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ORIGINAL CONTRIBUTIONS

Experimental ONCOLOGY

IMPACT OF COMBINATIONS OF EGF, TGFβ, 17β-OESTRADIOL, AND INHIBITORS OF CORRESPONDING PATHWAYS ON PROLIFERATION OF BREAST CANCER CELL LINES

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***Aim*: The impact of combinations of anti-cancer drugs and growth factors on tumour cells may differ from the assumed sum of the effects of each factor separately. Therefore it is important to study the effects of different combinations of various drugs and treatments. Our aim was to study the effects on breast cancer cell proliferation of EGF, TGFβ and 17β-oestradiol, three important regulators of breast tumourigenesis, and their respective inhibitors in different combinations. *Materials and Methods*: We screened the effects on proliferation of MCF7 and MDA-MB-231 cells of ninety different combinations of EGF, TGFβ and 17β-oestradiol, Iressa, SB431542 and Tamoxifen. Meta-data analysis of available clinical data was performed to validate observed proliferation data. *Results*: In MDA-MB-231 cells, TGFβ1 was found inhibitory when cells were simultaneously treated with EGF and 17β-oestradiol, with the effect potentiated by addition of all inhibitors combined. In the same cells, Iressa when combined with EGF was paradoxically stimulatory. Tamoxifen inhibited MCF7 cells co-treated with EGF or oestrogen, and enhanced the inhibitory effect of TGFβ in MDA-MB-231 cells. Meta-analysis of clinical gene expression studies confirmed several of these points, showing enhanced TGFβ and EGF expression in Tamoxifen-treated patients to correlate with decreased tumour size and grade respectively, and combined TGFβ-EGF expression to decrease the risk of metastasis. *Conclusion*: Our study shows significant differences in proliferation response to drugs and growth factors between MCF7 cells which do not have propensity to form metastases in animal models and MDA-MB-231 cells which may form metastases upon inoculation into animals. Several of these differences are unexpected and confirmed by clinical observations.**

***Key Words*: breast cancer, combinatorial treatment, EGF, oestrogen, TGFβ.**

Combinatorial treatments are a promising way to improve treatment outcomes in cancer with decreased resistance development and toxicity. However, cross-talk mechanisms between the drugs may produce unexpected toxicities [1]. Combined effects of drugs are difficult to predict and may show different synergistic or antagonistic effects depending on the signalling status of the cells being targeted [2].

In addition, tumour cells are under constant influence of physiological regulators which affect the tumourigenic properties of the cells and are often deregulated in cancer. There have been reported a number of studies of various combinations of cell regulators. For example, Sutherland et al. reported a study of IGF-1, EGF, TGFβ and bFGF effects on the proliferation of T-47D cells [3]. Additionally, the proliferative response of human breast cancer cells to polypeptide growth factors has been found to be modified by oestrogen signalling [4] and ER-EGF signalling cross-talk has been implicated in development of treatment resistance [5]. These and other similar studies have shown that combinations of regulators may have unexpected impacts on cell proliferation and that these impacts are combination-specific and not simply the sums of single treatments. An extensive intracellular signalling cross-talk may explain combinatorial effects, and signalling differences between patients may have an impact on differences in individual patient response [6].

Considering cross-talk both between the different drugs in a treatment combination and between cell signalling pathways in the patient treated, it is necessary to study the impact of drug combinations on cells in different signalling states in order to optimise treatment with combinatorial regimens.

EGF, TGFβ and oestrogen are among the most potent regulators of human breast epithelial tumourigenesis, and deregulation of all these pathways is associated with different steps in tumour progression. Oestrogen signalling drives proliferation in normal breast epithelial cells, and deregulation leads first to hyperplasia and thereafter to atypical hyperplasia, the stage before carcinoma *in situ*. It also contributes to genetic instability, a hallmark of cancer [7]. EGFR deregulation sustains proliferative signalling and contributes to development of hormone-independent growth in originally oestrogen-dependent cells. It also induces angiogenesis and metastasis and overexpression correlates with worse prognosis [8]. TGFβ1 is a factor inhibiting the growth of tumours at an early stage in breast cancer, but deregulation in later stages makes TGFβ contribute to development of metastasis [9]. Cross-talk mechanisms between these pathways have been studied [10–12], but there are no screening studies including both drugs and growth factors acting on the pathways. Our aim was

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Abbreviations used: bFGF – basic fibroblast growth factor; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; ER – oestrogen receptor; HER2 – human epidermal growth factor receptor 2; IGF-1 – insulin-like growth factor 1; PR – progesterone receptor; TGFα – transforming growth factor-alpha; TGFβ – transforming growth factor-beta.

thus to perform a combinatorial proliferation screening of both drugs and ligands acting on these pathways in order to find treatment combinations attractive for further study, which may aid in personalising cancer treatment.

