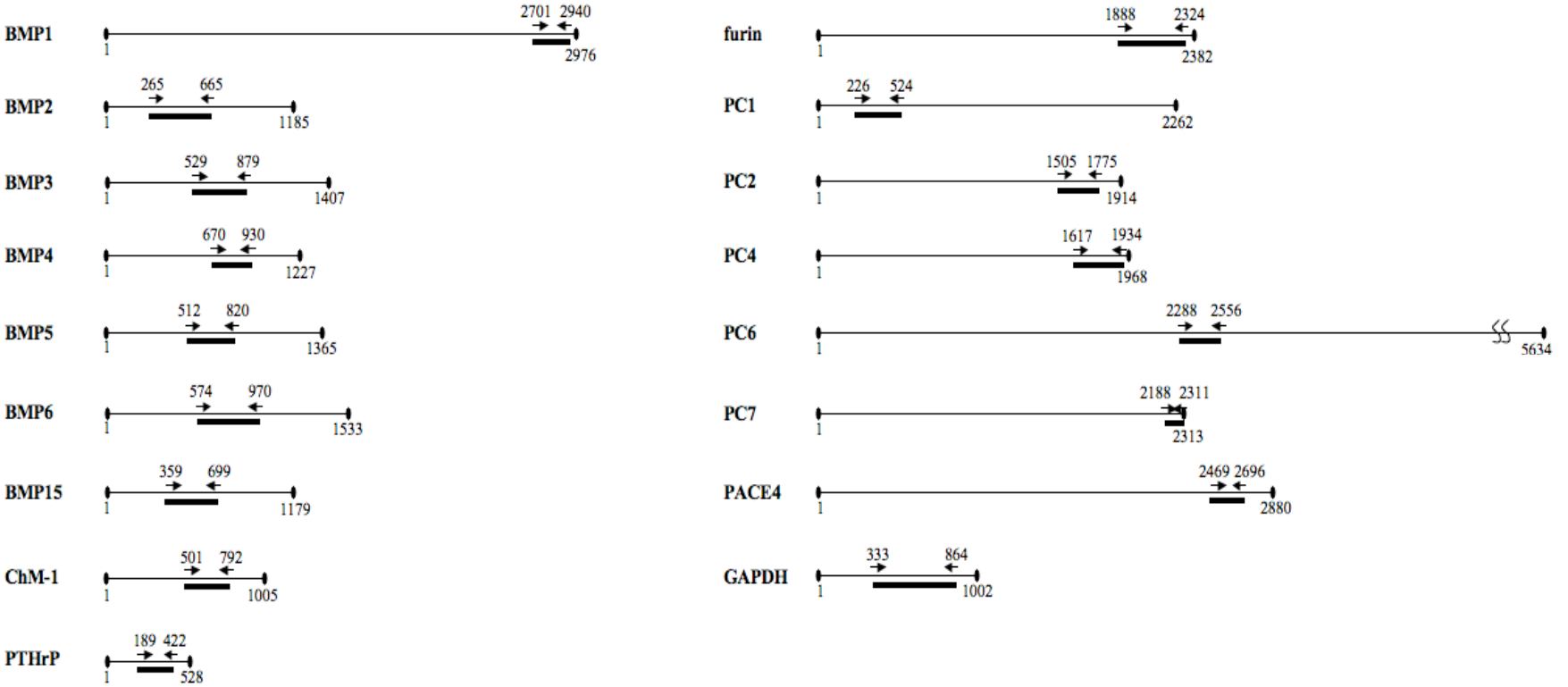

Fig 3. Location of the primer sets in cDNA of the target genes

2-5. PACE4 knockdown in MC3T3-E1 cells

MC3T3-E1 cells were grown in 35-mm dishes to 50% confluence and then transfected with 2 μg of a PACE4 or green fluorescent protein (GFP) shRNA-expressing vector [Yuasa *et al.,* 2009] using the FuGENE® 6 Transfection Reagent (Roche) according to the manufacturer's protocol. PACE4 and GFP shRNA-expressing vectors were generated by ligation of the sequence corresponding to shRNA against mPACE4 (G^{429}CGAAGTGACTCTCTTTATT^{448}) and the GFP target sequence (as recommended by the manufacturer) into the vector pSilencer 3.1-H1 neo (Ambion). After 24 h of cultivation, the used medium was replaced with fresh growth medium containing 400 μg/mL G418 (Nacalai Tesque). This selection step was continued for 2 weeks; the medium was changed every 3 days. The cells were then applied to the cell differentiation assay and subjected to qRT-PCR.

