

International Congress on Hormonal Steroids and Hormones and Cancer Up-regulation of LRP16 mRNA by 17 β -estradiol through activation of estrogen receptor α (ER α), but not ER β , and promotion of human breast cancer MCF-7 cell proliferation: a preliminary report

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Abstract

LRP16 is a novel gene cloned from lymphocytic cells, and its function is not known. The expression level of LRP16 mRNA was up-regulated by estrogen in breast cancer MCF-7 cells based on the computed aided serial analysis of gene expression (SAGE) analysis. In this study, we investigate the effect of 17 β -estradiol (17 β -E₂) on the expression of LRP16 mRNA and the effects of overexpression of LRP16 on the proliferation of cultured MCF-7 cells and the possible mechanisms involved. The expression level of LRP16 mRNA induced by 17 β -E₂ was determined by Northern blot analysis. LRP16 promoter-controlled luciferase expression vector (pGL3-S₀) was co-transfected with various nuclear receptors, including estrogen receptor α and β (ER α and ER β), glucocorticoid receptor α (GR α), androgen receptor (AR) and peroxisome-proliferator activated receptor γ and α (PPAR γ and PPAR α) into COS-7 cells, and the relative luciferase activity was measured using Dual-luciferase report assay systems. The effect of overexpression of LRP16 on MCF-7 proliferation was examined by the Trypan Blue exclusion method, and the cell cycle was analyzed by flow cytometry. The expression levels of cyclin E, p53 and p21^{WAF1/CIP1} proteins were determined by Western blot analysis. The results showed (1) 17 β -E₂ induced a five- to eightfold increase in LRP16 mRNA levels in MCF-7 cells; (2) the relative luciferase activities in the COS-7 cells co-transfected by pGL3-S₀ and ER α or AR were 7.8-fold and 11-fold respectively of those in the control cells transfected by pGL3-S₀ alone; (3) overexpression of LRP16 stimulated MCF-7 cell proliferation, and the numbers of cells in the S-phase of the cell cycle in cells transfected with LRP16 increased about 10% compared with the control cells; and (4) cyclin E levels were much higher in cells with overexpression of LRP16 than in the control cells, while the expression levels of p53 and p21^{WAF1/CIP1} were not different between the two groups of cells. From these results we concluded that estrogen up-regulates the expression level of LRP16 mRNA through activation of ER α and that overexpression of LRP16 promotes MCF-7 cell proliferation probably by increasing cyclin E.

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Introduction

Estrogen has very important effects on normal growth and differentiation in the mammary gland. Breast cancer development and progression are directly related to the effects of estrogen. The cloning of estrogen receptor alpha (ER α) from a breast cancer cell line, MCF-7, led to a greater understanding of its role in mediating the effects of estrogen (Green *et al.* 1986). ER α , as a transcription factor, binds to estrogen-response elements (EREs) in the promoter region of target genes (Tsai & O'Malley 1994) or interacts with proteins in other pathways (Batistuzzo de Medeiros *et al.* 1997, Galienand & Garcia 1997, Paech *et al.* 1997, Porter *et al.* 1999) to regulate the transcription of specific genes in estrogen-dependent cells. Numerous studies indicate that estrogen can induce and promote breast cancer and about 50% of primary breast cancers are ER α positive. For these reasons, decreasing estrogen production by aromatase inhibitors or blocking its action with anti-estrogens, e.g. tamoxifen and raloxifene, are the main treatments for ER α -positive tumors (Gradishar & Jordan 1997, Santen & Harvey 1999, Ali & Coombes 2000, Santen 2002). In addition to the direct effects of ER α as a nuclear transcription factor on breast cancer cells, there is growing evidence that estrogen has a proliferative effect on breast cancer cells via the regulation of various polypeptide growth factors, including insulin, insulin-like growth factor, transforming growth factor α (TGF α) and TGF β (Knabbe *et al.* 1987, Bates *et al.* 1988, Osborne *et al.* 2001), growth factor receptors or other signaling molecules (Huynh *et al.* 1996). ER α can also directly or indirectly through growth factors activate phosphatidylinositol-3-kinase and mitogen-activated protein kinase pathways to regulate cell proliferation (Kato *et al.* 1995, Simoncini *et al.* 2000, Santen *et al.* 2002b).