We report a systematic analysis of combinatorial treatments with TGFβ1, EGF, 17β-oestradiol, Tamoxifen, EGFR kinase inhibitor Iressa and TGFβ type I receptor kinase inhibitor SB431542 of MCF7 and MDA-MB-231 cells, studying the impact on cell proliferation of 90 different combinations of drug and growth factor. There was reported that these cells have different levels of tumourigenic transformation. Notably, in the most of studies, MCF7 cells do not have propensity to form metastases in animal models, while MDA-MB-231 cells may form metastases upon inoculation into animals. Despite that there were observations that both these cell lines may or may not form tumours and metastases, MCF7 cells are considered as cells at first steps of transformation, and MDA-MB-231 cells are considered as a model of advance transformed cells [13–15]. For validation of the screening findings, we performed meta-analysis of clinical data. This approach revealed several unexpected combinatorial effects which were found experimentally and validated through the meta-analysis, notably that the effects of TGFβ are dependent on cross-talk with EGF and oestrogen signalling. In conclusion, this study has pointed to a number of interesting synergistic and antagonistic interactions which may be further studied to aid in personalisation of cancer treatment.

MATERIALS AND METHODS

Cells and reagents. MDA-MB-231 and MCF7 cells were obtained from ATCC (Mannasas, VA) and cultured as recommended. MDA-MB-231 cells form metastases when injected in animals, while MCF7 may form tumours but do not form metastases. Cells were cultured in DMEM medium (Gibco, Carlsbad, CA) with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were monitored for absence of contaminations, e.g. mycoplasma, and for transformation status by measuring cells' colony formation, clonogenicity, contact inhibition, proliferation rates and morphology. Human recombinant EGF and human recombinant TGFβ1 were obtained from Peprotech (Rocky Hill, USA), and 17β-oestradiol was obtained from Sigma-Aldrich (Stockholm, Sweden). Tamoxifen, SB431542 and Iressa were obtained from Sigma-Aldrich.

Treatments. In total, 90 combinations were used for each cell line. 18 combinations of growth factors were used, with TGFβ1 at concentrations of 1 and 10 ng/ml, EGF at 50 and 100 ng/ml and 17β-oestradiol at 10 μM. The growth factors treatments (18 combinations) were combined with the following 5 combinations of drug treatments: none, 10 μM SB431542, 1 μM Tamoxifen, 10 μM Iressa and SB431542, Tamoxifen and Iressa together at the above concentrations.

MTT assay. Cell proliferation was measured by MTT assay (Promega, Madison, WI). The MTT assays were performed as described in earlier studies [16]. Cells were seeded in 96-well plates at 10 000 cells/well in culturing medium. The next day, fresh medium and treatments were added. Four wells were used as repeats for each condition. Cells were incubated for 48 h, whereafter MTT assay was performed and proliferation measured as absorbance at 570 nm in a spectrophotometer. Representative experiments of three repeats are shown in figures. Significance of differences was calculated by Student's t-test.

Clinical correlations. Meta-analysis of clinical data has previously been used to study drug interactions [17]. For relevant clinical correlations, we searched for microarray studies containing clinical outcome data and analysis of TGFβ1 and EGF ligand expression, since these ligands were included in our screening.

The study by Lyng et al. [18] comprised 108 ER-positive patients with invasive ductal or lobular carcinomas treated with Tamoxifen. Enhanced TGFβ1 and EGF expression were defined by two of three probes for each protein having expression values above the total patient average.

The study by Ellsworth et al. [19] included twenty breast cancer patients with lymph node metastases >2.0 mm of different histology and ER/PR/HER2 status and studied the difference in gene expression between primary tumours and paired metastatic lymph nodes. We calculated expression quotas between lymph nodes and primary tumour for each patient. Enhanced TGFβ1 expression was defined where the quota for one of two probes was above the average quota, and enhanced EGF expression was defined by the single EGF probe quota being above the average quota. Significant differences were calculated by Student's t-test in analysis of both studies.