3. Results

3-1. Differentiation of MC3T3-E1 cells induced by estrogen stimuli

To confirm the pharmacological effects of estrogen stimuli on ossification and to investigate their effect on the prevention of osteoporosis, the ATDC5 chondrogenic cell

line and the MC3T3-E1 osteoblastic cell line were used as typical model cells responsible for bone formation. Cells were cultured with the endogenous estrogen E2 or the phytoestrogen Diz and the S-form of Eq in differentiation medium containing CD-FBS. First, CD-FBS was prepared by CD treatment to reduce the amount of endogenous estrogens. The E2 concentration was less than the detection limit (<10 pg/mL); CD-FBS was used for cell cultivation to mimic the estrogen-poor condition observed in postmenopausal women. E2 is the predominant estrogen, and Diz is a typical soybean isoflavone with phytoestrogen activity. Diz is metabolized to Eq by intestinal bacteria such as *Lactococcus garvieae* [Uchiyama *et al.,* 2004]; Eq is known to be a potent soybean isoflavone. Because the structures of Diz and Eq are similar to that of E2 (Fig. 4), these compounds are able to bind to both ERα and ERβ [Usui *et al.,* 2006]. As shown in Figure 5, the presence of estrogen stimuli did not significantly affect chondrogenic differentiation of ATDC5 cells (Fig. 5a). On the other hand, in MC3T3-E1 cells, the cultivation period-dependent mineralization was significantly decreased in culture medium containing CD-FBS compared to that containing normal FBS. Moreover, the mineralization ability was restored by treatment with Eq, Diz, or

E2, even on culture in a medium containing CD-FBS (Fig. 5b). The differentiation of MC3T3-E1 cells by these estrogen stimuli was dose-dependent, and Eq was approximately 10-fold more effective than Diz (Fig. 5c). Furthermore, these inductive effects were inhibited by addition of the selective ER downregulator fulvestrant, which could bind to both ERα and ERβ [Tremblay *et al.,* 1997, Paige *et al.,* 1999] (Fig. 5d). As expected, these results suggest that the induction effect of isoflavones is dependent on the ER.

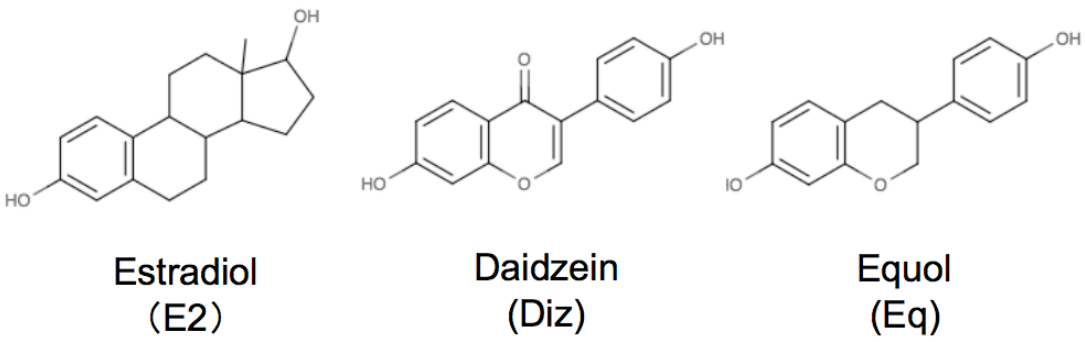

Fig. 4: Chemical structures of estradiol, daidzein, and equol.