LRP16 is a novel gene which was cloned from human lymphocyte cells by our group in 1999 using restriction length genomic scanning (RLGS), and then the cDNA was isolated using the rapid amplification of cDNA end (RACE) technique (GenBank Accession No. AF202922) (Yu *et al.* 2000). LRP16 contains an open reading frame for 325 amino acids. A Genbank search did not reveal a known protein with structural similarity to LRP16, and the detailed function of this gene is not exactly known. Based on a computer-aided SAGE (serial analysis of gene expression) pattern analysis, we found that the number of tags (5'-GAAGGAGATG-3') representing LRP16 mRNA was significantly increased in MCF-7 cells cultured with 17 β -estradiol (17 β -E $_2$) compared with cells cultured without 17 β -E $_2$. Thus we hypothesized that LRP16 may be a downstream gene of the ERs including ER α and ER β , and may contribute to MCF-7 cell proliferation after activation by ERs. In this study, we investigated the expression level of LRP16 mRNA in cultured MCF-7 cells, the regulation of the luciferase activity driven by LRP16 promoter and various nuclear receptors, and the effects of overexpression of LRP16 on MCF-7 cell proliferation.

Materials and methods

Materials

The vectors including pGL3-Basic, pRL-SV40, pGL3-CMV and pGEM-T-easy, Dual-luciferase report assay 1000 systems, PCR product extraction kit, plasmid purification kit and probe DNA labeling reagents were purchased from Promega. Nuclear receptor expression vectors including ER β , ER α , glucocorticoid receptor α (GR α), androgen receptor (AR) and peroxisome-proliferator activated receptor γ and α (PPAR γ and PPAR α) were kindly donated by Dr Hajime Nawata from Kyushu University of Japan. The transfection reagent SuperFect was purchased from Qiagen (Hilden, Germany). 17 α -Estradiol, testosterone, dexamethasone and fenofibrate were purchased from Sigma (St Louis, MO, USA). Troglitazone was obtained from Sankyo Pharmaceuticals (Tokyo, Japan). All the above mentioned compounds were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the cell growth medium was 0.1% (v/v). LA *Taq* DNA polymerase and various restriction enzymes were purchased from TaKaRa (Otsu, Shiga, Japan). Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), proteinase K, RNA extraction reagent Trizol and G418 were purchased from Gibco BRL (Grand Island, NY, USA). Superscript II RNase H⁻ reverse transcriptase was purchased from Invitrogen (Carlsbad, CA, USA). [α ³²P]dCTP was purchased from YaHui Chemical Co. (Beijing, China). All antibodies, including anti-human cyclin E, p53, p21^{WAF1/CIP1} and β -actin used for the Western blot analysis and the chemiluminescence luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The COS-7 cell line and human breast cancer MCF-7 cells were purchased from ATCC (Rockville, MD, USA). COS-7 cells were maintained in DMEM supplemented with 10% FCS and 100 U/ml penicillin-streptomycin, and MCF-7 cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 10 mg/ml bovine insulin and 100 U/ml penicillin-streptomycin in a humid atmosphere with 5% CO $_2$ at 37 °C.

Northern blot analysis

To study the time- and dose-dependent effects of 17 β -E $_2$ on the expression level of LRP16 mRNA in MCF-7 cells, the cells were treated with various concentrations of 17 β -E $_2$, from 10⁻⁹ M to 10⁻⁶ M, for 48 h, or were treated with 10⁻⁶ M 17 β -E $_2$ for 3 to 48 h. An equal volume of DMSO was added to the cultured MCF-7 cells as control. Total RNAs were prepared by the acid guanidium thiocyanate-phenol

chloroform method using Trizol purchased from Gibco BRL. Twenty micrograms total RNA were electrophoresed through a 1% agarose gel containing formaldehyde and were transferred to a Hybond N⁺ membrane (Amersham). The membrane was hybridized with ³²P-labeled LRP16 cDNA probe in 5 ml hybridization buffer containing 50% deionized formamide, 0.5 × SSPE, 0.5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 1 mg salmon sperm DNA (Sigma). After hybridization overnight at 50 °C, the membrane was washed according to the manufacturer's instructions and exposed to BioMax-MS film (Eastman Kodak) for 2 days at –70 °C.