RESULTS

Proliferation screening. We tested the effects of combinations of Iressa, SB431542, Tamoxifen, TGFβ1, EGF and 17β-oestradiol on cell proliferation (Fig. 1). The 90 tested conditions are indicated in the Material and Methods section. Here we describe those conditions which showed unexpected and novel responses of cells to treatments.

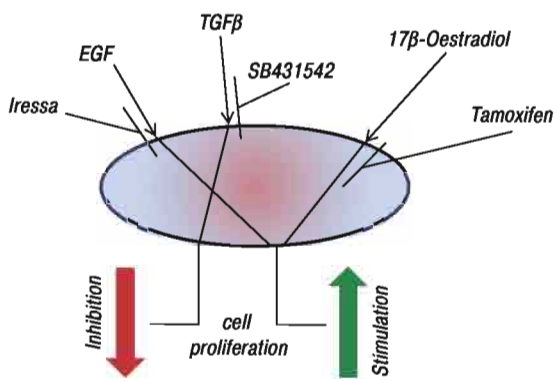

Fig. 1. Schematic presentation of the treatments. Links of EGF and 17β-oestradiol to growth-promoting, and TGFβ to growth-inhibitory effects are presented. Effects of Iressa, SB431542 and Tamoxifen on EGF, TGFβ and 17β-oestradiol regulated signalling are indicated

The effect of TGFβ1 is dependent on cross-talk with the other treatments. MCF7 cells generally showed a trend towards inhibition by TGFβ1, while in MDA-MB-231 cells, an inhibitory effect of TGFβ1 was only seen in cells co-treated with EGF and 17β-oestradiol (Fig. 2). This shows that an inhibitory effect may be enhanced by treatment with two traditional growth stimulators. Further support for the importance of oestrogen and TGFβ signalling cross-talk is provided by the fact that MDA-MB-231 cells treated with ER antagonist Tamoxifen were inhibited in a TGFβ1-concentration-dependent manner, while MCF7 cells on the other hand were inhibited upon addition of EGF or oestrogen regardless of TGFβ1 concentration (Fig. 3). Since MDA-MB-231 cells are ER-, this, as well as the stimulatory effect seen by Tamoxifen alone, may point to Tamoxifen off-ER-target effects.

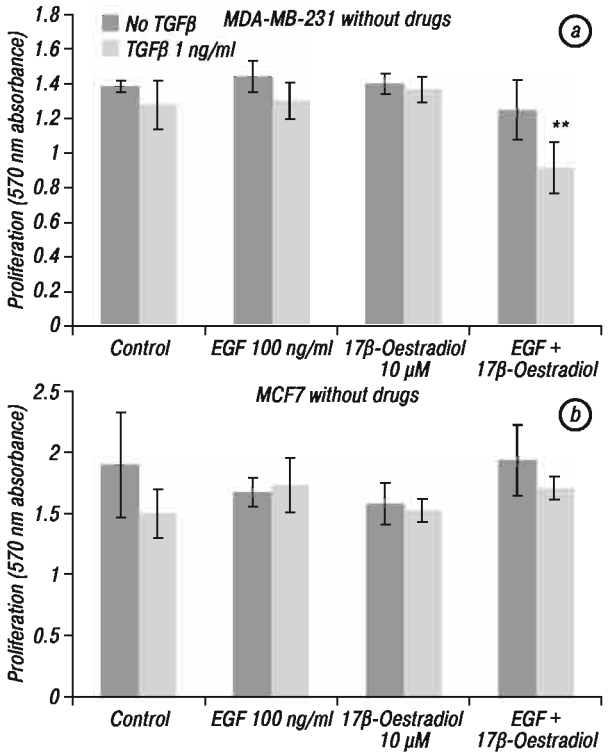

Fig. 2. Proliferation of MDA-MB-231 and MCF7 without addition of drugs shows the combination of EGF, 17β-oestradiol and TGFβ1 to inhibit proliferation in MDA-MB-231 but not in MCF7 cells. Treatment conditions are annotated. **($p < 0.01$) is in comparison with untreated cells

While Iressa did inhibit MDA-MB-231 cells, TGFβ1 did not have an additional inhibitory effect, but rather blocked the stimulatory effect of EGF, showing that the effect of TGFβ also depends on EGF signalling (Fig. 4).

