Detection of an elevated HER2 expression in MCF-7 breast cancer cells overexpressing estrogen receptor ß1

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Abstract. The estrogen receptor (ER) expression and HER2 amplification are important factors in determining the prognosis and therapy of breast cancer. Interactions between the two signaling pathways for example resulted in ERα-dependent regulation of HER2 expression in breast cancer cells. In this study, we investigated to what extent ERß is able to affect the HER2 expression. For this purpose, we analyzed HER2 levels in ERß1-overexpressing clones of the breast cancer cell lines MCF-7 and SK-BR-3 and of the ovarian cancer cell lines SK-OV-3 and OVCAR-3 by both RT-PCR and Western blot analysis. Treatment with ligand 17-ß estradiol diminished the HER2 expression in MCF-7 wild-type cells, an effect partially inhibited by treatment with 4-OH tamoxifen. MCF-7 breast cancer cells stably overexpressing ERß1 exhibited elevated >5-fold HER2 mRNA levels and elevated >3-fold HER2 protein levels even in the absence of estradiol. In contrast, ERß1 overexpression did not affect HER2 protein levels in the ERα-positive OVCAR-3 ovarian cancer cells and in the HER2 overexpressing, hormone-independent SK-BR-3 and SK-OV-3 cells. By demonstrating the elevated HER2 expression in a hormone-dependent breast cancer cell line overexpressing ERß1, our data suggest the presence of cross-talk between the two receptors. This is one of the molecular mechanisms underlying the significant ERß/HER2 co-expression observed in recent clinical studies.

Introduction

The growth of hormone-dependent breast and ovarian cancer cells can be regulated by estrogen receptors (ER) and receptor tyrosine kinases such as HER2. Estrogen receptors ERα and -ß are expressed in normal breast and ovary cancer cells and in malignancies derived from these tissues (1-3). Although the role of ERß in breast and ovary is not fully elucidated, the interaction of ERα and -ß is important for the normal development and function of these tissues. In animal studies, while ERα has been shown to be essential for normal mammary gland development, ERß effects are more subtle, with roles in terminal differentiation (4). ERß is the dominant estrogen receptor in normal breast tissues (5-7), but its expression declines during tumorigenesis (8,9) suggesting a role for ERß as a tumor suppressor in breast tissues (10). However, ~55-72% of primary breast cancer cases show a lower or higher expression of ERß (11-14). Our recent studies and others suggest that ERß has similar functions in ovarian carcinogenesis (15,16). Malignant ovarian tumors originating from the epithelial surface constitute ~90% of ovarian cancers and express low levels of ERß, compared to normal tissues. In addition, the restoration of ERß in ovarian cancer cells, leads to a strong inhibition of their proliferation and motility, while apoptosis is enhanced (15).

The receptor tyrosine kinase HER2 is overexpressed in breast and ovarian cancer tissues. In 20% of all ovarian epithelial cancer, HER2 is overexpressed (17-19) and may be associated with chemoresistance (20,21). The amplification or overexpression of the HER2 gene is found in 20-25% of human breast cancers (22) and is known as a poor prognostic marker and therapeutic target (23,24).

Recent studies demonstrated an interaction between the ERα and HER2 signal transduction (25,26). For example estradiol is able to reduce HER2 expression in an ERα-dependent manner (27). This may be a reason why the coexpression of the two receptors, which is correlated with a poor prognosis, is infrequent (28-30). In contrast, a significant positive correlation between HER2 overexpression and ERß expression was reported (11,13).

In this study, we elucidated the molecular mechanism underlying this co-expression of ERß and HER2. For this purpose, we examined the effect of the ERß overexpression on HER2 expression in breast and ovarian cancer cell lines.

Materials and methods

Materials. DMEM/F12 culture medium was obtained from Invitrogen (Karlsruhe, Germany), fetal calf serum (FCS) was purchased from PAA (Pasching, Austria) and 17-ß estradiol, 4-OH tamoxifen and serum replacement 2 (SR2) were obtained from Sigma (Deisenhofen, Germany). M-MLV-P

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Table I. Primer sequences used for RT-PCR amplification.

