Breast Cancer Res Treat (2008) 107:49–61
DOI 10.1007/s10549-007-9535-6

PRECLINICAL STUDY

Farnesol, a mevalonate pathway intermediate, stimulates MCF-7 breast cancer cell growth through farnesoid-X-receptor-mediated estrogen receptor activation

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Received: 31 October 2006 / Accepted: 30 January 2007 / Published online: 28 February 2007

Abstract Farnesoid X receptor (FXR) is a metabolic nuclear receptor expressed in the liver and traditionally considered as a bile acid sensor. Yet, FXR has been recently demonstrated in other tissues and cells, such as the kidneys, the adrenals, and arterial smooth muscle cells. Immunohistochemical data reported in this study point to the expression of FXR in human breast cancer. In addition, FXR expression was also found by Western blotting and immunofluorescence microscopy in breast-cancer-derived cell lines MCF-7 (estrogen receptor [ER]-positive) and MDA-MB-231 (ER-negative). The FXR activator farnesol, a mevalonate pathway intermediate, exerts a mitogenic effect on MCF-7 cells. The growth stimulation is completely suppressed by antiestrogens. In contrast, MDA-MB-231 cells appear farnesol-insensitive, suggesting an involvement of ER in farnesol mitogenicity. In accordance with this interpretation, farnesol induces in MCF-7 cells a decrease of ER level, consistent with a phenomenon of receptor downregulation. Farnesol also increases progesterone receptor (PgR) expression in MCF-7 cells and stimulates ER-mediated gene transactivation in MVLN cells (MCF-7 cells stably transfected with an ER reporter gene). Of note, both effects of farnesol on ER expression and activity are completely suppressed by antiestrogens. In addition, farnesol-induced PgR is markedly reduced by FXR gene silencing (siRNA), demonstrating the involvement of FXR in the estrogenic effects of farnesol. Finally, coimmunoprecipitation experiments (FXR immunoprecipitation followed by Western blot analysis of ER in the immunoprecipitate) produced definite evidence that FXR interacts with ER. Altogether, these observations reveal the hitherto unreported presence of FXR in breast cancer and show that the latter receptor functionally interacts with ER. The occurrence of such a crosstalk calls for some caution regarding the pharmacological use of FXR agonists.

Keywords Breast cancer · Farnesol · NR1H4 · Bisphosphonate · Receptor crosstalk

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Introduction

Breast cancer is the most common neoplasm in the female population of developed Western countries and is also a leading cause of cancer-related deaths [1]. Estrogen exposure has been known for long to contribute to the etiology of breast cancer [2]. Estrogens modulate the proliferation and/or differentiation of their target cells via specific receptors [3]. Approximately two-thirds of breast tumors diagnosed in clinics express estrogen receptor (ER) alpha [4]. The identification of ER in breast cancer has notably led to the

development of hormonal therapy based on estrogen antagonists (antiestrogens) and aromatase inhibitors [5, 6].

ER belongs to the superfamily of nuclear receptors which also includes receptors for other steroid hormones, thyroid hormone receptor, as well as receptors accepting vitamin derivatives [7]. Nuclear receptors primarily act as ligand-modulated transcription factors, endowed with the capacity of transactivating target genes when complexed with their cognate ligands [7]. Nuclear receptors are themselves targeted and posttranslationally modified by other signaling pathways (such as those triggered by membrane-bound receptors), and can, in some instances, interact with each other; this giving rise to a variety of crosstalk mechanisms [8–10].

The nuclear receptor superfamily not only includes receptors responding to extracellular mediators such as steroid hormones (''endocrine'' nuclear receptors), but also receptors specific for metabolic intermediates (''metabolic'' nuclear receptors) [11]. Examples of the latter are the peroxisome proliferator-activated receptors (PPARs), the liver X receptors, and the farnesoid X receptor (FXR/NR1H4). FXR was originally identified in the rat and named after the observation that it is activated by farnesol, an isoprenoid metabolic intermediate of the mevalonate pathway [12]. Subsequent work has identified primary bile acids as endogenous ligands for FXR and has revealed that this receptor plays a pivotal role in bile acid synthesis, conjugation, and transport [13, 14].

The mevalonate pathway is critical in higher eukaryotes since it leads to the synthesis of a variety of signaling compounds and vital cell constituents. Farnesyl pyrophosphate (FPP, doubly phosphorylated farnesol) is a key intermediate in this pathway since it is the last common intermediate for the synthesis of cholesterol, dolichol, and ubiquinone. Most importantly, FPP is also the substrate for protein prenylation, a posttranslational modification resulting in the membrane anchoring of proteins involved in subcellular signaling, such as Ras, Rho, and Rab. Thus, the mevalonate pathway and the subsequent protein prenylation are key targets for nitrogen-containing bisphosphonates [15], largely used for the treatment and for the prevention of cancer-induced osteolysis [16].

While examining the effect of mevalonate pathway intermediates on MCF-7 cells, an ER-positive breast carcinoma cell line, we observed that farnesol had a stimulatory effect on cell growth. Moreover, cell mitogenic response to farnesol was accompanied by a downregulation of ER and a triggering of ER-mediated gene transactivation. These intriguing findings prompted us to investigate further the mechanism underlying farnesol-induced MCF-7 cell stimulation. Data reported in this study indicate that FXR is expressed in MCF-7 cells, as well as in human breast and breast cancer tissue. Furthermore, we present here evidence of FXR–ER interactions that might account for FXR-mediated ER regulation in MCF-7 cells.

Material and methods

Breast cancer tissue specimens

Archival tumor samples of 65 patients with node-negative breast carcinomas collected at the Jules Bordet Institute were used for FXR evaluation. Twenty-eight samples were ER-negative and 37 were ER-positive, as assessed by immunohistochemistry with a mouse monoclonal anti-ER antibody (clone 6F11, Novocastra Laboratories, Newcastle upon Tyne, UK). For all samples, the proliferation markers Ki-67 (monoclonal mouse antibody, clone MIB1, DAKO, Glostrup, Denmark) and topoisomerase II alpha (monoclonal mouse antibody, clone Ki-S1, Chemicon, Temecula, CA) expression levels, also determined by immunohistochemistry, were available. The ethics committee of the Institute approved the use of the tissue material.

