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Agonistic Effect of Tamoxifen Is Dependent on Cell Type, ERE-Promoter Context, and Estrogen Receptor Subtype: Functional Difference between Estrogen Receptors α and β

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To investigate the functional differences between estrogen receptor (ER) α and β subtypes, we studied the expression and the transcription stimulating activities of these receptors. RT-PCR has demonstrated that ERα is expressed at a high level in MCF-7 cells derived from human breast cancer. Both ERα and ERβ were expressed at a lower level in HOS-TE85 and Saos2 cells derived from human osteosarcoma. Chloramphenicol acetyltransferase reporter assay detected the transcriptional activation by the endogenous receptor only in MCF-7 cells. Agonistic effect of tamoxifen was observed as strong as that of 17β-estradiol on ERE activation in MCF-7 cells at the concentration of 10^{-7}M when ERE-containing reporter is constructed with β-globin promoter. The effect of tamoxifen was not apparent when the reporter was constructed with thymidine kinase promoter, suggesting that the differential gene activation between tamoxifen and estrogen may take place depending upon ERE-promoter context. Agonistic activity of tamoxifen was also detected in COS-7 and Saos-2 cells, but not in HEC-1 cells derived from human endometrial carcinoma via exogenously expressed ER. Interestingly, this effect was ERα specific. Thus, we demonstrate that agonistic effect of tamoxifen depends on the cell type, ERE-promoter context, and ER subtype. These parameters would explain at least a part of the tissue specific effects of antiestrogens *in vivo*.

Estrogen receptor (ER) is a member of the steroid/thyroid hormone receptor superfamily. These receptors mediate their action by binding ligand dependently to the responsive elements present in the enhancer region of target genes and regulating their transcription directly (1, 2). Estrogen regulates the development of female organs and the growth, differentiation, and function of target cells. Estrogen also plays some important roles in non-reproductive systems such as the central nervous system (3-5), the skeletal system (6, 7) and the cardiovascular system (8, 9).

Antiestrogens, including tamoxifen and raloxifene, were developed for therapeutic agent of breast and uterine cancers (10-12). These act as an antagonist by competing the binding of estrogen to ER, inhibiting the effects of estrogen (10, 11). On the other hand, some tissue-specific estrogenic activities of antiestrogens have been reported. Tamoxifen exerts estrogen-like effects on bone metabolism (13), lipid metabolism (14) and uterine growth (15), whereas it dose not stimulate normal breast tissue growth (16). Raloxifene is known to have more specific estrogenic activities in lipid metabolism and bone metabolism (17). However, the mechanism of the tissue specific agonistic effect of some antiestrogens is not fully understood.

Recently, a new estrogen receptor subtype ERβ has been isolated from rat prostate (18) and human testis (19). This novel receptor also binds to estradiol and has the ligand dependent transcription stimulating activity via ERE. Interestingly, ERβ was shown to have different tissue distribution and different ligand binding specificity from ERα (20). In this study, the differential expression of ERα and ERβ in human cultured cells is analyzed using RT-PCR. In MCF-7 cells, which express ERα abundantly, estrogen and tamoxifen show para-

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Abbreviations: ER, estrogen receptor; ERE, estrogen responsive element; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse transcriptase polymerase chain reaction.

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doxical effects on the ERE activation and the cell growth. Then, we show that estrogenic effect of tamoxifen is dependent upon cell type, ERE-promoter context and ER subtype.

MATERIALS AND METHODS

Analysis of ERα and ERβ expression by RT-PCR in human cells. MCF-7, HEC-1, HOS-TE85 and Saos-2 cells were grown to a subconfluent state in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS). These cells were collected using ISOGEN (Nippon gene) and total RNA was prepared according to the manufacturer's instruction. Poly $(A)^+$ RNA was prepared using oligotex dT30 super (Takara). Using 0.2 μg of mRNA for each cell line, cDNA was synthesized using random primer of 1-st strand cDNA synthesis kit (Boehringer) according to the manufacturer's instruction. For the PCR reactions, an appropriate volume of the synthesized cDNA was added to a PCR reaction and 30 cycles of amplifications (1 min at 94 °C; 1 min at 55 °C; 1 min at 72 °C) were performed. The PCR products were separated on a 1 % agarose gel. The oligonucleotides, 5′-CTACATCATC-TCGGTTCCGC-3′ and 5′-GCTGTA-CAGATGCTCCTGGTGC-3′ were used for amplification of 292 bp fragment of ERα mRNA. The oligonucleotides, 5′-ATCTTTGACAT-GCTCCTGGC-3′ and 5′-ACGCTTCAGCTTGTGACCTC-3′ were used for amplification of 515 bp fragment of ERβ mRNA. The plasmids containing hERα and hERβ were used for positive controls of PCR reactions.