Target	Oligonucleotides	Sequences 5'-3'
ERα	ERα1	TGATGAAAGGTGGGATACGA
	ERα2	AAGGTTGGCAGCTCTCATGT
ERß1	ERß1	CAAGGCCGGTGTGTTTATCT
	ERß2	GGCGTCACTGAGACTGTGG
HER2	HER2-1	AACTGCACCCACTCCTGTGT
	HER2-2	TGATGAGGATCCCAAAGACC
ß-actin	actin-2573	CTGTGGCATCCACGAAACTA
	actin-2876	CGCTCAGGAGGAGCAATG

reverse transcriptase was purchased from Promega (Mannheim, Germany). The RNeasy mini kit, RNase-free DNase set and Quantitect SYBR-Green PCR kit were obtained from Qiagen (Hilden, Germany). PCR primers were synthesized at Metabion (Planegg-Martinsried, Germany). Platinum Pfx polymerase was purchased at Invitrogen (Karlsruhe).

Cell culture. MCF-7, SK-BR-3, OVCAR-3 and SK-OV-3 breast and ovarian cancer cells were obtained from the American Type Culture Collection (Manassas, USA). Cells were maintained in phenol red-free DMEM/F12 medium supplemented with 10% FCS. Cells were cultured with 5% CO_2 at 37˚C in a humidified incubator. Before treatment with E2 or 4-OH tamoxifen, FCS was replaced by 1x serum replacement 2 (SR2). Stimulation was carried out the following day using 3 nM E2 alone or in combination with 100 nM 4-OH tamoxifen for 48 h prior to RNA and protein extraction.

Reverse transcription and PCR. Total RNA was isolated by means of the RNeasy kit (Qiagen) according to the manufacturer's instructions. From 1 μg total RNA, cDNA was synthesized using 100 U M-MLV-P reverse transcriptase (Promega), 2.5 mM dNTP mixture and 50 pM random primers (Invitrogen). For real-time PCR detection of ERα, ERß1 or HER2 (primer sequences in Table I), 2 μl cDNA was amplified using the Quantitect SYBR-Green PCR kit (Qiagen) and the LightCyler PCR device (Roche Diagnostics, Mannheim, Germany). The PCR program was 95˚C for 15 min, followed by 35 PCR cycles (95˚C for 10 sec, 56˚C for 30 sec and 72˚C for 30 sec) and a final extension for 5 min at 72˚C, followed by a standard melting curve analysis. In all the RT-PCR experiments, a 190 bp ß-actin fragment was amplified as a reference gene using intron-spanning primers actin-2573 and -2876. After performing dilution experiments with a sample cDNA over a 100-fold range confirming the PCR efficiencies of all primer pairs to be approximately equal (31), data were analyzed using the comparative $\Delta\Delta C_T$ method (32) by calculating the difference between the threshold cycle (C_T) values of the target and reference gene of each sample and then comparing the resulting ΔC_T values between different samples. In these experiments, mRNA not subjected to reverse transcription was used as a negative control to distinguish cDNA and vector or genomic DNA amplification.

Antibodies and Western blot analysis. Cells were lysed in RIPA buffer 1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS) in phosphate-buffered solution (PBS) containing aprotinin and sodium orthovanadate. Aliquots containing 15 μg protein were resolved by 10% (w/v) SDS-polyacrylamide gel electrophoresis, followed by electrotransfer to a PVDF hybond (Amersham, UK) membrane. Immunodetection was carried out using the HER2 antibody NEU (C-18) (Santa Cruz Biotechnology, Santa Cruz, USA) or ß-actin antibody (8226, ABCAM, Germany) diluted at 1:5000 in PBS containing 5% skim milk (w/v) followed by horseradish peroxidase conjugated secondary antibody (diluted at 1:50000), which was detected using the chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK).

Results

Expression of ERα, ERß and HER2 in breast and ovarian cancer cell lines. To characterize the employed cancer cell lines, we first examined the expression of HER2, ERα and ERß in the cells on the mRNA level. The real-time RT-PCR analysis revealed a strong expression of HER2 in the SK-BR-3 breast and SK-OV-3 ovarian cancer cells, which are known to be HER2-overexpressing and estrogen-unresponsive. In contrast, the estrogen-responsive MCF-7 breast and OVCAR-3 ovarian cancer cells exhibited only relatively low HER2 mRNA levels (Fig. 1). All four cell lines tested expressed detectable levels of ERß1 mRNA, which were highest in the ERα-positive, estrogen-responsive MCF-7 and OVCAR-3 cells (Fig. 2). As expected ERß1 mRNA levels were shown to be strongly elevated in the ERß1-overexpressing (ERß$^+$) clones of these cell lines as previously described (15,33). The levels were generated by stable transfection with an ERß1 expression plasmid (Fig. 2). Real-time RT-PCR analysis of the ERα expression confirmed the previously described positive ERα status of MCF-7 and OVCAR-3 cells and the absence of functional ERα in the SK-BR-3 and SK-OV-3 cells (data not shown).