Immunohistochemical demonstration of FXR in breast cancer

A tissue microarray block containing 65 paraffin-embedded breast carcinoma samples (in duplicate) routinely fixed in neutral buffered formalin was cut, and tissue sections were mounted on poly-L-lysine-coated glass slides. Immunohistochemical staining was performed with an antibody raised against human FXR. Prior to immunostaining, antigen retrieval was achieved by microwave pretreatment (2×10 min at a power of 650 W) in citrate buffer pH 6. Thereafter, tissue sections were incubated for 30 min at 37 °C in the presence of the primary antibody (mouse monoclonal antihuman FXR/NR1H4 antibody, clone A9033A, R&D Systems, Minneapolis, MN) diluted 1:25. FXR antigen–antibody reaction was visualized using Ventana automated system with the highly sensitive Nexes reagents (Enhanced Nexes reagent, Ventana Medical Systems, Tucson, AZ). For negative control, primary antibody was replaced by phosphate-buffered solution (PBS). Nuclear staining was defined as positivity. FXR expression was scored from 0 to 8 by adding a score reflecting the proportion of positively

stained cells (none: 0, <1/100: 1, 1/100 to 1/10: 2, 1/10 to 1/3: 3, 1/3 to 2/3: 4, and >2/3: 5) and a score reflecting the staining intensity (none: 0, weak: 1, intermediate: 2, and strong: 3), as defined by Allred et al. [17]. The semiquantitative analysis was performed in a single-blind fashion by an experienced pathologist (D.L.).

Breast cancer cell lines

The ER-positive MCF-7 breast cancer cell line (ATCC HTB-22) was initially obtained in 1977 from the Michigan Cancer Foundation (Detroit, MI). MDA-MB-231 breast carcinoma cells (ATCC HTB-26) lacking ER expression were obtained from ATCC. MVLN cells (generously provided by Dr. M. Pons, INSERM U58, Montpellier, France) are MCF-7 cells stably transfected with the estrogen-response element cloned upstream of the luciferase reporter gene [18].

Cell culture conditions

Cells were cultured at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. For routine maintenance, cells were propagated in 75-cm^2 flasks containing Eagle's minimum essential medium (MEM) with Phenol Red, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and with L-glutamine, penicillin, and streptomycin (Gibco BRL, Life Technologies, Merelbeke, Belgium) at standard concentrations. Cells were harvested by trypsinization (0.1% trypsin–0.02% EDTA) and subcultured twice weekly. For experiments, cells were plated in steroid-free medium (SFM) consisting of MEM without Phenol Red supplemented with 10% dextran-coated charcoal-treated FBS, as previously described [19]. Of note, SFM is lipid-free and thus provides an environment suitable for the study of nuclear receptors such as FXR and ER. One day after seeding, the culture medium was replaced by fresh SFM containing farnesol (MP Biomedicals, Aurora, OH), 17β-estradiol (Sigma, St Louis, MO), 4-hydroxytamoxifen (Sigma, St Louis, MO), the raloxifen analog LY 117,018 (a gift from Eli Lilly & Co., Indianapolis, IN), fulvestrant (ICI 182,780, Tocris, Bristol, UK), ibandronate (a gift from Hoffmann-LaRoche, Basel, Switzerland), mevastatin (Sigma, St Louis, MO), or vehicle. Cells were treated for 1–3 days, with drugs alone or in combinations, as specified in ''Results.''

Western blot analysis (FXR, ER, and PgR)

FXR, ER, and progesterone receptor (PgR) amounts were determined by Western blotting. Cells were plated at a density of 10^4 cells/cm^2 in 60-cm^2 Petri dishes containing SFM, cultured for 24 h, and then incubated with compounds or vehicle as specified in ''Results.'' Cell monolayers were harvested and lysed using detergent cocktail, as previously described [20]. Protein concentrations in total cell lysates obtained by detergent extraction were determined by the BCA Protein Assay (Pierce, Rockford, IL) using bovine serum albumin as standard. Equal amounts of proteins were subjected to Western blotting using a rabbit polyclonal antihuman FXR/NR1H4 antibody (Abcam, Cambridge, UK) diluted 1:500, a mouse monoclonal antihuman ERα antibody (F-10, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5,000, or a mouse monoclonal antihuman PgR (A/B isoforms) (NCL-PGR-AB, Novocastra Laboratories, Newcastle upon Tyne, UK) diluted 1:500. Peroxidase-labeled antirabbit IgG antibody (1:10,000) or peroxidase-labeled antimouse IgG antibody (1:10,000) (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) were used as secondary reagents to detect corresponding primary antibodies. Bound peroxidase activity was revealed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemicals Co.). Immunostaining signals were digitalized with a PC-driven LAS-3000 CCD camera (Fujifilm, Tokyo, Japan), using a software specifically designed for image acquisition (Image Reader, Raytest®, Straubenhardt, Germany). Immunoreactive band intensities were quantified using the software AIDA® Image Analyser 3.45 (Raytest®, Straubenhardt, Germany).

Immunofluorescence microscopy (FXR and ER)

MCF-7 cells in SFM were plated at a density of 10^4 cells/cm^2 on sterile round glass coverslips in 12-well dishes. Two days after seeding, cells were fed fresh SFM containing compounds or vehicle as specified in ''Results.'' After 24 h of incubation, cell monolayers were rinsed with Dulbecco's PBS (DPBS) and fixed for 15 min with 4% paraformaldehyde (PAF) in DPBS. After fixation, PAF was changed for DPBS, where the cell cultures were kept at 4 °C until immunostaining.

Demonstration of FXR and ER by immunofluorescence was achieved as detailed in a previous publication [21], except that cells were preincubated for 20 min in PBS containing 0.05% casein (Sigma, St Louis, MO) (PBS–CAS) and 0.05 M NH_4Cl to prevent nonspecific adsorption of immunoglobulins. A mouse monoclonal antihuman FXR/NR1H4 antibody (R&D Systems, Minneapolis, MN) or a rabbit polyclonal antihuman ER (HC-20, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary reagent. Cells were exposed for 60 min to one of the primary antibodies diluted 1:50 in PBS–CAS. Thereafter, the

cell preparations were successively exposed to EnVision™ (Dakopatts, Glostrup, Denmark), rabbit antiperoxidase antiserum (Laboratory of Hormonology, Marloie, Belgium), biotinylated swine antirabbit immunoglobulins antibodies (Dakopatts), and Texas Red-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After final rinses in PBS, the coverslips were mounted on glass slides using commercial antifading medium (Vectashield®, Vector Laboratories). Negative controls were produced by omitting the primary antibody.

The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi illumination. Excitation wavelength of 596 nm and emission wavelength of 615 nm were used for the observation of Texas Red fluorescence. The appearance of immunostained cell preparations was documented using a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland). Microscopic fields were digitalized and stored thanks to a software specifically designed for image acquisition (Leica IM 50).

Gene silencing with small interfering RNA (siRNA)

The siRNA targeting the human FXR with the cDNA sequence 5′-GAGGAUGCCUCAGGAAAUA-3′ was synthesized and annealed by Eurogentec (Seraing, Belgium). The siRNA duplex negative control (scramble; cDNA sequence 5′-AAAGCGUCUGG AAAAGUCG-3′) from Eurogentec was used to evaluate the nonspecific effects on gene expression. MCF-7 cells (10^6 cells in 60-cm^2 Petri dishes) were cultured in SFM for 16 h and transfected for 6 h with 50 nM siRNA duplex using jetSI-ENDO (Eurogentec) in OptiMEM (Gibco BRL, Life Technologies, Merelbeke, Belgium) according to the manufacturer's instructions. Transfected cells were fed fresh SFM, further cultured for 16 h, and then exposed to 50 μM farnesol for 24 h before Western blot analysis for PgR expression.