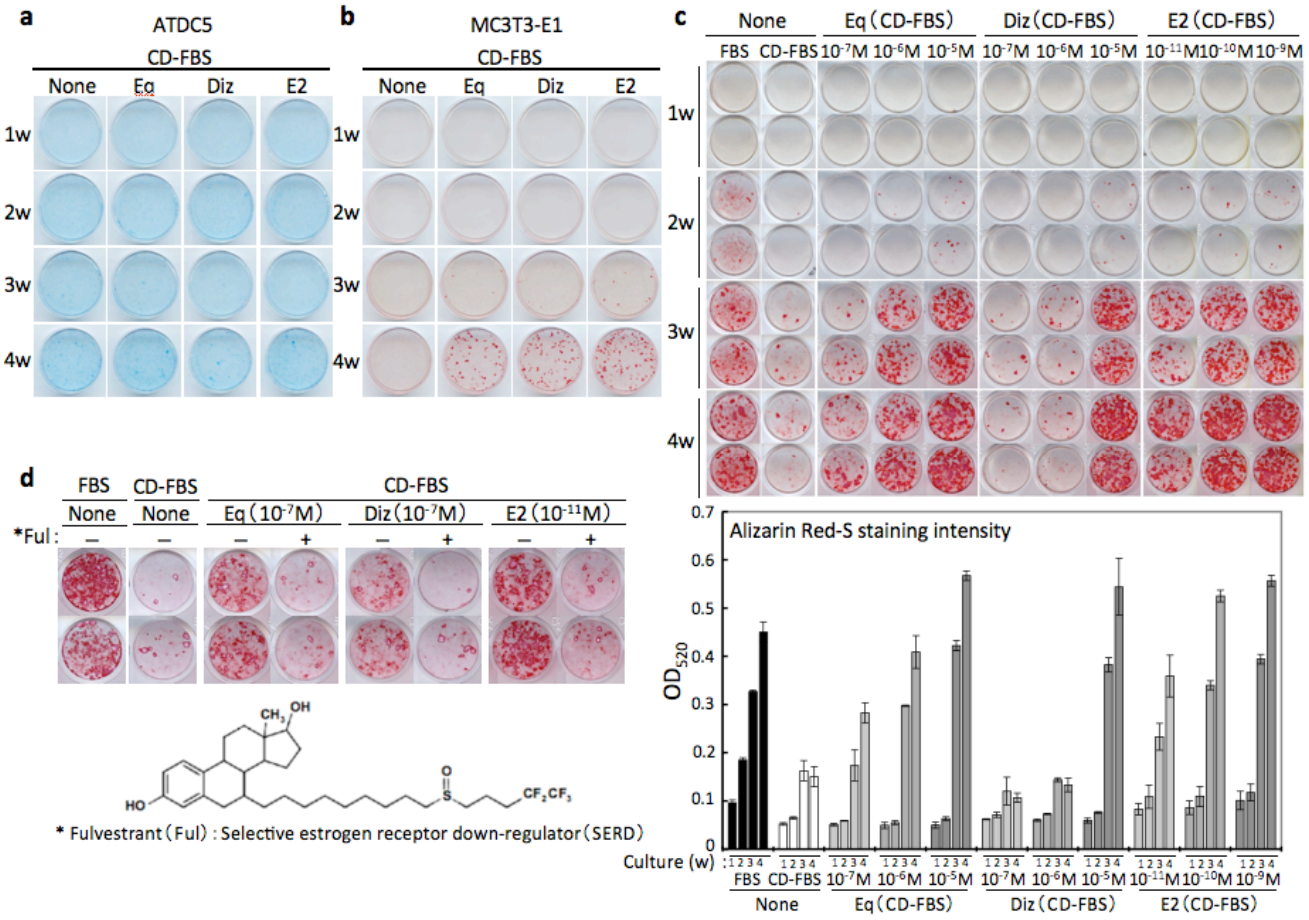

Fig. 5: Effects of the estrogenic agents equol, daidzein, and estradiol on the differentiation of ATDC5 cells and MC3T3-E1 cells.

Both cell lines were cultured in the respective differentiation medium containing charcoal-dextran-treated fetal bovine serum (CD-FBS) with no estrogen stimulus, 10^{-5} M equol (Eq), 10^{-5} M daidzein (Diz), or 10^{-9} M estradiol (E2). After each cultivation period, the differentiation of ATDC5 and MC3T3-E1 cells was detected by alcian blue staining (**a**) and alizarin red S staining (**b**), respectively. **c** To investigate the dose response of the estrogen stimuli in MC3T3-E1 cells, the cells were cultured in differentiation medium containing FBS only, CD-FBS only, CD-FBS with Eq (10^{-7}, 10^{-6}, or 10^{-5} M), CD-FBS with Diz (10^{-7}, 10^{-6}, or 10^{-5} M), or CD-FBS with E2 (10^{-11}, 10^{-10}, or 10^{-9} M) (upper picture). Subsequently, MC3T3-E1 cells were stained by alizarin red S to confirm mineralization, and the staining intensity was quantified by measuring the absorbance at 520 nm (n = 2) (lower graph). **d** To investigate the effect of fulvestrant (Ful) on the differentiation induced by estrogenic agents, MC3T3-E1 cells were co-cultured for 4 weeks with each estrogenic agent (10^{-7} M Eq, 10^{-7} M Diz, and 10^{-11} M E2) in differentiation medium containing CD-FBS and 10^{-6} M Ful.

3-2. Effect of estrogen stimuli on the expression patterns of bone-related genes in MC3T3-E1 cells

The molecular effects of estrogen stimuli on osteoblast differentiation were investigated by analyzing the expression patterns of bone-related genes in MC3T3-E1 cells. Bone-related genes include the following factors: ER and glucocorticoid receptors responsible for steroid hormone signaling (ERα, ERβ, and GR), collagens that are a type of differentiation markers of osteoblasts (type I collagen [Col-I], type II collagen [Col-II], and type X collagen [Col-X]), transcription factors related to mesenchymal stem cell differentiation (SOX5, SOX9, RUNX2, and osterix), extracellular matrix proteins (osteopontin and osteocalcin), fibroblast growth factors (FGFR1, FGFR2, FGFR3, and FGF23), factors associated with the differentiation of osteoclasts (PTHrP, RANKL, and osteoprotegerin), BMPs (BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, and BMP15), SPCs (furin, PC1, PC2, PC4, PC6, PACE4, and PC7), chondromodulin-1 (ChM-1), which promotes chondrocyte differentiation, and the bone differentiation marker alkaline phosphatase (ALP). Transcription levels of several genes changed during the differentiation of MC3T3-E1 cells (Figs. 6a-6d). For example, the transcription of Col-II decreased, while those of osteocalcin and