Plasmid construction and luciferase analysis

An LRP16 promoter fragment about 2.6 kb upstream from the translation start site (–2623 to –23 bp) was generated from genomic DNA of mononuclear cells from a healthy adult by PCR. The sequences of sense and antisense primers to amplify this fragment were 5'-GAGCTCCGCGCTGGCGTTTGAACATGG-3' and 5'-AAGCTTCCGCCACTTGGACTCTATTT-3' respectively. The PCR products were subcloned into pGEM-T-easy vector and were sequenced to ensure that no misincorporated mutations were introduced into the PCR products during amplification. The reporter recombinant pGL3-S₀ was generated by inserting the promoter fragment into the SacI and HindIII sites of pGL3-basic vector. Transfection was performed using SuperFect reagents according to the manufacturer's instructions. Briefly, COS-7 cells were seeded in 35 mm dishes (1 × 10⁵ each dish) 12 h prior to transfection, and they were co-transfected with 1 µg pGL3-S₀ and 1 µg of various nuclear receptors including ERα, ERβ, GRα, AR, PPARγ or PPARα. One hundred nanograms renilla luciferase control vector pRL-SV40 (as an internal standard) were added to each dish to assess the transfection efficiency. After 12 h of transfection, 10^{–6} M of various ligands including 17β-E₂, testosterone, dexamethasone, troglitazone or fenofibrate were added to the dishes. On the next day of transfection, the cells were lysed and harvested using the Dual-luciferase reporter assay system and the luciferase activity was analyzed using Lumat LB 9507. The promoter activity was expressed as fold increase in luciferase activity normalized for renilla luciferase activity (termed relative luciferase activity).

Construction of LRP16 expression vector and stable transfection

The entire coding region of LRP16 was generated from cDNA extracted from MCF-7 cells by RT-PCR using the sense/antisense (5'-TGGGCGGAGAGCCGTCCCGCG-3'/5'-CGGGGCGCGGAGGGAAAGAAG-3') primers, the design of which was based on our previous reported

sequence (Han *et al.* 2001a,b). The PCR products were subcloned into pGEM-T-easy vector and sequenced to ensure that no misincorporated mutations were introduced into PCR products during amplification. The full coding region of LRP16, which was a 1134 bp fragment, was digested with KpnI and BamHI from pGEM-LRP16 and was inserted into the same sites of the pcDNA3.1 vector. The construction was confirmed by enzymatic digestion, and was sequenced to verify the correct reading frame. The construct (pcDNA-LRP16) was transfected into MCF-7 cells by the SuperFect transfection reagent. Two days after transfection, the cells were treated with G418 at 1 mg/ml for 3 weeks and then were continuously cultured with 0.5 mg/ml G418. For a cell proliferation assay, the cells transfected with LRP16 or empty vector were plated onto 24-well plates at 1 × 10⁴ cells/well in the culture medium and the medium was changed every 2 days. After washing with PBS two times, the cells were trypsinized and then counted by the Trypan Blue exclusion method using a hemocytometer every 24 h for 5 days.

