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Comparative effects of raloxifene, tamoxifen and estradiol on human osteoblasts *in vitro*: Estrogen receptor dependent or independent pathways of raloxifene

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ABSTRACT

SERMs bind to both estrogen receptor (ER)α and β, resulting in tissue dependent estrogen agonist or antagonist responses. Both raloxifene and tamoxifen are most frequently used SERMs and exert estrogen agonistic effects on human bone tissues, but the details of their possible direct effects on human bone cells have remained largely unknown. In our present study, we examined the comparative effects of raloxifene, tamoxifen, and native estrogen, estradiol on human osteoblast cell line, hFOB *in vitro*. Both the cell numbers and the ratio of the cells in S phase fraction were significantly increased by the treatment of raloxifene or tamoxifen as well as estradiol treatments in hFOB. Gene profile patterns following treatment with raloxifene, tamoxifen, and estradiol demonstrated similar patterns in a microarray/hierarchal clustering analysis. We also examined the expression levels of these genes detected by this analysis using quantitative RT-PCR. MAF gene was induced by raloxifene treatment alone. GAS6 gene was induced by raloxifene and tamoxifen as well as estradiol. An estrogen receptor blocker, ICI 18, 286, inhibited an increase of GAS6 gene expression but not the levels of MAF gene mRNA expression. Results of our present study demonstrated that raloxifene exerted direct protective effects on human osteoblasts in both estrogen receptor dependent and independent manners.

1. Introduction

It is well known that sex steroids such as estrogen and androgen play an important role in maintenance of human bone tissues. For instance, reductions in circulating estrogen levels at the menopause are related with a rapid deterioration of bone density [1]. Various studies using experimental rodents demonstrated that estrogen depletion led to high-resorption osteoporosis caused by activation of osteoclasts which have estrogen receptor (ER)α/β mRNA or protein [2–5]. ERα/β mRNA or protein was also detected in osteoblasts [5–8]. Therefore, estrogen has considered having direct effects on osteoblasts as well as osteoclasts in human skeletal systems. Estrogen replacement therapy is very useful for bone health of postmenopausal osteoporosis patients [9] but side effects such as increased incidences of breast cancer and gynecological disorders prevented the practice of this replacement [10,11].

The selective estrogen receptor modulators (SERMs) are chemical compounds, which activate the estrogen receptors with different estrogenic and antiestrogenic tissue specific effects [12]. Results of several clinical trials demonstrated the effectiveness of raloxifene in the treatment of postmenopausal osteoporosis [13–15]. Raloxifene binds with high affinity to both ERα and ERβ as in estradiol, but in general does not increase the incidence of breast cancer and gynecological symptoms including endometrial carcinoma [16]. Results of several *in vitro* studies also demonstrated the direct effects of raloxifene on human or rodent primary osteoblast or osteoblast-like (osteosarcoma) cells [17–19]. Tamoxifen, which is also one of well-established SERMs, has been demonstrated to have partial estrogen agonistic effects on uterus as well as bone tissues [20]. In contrast to tamoxifen, raloxifene has anti-estrogenic effect on uterus as well as breast tissues [21,22]. Results of clinical trial which compared the clinical effects of raloxifene and tamoxifen on the risks of developing invasive breast carcinoma demonstrated that there were no differences between raloxifene and tamoxifen treatment groups in the total number of fracture in both hip and spine colles [15]. Results of *in vitro* study using microarray analysis demonstrated that the gene expression patterns induced by raloxifene, tamoxifen, and estradiol treatments in osteosarcoma U2OS transected with ERα (U2OS/ERα) and U2OS/ERβ [23]. However, comparative direct effects of raloxifene and tamoxifen on cell proliferation and gene expression patterns in human non-neoplastic osteoblasts have remained largely unknown.

Therefore, in this study, we first examined effects of raloxifene, tamoxifen, and estradiol on cell proliferation and cell cycle of

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normal human osteoblast-like cells. We then evaluated raloxifene, tamoxifen, and estradiol responsive genes using a microarray analysis in order to further characterize the possible differential genomic effects of these compounds on human osteoblasts. In this study, hFOB was employed in order to examine the effects on native status of human osteoblasts.

2. Materials and methods

2.1. Chemicals

Raloxifene HCl (LY139481) was kindly provided by Eli Lilly and Company (Indianapolis, IN, USA). Tamoxifen and estradiol (β-estradiol) were commercially purchased from Sigma–Aldrich Co. (MO, USA). ICI 182,780 were purchased from Tocris Cookson Inc. (MO, USA). All test materials were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical industries, Ltd. Osaka, Japan). The final concentrations of DMSO used in this study did not exceed 0.05% in any of the experiments preformed.

2.2. Osteoblast cell line and culture conditions

Human osteoblast cell, hFOB 1.19 cell line (CRL-11372) was obtained from American Type Culture Collection (VA, USA). hFOB 1.19 cell was cultured based on the protocol of the previously study [24]. Cells were maintained in culture at 34 °C, 95% relative humidity and 5% CO_2 in room air. The cells were plated on 96-well plates or 100 mm culture dishes at initial concentration of 5×10^4 cells/ml. Different concentrations of test compounds were added, and the assay was terminated by removing the medium from wells. ICI 182,780 was added simultaneously. In hFOB cell, expression of ERβ mRNA was more predominant than that of ERα mRNA [25].

2.3. Immunocytochemistry

Antibodies for ERα (NCL-ER-6F11) and ERβ (ER-β-14C8) were purchased from Novocastra Laboratories Ltd. (Newcastle, UK) and GeneTex, Inc. (Texas, USA), respectively. Cells were grown directly on chamber glass slides (4 well glass slide, Lab-Tek II, Nalgene Nunc International, NY, USA) under culture conditions described above. Glass slides were fixed with 10% formaldehyde for 10 min. Cells were immunostained by a biotin–streptavidin method using Histofine kit (Nichirei Co. Ltd, Tokyo, Japan) and have been previously described in detail [26]. The antigen–antibody complex was then visualized with 3,3′-diaminobenzidine solution (Dojindo Laboratories, Kumamoto, Japan). Human breast carcinoma cell line, T-47D (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) was used as positive control.