Plasmids construction. Human ERα expression vector pSSRα-hERα was constructed by ligating a 2.7 kb EcoRI fragment of HEG0 (21) into the EcoRI sites of the eukaryotic expression vector, pSSRα (22). Human ERβ expression vector pSSRα-hERβ was constructed using ERβ cDNA isolating by phage screening and RT-PCR. The open reading frame sequence of the ERβ was completely corresponded with the published one (19). The oligonucleotides containing the wild type ERE of Xenopus vitellogenin gene A2 enhancer (vit-ERE) (−338/−310) (23) was synthesized as described (24). These oligonucleotides were annealed and inserted into the SacI site of pUC18. The ERE-tk-CAT and ERE-GCAT were constructed by ligating the HindIII and the SalI fragment of vit-ERE into the appropriate sites of the pBLCAT (25) and pGCAT (26).

Cell culture and chloramphenicol acetyltransferase (CAT) analysis. CAT analysis was performed as follows. Briefly, 1×10^6 cells were plated in 60 mm Petri dishes and maintained in DMEM containing 10 % FBS for 24 hr. One hour prior to transfection, the medium was changed to phenol red-free DMEM containing 10 % dextran coated charcoal treated FBS (27). Cells were transfected by the calcium phosphate precipitation method with 0.2 μg of ER expression vector, 2 μg of reporter plasmids and 2 μg of PCH110 β-galactosidase expression vector (Pharmacia), used as an internal control to normalize for variation in transfection efficiency. In some experiments, ER expression vector was omitted. The total amount of transfected DNA was made up to 20 μg with carrier DNA pGEM3Zf(−) (Promega). After 12 hr of incubation, the cells were cultured further in the absence or presence of various amounts of 17β-estradiol, tamoxifen and raloxifene for 24 hr. Cell extracts were prepared and assayed for β-galactosidase and CAT activities. The results were analyzed by BAS2000 Bio Image Analyzer (Fuji Film) and the signal intensities were quantified.

Statistical analysis. Data are presented as mean ± standard deviation (SD). The differences between control and each treated value in CAT analysis were evaluated using Student's *t*-test. The growth differences between control and each treated sample were analyzed using one-way analysis of variance, followed by the nonparametric Dunnett's test.

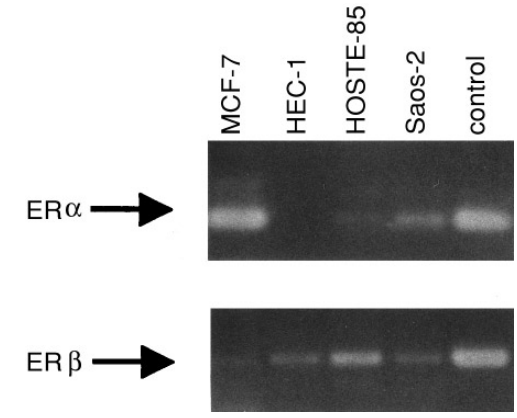

FIG. 1. The expression of ERα and ERβ mRNA in human cultured cells. cDNAs were synthesized using 0.2 μg of mRNA from MCF-7 (human breast cancer), HEC-1 (human endometrial carcinoma), HOS-TE85, and Saos-2 (human osteosarcoma) cells. RT-PCR was performed using 0.1 volume of each cDNA. As the positive controls, full length human ERα and β containing plasmids were used for the PCR. The 0.2 volume of the products was loaded on a 1% agarose gel. The 292 bp fragment for ERα and the 515 bp fragment for ERβ were indicated by arrows.

RESULTS

Expression of ERα and ERβ in human cultured cells. To investigate the expression of ERα and β mRNA, mRNA was isolated from human cells and used for RT-PCR. Although it is difficult to estimate the accurate quantity of mRNA by RT-PCR, the expression patterns of ERα and β were quite different (Fig. 1). For ERα the intensities of the bands were highest in MCF-7 cells and lower in HOS-TE85 and Saos-2 cells. The signals for ERβ were detected at a low level in HOS-TE85, Saos-2 and HEC-1 cells. We could hardly detect the signal of ERα in HEC-1 cells and that of ERβ in MCF-7 cells.