BMP4 increased as differentiation progressed. Most genes were not affected by estrogen stimuli. However, I found that the transcription of PACE4 was upregulated by estrogen stimuli as differentiation progressed (Fig. 6e). Other SPCs such as furin and PC7 were also expressed in MC3T3-E1 cells, but their expression did not depend on estrogen stimuli. No significant expression of other SPCs was observed. Subsequently, I quantitatively estimated the relative transcription level of the PACE4 gene in a time course study in the presence and absence of estrogen stimuli (Fig. 7a). As shown in Figure 7a, PACE4 gene transcription was significantly induced by Diz, Eq, and E2 at the late stage of osteoblast differentiation. Interestingly, sequencing of the amplicon of the PACE4 gene fragment revealed that the PACE4 isoform expressed in MC3T3-E1 cells was PACE4A-II, the expression of which has been confirmed in placenta and HepG2 cells [Mori *et al.,* 1999] (GenBank Acc. No. NM_138319.2). Moreover, because I found that the ERα mRNA levels tended to increase in response to the lack of estrogen stimuli (Fig. 6e), I quantitatively analyzed the mRNA levels of ERα and ERβ (Figs. 7b and 7c). ERα expression was upregulated over time in the absence of estrogen stimuli. However, in medium containing endogenous estrogen or in the

presence of the exogenous estrogens E2, Eq, and Diz, its expression level in MC3T3-E1 cells was unchanged or decreased as osteoblast differentiation progressed. However, the mRNA level of ERβ did not show such a response to the presence of estrogens.

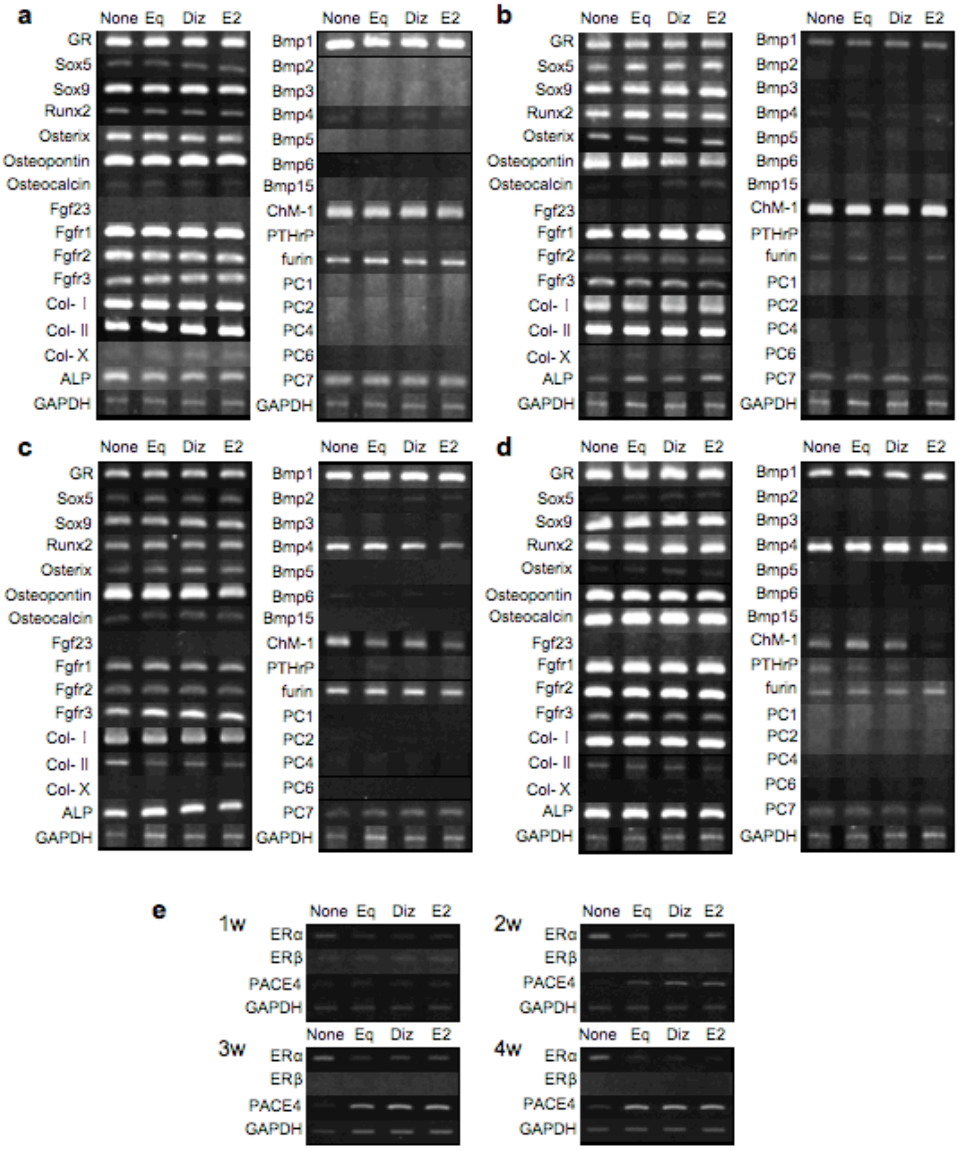

Fig. 6: Time course of the transcriptional expression patterns of bone-related genes.