2.4. Cell proliferation assay

hFOB cells were treated with steroids and test compounds after 24, 48, and 72 h when the specimens were harvested and evaluated for cell proliferation using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] method (Cell Counting Kit-8; Dojindo Laboratories). Ten microliters of 5 mM WST-8 were added to 100 l of cells, which were then incubated for 2 h at 37 °C [26]. Optical densities (OD, 450 nm) were obtained with a SpectraMax 190 microplate reader (Molecular Devices, Corp., CA, USA) and Softmax Pro 4.3 microplate analysis software (Molecular Devices, Corp.). The status of cell proliferation (%) was calculated according to the following equation: (cell OD value after test materials treated/vehicle control cell OD value) × 100.

2.5. Cell cycle analysis

In order to examine the status of cell cycle regulation of hFOB, we evaluated the percentage of cells in G0–G1, S, and G2/M phase. Cells were tripsinized and collected by centrifugation. Cells were resuspended in PBS and fixed 70% ethanol. The samples were then kept at −30 °C until use. Fixed cells were incubated with RNase (Sigma–Aldrich) for 60 min at room temperature and stained with propidium iodide (Sigma–Aldrich). Flow cytometry was then performed with FACSCalibur (BD Biosciences, San Jose, CA USA) flow cytometer. Acquisition was performed using CellQuest software (BD Biosciences) and the percentage of cells in each cell cycle phases was evaluated on a DNA linear plot using ModFit software (BD Biosciences), which counted 50,000 nuclei per sample. Mononuclear cells were used as a DNA diploid control.

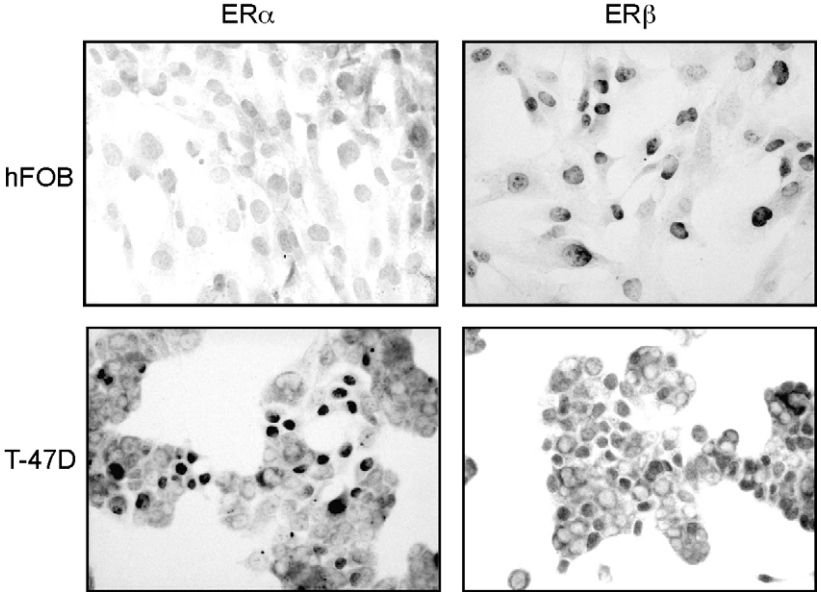

Fig. 1. Immunocytochemistry of ERα and ERβ in hFOB cells. Imunoreactivity of ERα was weak or negative in hFOB cells (*top*, *left*). Imunoreactivity of ERβ was markedly detected in nuclei of hFOB cells (*top*, *right*). Marked ERα (*bottom*, *left*) and weak ERβ (*bottom*, *right*) immunoreactivities were detected in the nuclei of T-47D cells, which is positive control of ER.