Analysis of transcriptional activation and growth induction in MCF-7 cells. Using the cells described above, CAT assays were carried out to see the function of endogenous ER. We could detect estrogen dependent transactivation via ERE linked to β-globin promoter only in MCF-7 cells (Fig. 2A). Notably, both tamoxifen and raloxifene showed the agonistic activities under these conditions. The transcription activation capability of tamoxifen, in particular, was as high as that of 17β-estradiol at 10^{-7}M. At the same concentration, 17β-estradiol promoted the growth of MCF-7 cells (Fig. 2B). Despite the strong agonistic activity of tamoxifen observed in the CAT assay, it had no effect on the growth of MCF-7 cells under the same condition (Fig. 2B). When we replaced the promoter of ERE-containing reporter to the ERE-tk-CAT, no significant agonistic activities of tamoxifen and raloxifene could be observed in MCF-7 cells any more (Fig. 2C).

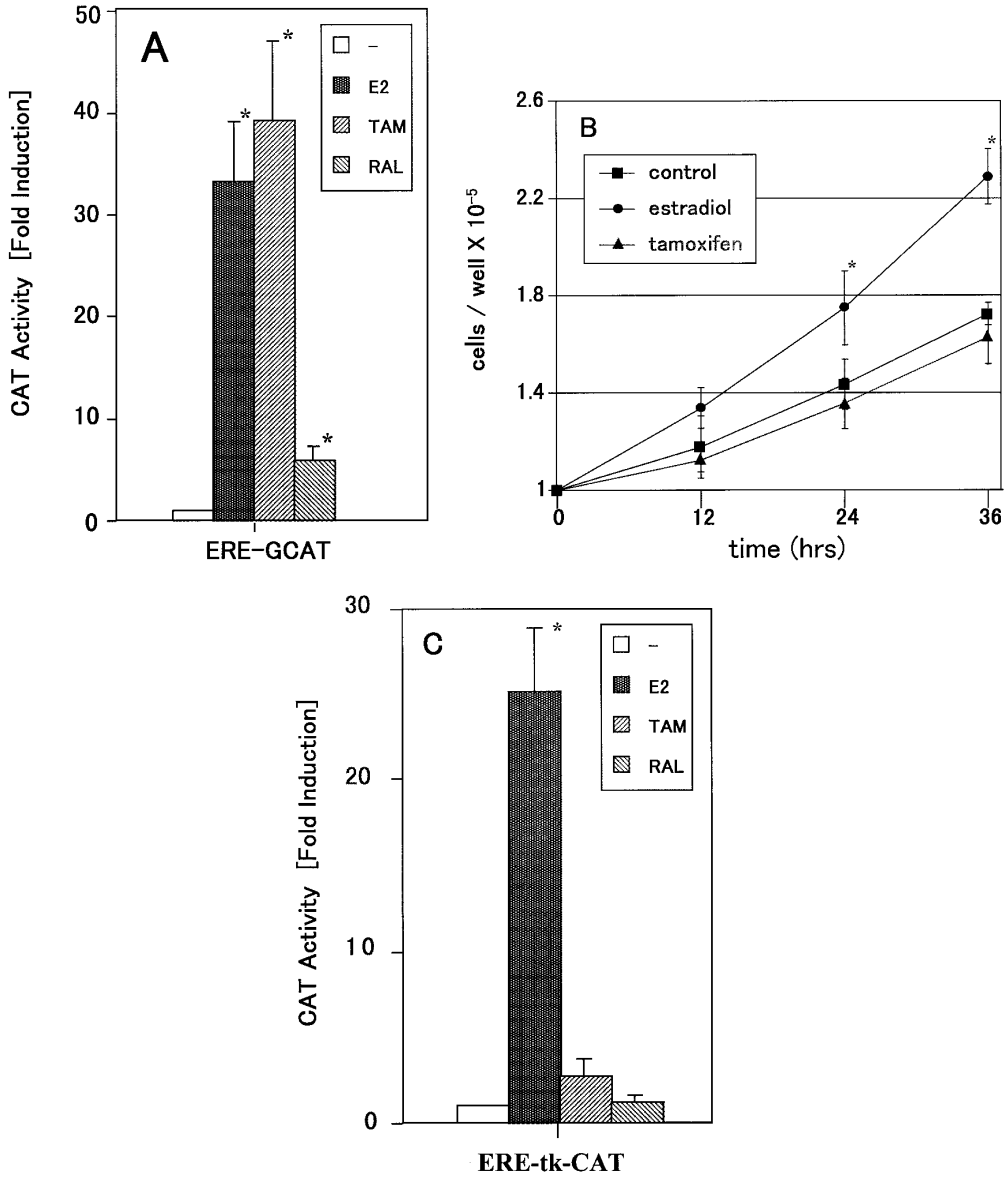

FIG. 2. Activation of transcription and growth by estrogen and antiestrogens in MCF-7. MCF-7 cells were transfected with the reporter plasmid, ERE-GCAT (A) or ERE-tk-CAT (C), with an internal control PCH110 plasmid. After transfection, cells were incubated for 24 h in the presence or absence of 100 nM 17β-estradiol (E2,) tamoxifen (TAM), or raloxifene (RAL). Results were indicated as fold induc-tion over the basal level in the absense of 17β-estradiol. (B) 1×10^5 cells were incubated in the presence or absence of 100 nM 17β-estradiol or tamoxifen. Cells were harvested and counted at the indicated times after treatment with agents. Each bar and point represent means $\pm$ SD (n = 3). *, $P < 0.05$ *vs.* basal level.