Luciferase induction assay

MVLN cells were used to study the transcriptional activity of ER by determining ER-induced luciferase activity [18] using the Luciferase Assay System from Promega (Madison, WI). Cells were plated in 6-well plates at a density of 10^4 cells/cm^2 in SFM, cultured for 72 h, and then treated for 24 h with farnesol, 17β-estradiol, antiestrogens, or vehicle as described in ''Results.'' At the end of the treatment, the medium was removed, and cell monolayers were rinsed twice with PBS. Diluted lysis solution (250 μl, Promega E153A) was added, and the cultures were submitted to mild agitation for 20 min in order to extract luciferase. Detergent-lysed cells were scraped, and suspensions were clarified by centrifugation (5 min, 10,000 × g). Finally, 20 μl of extracts were mixed at room temperature with 100 μl of luciferase reagent mixture (Promega E151A/E152A) prepared according to the manufacturer's protocol. Luminescence was measured in a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Luciferase induction was expressed in arbitrary units (relative luciferase units), calculated per mg of protein, and data are given as percentages of the mean value obtained from untreated cells.

Receptor coimmunoprecipitation

The occurrence of FXR/ER complexes was established by coimmunoprecipitation experiments, using first an anti-FXR antibody to immunoprecipitate FXR, followed by an anti-ER antibody to demonstrate by Western blotting the presence of ER in the immunoprecipitates. MCF-7 cells were plated in 60-cm^2 Petri dishes (10^6 cells per dish) in SFM and cultured for 24 h. Control cells were not further incubated, while treated cells were exposed to farnesol for 10, 20, 30, and 60 min as indicated in ''Results.'' Cell monolayers were rinsed, harvested, and lysed using detergent cocktail, as described in ''Western blot analysis (FXR, ER, and PgR).'' Clarified supernatants containing equivalent amounts of proteins (1 mg) were diluted with lysis buffer up to 500 μl; aliquots were stored for total FXR and ER expression determinations (see ''Western blot analysis (FXR, ER, and PgR)''). In order to remove proteins that may otherwise crossreact at the time of immunoprecipitation, supernatants were incubated with 100 μl of antirabbit IgG antibody-agarose (Sigma, St Louis, MO) for 2 h under agitation, and then centrifuged. Supernatants were, therefore, incubated overnight with the rabbit polyclonal antihuman FXR/NR1H4 antibody (Abcam, Cambridge, UK) diluted 1:100. FXR–antibody complexes were precipitated with 100 μl of antimouse IgG antibody-agarose for 2 h under agitation, and collected by centrifugation. Pellets were washed four times with the lysis buffer (see ''Western blot analysis (FXR, ER, and PgR)''), suspended in 60 μl electrophoresis sample buffer, and boiled for 5 min. Samples were finally subjected to Western blotting to assess ER levels, using the mouse monoclonal antihuman ERα antibody F-10, as described above. Nonspecific interactions were

evaluated by omitting the anti-FXR antibody in the immunoprecipitation step.

Crystal violet growth assay

Cell number was assessed indirectly by staining with crystal violet dye as previously described [22]. Briefly, cancer cells were seeded in 96-well plates (density 5,000 cells/well) in SFM, and cultured for 24 h. Cells were seeded in 96-well plates at a density of 5,000 cells/well in SFM and cultured for 24 h. Cells were then exposed to farnesol, 17β-estradiol, antiestrogens, or vehicle as described in ''Results.'' Medium was removed, cells were gently washed with PBS, fixed with 1% glutaraldehyde/PBS for 15 min, and stained with 0.1% crystal violet (w/v in ddH_2O) for 30 min. Cells were destained under running tap water for 15 min and subsequently lysed with 0.2% Triton X-100 (v/v in ddH_2O). The absorbance was measured at 550 nm using a Microplate Autoreader EL309 (BIO-TEK Instruments, Winooski, VT). Blank wells lacked cells and drugs. The EC_{50} value refers to drug concentrations producing half maximal stimulation of growth.

Statistical analysis

For immunohistochemistry, the correlations between FXR staining and ER, as well as Ki-67 and topoisomerase II alpha expressions (% of positively stained cells) were determined through the calculation of nonparametric Spearman's rank correlation coefficients. Confidence intervals (CIs) were obtained with Fisher's transformation.

Other data are reported as means ± SD, and statistical analysis was performed by analysis of variance (ANOVA). Dunnett post hoc test was used to compare treated conditions to the untreated condition (control) and Tukey post hoc test was performed for multiple comparisons between groups. The level of statistical significance was arbitrarily set at 0.01. All analyses used SPSS software (Paris, France).

Results

Expression of FXR in breast cancer tissue

Figure 1 illustrates FXR immunostaining in human breast tissue and breast cancer samples. FXR was strongly expressed in normal breast (Fig. 1a), and was also found in some (Fig. 1b and c), but not all (Fig. 1d) cases of breast cancers. In addition, FXR expression was evaluated by immunohistochemistry in 65 breast cancer samples. Thirty-nine of the 65 samples (60%) showed at least a minimal immunostaining (i.e. ≥ 10% of positive cells) with median FXR score of 4 for the 65 samples (as defined in ''Material and methods''). FXR was weakly expressed in only 50% of the ER-negative subgroup, while it was detected in 70% of the ER-positive subgroup with a median FXR score of 5. Thus, a statistically significant correlation coefficient was observed between FXR expression and ER expression (r = 0.311, 95 CI = 0.07–0.52). A trend for a significant correlation was observed between FXR expression and that of the proliferative marker Ki-67 (r = 0.229, 95 CI = 0.02 to 0.45), whereas a highly significant