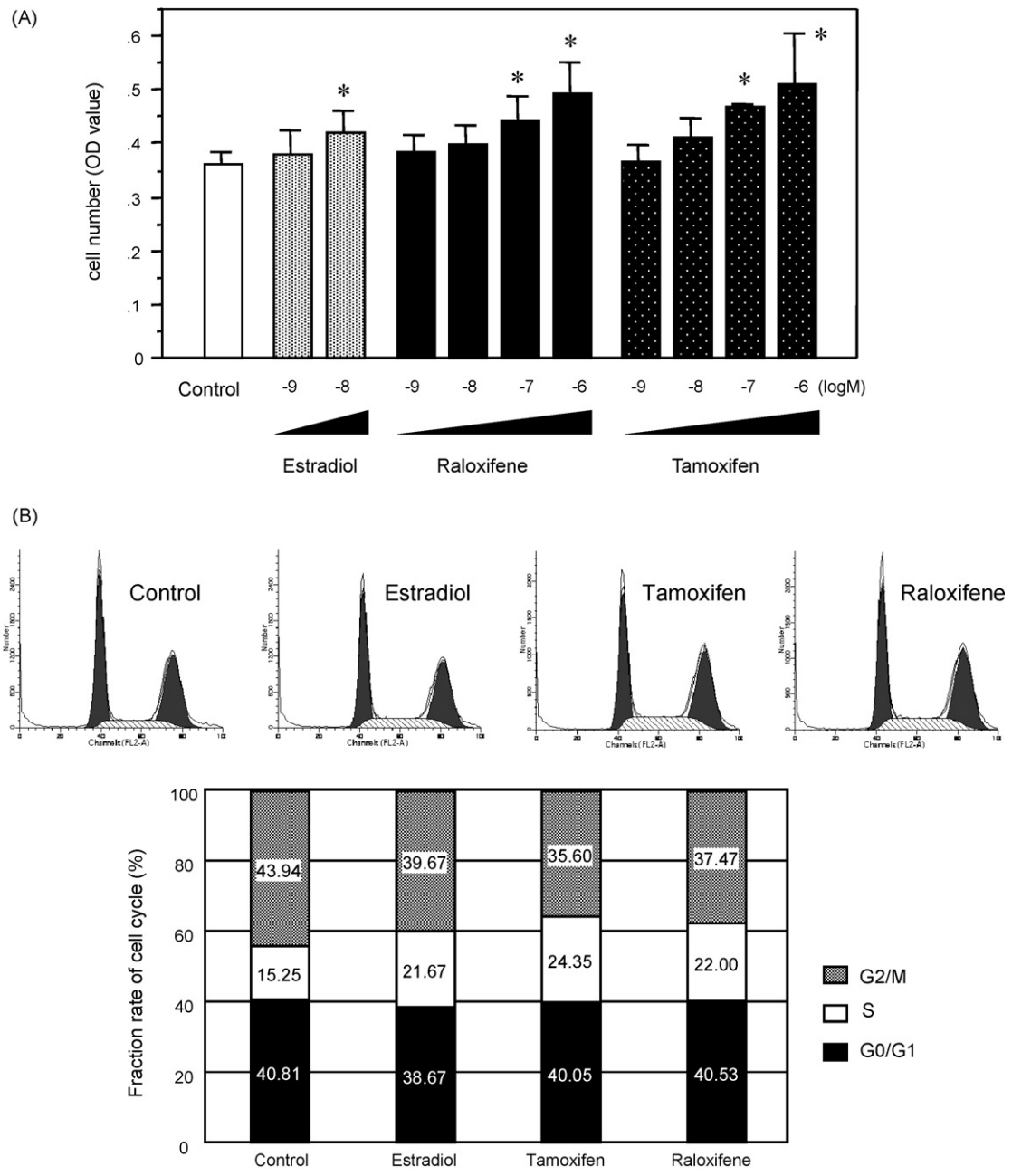

Fig. 2. A: Cell proliferation of hFOB cells treated by estradiol (10^{-9} and 10^{-8} M), raloxifene (10^{-9} to 10^{-6} M), and tamoxifen (10^{-9} to 10^{-6} M) for 72 h. All data were demonstrated as mean ($n = 3$) ± SD. $^{*}p < 0.05$ *vs.* control (0.05% DMSO). B: Cell cycle analysis of hFOB cells treated by estradiol (10^{-8} M), raloxifene (10^{-7} M), and tamoxifen (10^{-7} M) for 48 h. Similar results were obtained in at least two independent experiments.

2.6. Microarray analysis

Cell lysates were prepared using RLT buffer (QIAGEN GmbH, Hilden, Germany). Total RNA was extracted using Rneasy Mini Kit (QIAGEN). First-strand cDNA was synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA), 100 pmol T7-$(dT)_{24}$ primer (Invitrogen). Ten units of T4 DNA polymerase (Invitrogen) were then added, and the dsDNA was mixed with T7 RNA polymerase (Invitrogen). The purified cRNA was fragmented at 300–500 bp as target solution. Test samples and reference samples were labeled with cyanine-5 (Cy5)- and cyanine-3 (Cy3)-labeled CTP (PerkinElmer Inc., Waltham, MA, USA), respectively. Cy3- or Cy5-labeled cRNA probes were hybridized on the Human 1A ver. 2.0 (Agilent Technologies, Inc., Santa Clara, CA, USA) including 22,000 genes. The reacted arrays were then scanned as digital image files with GenePix 4000A (Axon Instruments, Foster City, CA, USA). Relative levels of gene expression were calculated by global normalization. The ratio of Cy3 and Cy5 signal intensity of each spot was quantitatively calculated using GenePix Pro 5.0 (Axon Instruments). Data were subjected to hierarchical clustering analysis and visualization using the Cluster and TreeView programs (Stanford University) [27] in order to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each sample [25].

2.7. Quantitative RT-PCR

Quantitative RT-PCR was carried out using the LightCycler System and the FastStart DNA Master SYBR Green I with

Table 1
Genes induced by raloxifene treatment in hFOB—2.0 higher or lower.

Category	GB#	Gene title	Symbol	Ratio		
				E2	TAM	RAL
Up	AF055376	v-Maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF[a]	1.14	1.60	3.61
	AW006750	Kelch-like 24 (Drosophila)	DRE1	1.12	1.22	3.42
	BC030005	Multiple C2 domains, transmembrane 1	MCTP1	1.54	1.61	3.36
	AK027160	BCL2-like 11 (apoptosis facilitator)	BCL2L11[b]	1.83	1.04	3.18
	AI469884	Carboxypeptidase M	CPM[a,b]	1.63	1.90	3.11
	AL832710	Plexin D1	PLXND1	0.85	0.97	3.03
	AI479082	Growth arrest-specific 6	GAS6[a,b]	0.92	1.53	2.97
	AW504458	Guanine nucleotide binding protein (G protein), beta polypeptide 4	GNB4	1.49	1.99	2.93
	BC026009	G protein-coupled receptor 125	GPR125	1.37	0.90	2.90
	AL575337	RAB11B, member RAS oncogene family	RAB11B[a]	0.71	0.89	2.82
	AF152504	Protocadherin gamma subfamily A, 3	PCDHGA3	1.31	1.28	2.78
	AL563460	GATA binding protein 2	GATA2	1.25	0.95	2.75
	BF433902	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFRSF11B	0.70	0.66	0.15
Down	NM018495	Caldesmon 1	CALD1	0.88	0.50	0.16
	BC018898	Lymphotoxin beta (TNF superfamily, member 3)	LTB	1.05	0.94	0.23
	NM014421	Dickkopf homolog 2 (Xenopus laevis)	DKK2	0.59	0.63	0.24
	BC014029	Ubiquitin protein ligase E3C	UBE3C	1.07	1.10	0.29
	BE566136	Transcription factor CP2-like 2	TFCP2L2	1.02	0.77	0.30
	AL136528	Tumor protein p73	TP73	0.89	0.68	0.32
	NM006290	Tumor necrosis factor, alpha-induced protein 3	TNFAIP3	0.59	0.72	0.33
	NM000882	Interleukin 12A	IL12A	0.88	0.83	0.36
	M19701	Retinoblastoma 1 (including osteosarcoma)	RB1	0.91	1.05	0.37
	BE962027	SMAD specific E3 ubiquitin protein ligase 2	SMURF2	0.99	1.01	0.37
	H48516	Deleted in lymphocytic leukemia, 2	DLEU2	0.91	1.90	0.38