Antagonistic effects of tamoxifen and raloxifene against human ERα and ERβ. To investigate the functional differences between ERα and ERβ, effects of 17β-estradiol and antagonistic effects of tamoxifen and raloxifene were studied using exogenously expressed human ERα and ERβ in COS-7 cells. In these cells, 17β-estradiol stimulated transcription by both ERα and ERβ via ERE-GCAT. Tamoxifen and raloxifene showed dose-dependent transcriptional inhibition against this ERE activation by both ERα and ERβ to similar extents (Fig. 3).

ERα selective and cell type specific agonistic effect of tamoxifen in COS-7, Saos-2, and HEC-1 cells. The agonistic effects of tamoxifen and raloxifene were analyzed using ERE-GCAT in several human cells. While some agonistic effect of tamoxifen was observed for ERα, this effect was not observed for ERβ in COS-7 cells (Fig.4A). This ERα selective agonistic effect of tamoxifen was also detected in Saos-2 (Fig. 4B) and other cells, MG63, HHUA and HeLa cells (data not shown). However, this effect was not observed in HEC-1 cells (Fig. 4C). In addition, the agonistic effect of tamoxifen was lower than the

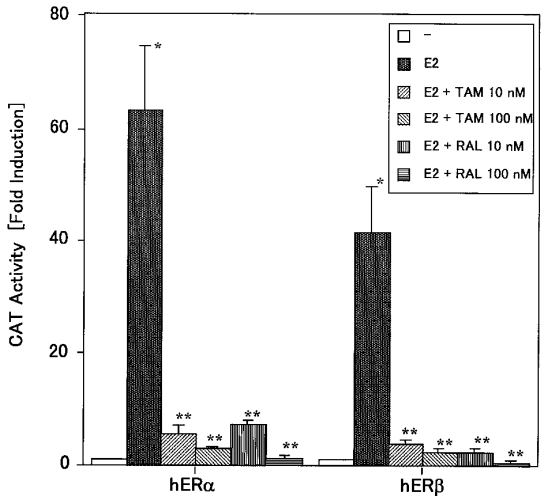

FIG. 3. Effect of 17β-estradiol and antagonistic effect of tamoxifen and raloxifene against exogenously expressed human ERα and ERβ in COS-7 cells. COS-7 cells transfected with ERE-GCAT, PCH110, and pSSRα-ERα or pSSRα-ERβ were incubated for 24 h in the presence or absence of 100 nM 17β-estradiol and the cell extract was assaayed for CAT activity. Dose response of tamoxifen (TAM) and raloxifene (RAL) in the presense of 100 nM 17β-estradiol (E2) was plotted. Results were indicated as fold induction over the basal level in the absense of 17β-estradiol. Each bar represents the mean ± SD (n = 3). *, $P < 0.05$ *vs.* basal level. **, $P < 0.05$ *vs.* 17β-estradiol treatment.

effect of 17β-estradiol in these cells. No agonistic effects of raloxifene were detected in any of cells examined here (Fig. 4A, B, C). Tamoxifen agonism was not significant in ERα-transfected COS-7 cells when ERE-tk-CAT reporter was used (Fig. 4D).