Effect of ERß overexpression on HER2 expression in breast and ovarian cancer cell lines. We analyzed the expression of the HER2 gene in the ERß1-overexpressing breast and

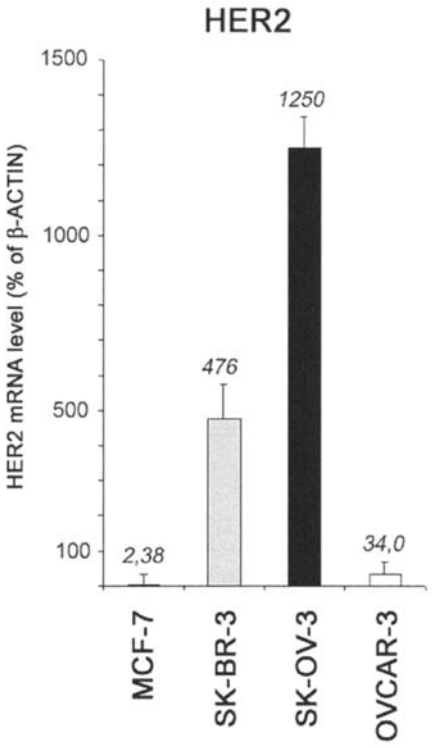

Figure 1. HER2 mRNA levels in breast and ovarian cancer cell lines. Total RNA isolated from the indicated cancer cell lines was subjected to real-time RT-PCR. Relative HER2 mRNA levels are expressed in percent of ß-actin control (n=4).

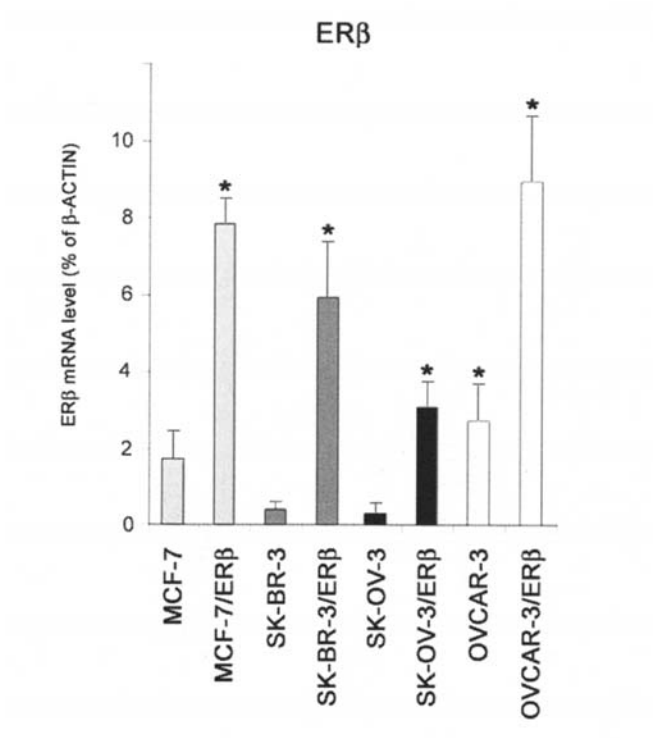

Figure 2. Estrogen receptor ß mRNA levels in wild-type and stably transfected ERß+ cell lines. Total RNA isolated from the indicated cancer cell lines and clones was subjected to real-time RT-PCR. Relative ERß mRNA levels are expressed in percent of ß-actin control (n=4); *p<0.01 vs. wild-type.

ovarian cancer cell lines on the mRNA and protein levels. HER2 mRNA levels were elevated >5-fold in the MCF-7/ERß+ cells (Fig. 3a) and elevated ~4-fold in the SK-OV-3/ERß+ cells (Fig. 4a) cultured in serum- and steroid hormone-free medium when compared to wild-type cells. The addition of 17-ß estradiol (E2) significantly decreased the HER2 mRNA levels in the hormone-dependent, ERα-positive breast cancer cell line MCF-7 down to 59.7% (Fig. 3a), but not in the hormone-independent ovarian cancer cell line SK-OV-3. This effect could be partially inhibited in the MCF-7 cells by treatment with 4-OH tamoxifen and was also present in the MCF-7/ERß+ cells. In contrast, the HER2 mRNA levels were not altered in the SK-BR-3/ERß+ breast cancer and OVCAR-3/ERß+ ovarian cancer cells.