E2: estradiol, TAM: tamoxifen, RAL: raloxifene.
[a] Genes performed quantitative RT-PCR.
[b] Genes have estrogen receptor response element located its promoter lesion.

software version 3.5.3 (Roche Diagnostics GmbH, Mannheim, Germany). PCR was set up at 3 mM $MgCl_2$, 10 pmol/l of each primer. The primer positions used in this study are as follows; RPL13A (NM_012423) [25]; MAF (NM_005360), Forward 1943–Reverse 2077; RAB11B (NM_004218), Forward 289–Reverse 444; ITGB1 (NM_002211), Forward 2201–Reverse 2320; DLX (NM_005222), Forward 338–Reverse 466; PDE3B (NM_000753), Forward 443–Reverse 579; CPM (NM_001874), Forward 762–Reverse 907; GAS6 (NM_000820), Forward 1317–Reverse 1480; SAFB (NM_002967), Forward 1563–Reverse 1713; BGN (NM_001711), Forward 951–Reverse 1087; ATF7IP (NM_018179), Forward 2284–Reverse 2444; RAB40C (NM_021168), Forward 549–Reverse 741. All primer sets except for RPL13A were designed using OLIGO Primer Analysis Software (TAKARA Bio Inc., Shiga, Japan). An initial denaturing step of 95 °C for 10 min was followed by 35 cycles, respectively, of 95 °C for 10 min; 15 s annealing at 68 °C (RPL13A, MAF, DLX, GAS6, ITGB1, ATF7IP, RAB11B, RAB40C) or 64 °C (PDE3B, SAFB, CPM, BGN); and extension for 15 s at 72 °C. Negative control experiments included those lacking cDNA substrates to confirm the presence of exogenous contaminant DNA. No amplified products were detected under these conditions. The mRNA levels in each case were represented as a ratio of RPL13A, and evaluated as a ratio (%) compared with that of each control [25,26].

2.8. Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed with the StatView 5.0 J software (SAS Institute Inc., NC, USA). All data were analyzed by analysis of variance (ANOVA) followed by post hoc Bonferroni/Dunnet multiple comparison

Table 2
Genes induced by raloxifene and tamoxifen treatment in hFOB—2.0 higher or lower.

Category	GB#	Gene title	Symbol	Ratio		
				E2	TAM	RAL
Up	AA215854	Integrin, beta 1	ITGB1[a]	0.56	3.22	3.86
	BE178502	JNK/SAPK-inhibitory kinase	JIK	1.80	3.04	3.41
	AW504458	Guanine nucleotide binding protein (G protein), beta polypeptide 4	GNB4	1.49	2.99	2.93
	NM000798	Dopamine receptor D5	DRD5	1.14	3.24	2.91
	AI769566	Scaffold attachment factor B	SAFB[a]	1.54	2.72	2.89
	NM001340	Cylicin, basic protein of sperm head cytoskeleton 2	CYLC2	1.44	2.44	2.87
	NM001531	Major histocompatibility complex, class I-related	MR1	0.99	0.25	0.29
Down	AK026133	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B	SEMA4B	0.81	0.26	0.32
	AF465843	Sterile alpha motif and leucine zipper containing kinase AZK	ZAK	0.60	0.34	0.35
	BU683892	Chromobox homolog 3 (HP1 gamma homolog, Drosophila)	CBX3	0.96	0.25	0.36
	Z98752	l (3) mbt-like (Drosophila)	L3MBTL[b]	0.61	0.34	0.36

E2: estradiol, TAM: tamoxifen, RAL: raloxifene.
[a] Genes performed quantitative RT-PCR.
[b] Gene has estrogen receptor response element located its promoter lesion.

Table 3
Genes induced by raloxifene and estradiol treatment in hFOB—2.0 higher or lower.

Category	GB#	Gene Title	Symbol	Ratio		
				E2	TAM	RAL
Up	AA040332	Distal-less homeobox 6	DLX[a]	3.14	1.49	3.92
	D12625	Neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)	NF1	3.05	1.19	3.34
	NM000753	Phosphodiesterase 3B, cGMP-inhibited	PDE3B[a,b]	2.87	1.36	3.29
	NM001711	Biglycan	BGN[a]	3.03	1.74	3.29
	NM016950	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	SPOCK3	2.80	1.03	2.74
	NM001145	Angiogenin, ribonuclease, RNase A family, 5	ANG	2.78	1.42	2.74
	AU156721	Pregnancy-associated plasma protein A, pappalysin 1	PAPPA	0.27	1.09	0.19
Down	NM003790	Tumor necrosis factor receptor superfamily, member 25	TNFRSF25	0.29	0.78	0.24
	BG494416	Phosphodiesterase 5A, cGMP-specific	PDE5A	0.29	0.96	0.27
	AI002966	BLZF1 and Name: basic leucine zipper nuclear factor 1 (JEM-1)	BLZF1	0.37	1.08	0.37

E2: estradiol, TAM: tamoxifen, RAL: raloxifene.
[a] Genes performed quantitative RT-PCR.
[b] Gene has estrogen receptor response element located its promoter lesion.

test. A p-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Expressions of ERα and ERβ in hFOB cell line

Representative findings of immunocytochemistry of ERα and ERβ are illustrated in Fig. 1. Imunoreactivity of ERβ was marked in the nuclei of hFOB cells, whereas that of ERα was weak or negative in hFOB cells. Marked ERα and weak ERβ immunoreactivity was detected in the nuclei of T-47D cells.