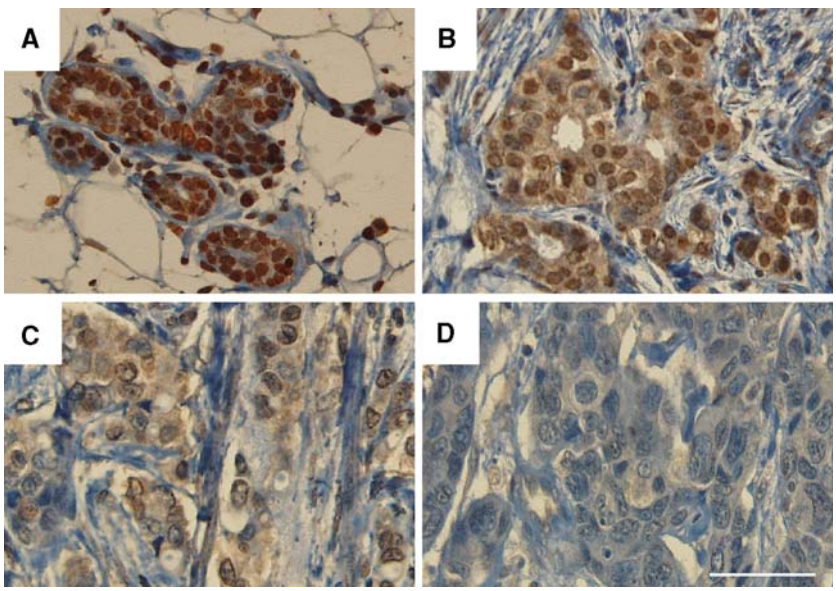

Fig. 1 Demonstration of FXR expression in breast cancer tissue specimens. Normal breast tissue (**a**) and breast carcinomas (**b**–**d**) were subjected to immunohistochemical analysis as described in ''Material and methods.'' FXR was highly expressed in normal breast as well as tumor B, moderately expressed in tumor C, and not expressed in tumor D. Magnification bar, 50 μm

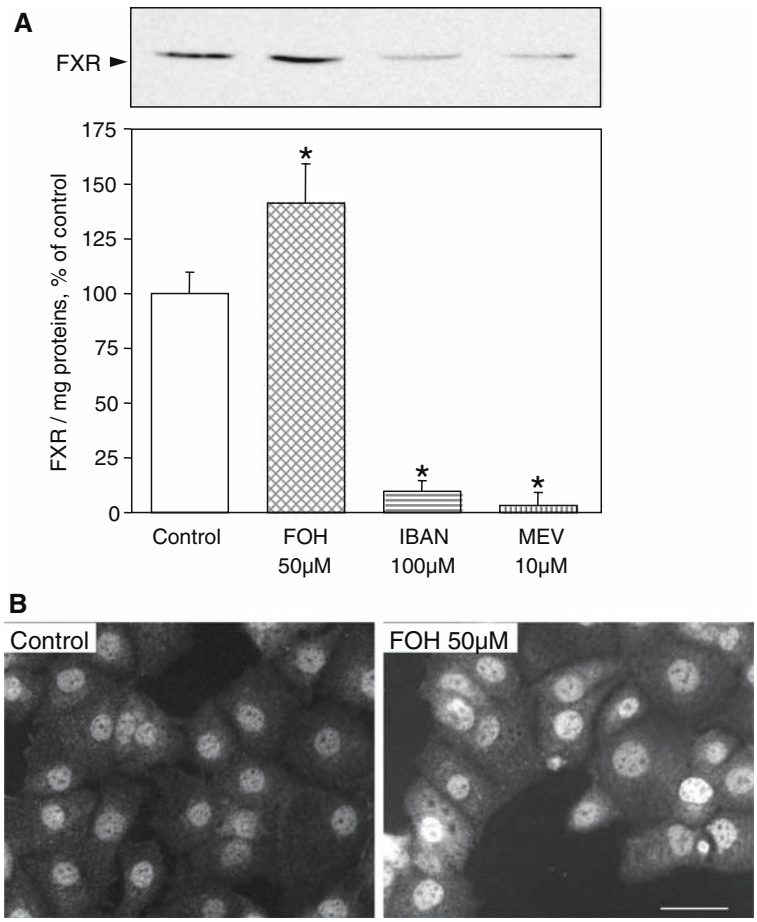

Fig. 2 Expression and regulation of FXR in MCF-7 cells determined by Western blotting (**a**) and immunofluorescence (**b**). (**a**) MCF-7 cells were incubated for 24 h with 50 μM farnesol (FOH), 100 μM ibandronate (IBAN), 10 μM mevastatin (MEV), or vehicle (control) in SFM. Equal quantities of proteins (50 μg) were submitted to 10% SDS–PAGE and electrotransferred onto nitrocellulose membranes. Immunodetection was performed with a rabbit polyclonal antihuman FXR antibody. Quantitative data were obtained from densitometric analyses ($n = 4$) and are presented as percentages of control values (mean ± SD). *ANOVA, $p < 0.05$ vs. control. (**b**) FXR demonstration by immunofluorescence microscopy. A mouse monoclonal antihuman FXR was used as the primary reagent. Other experimental conditions as in **a**. Texas Red labeling. Magnification bar, 50 μm

correlation ($r = 0.459$, 95 CI = 0.24–0.63) was observed between FXR expression and that of topoisomerase-II alpha, a proliferation marker specifically associated with the G2/M phase.

FXR expression and regulation in breast cancer cell lines

To further investigate the properties of FXR in mammary cancer cells, the expression of this nuclear receptor was examined by Western blotting and immunofluorescence microscopy in human breast cancer cell lines. Immunoblot analysis performed on MCF-7 cells revealed the presence of a FXR-immunoreactive protein band at ~60 kDa (Fig. 2a), while cells processed for FXR immunofluorescence exhibited a distinctive nuclear signal (Fig. 2b). A similar FXR expression was detected in MDA-MB-231 cells (data not shown), indicating that in breast cancer cell lines FXR may be expressed independent of the ER status. In MCF-7 cells, incubation for 24 h with the FXR activator farnesol increased the level of the receptor, while incubations with the bisphosphonate ibandronate or with mevastatin—inhibitors of the mevalonate pathway which reduce intracellular farnesol production [15, 23]—induced FXR downregulation (Fig. 2a).

Effects of farnesol on breast cancer cell growth

The effect of farnesol on the growth of MCF-7 and MDA-MB-231 cells, cultured in lipid-free medium (SFM), was assessed by crystal violet staining after 72 h