Western blot analysis

The expression of the cell cycle-related proteins including p53, p21^{WAF1/CIP1} and cyclin E, and β-actin were examined by Western blot analysis, as described previously (Mu *et al.* 2001), using specific antibodies. The MCF-7 cells transfected with LRP16 or empty vector were washed with PBS and lysed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.2 mM/l phenylmethylsulfonyl fluoride supplemented with 0.2 trypsin inhibitory units/ml aprotinin, 0.7 µg/ml pepstatin, and 1 mg/ml leupeptin). The samples were sonicated for a few seconds to shear the DNA and reduce the viscosity. Twenty micrograms protein were mixed with an equal volume of 2 × electrophoresis buffer, then boiled for 3 min. The samples were electrophoresed on a 12% SDS-polyacrylamide gel, and proteins were transferred to a Hybond-P, polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Blots were rinsed in Tris-buffered saline–0.1% Tween-20 and blocked with 4% BSA overnight at 4 °C. The blots were incubated with mouse antihuman p53 (1:1000), goat antihuman p21^{WAF1/CIP1} (1:1000), rabbit antihuman cyclin E (1:1000), or mouse antihuman β-actin (1:200) antibodies for 1 h at room temperature. After washing with Tris-buffered saline–0.1% Tween-20 three times, blots were probed with horseradish peroxidase-labeled antimouse, antigoat or antirabbit IgG (1:1000) in blocking buffer. Proteins were detected using a chemiluminescence luminol reagent, and the bands were visualized by autoradiography.

Statistical analysis

For the cell proliferation assay, experiments were performed in triplicate, and the results were expressed as the mean + s.d.

of three independent experiments. A statistical analysis was performed using Statview software. All data were evaluated for paired variables to compare two groups. $P < 0.05$ was considered to be statistically significant.

Results

17 β -E₂ increases the expression level of LRP16 mRNA in MCF-7 cells

MCF-7 cells were treated with various concentrations of 17 β -E₂ from 10⁻⁹ to 10⁻⁶ M/l for 48 h, then the total RNA was extracted and the expression level of LRP16 mRNA was determined by Northern blot analysis. As shown in Fig. 1, the expression levels of LRP16 mRNA in the cells treated with various concentrations of 17 β -E₂ were two- to threefold higher than those in control cells. The time course for the effect of 10⁻⁶ M/l 17 β -E₂ on the expression level of LRP16 mRNA was investigated over a 48-h time period. The significant increase in LRP16 mRNA levels was observed as early as 3 h after addition of 17 β -E₂. The expression levels of LRP16 mRNA in the cells treated with 17 β -E₂ for 3 to 48 h were five- to eightfold higher than those in the cells before treatment with 17 β -E₂. This stimulatory effect of 17 β -E₂ was neither dose- nor time-dependent.

17 β -E₂ up-regulates LRP16 mRNA through activation of ER α

To investigate whether or not the elevation of LRP16 mRNA by 17 β -E₂ was associated with activation of ERs, and whether various other nuclear receptors including AR, GR α , PPAR γ and PPAR α also regulate the transcription of LRP16 mRNA, we made the LRP16 promoter expression construct, pGL3-S₀, and determined the luciferase activity. As shown in Fig. 2, the luciferase activity in the cells co-transfected with pGL3-S₀ and ER α or AR were 11- and 7.8-fold higher,

respectively, than that in the cells transfected with pGL3-S₀ alone. Other nuclear receptors including ER β , GR α , PPAR γ and PPAR α had either no or only a slight effect on the luciferase activity driven by the LRP16 promoter. These results indicate that 17 β -E₂ may up-regulate LRP16 mRNA by activation of ER α , but not ER β , and that LRP16 mRNA transcription was specifically regulated by ER α and AR.

Overexpression of LRP16 promotes MCF-7 cell proliferation

To determine whether overexpression of LRP16 promotes MCF-7 cell proliferation, the entire coding region of LRP16 was cloned into pcDNA3.1 vector under the control of the cytomegalovirus (CMV) promoter and the resulting plasmid was transfected into MCF-7 cells. The empty vector was used as a negative control. The cells were then treated with 1 mg/ml G418 for 3 weeks. All of the MCF-7 parental cells were killed by G418 within this period. In response to overexpression of LRP16, we observed profound alterations in cell proliferation. As shown in Fig. 3, cell proliferation was markedly promoted after LRP16 transfection. On the third day of observation, the number of cells transfected with LRP16 was about twofold greater than the control cells. The effects of overexpression of LRP16 on cell differentiation were analyzed by flow cytometry. Overexpression of LRP16 induced a decrease of about 10% in cell numbers in G₀/G₁ phase, and an increase of about 10% in cell numbers in S phase.