3.2. Cell proliferation and cell cycle

Results of the cell proliferation assays are summarized in Fig. 1A. There was a significant increase in the cell number after 72 h in hFOB cells treated with 10^{-8} to 10^{-6} M estradiol (Fig. 1A). The cell number of hFOB treated by 10^{-7} to 10^{-6} M tamoxifen or raloxifene for 72 h was also significantly higher than vehicle control employed in this study.

Results of the cell cycle assays are summarized in Fig. 1B. Flow cytometry analysis demonstrated that the ratio of the cells at S fraction was significantly increased after 24 h (data not present) and 48 h (Fig. 1B) in hFOB cells treated with 10^{-7} M raloxifene, 10^{-7} M tamoxifen and 10^{-8} M estradiol, respectively.

3.3. Microarray/hierarchical clustering analysis

Results of the microarray/hierarchical clustering analysis are summarized in Fig. 2. In hFOB cells, the hierarchical clustering analysis contains 367 genes, which demonstrated expression ratios above 2.0-fold and below 0.5-fold compared with vehicle control cells after 12 h of each gene treated with 10^{-7} M raloxifene, 10^{-7} M tamoxifen, or 10^{-8} M estradiol. There were marked similarities between the expression profiles induced by raloxifene, tamoxifen and estradiol (Fig. 2A). In the hierarchical clustering analysis containing 134 genes, which demonstrated expression ratios above 2.0-fold, the expression profiles of raloxifene-treated cells were closely related to those of estradiol (Fig. 2B). In the hierarchical clustering analysis containing 231 genes, which demonstrated expression ratios below 0.5-fold, the expression profiles of tamoxifen-treated cells were also closely related to those of estradiol (Fig. 2C).

The lists of the genes with known biological functions, which demonstrated highest alterations by raloxifene, tamoxifen, and/or estradiol are summarized in Tables 1–4. The genes induced by raloxifene treatment, which were all up- or down-regulated twice or more than control are summarized in Table 1. The genes induced by raloxifene, tamoxifen and estradiol treatments, which were all up- or down-regulated twice or more than control are also summarized in Table 2 (raloxifene and tamoxifen), Table 3 (raloxifene and estradiol) and Table 4 (raloxifene, tamoxifen, and estradiol), respectively. Among these genes detected in our present study, we selected 11 genes, which were biologically known to bone homeostasis or cell proliferation after the extensive literature search. We then examined whether these 11 genes were increased by raloxifene, tamoxifen, or estradiol treatments using quantitative RT-PCR in hFOB cells. The genes which we examined using quantitative RT-PCR were as follows: MAF, CPM, GAS6, RAB11B, ITGB1, SAFB, DLX, PDE3B, BGN, ATF7IP, and RAB40C.

3.4. Validation of microarray analysis using quantitative RT-PCR

Results of the validation of microarray analysis are summarized in Fig. 3. All of these 11 genes summarized in Tables 1–4 were

Table 4
Genes induced by raloxifene, tamoxifen, and estradiol treatment in hFOB—2.0 higher or lower.

Category	GB#	Gene title	Symbol	Ratio		
				E2	TAM	RAL
Up	NM018005	Activating transcription factor 7 interacting protein	ATF7IP[a]	3.00	3.74	3.83
	NM006927	ST3 beta-galactoside alpha-2,3-sialyltransferase 2	SIAT4B	3.25	3.42	3.39
	AI955239	Oxysterol binding protein-like 7	OSBPL7	3.07	3.26	3.31
	BC014531	RAB40C and Name: RAB40C, member RAS oncogene family	RAB40C[a]	3.23	3.27	3.30
	AL137798	Macrophage stimulating 1 (hepatocyte growth factor-like)	MST1	2.15	2.80	2.76
Down	NM005084	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	PLA2G7	0.05	0.16	0.14
	NM152573	RAS and EF-hand domain containing	RASEF	0.10	0.36	0.28
	BC036029	ADAM metallopeptidase domain 22	ADAM22	0.31	0.18	0.39

E2: estradiol, TAM: tamoxifen, RAL: raloxifene.
[a] Genes performed quantitative RT-PCR.