Treatment of cells with all three inhibitors — Iressa, Tamoxifen and SB431542 — showed the same trends in effects on proliferation as untreated cells (see Fig. 2), with the difference that EGF + 17β-oestradiol treatment inhibited MDA-MB-231 cells regardless of TGFβ treatment (Fig. 5), providing further evidence that the effect of drugs in combination is not simply an addition of the single drug effects, but the result of signalling cross-talk.

Meta-analysis of clinical correlations. Through meta-analysis of original data reported by Ellsworth et al. [19], which focused on gene signatures predicting metastasis, we found that an increased rate of TGFβ1 and EGF ligand co-expression in lymph node metastases compared with primary tumours from the same patients significantly correlated with a lower number of positive lymph nodes (Fig. 6, *a*), implying that in the metastatic process, cells co-expressing TGFβ and EGF are less prone to metastasize. While the original data did not allow the evaluation of an additional impact of oestrogen signalling, the available meta-data confirm our observations of the inhibitory effects on cell proliferation by TGFβ1 being dependent on EGF (see Fig. 2).

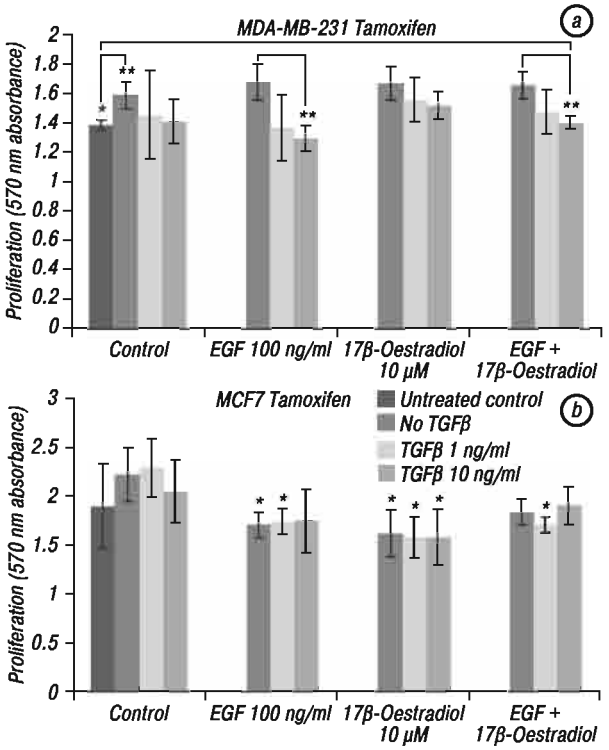

Fig. 3. Proliferation of MDA-MB-231 and MCF7 cells treated with 1 μM Tamoxifen shows MDA-MB-231 proliferation to decrease in a TGFβ1-dependent manner. MCF7 cells are inhibited by 17β-oestradiol and EGF independent of TGFβ1. Treatment conditions are annotated. For MCF7, significant differences are in comparison to Tamoxifen only (*p < 0.05; **p < 0.01)

Meta-analysis of the study by Lyng et al. [18], comprising 108 ER+ patients treated with Tamoxifen, found that patients with above average TGFβ1 expression had smaller tumours than both patients where TGFβ1 and EGF were co-expressed and the total patient average (Fig. 6, *b*), while patients with above average EGF expression had a lower grade of malignancy (Fig. 6, *c*). Other variations in clinical observations and TGFβ and EGF activities were found to be not significant. These data support our observations where the proliferation in both cell lines was decreased when Tamoxifen was combined with TGFβ1 (MDA-MB-231) or EGF (MCF7) (see Fig. 3).