DISCUSSION

In this study, we have shown the expression of ERα and ERβ mRNA in human cultured cells that are assumed to be estrogen target cells (28-30). Our results indicate that MCF-7 cells, derived from a mammary carcinoma, express only ERα, but two osteosarcoma cell lines examined here express both ERα and ERβ. While we showed expression of ERα and ERβ mRNA in these cells by RT-PCR, we could detect transcriptionally active ER only in MCF-7 cells by CAT analysis. The inability for ERE activation in other human cell lines may be due to the low expression of ER and/or the low sensitivity of CAT assay. Alternatively, we may be detecting by RT-PCR splicing variant that cannot activate ERE-GCAT reporter plasmid. Because ERα and ERβ are highly conserved in some region, ERα probe may have detected ERβ signals in past studies. While both ERα and ERβ could be detected widely in cultured cells, we may have to re-evaluate the previous results of ER expression carefully.

In MCF-7 cells, transcriptional activation by tamoxifen was as high as that by 17β-estradiol. Many studies indicated that the growth of MCF-7 cells was stimulated by 17β-estradiol and this stimulation was antagonized by tamoxifen (31, 32). The transactivation by 17β-estradiol via ERE-linked reporter and mixed agonist/antagonistic effect of tamoxifen have also been reported (33). However, the relationship between the growth effect and the transcriptional effect was not well analyzed so far. Interestingly, in spite of high transcriptional activation of ERE-GCAT by tamoxifen which is as high as 17β-estradiol, only 17β-estradiol but not tamoxifen showed the stimulation of cell growth. This paradoxical effect of tamoxifen may be dependent on the promoter specificity of tamoxifen activation as shown by the experiments comparing ERE-GCAT and ERE-tk-CAT reporter. This may be due to the different composition of the co-activators which transmit the effect of ER-ligand complex to the transcription initiation complex at the promoter of growth related genes. Differential gene activation via different promoter can thus take place in the cells between tamoxifen and 17β-estradiol. Actually, such differential gene activation has recently been reported in GC3 (34) and MCF-7 cells (35).

We compared the transcriptional response of ERα and ERβ against 17β-estradiol and two antiestrogens in several human cells. The differential response between ERα and ERβ was only observed in the agonistic effect of tamoxifen. This agonistic effect of tamoxifen was also cell type specific. The effect of tamoxifen was lower than that of 17β-estradiol in COS-7 and Saos-2 cells and was not detected in HEC-1 cells. In MCF-7 cells, agonistic effect of tamoxifen was strongly observed and raloxifene also showed a lower agonistic activity. It is possible that the balance of intracellular ERs and co-factors is important for these agonistic transcriptional activation by antiestrogens.

ER has two transcriptional activation functions; AF-1 is present in the N-terminal region of the receptor and AF-2 is in the C-terminal ligand binding domain (36). While one of the antiestrogen, ICI16384 (ICI) inhibits ER actions through both AF-1 and AF-2, tamoxifen inhibits only through AF-2 function (37). It has also been reported that agonistic activity of tamoxifen is due to AF-1 activity of ER and that this activity is cell type and promoter context specific (38). Tamoxifen is assumed to allow AF1 to function, namely it has agonistic activity in the cells in which AF1 becomes active. The homology between human ERα and ERβ of DNA binding domain is 96 % and that of ligand binding domain including AF-2 is 58 %. However, that of N-terminal A/B domain including AF-1 is only 22 %. ERα specificity of agonistic effect of tamoxifen may be due to the difference between N-terminal AF-1 region of ERα and ERβ.