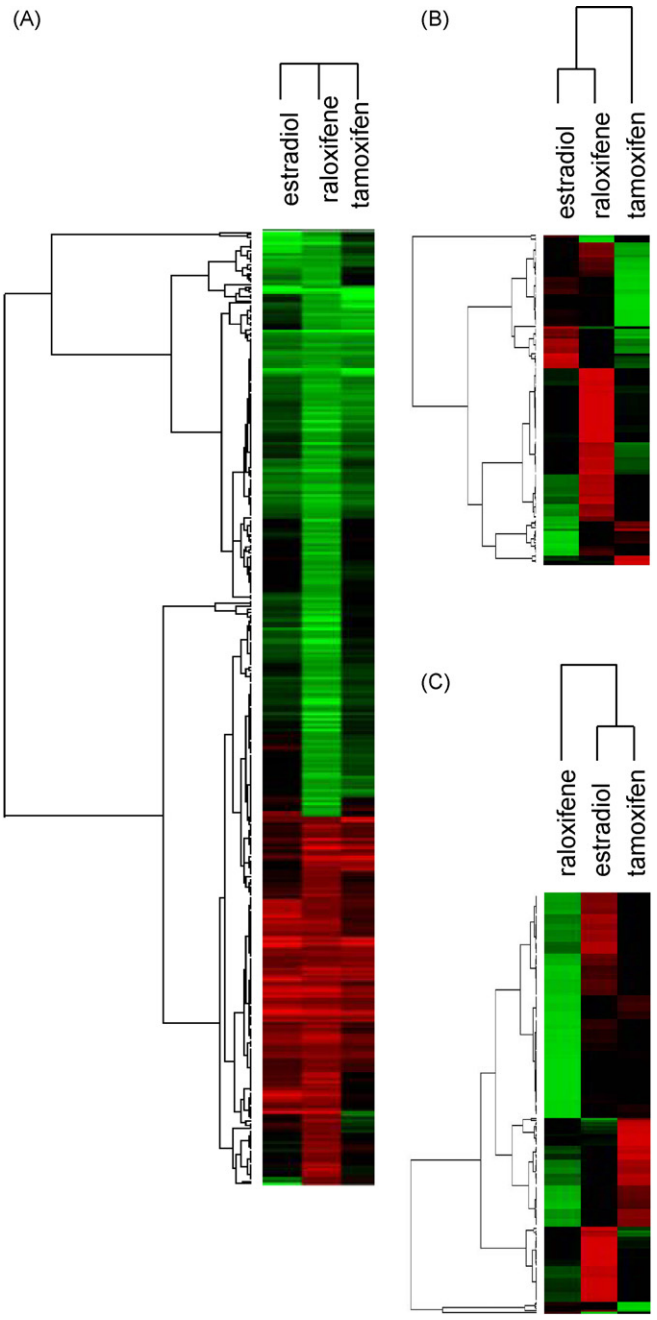

Fig. 3. In microarray/hierarchical clustering analysis of the expression levels of each genes in hFOB treated with raloxifene (10^{-7} M), tamoxifen (10^{-7} M), and estradiol (10^{-8} M) for 12 h. A: The hierarchical clustering analysis contains 367 genes, which demonstrated expression ratios above 2.0-fold and below 0.5-fold compared with vehicle control. B: The hierarchical clustering analysis contains 134 genes, which demonstrated expression ratios above 2.0-fold. C: The hierarchical clustering analysis contains 231 genes, which demonstrated expression ratios below 0.5-fold.

significantly increased by 10^{-7} M raloxifene treatment, and 3/11 genes (GAS6, ATF7TP, and RAB40C) were also significantly increased by both 10^{-7} M tamoxifen and 10^{-8} M estradiol treatments, respectively. 1/11 gene (SAFB) was also significantly increased by 10^{-7} M tamoxifen but not estradiol treatment, and 2/11 genes (DLX and PDE3B) was also significantly increased by 10^{-8} M estradiol but not tamoxifen treatment. In microarray analysis, GAS6 gene expression level did not change by tamoxifen and estradiol treatments, respectively.

3.5. Effects of estrogen receptor blocker on hFOB treated with SERMs and estradiol

We examined the effects of specific estrogen receptor blocker on the increases of MAF and GAS6 gene levels in hFOB treated with raloxifene, and/or tamoxifen and estradiol.

Results of the effects of estrogen receptor blocker, ICI 182,780 are summarized in Fig. 4. The cell proliferation induced by raloxifene, tamoxifen, and estradiol treatments was significantly diminished by ICI 182,780 (10^{-6} M) treatment. The increase of the GAS6 gene expression in hFOB by raloxifene, tamoxifen, and estradiol treatments was also significantly diminished by ICI 182,780 treatment. However, ICI 182,780 treatment did not influence an increase of the MAF gene expression by raloxifene treatment (Fig. 5).

4. Discussion

Results of previous studies on the gene regulation by SERMs on human osteosarcoma cell line or primary culture of osteoblasts have not necessarily been consistent. Kian et al. [23] reported that the great majority of the genes regulated in osteosarcoma U2OS/ERα in response to estradiol, raloxifene, and tamoxifen were distinct from those regulated in U2OS/ERβ in their microarray analysis. They also demonstrated that the pathways, which induced by raloxifene, and tamoxifen treatments diverge at the level of gene expression in U2OS/ERα and/ERβ, respectively [23]. Sixteen out of 30 bone homeostasis related genes were activated by three compounds, respectively in the U2OS/ERβ cells. 6/16 genes were induced by raloxifene alone, whereas 0/16 genes were co-regulated with three compounds treatments [23]. However, it is also true that the regulation patterns of the genes with an exception of 30 bone related-genes induced by raloxifene, tamoxifen, and estradiol have remained unknown. Optimal dose of estradiol employed in estrogen receptor reporter gene assay are generally considered 10 nM in *in vitro* studies, and that of raloxifene and tamoxifen are 10–100 nM in these studies [28,29]. Therefore, results of our research were considered as the pharmacological effects of estradiol and SERMs on osteoblasts *in vitro*. In our present study, however, these 30 genes reported by ERs transfected osteosarcoma cells were not up- or down-regulated in human normal osteoblasts. Both estradiol (10^{-8} to 10^{-6} M) and raloxifene (10^{-7} and 10^{-6} M) were also reported to result in a significant reduction in IL-6 production, although these effects were more pronounced with estradiol in osteosarcoma cell line (Saos-2), primary human osteoblast (HOB), and human bone marrow stromal cell line (HCC1) [30]. HOB cells were obtained from trabecular bone of four different patients (two males and two females) [30]. The study using primary human osteoblasts obtained from trabecular bone of postmenopausal woman, however, demonstrated that the effects of estradiol (10^{-8} M) or tamoxifen (10^{-8} M) did not change IL-6 expression, but raloxifene (10^{-8} M) produced a significant decrease in IL-6 mRNA expression level [31]. These discrepancies regarding down-regulation of IL-6 expression induced by SERMs and estradiol may be due to the potential differences of expression levels and patterns of ER and its isoform in human osteoblasts and its alternative osteoblast-like cells.