MC3T3-E1 cells were cultured in differentiation medium containing charcoal-dextran-treated fetal bovine serum with no estrogen stimulus, 10^{-5} M equol (Eq), 10^{-5} M daidzein (Diz), or 10^{-9} M estradiol (E2). Over a period of 4 weeks, total RNA was isolated from cultured cells once a week, and the mRNA levels of bone-related genes were analyzed by reverse transcription-polymerase chain reaction: **a** 1 week; **b** 2 weeks; **c** 3 weeks; and **d** 4 weeks.

e The results of two genes that showed remarkable changes depending on the estrogen stimuli and those of the estrogen receptor β (ERβ) gene (for comparison) are shown separately. The endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control.

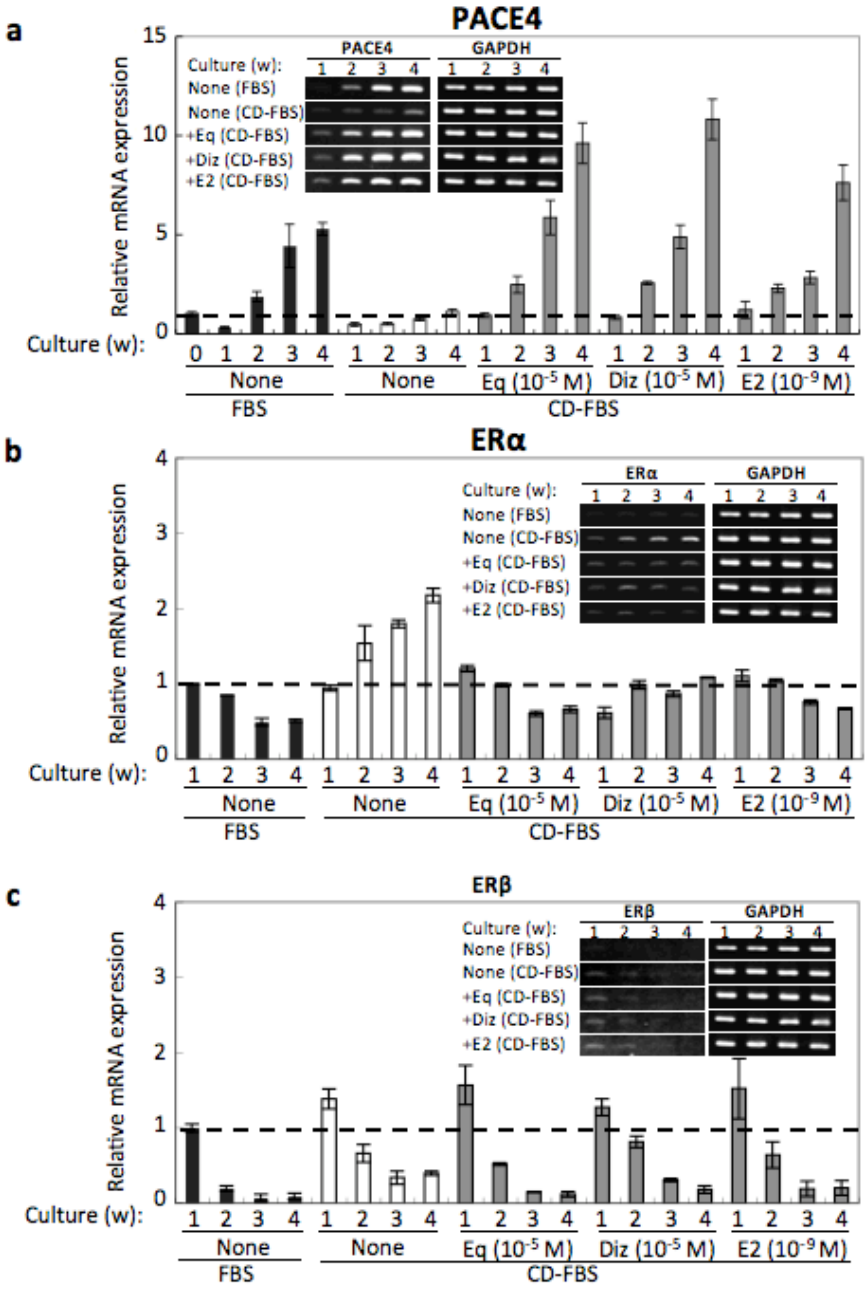

Fig. 7: Time course of the expression patterns of PACE4, ERα, and ERβ in the presence or absence of estrogen stimuli.