The HER2 protein levels as assessed by Western blot analysis were elevated >3-fold in the MCF-7 cells overexpressing ERß1 (Fig. 3b and c). This effect was not dependent on the presence of E2 and was not affected by the addition of 4-OH tamoxifen. In contrast, in the SK-OV-3/ERß+ cells exhibiting increased amounts of HER2 transcripts, we detected only a marginal increase of the HER2 protein level which was not significant (Fig. 4b and c). SK-BR-3 and OVCAR-3 cells overexpressing ERß1 did not exhibit changes in the HER2 expression on the protein level.

Discussion

In this report we demonstrated that elevated ERß receptor levels can trigger a significant increase of HER2 expression in hormone-dependent breast cancer cells. Our findings are one explanation for the recently published observation that ERß and HER2 expression is strongly and positively correlated in primary breast cancer (11,13).

The interactions between the ERα-mediated cellular estrogen response and HER2 signal transduction have previously been reported. The activity of ERα and estrogen-related receptor α1 is modulated by the HER2 signaling pathway (25,26). On the other hand, the HER2 expression is known to be suppressed by ERα-dependent signaling mechanisms (27). We also examined whether cross-talk exists between ERß and HER2 in breast and ovarian cancer cells.

For this purpose, we used a set of four hormone-dependent and -independent breast and ovarian cancer cell lines exhibiting different levels of ERα including the HER2-overexpressing cell lines SK-BR-3 and SK-OV-3. We compared the HER2 expression level of these wild-type cells to the expression of this receptor tyrosine kinase in ERß1-overexpressing clones of these cell lines generated by stable transfection with an ERß1-expression plasmid as previously described (15,33). The ERß1-overexpressing clones used in this study exhibited elevated ERß mRNA levels which are still in a physiological range resembling the ERß expression level of tumor cell lines such as MDA-MD-231 (34) and are characterized by a significantly slowed proliferation, increased basal apoptosis and impaired motility (15,33). These characteristics of our ERß+ clones are in line with reports from other groups demonstrating that ERß may negatively regulate cellular proliferation, promote apoptosis and thus may have a protective role in hormone-dependent tissues such as breast and prostate (35-37). Similar observations