Overexpression of LRP16 increases cyclin E

The expression levels of p53, p21^{WAF1/CIP1} and cyclin E were measured by Western blot analysis to determine if these proteins, which play important roles in the G₀/G₁ checkpoint control of cell differentiation, were changed by overexpression of LRP16. As shown in Fig. 4, the expression levels of p53 and p21^{WAF1/CIP1} were not different in the cells showing

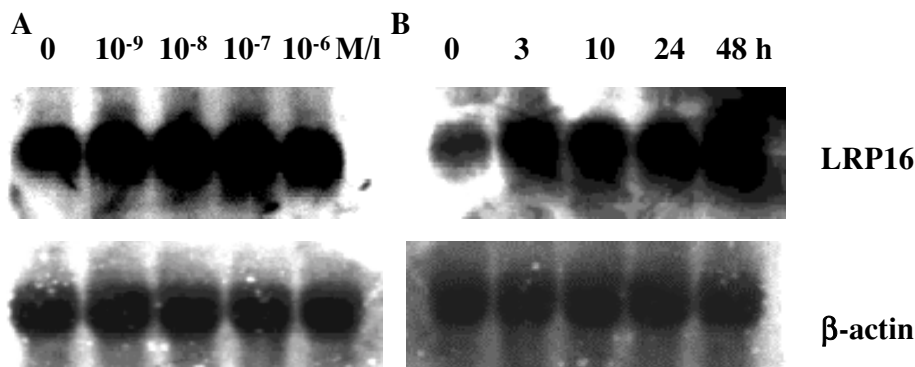


Figure 1 Effect of 17 β -E₂ on the expression of LRP16 mRNA in MCF-7 cells. MCF-7 cells were treated with various concentrations of 17 β -E₂ from 10⁻⁹ to 10⁻⁶ M/l for 48 h (A), or were treated with 10⁻⁶ M/l 17 β -E₂ for 3 to 48 h (B). Total RNA was extracted and the expression of LRP16 mRNA was determined by Northern blot analysis. β -Actin was used as a control.

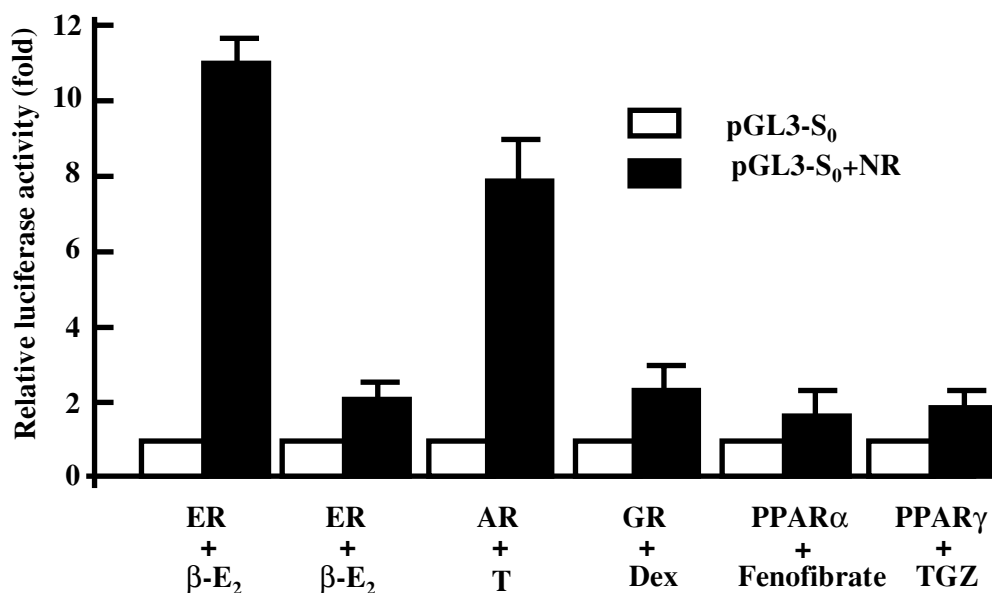


Figure 2 Effects of various nuclear receptors on the luciferase activity controlled by LRP16 promoter. LRP16 promoter (–2.6 kb) controlled luciferase reporter vector (pGL3-S₀) and various nuclear receptor (NR) expression vectors were co-transfected into COS-7 cells. After 12-h transfection, 10⁻⁶ M/l of various ligands as indicated were added. The relative luciferase activity was determined using the Dual-luciferase reporter assay system after 48 h transfection. β-E₂, 17β-E₂; T, testosterone; Dex, dexamethasone; TGZ, troglitazone.