Another pathway of tamoxifen agonism has been shown to be the ER/AP-1 pathway which is also cell type specific (39). In this report, we showed that the

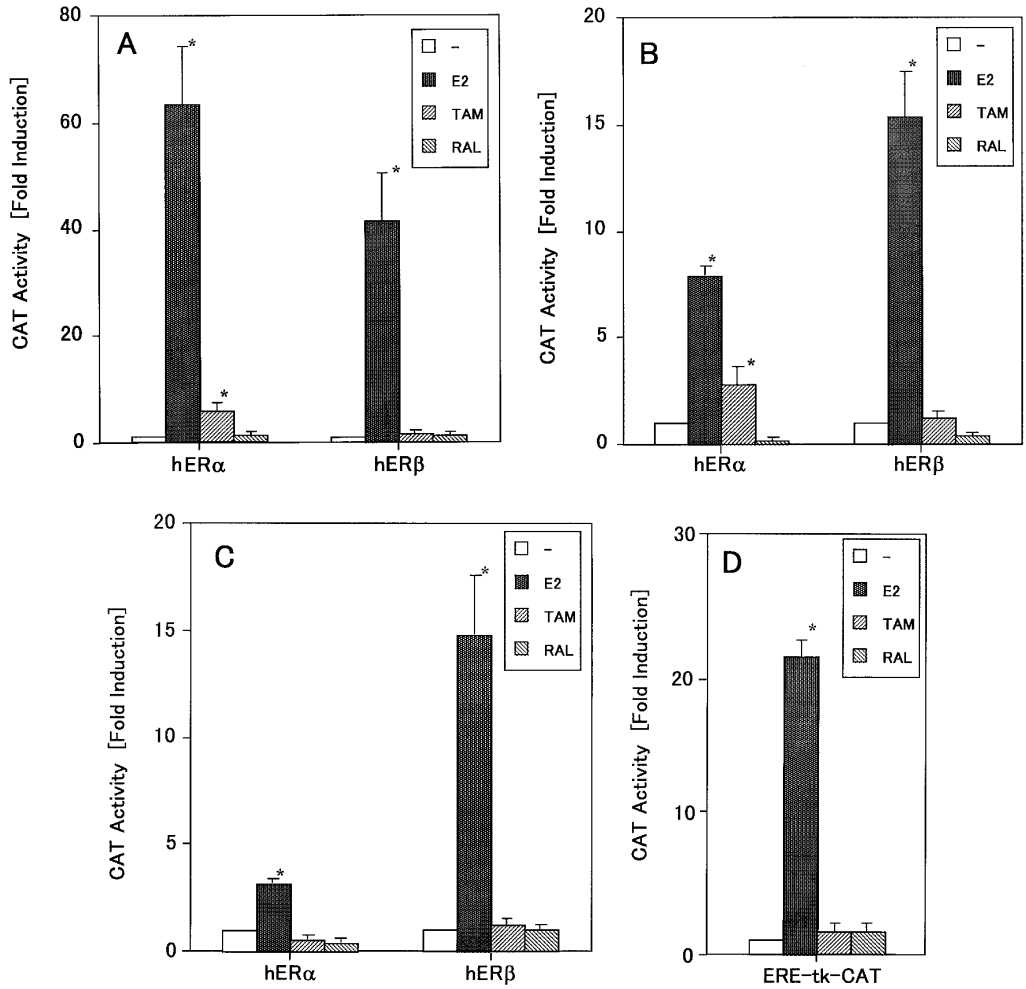

FIG. 4. Effects of 17β-estradiol, tamoxifen, and raloxifene on exogenously expressed human ERα and ERβ in COS-7, Saos-2, and HEC-1 cells. COS-7 (A), Saos-2 (B), and HEC-1 cells (C) transfected with ERE-GCAT, PCH110, and pSSRα-ERα or pSSRα-ERβ were incubated for 24 h in the presence or absence of 100 nM 17β-estradiol (E2), tamoxifen (TAM), or raloxifene (RAL). (D) COS-7 cells transfected with ERE-tk-CAT, PCH110, and pSSRα-ERα were incubated for 24 h in the presence or absence of 100 nM 17β-estradiol (E2), tamoxifen (TAM), or raloxifene (RAL). Results were indicated as fold induction over the basal level in the absence of E2. Each bar represents the mean $\pm$ SD (n = 3). *, $P < 0.05$ *vs.* basal level.

agonistic activity of tamoxifen was ERα specific and ERE-GCAT reporter specific. The vitellogenin ERE and rabbit β-globin promoter used here do not contain AP-1 like sequences (data not shown), suggesting that our result of tamoxifen agonism may not be mediated by the ER/AP-1 pathway.

In this report, we suggested that the promoter context selective transactivation of tamoxifen could explain the inability of tamoxifen to promote the growth of MCF-7 cells. At least a part of tissue specific agonistic effects of antiestrogens may be explained by the promoter context and ER type specific transcriptional activation by these agents.

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