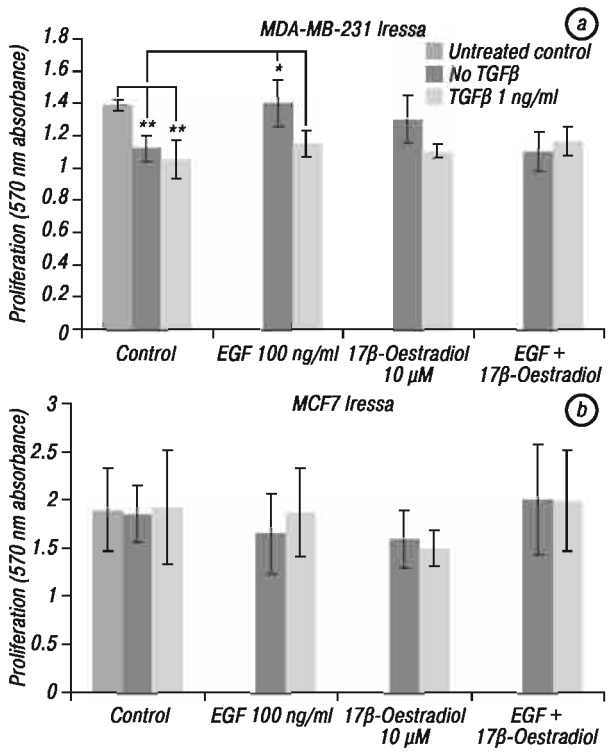

Fig. 4. Proliferation of MDA-MB-231 and MCF7 cells upon treatment with 10 μM Iressa shows EGF to have a stimulatory effect, which is counteracted by addition of TGFβ1, in the presence of Iressa in MDA-MB-231 cells. "Untreated control" indicates condition with no treatments. Treatment conditions are annotated (*$p < 0.05$, **$p < 0.01$)

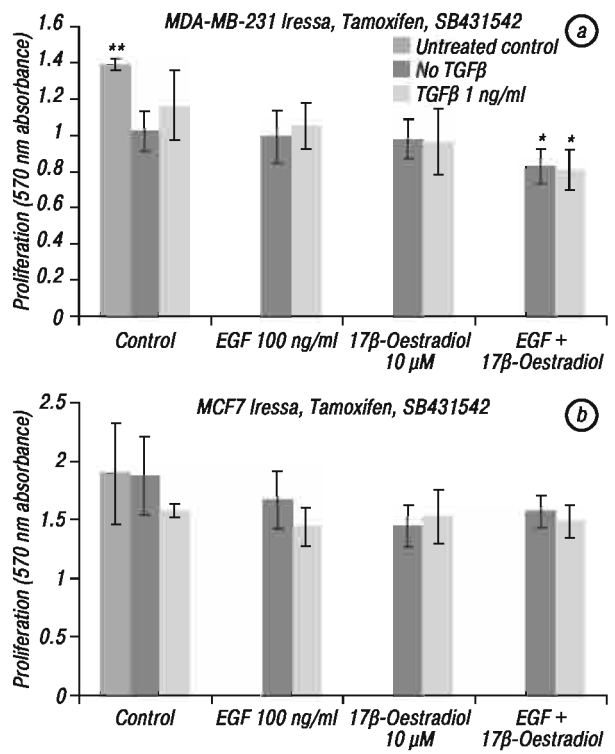

Fig. 5. Proliferation of MDA-MB-231 and MCF7 cells exposed to 10 μM Iressa, 1 μM Tamoxifen and 10 μM SB431542 shows combined application of EGF and 17β-oestradiol to inhibit MDA-MB-231 cells. Treatment conditions are annotated. "Untreated control" indicates condition with no treatments. Significant differences *($p < 0.05$) and **($p < 0.01$) are in comparison with cells treated only with the three inhibitors