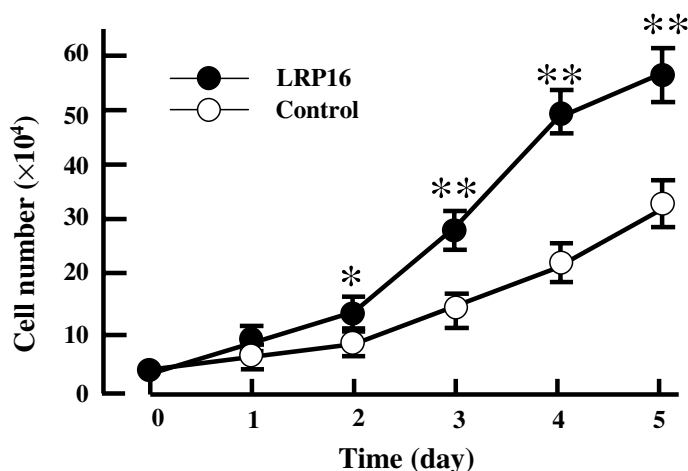


Figure 3 Effect of LRP16 overexpression on MCF-7 cell proliferation. MCF-7 cells were transfected with LRP16 or empty vector (control). After G418 selection, the cell number was determined by the Trypan Blue exclusion method. **P* < 0.05, ***P* < 0.01 compared with control.

overexpression of LRP16 compared with control cells, while cyclin E was much higher in the cells with overexpression of LRP16 compared with control cells.

Discussion

We cloned LRP16 from lymphocytes with the purpose of finding a leukemia relapse related gene, but there was no

difference between patients diagnosed with acute myeloid leukemia and relapsed patients in LRP16 mRNA expression levels as determined by semi-quantitative RT-PCR (Han *et al.* 2002). Computer-aided SAGE analysis indicated that LRP16 might be important for breast cancer cell proliferation because 17β-E₂ induced a significant increase in LRP16 mRNA in MCF-7 cells. In the present study, we investigated the regulation of LRP16 mRNA by 17β-E₂ in cultured

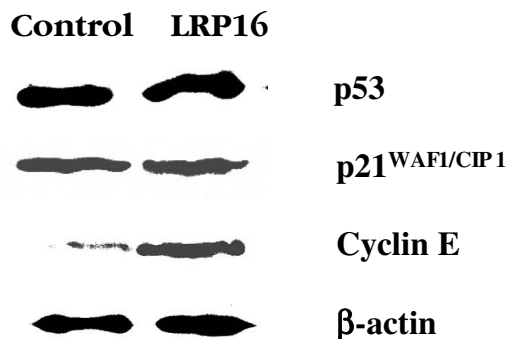


Figure 4 Expression of p53, p21^{WAF1/CIP1}, cyclin E and β -actin determined by Western blot analysis. Proteins were extracted from the cells transfected with LRP16 or empty vector (control) and a Western blot analysis was performed with the specific antibodies as indicated.

MCF-7 cells, the effects of ERs on LRP16 transcription, and the possible mechanisms involved. The MCF-7 breast cancer cell line is an estrogen-dependent cell line and expresses a high level of intracellular ER α . Consistent with the results of SAGE analysis, we demonstrated that 17 β -E₂ up-regulated LRP16 mRNA expression levels in MCF-7 cells, and this effect was observed as early as 3 h after addition of 17 β -E₂. After 3 h treatment, the expression of LRP16 mRNA was at a persistently high level and there was no further change over 48 h, indicating that the effect of 17 β -E₂ was rapid and that regulation might be at the transcription level of the LRP16 gene. All of the concentrations of 17 β -E₂, from 10⁻⁹ to 10⁻⁶ M/l, employed in this study presented similar stimulating effects, indicating that 17 β -E₂ was a strong regulator for LRP16 transcript; concentrations lower than 10⁻⁹ M/l 17 β -E₂ might have a dose-dependent effect as has been shown for cell proliferation in MCF-7 cells. Further studies are needed to clarify these points.