MC3T3-E1 cells were cultured in differentiation medium containing fetal bovine serum (FBS) only, charcoal-dextran-treated FBS (CD-FBS) only, CD-FBS with 10^{-5} M equol (Eq), CD-FBS with 10^{-5} M daidzein (Diz), or CD-FBS with 10^{-9} M estradiol (E2) for 4 weeks. The transcriptional levels of PACE4 (**a**) (n = 3) and those of estrogen receptor α (ERα) (**b**) and ERβ (**c**) were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR(n = 2) using cDNA prepared at each indicated time point as templates

3-3. Requirement of PACE4 for the differentiation of MC3T3-E1 cells

PACE4 is a member of the SPCs, which are required for the maturation/activation of bone formation factors such as BMPs [Constam *et al.,* 1999, Akamatsu *et al.,* 1999] and osteocalcin [Viegas *et al.,* 2013]. To examine whether the expression of PACE4 is only the result of osteoblast differentiation or if it plays an important role during differentiation and mineralization, I prepared *PACE4*-knockdown MC3T3-E1 cells. The PACE4- or GFP-specific shRNA expression vector was transfected into MC3T3-E1 cells, and selection culture with G418 was carried out for 2 weeks. GFP-specific shRNA-expressing MC3T3-E1 cells were used as mock cells. G418-resistant cells were cultured in differentiation medium containing FBS, CD-FBS, CD-FBS with Eq, CD-FBS with Diz, or CD-FBS with E2 until the late differentiation stage. Then, we quantitatively monitored the transcriptional levels of PACE4, furin, and PC7 expression in MC3T3-E1 cells cultured in differentiation medium containing FBS (Fig. 8a). The mRNA level of PACE4 was clearly downregulated in PACE4 shRNA-expressing MC3T3-E1 cells compared to that in mock cells. The shRNA targeting PACE4 used in this experiment was confirmed to be specific for the mRNA of the PACE4 gene because PACE4-specific shRNA caused almost no significant

suppression of the transcription of furin or PC7. In the mineralization assay of *PACE4*-knockdown cells and mock cells cultured in the presence or absence of estrogen stimuli using alizarin red S staining, *PACE4*-knockdown cells showed a strong decrease in mineralization, even in the presence of estrogen stimuli, whereas the differentiation of mock cells had significantly progressed in the presence of FBS or the estrogen stimuli Eq, Diz, and E2 with CD-FBS at the late differentiation stage, similar to normal MC3T3-E1 cells (Fig. 8b). This result strongly suggests that PACE4 is required for the differentiation of osteoblasts.

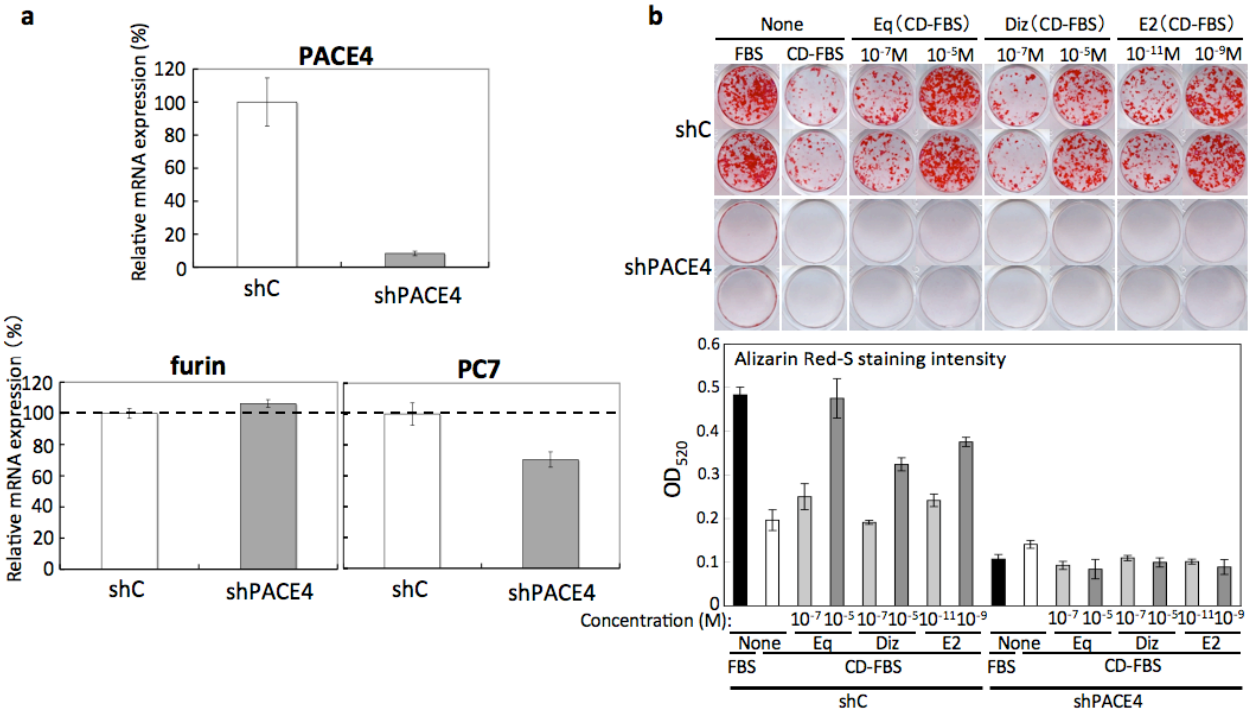

Fig. 8: Effect of PACE4 knockdown on the mineralization of MC3T3-E1 cells.