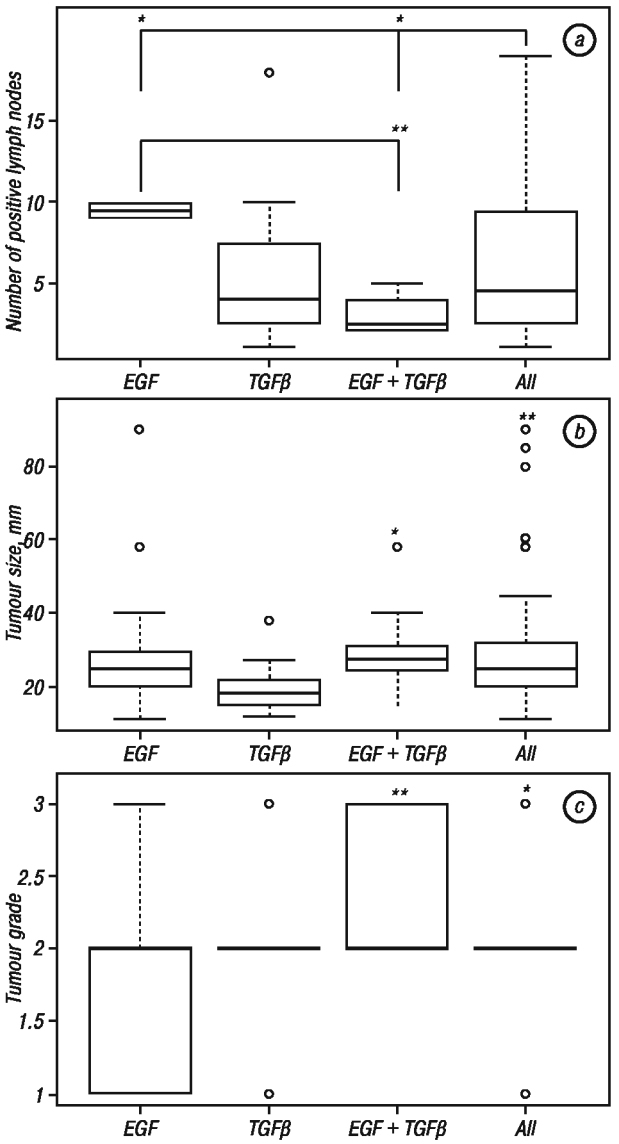

Fig. 6. Clinical outcomes of TGFβ and EGF co-expression. *a*) Meta-analysis of data reported by the study by Ellsworth et al. [19] shows TGFβ1 and EGF co-expression in lymph node metastases to correlate with fewer positive lymph nodes. *b*) Meta-analysis of data reported by the study by Lyng et al. [18] on Tamoxifen-treated patients shows TGFβ1 expression to correlate with smaller tumours. Significant differences *($p < 0.05$) and **($p < 0.01$) are in comparison with the group showing enhanced TGFβ expression. *c*) In the study by Lyng et al. [18], EGF expression was associated with a lower grade of malignancy. **($p < 0.01$) and *($p < 0.05$) are in comparison with EGF condition

DISCUSSION

It is an accepted fact that combinations of multiple cellular regulators may have different impacts as compared to the sum of the same regulators applied individually. Thus, it is necessary to study multiple interactions not only between potential drug candidates but also the interplay between the drugs and the different growth factors whose signalling is deregulated in cancer cells. Our study focused on exploring the effects on cell proliferation by TGFβ1, EGF, 17β-oestradiol, Tamoxifen, Iressa and SB431542. We observed differences in response between the ER^- MDA-MB-231 and ER^+ MCF7 cell lines.

The main novel findings were 1) that TGFβ1 inhibited proliferation of MDA-MB-231 cells when the cells are also treated with EGF and oestrogen, 2) that exposure of MDA-MB-231 cells to Tamoxifen, EGF and/or 17β-oestradiol promoted the inhibitory effect of TGFβ1, while combinations of Tamoxifen, EGF and/or 17β-oestradiol inhibited MCF7 proliferation irrespectively of TGFβ1 addition, 3) that addition of EGF to Iressa had a stimulatory effect, which was blocked by TGFβ1, in MDA-MB-231 cells, and 4) that co-treatment with Iressa, Tamoxifen and SB431542 showed the same general response trend as untreated cells. The latter fact implies a cross-inhibition between the drugs, further underlining the fact that addition of new drugs, proven effective when used alone, to existing treatment regimens, may have a negative effect. Tamoxifen alone somewhat increased MDA-MB-231 cell proliferation. Since these cells are ER^-, this is not completely unexpected. Tamoxifen has earlier been observed to increase the proliferation of Tamoxifen-resistant breast cancer cells [20].

The correlations between TGFβ and EGF gene expression and clinical outcomes confirm certain of our findings, showing impact on tumour size and grade when EGF or TGFβ are combined with Tamoxifen, and a reduction in lymph node metastasis upon TGFβ-EGF co-expression.

Our findings underline the need for in-depth study of drug and growth factor interactions before application of any combinatorial regimen in cancer treatment. This study particularly highlights the different roles of TGFβ in regulating cell proliferation, and how the impact of TGFβ is dependent on cell type and cross-talk with EGF and oestrogen signalling.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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