Therefore, in our present study, we focused on up-regulated genes induced by SERMs and estradiol using microarray analysis in hFOB osteoblasts. Over expression of MAF, which turned out to be raloxifene-specific inducible gene in our present study, selectively inhibits transcriptional activation of both IL-12 p40 and IL-12 p35 genes [32]. ERβ but not ERα are predominantly detected in osteoblasts located on human cancellous bone using immunohistochemical analysis [33]. In osteoblast-like cell lines, osteosarcoma Saos-2 and MG-63, relatively higher ERα mRNA expression was detected than that of ERβ [25]. In hFOB cell, both mRNA and protein of ERβ were more predominant than those of ERα. Therefore, hFOB

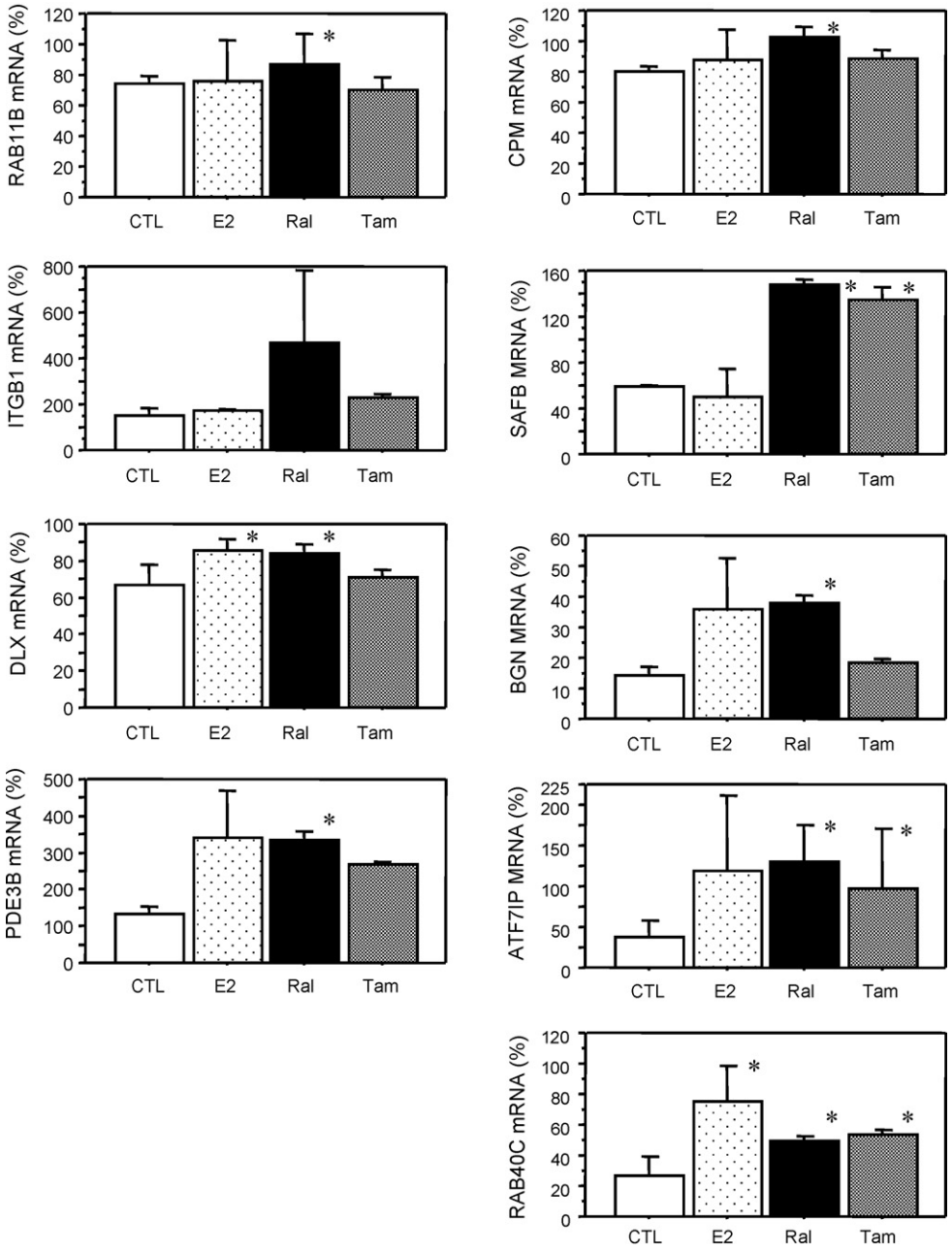

Fig. 4. Validation of microarray analysis using quantitative RT-PCR. All data were demonstrated as mean ($n = 3$) ± SD. Similar results were obtained in at least two independent experiments. CTL: vehicle (0.05% DMSO) control, E2: 10^{-8} M estradiol, RAL: 10^{-7} M raloxifene, TAM: 10^{-7} M tamoxifen, $^{*}p < 0.05$ *vs.* CTL.

examined in our present study is considered to maintain relatively native status of sex steroids pathways present in human osteoblasts and is considered suitable *in vitro* model for examining effects of steroids on human non-pathological osteoblasts.