A number of studies have demonstrated that breast cancer is a hormone-dependent multistep process that can be induced by a variety of compounds and mechanisms, i.e. hormones, chemicals, radiation and viruses, in addition to or in combination with genetic factors (Russo *et al.* 2002). Although estrogens have been shown to play a central role in breast cancer development, their carcinogenicity on human breast epithelial cells has not yet been clearly demonstrated. Perhaps the most important pathway for the action of estrogen on the target cells is mediated via ER α and/or ER β , which function as ligand-activated transcription factors regulating gene expression at specific EREs in the DNA (Katzenellenbogen & Katzenellenbogen 2000). Our study demonstrated that LRP16 transcription was specifically regulated by ER α , but not by ER β , since the luciferase activity driven by LRP16 promoter was significantly up-regulated by co-transfection with ER α , while co-transfection with ER β had little effect on the activity. These results indicate that

LRP16 is a specific downstream gene of ER α , which may play an important role in breast cancer proliferation. Even though many functions have been suggested for ER β in the breast (Gustafsson & Warner 2000, Knowlden *et al.* 2000, Speirs & Kerin 2000, Warner *et al.* 2000), no clear picture has emerged about its role in breast cancer. Previous studies have demonstrated that when ovariectomized mice were implanted s.c. with pellets composed of estrogen and progesterone, mammary glands in the ER β -/- mice were indistinguishable from those of similarly treated wild-type mice, indicating that ER β is not necessary for estrogen-induced proliferation of the mammary gland (Palmieri *et al.* 2002). Accumulating evidence indicates that androgens and the AR modulate the development and progression of breast cancer; however, the precise role and actions remain poorly defined. AR is expressed in approximately 35–75% of breast cancers (Ellis *et al.* 1989, Kuenen-Boumeester *et al.* 1992). The number of AR-positive samples and the level of AR mRNA were significantly higher among the cancer samples than among normal samples. The expression level of both AR gene and AR protein in nuclei was found to be positively correlated with tumor invasiveness (Brys *et al.* 2002). Our study showed that AR had a similar effect as ER α in the up-regulation of LRP16 transcription. But the significance of this up-regulative effect on LRP16 is as yet unknown. Further studies are needed to determine the mechanisms of action of the effects of ER α and AR: (1) the effects of ER α and AR on LRP16 transcription may be direct by binding to their response elements in the LRP16 promoter or indirect by activation of growth factors or other pathways; (2) the stimulatory effect of androgen on LRP16 transcription may be mediated by AR or by aromatization of androgen to estrogen and then binding to ER α . Computer-aided analysis showed that there are at least nine imperfectly palindromic estrogen response elements and 3 1/2ERE(n)_x SP1 sequences (Safe 2001) in the 2.6 kb LRP16 promoter employed in this study.

Up-regulation of LRP16 transcription by ER α in MCF-7 cells implied that this gene may contribute to cell proliferation. This hypothesis was demonstrated in the present study. Overexpression of LRP16 promoted MCF-7 cell proliferation and this effect might be induced by increased cyclin E protein which is an important protein for G₀/G₁ checkpoint control of cell differentiation. Overexpression of cyclin E has been demonstrated to promote MCF-7 cell proliferation by stimulating G(1)/S transition (Sgambato *et al.* 1997, Huang *et al.* 2000, Chappell *et al.* 2001).

The understanding of how estrogen induces breast cancer development and progress is important for creating strategies and targets for breast cancer prevention and treatment. In this study we demonstrated, for the first time, that LRP16 is a new downstream gene of ER α and AR. The stimulative effect of overexpression of LRP16 on MCF-7 cell proliferation indicates that LRP16 is a new candidate for investigating the mechanism of breast cancer development. Blocking

the effect of LRP16 via its antisense oligonucleotide or in other ways may also provide a novel therapy for estrogen-dependent diseases.

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