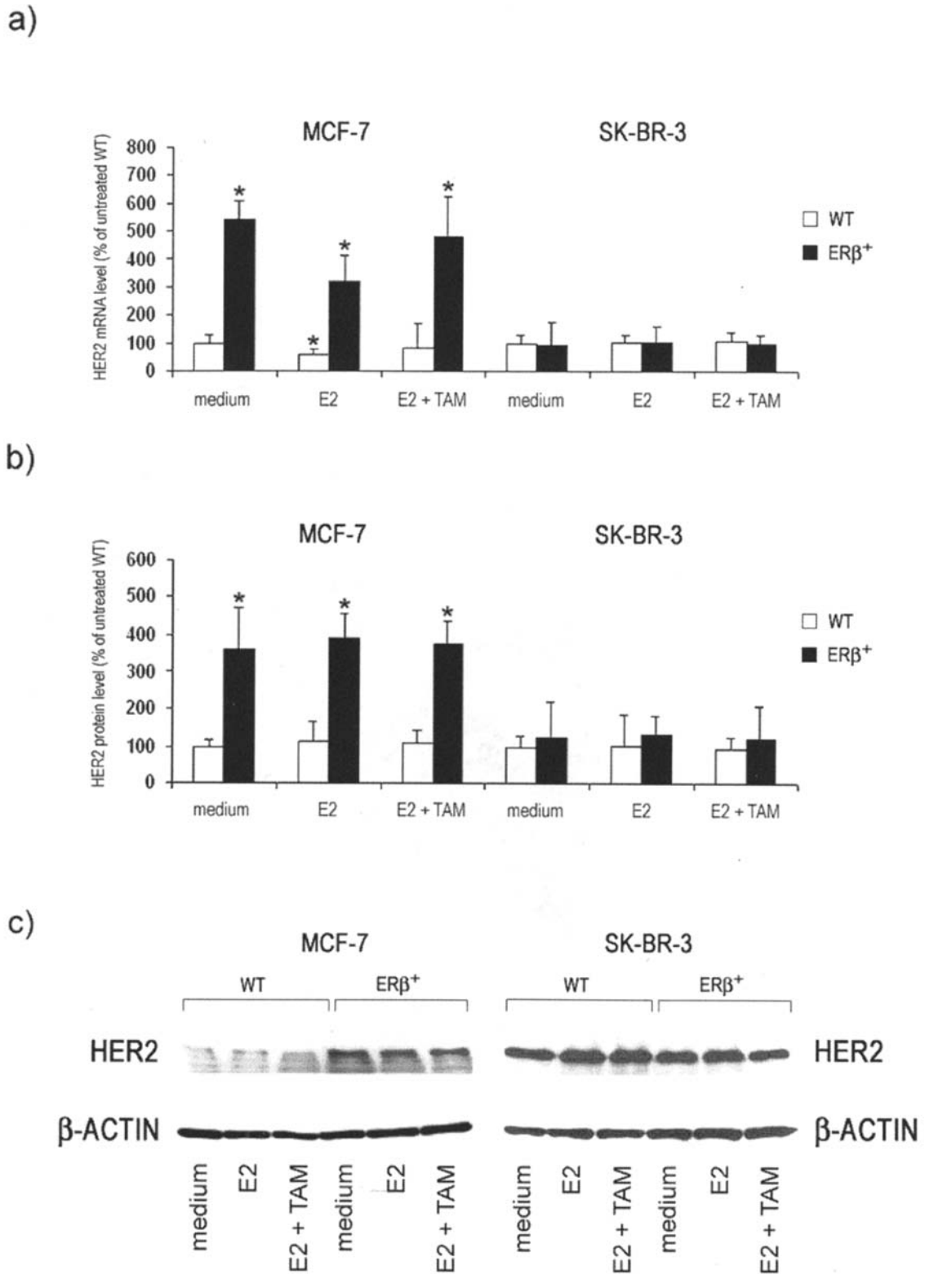

Figure 3. HER2 expression in wild-type and ERß-transfected breast cancer cell lines. The indicated cell lines and clones were cultured in steroid-hormone-free medium and treated with 3 nM 17-ß estradiol (E2) alone or in combination with 100 nM 4-OH tamoxifen (TAM) for 48 h prior to RNA and protein preparation. (a) HER2 mRNA levels expressed in percent of the ß-actin control. (b) HER2 Protein level in percent of the untreated wild-type control. (c) Representative Western blot (n=4); $^{*}p<0.01$ vs. wild-type.

were made in studies analyzing the function of ERß in ovarian cancer cells (15). These observations taken together with the fact that the ERß expression is reported to decrease during tumorigenesis suggest that ERß has a tumor-suppressor function in hormone-dependent tissues. In contrast, the HER-2 overexpression and/or amplification in breast cancer with positive hormone receptor status is correlated with an aggressive tumor phenotype (38). Thus, our findings, which