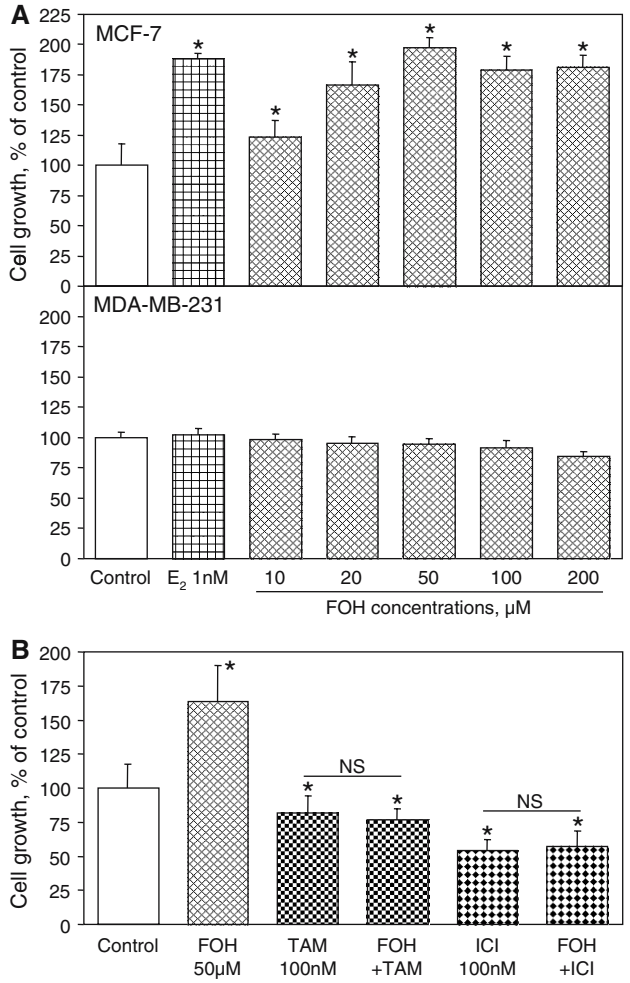

Fig. 3 Effects of farnesol on MCF-7 and MDA-MB-231 cell growth (**a**) and modulation of farnesol activity by antiestrogens (**b**). (**a**) Breast cancer cells were treated for 72 h with increasing concentrations of farnesol (FOH) (10–200 µM), 1 nM 17β-estradiol (E_2), or vehicle (control) in SFM. Cell proliferation was determined by crystal violet staining assay. Data are presented as percentages of control values (mean ±SD). Mean of results pooled from four experiments ($n = 24$). *ANOVA, $p < 0.05$ vs. control. (**b**) MCF-7 cells were exposed for 3 days to 50 µM farnesol (FOH), 100 nM 4-hydroxytamoxifen (TAM), FOH + TAM, 100 nM fulvestrant (ICI), FOH + ICI, or vehicle (control) in SFM. Cell growth was assessed as described above. *NS* nonsignificant

of exposure (Fig. 3a). Farnesol enhanced the growth of MCF-7 cells in a dose-dependent manner. The EC_{50} value was estimated at 15 µM and the concentration for maximal growth stimulation (~100%) was 50 µM. Of note, in similar experimental conditions, 1 nM 17β-estradiol also stimulated MCF-7 cell growth by twofold. In contrast, farnesol, as well as 17β-estradiol, had no effect on the ER-negative/FXR-positive MDA-MB-231 cells, suggesting that ER could be of importance in the mediation of the mitogenic effect of farnesol. To test this hypothesis, ER antagonists were used in combination with farnesol. As illustrated in Fig. 3b, both partial (4-hydroxytamoxifen) and pure antiestrogens (fulvestrant) completely suppressed the growth stimulation induced by farnesol in MCF-7 cells. These data demonstrate the crucial role played by ER as a mediator in the proliferative action of farnesol on MCF-7 cells. In addition, the effects of the FXR ligand chenodeoxycholic acid (CDCA) were examined in MCF-7 and MDA-MB-231 cells (data not shown). CDCA stimulated the proliferation of MCF-7 cells, while it had no effects in MDA-MB-231 cells; the antiestrogens completely suppressed the growth stimulation induced by CDCA in MCF-7 cells.

Of note, it has been recently reported that high concentrations of FXR ligands exert an antiproliferative effect on breast carcinoma cell lines, regardless of their ER status [24]. It must be noted, however, that these observations, which are at variance with the data reported here, were performed using cells cultured in serum-free medium, an experimental condition which is likely to compromise cell proliferation and ER-mediated growth responses [19]. This could have obscured interactions between FXR and ER.

Regulation of ER expression and transcriptional activity by farnesol in breast cancer cells

The effect of FXR activation on ER level in MCF-7 cells was examined by Western blotting (Fig. 4a) and immunofluorescence (Fig. 4b). Incubation with farnesol for 24 h downregulated ER by 50%. When used as a positive control at a concentration that induced a similar stimulation of cell growth, 17β-estradiol decreased ER content by 90%. Besides, farnesol in combination with 17β-estradiol did not modify ER downregulation induced by the estrogen alone. The selective estrogen receptor modulators (SERMs) 4-hydroxytamoxifen and LY 117,018 (data not shown for the latter antiestrogen) which are known to stabilize ER, completely suppressed the ER down-regulation induced by farnesol. These data indicate that farnesol only acts on free ER and fails to affect the ligand-bound form of the receptor, suggesting indirect interactions between farnesol and ER. Indeed, further experiments revealed that farnesol did not compete with [^{3}H]-17β-estradiol for binding to human recombinant ER immobilized on hydroxylapatite gel (data not shown).

The effect of farnesol on ER-mediated gene transactivation was first examined in MCF-7 cells by evaluating the expression level of the estrogen-inducible PgR gene (Fig. 5a). Incubation with farnesol for 24 h