In our present study, we focused on raloxifene-specific inducible gene, MAF and estrogen-related GAS6 genes in hFOB microarray analysis in order to further characterize the potential differences of bone-sparing effects between raloxifene and estradiol. The transcription factor MAF is required for normal chondrocyte differentiation during endochondral bone development in mice [34]. MAF was also reported to be detected in mice osteoblast [34], but its significance has still remained unclear. In multiple myeloma cells or bone marrow stroma cells, MAF up-regulates cycline D2, a promoter of cell cycle progression, and integrin β7, an adhesion molecule, respectively [35]. Therefore, MAF gene induced by only raloxifene treatment in our present study may be involved in the process of regulating cell growth or cell–cell interaction in human normal osteoblasts but it awaits further investigations for clarification. The expression of GAS6 mRNA transcript was also reported in mice osteoblasts, osteoclasts, and bone marrow cells [36]. GAS6 protein is a secreted protein originally identified as the ligand for the tyrosine kinase receptor Axl, and Gas6 was reported to be able to protect NIH3T3 cell apoptosis induced by growth factor depletion [37]. The ER-specific antagonist, ICI182,780 demonstrated no inhibitory effects on MAF expression increased by only raloxifene treatment in hFOB cells. In quantitative RT-PCR analysis, GAS6 expression was increased by raloxifene, tamoxifen, and estradiol treatments and this increase was completely inhibited by ICI182,780 treatment. GAS6 gene has estrogen response element (ERE) in its promoter lesion [38], and is considered to be primary target gene of ER in mammary epithelial cells [39]. In our present study, an increment of GAS6 mRNA levels by E2 and tamoxifen treatments could not

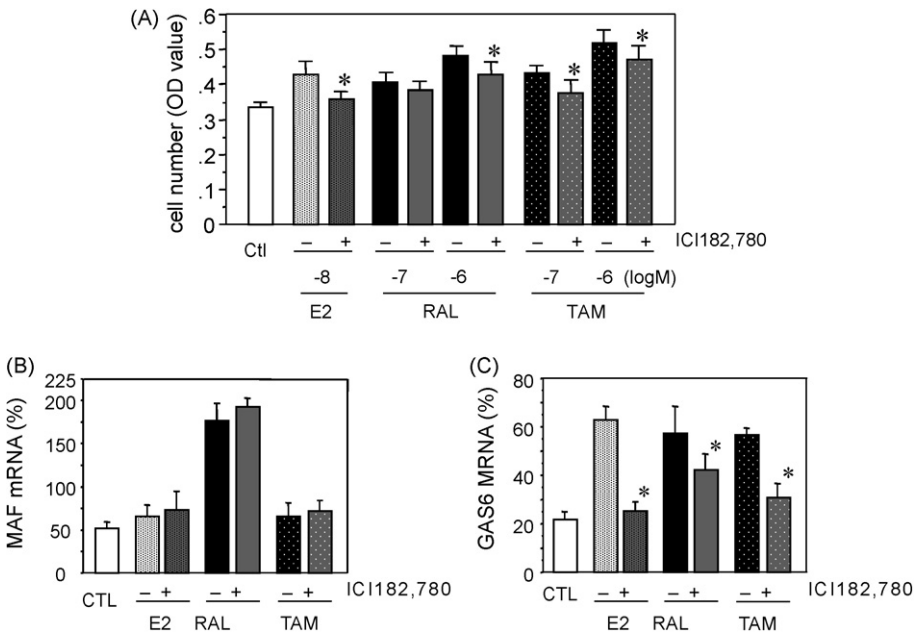

Fig. 5. A: Effects of ICI 182,780 (10^{-6} M) on raloxifene (10^{-7} and 10^{-6} M), tamoxifen (10^{-7} and 10^{-6} M), and estradiol (10^{-8} M) stimulated the cell proliferation of hFOB. B: Effects of ICI 182,780 (10^{-6} M) on MAF gene expression in hFOB. C: Effects of ICI 182,780 (10^{-6} M) on MAF gene expression in hFOB. All data were demonstrated as mean ($n = 3$) ± SD. Similar results were obtained in at least two independent experiments. CTL: vehicle (0.05% DMSO) control. E2: 10^{-8} M estradiol, RAL: 10^{-7} M raloxifene, TAM: 10^{-7} M tamoxifen, +: with ICI 182,780, −: without ICI 182,780, $^{*}p < 0.05$ *vs.* CTL.

be detected using microarray analysis. This discrepancy may be explained, in part, by the greater dynamic range afforded by quantitative RT-PCR [40]. In addition, the differences in hybridization conditions for each gene-specific primer set and the corresponding cDNA target may also contribute to this discrepancy [41]. hFOB cell growth induced by raloxifene treatment was not completely inhibited by ICI182,780 treatment in our present study. These results all suggest that raloxifene may stimulate hFOB cell proliferation through both ER dependent and independent pathways such as MAF and GAS6 pathways. Endogenous estradiol metabolites induce potent biological effects through ER independent pathways in several cell lines including osteosarcoma cells [42,43]. Maran et al. [43] also reported that estradiol leads to the activation of signal transducers and activators of transcription 1 (STAT1) protein in ER negative osteoblast cell line. Furthermore, tamoxifen also has potential effects on transcriptional factors via other nuclear receptors such as steroid and xenobiotic receptor [44], which is expressed in osteoblast [45]. Results form these reports suggest that raloxifene including its metabolites may activate transcriptional factors directly in human osteoblasts. However, the mechanisms of direct effects of both MAF and GAS6 gene on hFOB cell proliferation induced by raloxifene require further investigations for clarification.

In normal bone remodeling, bone formation by osteoblasts follows bone resorption by osteoclasts and occurs in a precise and quantitative manner (coupling) [33]. In this coupling between bone formation and resorption, a coupling factor that induces bone formation is considered to be released during the process of osteoclastic bone resorption [33]. Estrogens are well known to exert pro-apoptotic effects on osteoclasts and anti-apoptotic effects on osteoblast and osteocytes [46]. This study has focused on the specific effects on osteoblast cells. However, it is true that there were significant increases in both serum bone formation and resorption markers in postmenopausal women administered with raloxifene treatment [14]. In CD14+ monocyte, tamoxifen but not raloxifene directly inhibited both human osteoclast formation and bone resorption [47]. In the co-cultures studies employing either SaOS-2 or MG-63 cells, raloxifene as well as tamoxifen inhibited osteoclast formation of CD14+ monocyte [47]. These findings as well as those of our present studies all suggest that raloxifene exert beneficial of bone-sparing effects on human bone including the modulation of an interaction between osteoblasts and osteoclasts with different pathway from tamoxifen treatment.

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