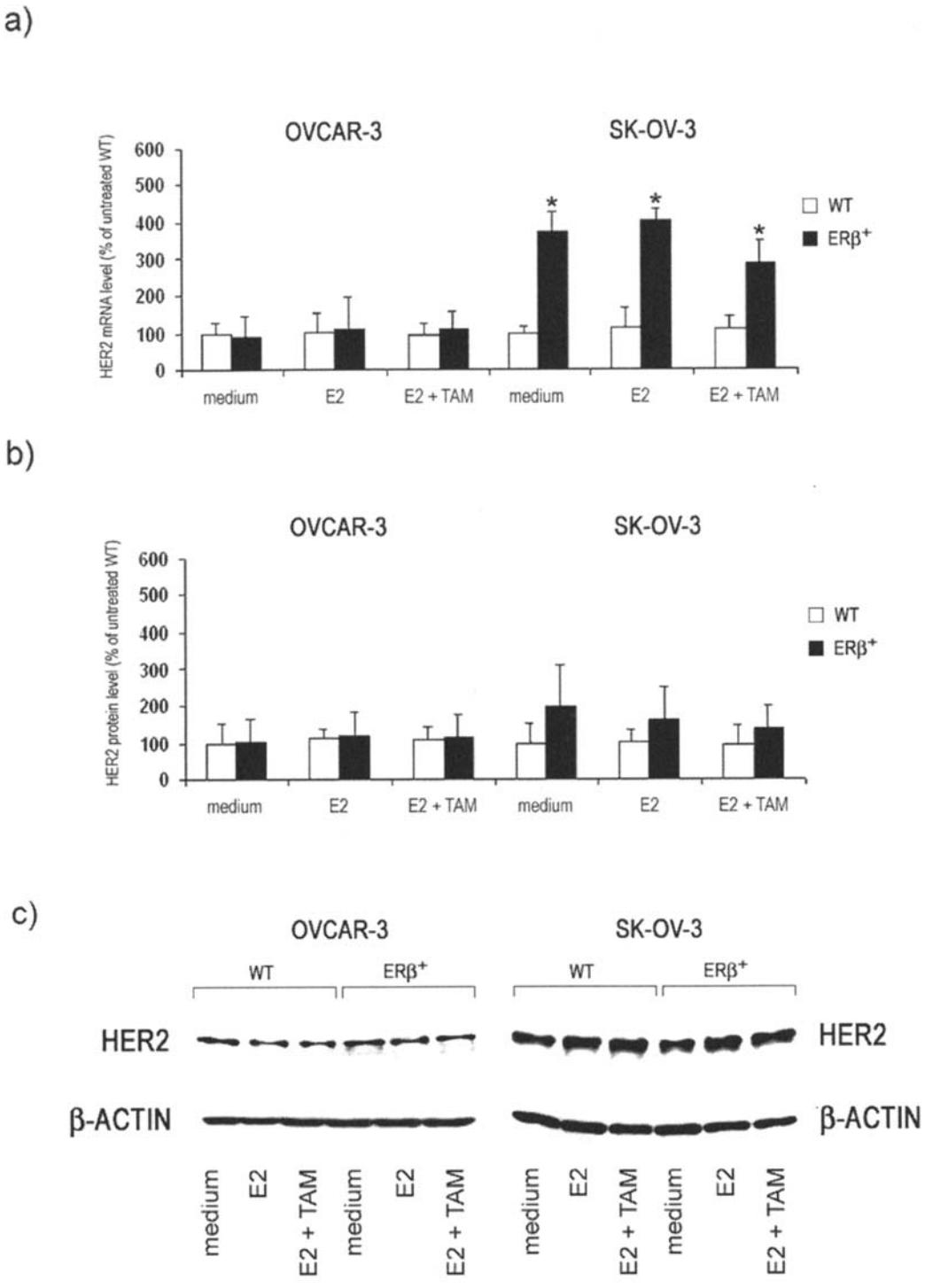

Figure 4. HER2 expression in wild-type and ERß-transfected ovarian cancer cell lines. The indicated cell lines and clones were cultured in steroid-hormone-free medium and treated with 3 nM 17-ß estradiol (E2) alone or in combination with 100 nM 4-OH tamoxifen (TAM) for 48 h prior to RNA and protein preparation. (a) HER2 mRNA levels expressed in percent of the ß-actin control. (b) HER2 Protein level in percent of the untreated wild-type control. (c) Representative Western blot (n=4); *p<0.01 vs. wild-type.

demonstrate that an overexpression of ERß leads to the elevation of HER2 expression in ERα-positive MCF-7 breast cancer cells, are noteworthy. However, results from our previous studies demonstrating a diminished proliferation of the MCF-7/ERß+ clone when compared to wild-type MCF-7 cells clearly suggest that the up-regulation of HER2 is not sufficient to trigger increased cell growth.

We confirmed the results of previous studies by showing that treatment with E2 is able to diminish the HER2 expression in MCF-7 wild-type cells expressing ERα (27). Given that the overexpression of ERß1 was able to increase the HER2 expression in this cell line, it is tempting to speculate that the ERα-triggered down-regulation of HER2 was counteracted by a high expression of ERß1 in a ligand-independent

manner. This interpretation of our observations would be in line with previous reports demonstrating that ERß acts as an ERα antagonist in specific settings (39,40).

The fact that we did not observe any significant effect of ERß1 overexpression on HER2 protein levels of SK-OV-3 and SK-BR-3 cells could simply be explained by the strong HER2 overexpression already present in the two cell lines which did not allow for the detection of possible ERß actions (41,42). The second reason for the observed unchanged HER2 expression in these cell lines was the known loss of functional ERα in SK-OV-3 and SK-BR-3 cells which does not allow for the observation of antagonistic ERß effects (39,40).

In this study, we reported the up-regulation of the HER2 receptor tyrosine kinase expression in estrogen-responsive MCF-7 breast cancer cells overexpressing ERß1. This effect could be one of the reasons for the significantly correlated co-expression of the two receptors observed in recent clinical reports.

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