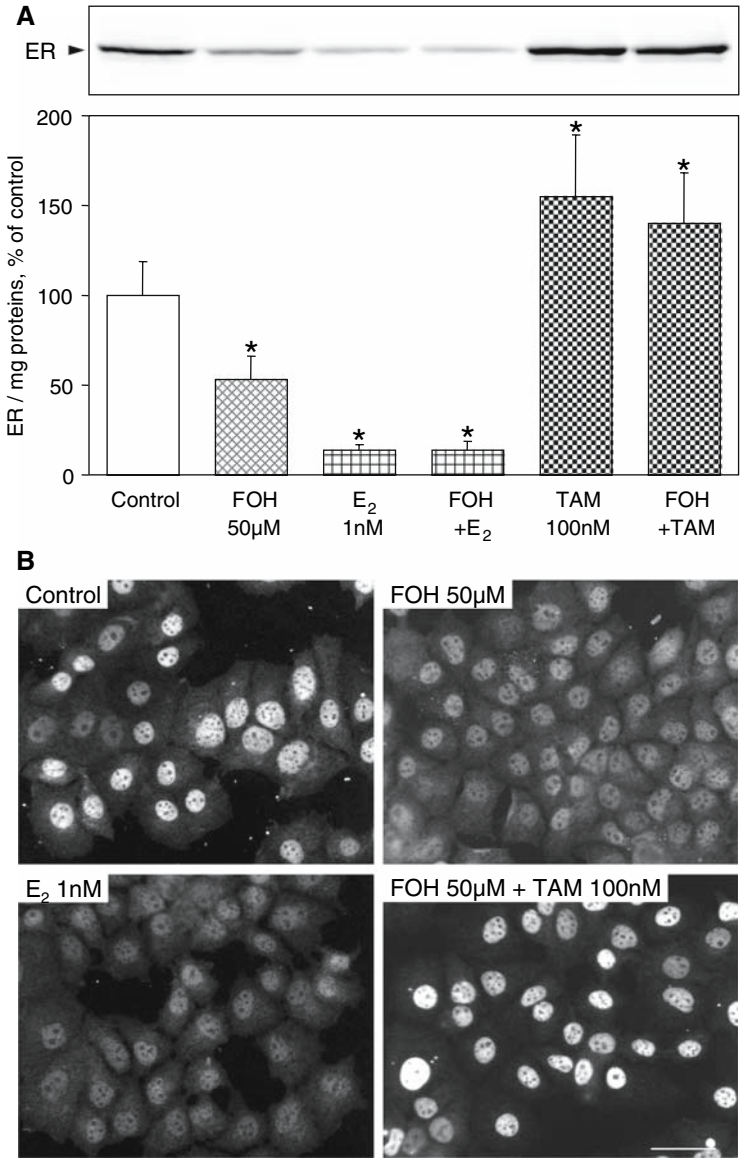

Fig. 4 Regulation of ER levels in MCF-7 cells exposed to farnesol, 17β-estradiol or 4-hydroxytamoxifen, as assayed by Western blotting (**a**) and immunofluorescence (**b**). (**a**) MCF-7 cells were incubated for 24 h in SFM containing 50 μM farnesol (FOH), 1 nM 17β-estradiol (E_2), FOH + E_2, 100 nM 4-hydroxytamoxifen (TAM), FOH + TAM, or vehicle (control). Equal quantities of proteins (20 μg) were subjected to 10% SDS–PAGE and electrotransferred onto nitrocellulose membranes. Immunodetection was performed with a mouse monoclonal antihuman ER antibody. Quantitative data were obtained from densitometric analyses ($n = 4$) and are presented as percentages of control values (mean ± SD). *ANOVA, $p < 0.05$ vs. control. (**b**) ER demonstration by immunofluorescence microscopy. A rabbit polyclonal antihuman ER antibody was used as the primary reagent. Other experimental conditions as in **a**. Texas Red labeling. Magnification bar, 50 μm

increased by 265% the expression of the B isoform of PgR (114 kDa, transcriptional activator). Of note, the A isoform of PgR (94 kDa, transcriptional inhibitor) is known to be weakly expressed and induced in MCF-7 cells, as previously discussed [20]. PgR induction by farnesol was quite similar to that observed with estradiol and was not enhanced by combining estradiol and farnesol. The effect of farnesol on PgR expression was again completely suppressed by using antiestrogens (4-hydroxytamoxifen or fulvestrant). The effect of farnesol on the transcriptional activity of ER was also examined in MVLN cells obtained by stable transfection of MCF-7 cells with an estrogen-responsive luciferase reporter gene (Fig. 5b). Exposure to farnesol for 24 h stimulated the transcription of the estrogen-responsive reporter gene by 180%. Luciferase gene

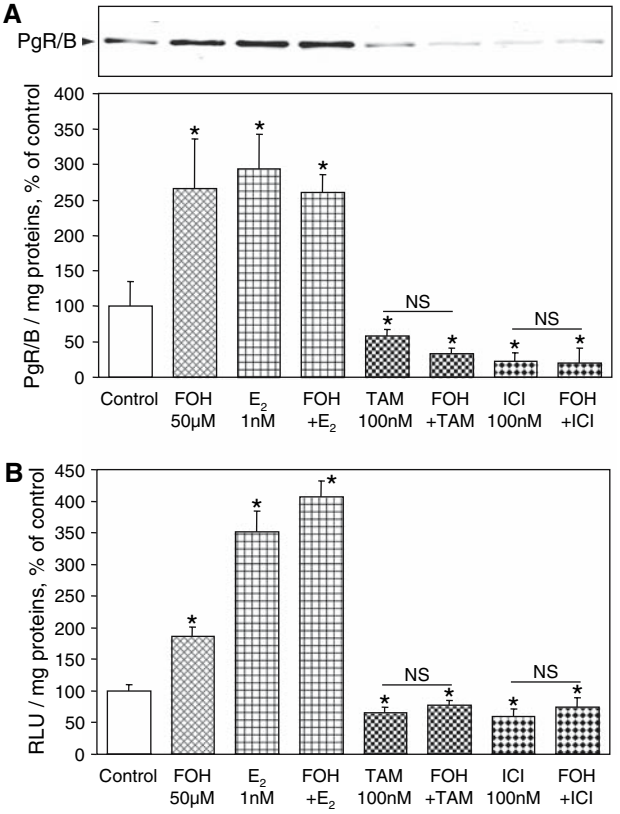

Fig. 5 Transcriptional activity of ER in MCF-7 cells treated with farnesol, 17β-estradiol, antiestrogens, or combinations thereof, as documented by PgR Western blot analysis (**a**) and luciferase induction assay (**b**). (**a**) MCF-7 cells were incubated in SFM for 24 h with 50 μM farnesol (FOH), 1 nM 17β-estradiol (E_2), 100 nM 4-hydroxytamoxifen (TAM), 100 nM fulvestrant (ICI), or combinations thereof. Control: cells incubated in the presence of vehicle. Equal amounts of proteins (50 μg) were subjected to 8% SDS–PAGE and electrotransferred onto nitrocellulose membranes. Immunodetection was performed with a mouse monoclonal antihuman PgR antibody. Quantitative data were obtained from densitometric analyses ($n = 4$) and are presented as percentages of control values (mean ± SD). *ANOVA, $p < 0.05$ vs. control. (**b**) MVLN cells were treated as above. Luciferase activities were normalized with respect to protein levels. Data are given as percentages of control values (mean ± SD). Experiments were performed four times in replicate ($n = 8$). *ANOVA, $p < 0.05$ vs. control. *NS* nonsignificant

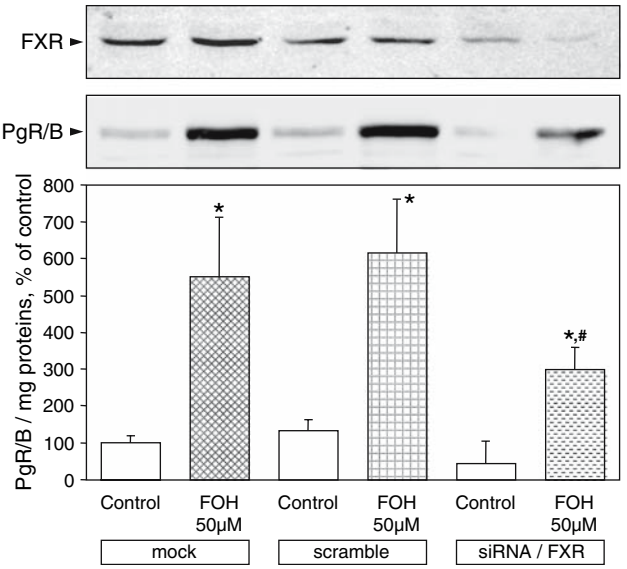

Fig. 6 Effect of FXR gene silencing on farnesol-induced PgR in MCF-7 cells. MCF-7 cells were transfected or not (mock, negative control) for 6 h with 50 nM siRNA duplex against FXR or corresponding scramble (negative control). Sixteen hours after transfection, cells were incubated for 24 h in SFM containing 50 μM farnesol (FOH) or vehicle (control). Proteins were subjected to Western blotting for FXR or PgR as described in Fig. 2a or Fig. 5a, respectively. *ANOVA, $p < 0.05$ vs. respective control; #ANOVA, $p < 0.05$ vs. farnesol effects in mock and scramble conditions

transactivation induced by 17β-estradiol was stronger (350%), but was not significantly different from the stimulation induced by farnesol plus estradiol. In addition, the stimulation of the transcriptional activity by farnesol was completely abrogated when the latter was combined with 4-hydroxytamoxifen or fulvestrant. In the latter case, reporter gene transactivation was not different from that observed in the presence of either antiestrogen alone. Altogether, these data confirm the estrogenic effect of farnesol on MCF-7 cells.