MC3T3-E1 cells transfected with the PACE4-specific short hairpin RNA (shRNA) expression vector (shPACE4) or the green fluorescent protein (GFP)-specific shRNA expression vector (shC) were selected by cultivation in the presence of G418 for 2 weeks. Cells were then cultured in differentiation medium containing fetal bovine serum (FBS), charcoal-dextran-treated FBS (CD-FBS), CD-FBS with equol (Eq) (10^{-7}, 10^{-5} M), CD-FBS with daidzein (Diz) (10^{-7}, 10^{-5} M), or CD-FBS with estradiol (E2) (10^{-11}, 10^{-9} M) until the late differentiation stage, respectively. **a** The expression levels of subtilisin-like proprotein convertases such as PACE4, furin, and PC7 in shC and shPACE4 after 4 weeks of cultivation in differentiation medium containing FBS were analyzed by quantitative reverse transcription-polymerase chain reaction (n = 3) to determine whether shPACE4 was specific to PACE4. **b** To investigate the requirement of PACE4 expression for cell differentiation, transfected cells were stained with alizarin red S (upper picture) after 3 weeks of cultivation in differentiation medium containing FBS, CD-FBS, or CD-FBS with each estrogenic agent, and the mineralization level was quantified by measuring the absorbance at 520 nm (n = 3) (lower graph).

4. Conclusion

With aging and decreases in the blood estrogen level, osteoporosis can adversely affect the quality of life of elderly people, especially postmenopausal women. Estrogen replacement therapy is regarded as an effective treatment for preventing osteoporosis. Moreover, the intake of soybeans containing isoflavones, which are phytoestrogens that mimic the action of estrogen, is an effective and attractive way to further increase endogenous estrogen levels. Recently, it has been suggested that people in whom Diz is metabolized to Eq by intestinal bacterial flora have better bone health owing to the maintenance of bone mass, compared to those in whom intestinal bacteria do not produce Eq [Wu *et al.,* 2007]. It is well known that most stages of the bone formation process depend on endochondral ossification, where the cartilage formed by chondrocytes is replaced with bone derived from osteoblasts. Therefore, we investigated which step of the cell differentiation process is regulated by estrogen stimuli, i.e., the induction efficiency of cell differentiation by estrogen stimuli was compared between ATDC5 and MC3T3-E1 cells. As shown in Figures 5a and 5b, compared to ATDC5 cells, differentiation of MC3T3-E1 cells was clearly under the control of estrogen stimuli, i.e., it was induced by E2 and the phytoestrogens Diz and

Eq. I also investigated the dose-dependent effect of E2 and phytoestrogens on the differentiation of MC3T3-E1 cells. As shown in Figures 5c and 5d, Eq facilitated osteoblast differentiation more effectively than Diz via the ER; however, E2 was a significantly stronger inducer than these phytoestrogens. Among the different ERs, ERα has been reported to have a role in osteoblast mechanotransduction via activation of the β-catenin-mediated Wnt signaling pathway, which is involved in bone formation [Armstrong *et al.,* 2007]. As shown in Figure 6, the mRNA level of ERα was higher than that of ERβ. Furthermore, the expression of ERα was more sensitive to the absence of estrogen stimuli, i.e., in the absence of estrogen stimuli, ERα levels increased over time in MC3T3-E1 cells cultured in medium containing CD-FBS. However, the expression of ERβ was not affected by the presence of estrogen stimuli (Figs. 7b and 7c). Thus, my experimental results also suggest that ERα is the primary receptor responsible for estrogen stimuli to facilitate bone formation. Moreover, although both Diz and Eq showed higher affinity to ERβ than to ERα, the binding affinities of Eq to both ERα and ERβ were higher than those of Diz [Usui *et al.,* 2006, Muthyala *et al.,* 2003, Morito *et al.,* 2001]. These findings may provide an

explanation for the results shown in Figure 5c, which shows that Eq is more effective than Diz in inducing differentiation of MC3T3-E1 cells.

I also investigated the time course of the expression of bone formation-related genes in MC3T3-E1 cells by RT-PCR. The expression levels of several genes such as Bmp4 and osteocalcin increased during cell differentiation, but their expression was not responsive to estrogen stimuli. Of these genes, the expression of PACE4, a member of the SPC family, changed considerably in response to estrogen stimuli and increased as osteoblast differentiation progressed; however, the transcription of other SPCs such as furin and PC7 was independent of estrogen stimuli (Fig. 6 and Fig. 7a). Unlike furin, which is a ubiquitously and constitutively expressed membrane-bound SPC, PACE4 is localized in the extracellular matrix and its expression levels change markedly during development and differentiation [Yuasa *et al.,* 2009]. SPCs can cleave the precursors of BMPs, which regulate skeletogenesis, to convert them to the active forms. For example, furin and/or PC6 proteolytically activate BMP4 [Cui *et al.,* 1998] and PACE4 activates BMP6 [Yuasa *et al.,* 2012]. In addition, it has been reported that the transcription of BMP2 is increased by treatment with E2 after 24 h in mouse

mesenchymal stem cells that differentiated into osteoblasts [Zhou *et al.,* 2003]; furthermore, furin has been shown to convert proBMP2 to mature BMP2 [Felin *et al.,* 2010]. In the present study, mRNA expression of BMP2 and BMP6 in MC3T3-E1 cells was confirmed at trace levels; however, their expression levels were not linked with the cell differentiation induced by estrogen stimuli. Although the mRNA expression of BMP4 significantly increased as differentiation progressed, the increase was not a response to estrogen stimuli. Therefore, it is difficult to explain the correlation between osteoblast differentiation and estrogen stimuli only on the basis of the SPC/BMP substrate relationship identified to date. As shown in Figures 6 and 7a, among the SPCs expressed in MC3T3-E1 cells, only the increase in the expression of PACE4 can be linked to osteoblast differentiation. In addition, knockdown of PACE4 resulted in delayed osteoblast differentiation (Fig. 8b). PACE4 plays a key role in chondrogenic cell differentiation via processing of BMP6 [Yuasa *et al.,* 2012]; therefore, it is reasonable that PACE4 also regulates the differentiation of osteoblasts related to bone formation. However, the differentiation of chondrogenic cells was not affected by estrogen stimuli, as shown in Figure 5a. Moreover, the PACE4 isoform expressed

in osteoblastic cells was isoform A-II, which is a rare isoform of PACE4; to date, it has been confirmed to be present in HepG2 cells and the placenta. Therefore, the expression of PACE4 seems to be regulated by different independent systems in chondrogenic cells and osteoblastic cells, and PACE4 may control osteoblast differentiation via combinations of substrate(s) that are different from those in chondrogenic cells. After binding of the estrogenic ligand to its receptor to regulate the expression of target genes, the ER-ligand complex translocates to the nucleus, where it activates transcription directly by binding to the response element or indirectly via other DNA-binding proteins [Björnström *et al.,* 2005]. The ER can bind to DNA in less than 1 h after ligand binding [Métivier *et al.,* 2005]. However, it took several weeks to show a significant increase in the transcription of PACE4 in response to estrogen stimuli; furthermore, there are no typical ER response elements in the upstream region of the PACE4 gene. Therefore, it is thought that PACE4 may be indirectly regulated by some other gene(s) that is directly targeted by estrogen stimuli, or by post-transcriptional regulation, e.g., mRNA stabilization/destabilization, governed by RNA-binding proteins [Lal *et al.,* 2004]. Among the SPCs, a significant correlation

between ER content and the gene expression of PACE4 has only been observed in human breast cancer [Cheng *et al.,* 1997]; however, details of the correlation between the dynamic states of ER and PACE4 are still unclear. Therefore, further studies are required to explain the mechanism underlying the transcriptional regulation of PACE4 by estrogen stimuli, which was identified in this work. Moreover, the substrate(s) leading to cell differentiation after activation by PACE4 in the presence of estrogen stimuli has not yet been identified in osteoblastic cells. One candidate of interest is osteocalcin, an important marker for differentiated osteoblasts; its expression increased during the time course, similar to the expression of PACE4 at the late stage of cell differentiation (Fig. 6). It is also known that osteocalcin requires cleavage at the SPC recognition motif (Table 1) in order to convert into the mature active form. Therefore, further investigations are necessary to clarify the role of PACE4 in osteoblast differentiation.

In conclusion, the results from my present study strongly suggest that estrogen stimuli, including physiological estrogen and phytoestrogens such as Diz and Eq, induce osteoblast differentiation through upregulation of PACE4 mediated by the ER.

ABBREVATIONS

ALP, alkaline phosphatase; **BMPs**, bone morphogenetic proteins

CD-FBS, charcoal/dextran-treated FBS; **ChM-1**, chondromodulin-1

Col-Ⅰ, type Ⅰ collagen; **Col-Ⅱ**, type Ⅱ collagen; **Col-Ⅹ**, type Ⅹ collagen

Diz, daidzein; **E2**, 17 β -estradiol; **ER**, estrogen receptor; **Eq**, equol

FBS, fetal bovine serum; **FGFR**, fibroblast growth factor receptor

Ful, fulvestrant; **GFP**, green fluorescent protein; **GR**, glucocorticoid receptors

MSC, mesenchymal stem cell; **PBS**, phosphate-buffered saline

RT-PCR, reverse transcription PCR; **shRNA**, short hairpin RNA

SPC, subtilisin-like proprotein convertase; **qRT-PCR**, quantitative real-time PCR

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Estrogens activate bone morphogenetic protein-2 gene transcription in mouse mesenchymal stem cells. Mol Endocrinol 17:56-66

LIST OF PUBLICATIONS

I. **Kim H**, Tabata A, Tomoyasu T, Ueno T, Uchiyama S, Yuasa K, Tsuji A, Nagamune H（2014）Estrogen stimuli promote osteoblastic differentiation via the subtilisin-like proprotein convertase PACE4 in MC3T3-E1 cells. JBMM (in press)

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