Demonstration of FXR involvement in the estrogenic effect of farnesol

In order to establish whether the induction of the PgR expression by farnesol in MCF-7 cells occurs via FXR, FXR gene silencing experiment was performed. Cancer cells transfected with siRNA against FXR showed a decrease in the expression of FXR protein, as documented by Western blot analysis (Fig. 6). As expected, farnesol exposure for 24 h increased the expression of PgR in negative controls (mock and scramble), showing that transfection experiment did not induce nonspecific effects on gene expression. FXR silencing significantly inhibited the PgR induction by the FXR agonist (Fig. 6). These data demonstrate that FXR mediates the estrogenic effect of farnesol in MCF-7 cells.

Evidence for interaction between FXR and ER

In order to unravel possible interaction between FXR and ER, and to address the mechanism involved in the estrogenic effect of farnesol, MCF-7 cell extracts were

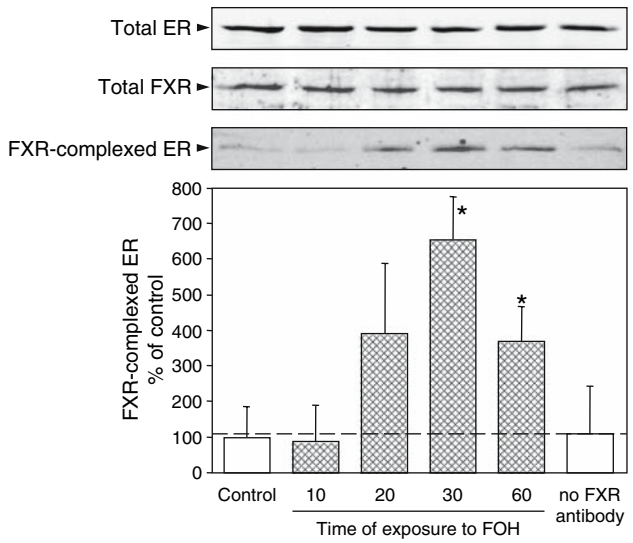

Fig. 7 Western blotting of ER in immunoprecipitated FXR preparations. MCF-7 cells were incubated in SFM with 50 μM farnesol (FOH) or vehicle (control) for 10, 20, 30, or 60 min. Solubilized protein preparations were submitted to FXR immunoprecipitation, as described in ''Material and methods.'' ER expression was assayed in the immunoprecipitates by Western blotting as described in Fig. 4a. The second control ''no FXR antibody'' means that no primary antibody was used for immunoprecipitation. The discontinued line refers to the nonspecific signal level. Quantitative data were obtained from densitometric analyses ($n = 3$) and are presented as percentages of control values (mean ± SD). *ANOVA, $p < 0.05$ vs. control. Total ER and FXR levels were determined by Western blotting in cell extracts (see Figs. 2a and 4a) before immunoprecipitation

subjected to FXR immunoprecipitation and subsequent ER Western blotting (Fig. 7). These coimmunoprecipitation experiments revealed a time-dependent increase in the amount of FXR/ER complexes in MCF-7 cells incubated with 50 μM farnesol, a finding consistent with a positive crosstalk between both nuclear receptors. The relative amount of FXR-ER complexes reached a maximum after 30 min of exposure to farnesol, but decreased after a longer exposure time (1 h), probably because of the initiation of farnesol-induced ER downregulation. Moreover, treatments with SERMs, and even more with the pure antiestrogen fulvestrant, dramatically decreased the level of FXR/ER complexes (data not shown), an indication that ligand binding completely suppresses ER crosstalk with the farnesol/FXR signaling pathway, independent of ER stabilization (SERMs) or downregulation (fulvestrant). Altogether, these data point to the involvement of FXR in the activation of ER by farnesol and reveal a previously unrecognized interaction between ER and a metabolic nuclear receptor.

Discussion

Immunohistochemistry, Western blotting, and immunocytochemical data reported in this study reveal that FXR is produced in human breast tumor samples and in breast carcinoma cell lines. FXR is also expressed in normal breast tissues, indicating that it is not specifically associated with neoplastic transformation. Similar findings concerning the expression of FXR in breast carcinoma and normal breast tissue have been reported very recently [24]. In addition, our immunohistochemical analyses on 65 breast carcinoma samples establish significant correlations between FXR expression and ER, Ki-67, and topoisomerase-II alpha expressions. These data suggest that FXR expression could be associated with a poor prognosis subgroup (highly proliferative) of ER-positive breast carcinomas. In this respect, our in vitro data demonstrate that farnesol-induced FXR activation causes mitogenicity in MCF-7 cells through a positive crosstalk with ER.

The present observations extend recent work demonstrating FXR protein in the MDA-MB-231 cell line [25]. FXR demonstration by immunofluorescence shows a nuclear localization, similar to that already described for other nuclear receptors. In addition, farnesol exposure results in an increase of FXR protein level, suggesting activation-induced receptor upregulation. In this regard, a number of recent studies have investigated the modalities of ligand-induced nuclear receptor regulation at the posttranslational level. Most of these studies focused on endocrine nuclear receptors like ER, PgR, glucocorticoid receptor, and thyroid hormone receptor. In these cases, it has been found that exposure to agonist ligands leads to receptor downregulation by proteasome-mediated degradation [20, 26]. Thus, our observation that farnesol upregulates FXR in MCF-7 cells may seem at first sight surprising, but in accordance with another study showing that natural and synthetic FXR ligands increase the expression of FXR in HepG2 cell line, suggesting the existence of an autoregulatory loop at transcriptional level [27]. In addition, as revealed by previous work, FXR is not unique in this respect. Another metabolic nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ), has been reported to undergo upregulation in hepatocytes exposed to the thiazolidinedione agonist ligand troglitazone. Increase of PPARγ expression induced by troglitazone was associated with an enhancement of PPARγ gene transcription [28].

We present here evidence that the mevalonate pathway intermediate farnesol stimulates MCF-7 cells; this stimulatory effect most probably occurring through

an FXR-mediated activation of ER. In fact, it must be pointed out that farnesol, albeit known as an FXR activator, is not considered as a true FXR ligand since it has not been found to physically interact with this receptor [12]. Thus, we must surmise that MCF-7 cell response to farnesol is not a direct consequence of farnesol binding to FXR but rather involves the activity of yet unidentified farnesol-derived or -induced FXR ligand(s). Nevertheless, FXR appears to be the key mediator of the effects of farnesol since the FXR ligand chenodeoxycholic acid provoked closely similar mitogenic effects in MCF-7 cells (data not shown).

Even though there is still uncertainty concerning the mechanism of action of farnesol on MCF-7 cells, it must be emphasized that, in these cells, FXR expression depends on the integrity of the mevalonate pathway. As shown here, cell treatment with ibandronate or mevastatin provokes a loss of FXR, as documented by Western blotting. Ibandronate is a nitrogen-containing bisphosphonate which acts as an analog of isoprenoid diphosphate lipids and inhibits farnesyl diphosphate synthase, a key enzyme of the mevalonate pathway [15]. Mevastatin is an inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase which catalyzes the first step in the mevalonate pathway [23]. In a similar way, a reduction of FXR expression/DNA-binding activity has been observed in liver tissue and in HepG2 hepatoma cell line upon treatment with simvastatin [29]. These results suggest that, in breast carcinoma and other cells, intermediates of the mevalonate pathway somehow exert a control on FXR expression and/or stability.

As demonstrated by our data, ER downregulation induced in MCF-7 cells by farnesol exposure was accompanied by a proliferative response similar to that induced by estrogen agonists. In addition, farnesol increased PgR expression (used as a marker of ER-mediated gene transactivation) in MCF-7 cells. The use of MVLN cells (MCF-7 cells stably transfected with an ER-driven reporter gene) confirmed that farnesol enhanced ER-induced gene transactivation. The first explanation that comes to mind is that farnesol simply behaves as an agonist ligand for ER and is thereby endowed with a weak estrogenic activity. Such an interpretation would be consistent with the suppressing effect of antiestrogens. However, a direct effect of farnesol on ER is highly unlikely since this compound lacks the structural features (polycyclic structure, aromatic ring, hydroxyl group, etc.) that are known to be critical for ligand interaction with the ER binding pocket [30]. Moreover, we have determined that farnesol does not bind ER since it was unable to inhibit [^{3}H]-estradiol binding to human recombinant ER, as assayed by hydroxylapatite separation method (data not shown). In addition, similar effects on ER (i.e., downregulation and increase of transactivation activity) have been shown previously to be induced by chenodeoxycholic acid, which is a bona fide FXR ligand [31]. Finally, the definite evidence that farnesol exerts estrogenic effect via FXR was obtained by gene silencing experiment using siRNA against FXR. Indeed, transient inhibition of FXR expression in MCF-7 cells decreased the PgR induction by farnesol.

Taken together, these data suggest a positive crosstalk between FXR and ER, which might account for farnesol-induced ER activation. This hypothesis was confirmed by the immunochemical demonstration that physical interactions between both receptors were promoted by FXR activation. In this regard, it is noteworthy that FXR only associates with the unliganded form of ER, since treatment with 4-hydroxytamoxifen and raloxifen analog, nonsteroidal partial antiestrogens which do not induce ER downregulation, totally abrogated FXR–ER interactions (data not shown).

Although ER mechanism of ligand-induced transactivation normally involves receptor homodimerization, ER association with another intracellular receptor is not unheard of. A well-documented example concerns ER crosstalk with the aryl hydrocarbon receptor (AhR), a basic loop–helix–loop ligand-modulated transcription factor targeting genes involved in xenobiotic detoxification. AhR has been demonstrated in a variety of tissues, including ER-expressing tissues such as the endometrium, the mammary gland, and also breast cancer cell lines [32]. As reported previously, MCF-7 cell exposure to the AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin results in ER downregulation [33]. Besides, AhR has also been found to physically interact with ER [9, 34] and to exert a weak estrogenic effect in MCF-7 cells. Of note, AhR–ER association is promoted by AhR ligand binding [9]. Yet, there are major differences between AhR–ER and FXR–ER crosstalks. First, in the presence of an estrogen agonist, AhR acts like an antiestrogen [32]; this is not the case for FXR since farnesol does not interfere with ER-mediated signaling. Secondly, AhR is downregulated (and not upregulated) upon ligand binding [35].

We are well aware that observations reported here raise a number of issues which still remain unresolved. These issues pertain to the basic aspects as well as to the clinical relevance of FXR expression and activity in breast carcinoma cells. In this context, a knowledge of the role of FXR in breast and breast tumor tissue requires additional studies aiming at the identification of endogenous, physiological ligand(s) in that particular

tissue environment. With regard to breast cancer etiology, the presence of high plasma levels of deoxycholic acid in postmenopausal breast cancer patients has been documented, suggesting that this bile acid might be involved in the onset and development of breast tumors [36]. Moreover, accumulation of bile acids from serum has been reported in breast cyst fluid and has been discussed as potential risk factor of developing breast cancer [37–39]. That would of course imply that bile acids act as FXR ligands in breast tissue, a fact which is not established.

As mentioned above, FXR functions as a bile acid sensor in the liver and intestine, and plays a pivotal role in the regulation of genes involved in bile acid and cholesterol homeostasis, as well as in triglyceride and carbohydrate metabolism [14, 40]. As a well-known example, FXR negatively regulates the transcription of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in cholesterol to bile acid conversion, and thus inhibits bile acid production from cholesterol. Because of this and other regulatory effects on metabolism, FXR is considered as an attractive drug target for the treatment of liver diseases such as cholestasis and liver fibrosis, and, more generally, for the treatment of medical conditions related to lipid and glucose disorders [40–42]. In addition, the discovery of FXR in vascular smooth muscle cells suggests that it could also be a target for the prevention/management of cardiovascular diseases [43]. These considerations have led to the development of potent nonsteroidal (GW4064, fexaramine) and steroidal (INT-747) FXR agonists. On the basis of the antiproliferative effects of FXR agonists on breast carcinoma cells in vitro, FXR has even been proposed as a novel therapeutic target for the management of breast cancer [24]. Yet, our demonstration of potential FXR crosstalk with ER in breast cancer cells calls for some caution regarding the clinical use of FXR ligands, because of possible mitogenic effects on breast cancer tissue.

Acknowledgments This study received financial support from the Fondation Medic, from Hoffmann-LaRoche (Basel, Switzerland), from the Fondation contre le Cancer, from the Belgian Fund for Medical Scientific Research (grants no. 3.4563.02 and 3.4512.03), and from Les Amis de l'Institut Bordet. Guy Laurent is Senior Research Associate of the National Fund for Scientific Research (Belgium).

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