Clinical Endocrinology (2006) 64, 20–28 doi: 10.1111/j.1365-2265.2005.02408.x

ORIGINAL ARTICLE

Breast cyst fluids increase the proliferation of breast cell lines in correlation with their hormone and growth factor concentration

Pablo J. Enriori*¶, Stella Maris Vázquez*, Violeta Chiauzzi*, Cecilia Pérez*, Carlos R. Fischer†, Jorge R. Gori†, Alberto E. Etkin‡, Eduardo Charreau*, Ricardo S. Calandra*§ and Isabel A. Lüthy*

**Instituto de Biología y Medicina Experimental, †Hospital Alemán and ‡Hospital Durand, Buenos Aires and §Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina*

Summary

Objective and design Gross cystic disease (GCD) of the breast is reported to occur in 7% of women in the developed world and, although not premalignant, is thought to be associated with an increased risk of breast cancer. Hormone and growth factor concentration levels were measured in breast cyst fluid (BCF) to correlate them with their mitogenic activity in tumour (MCF-7) or nontransformed (MCF-10A) cells.

Results Oestradiol (E_2), oestrone (E_1), E_2-sulfate (E_2-S), E_1-sulfate (E_1-S) and epidermal growth factor (EGF) concentrations were, as expected, significantly higher in type I than in type II cysts, while transforming growth factor-beta 2 (TGF-β2) showed higher levels in type II cysts. Fifty per cent of the BCF samples stimulated [^{3}H]-thymidine incorporation into MCF-7 cells while 34·5% inhibited this parameter. In MCF-10A cells, most BCF samples were stimulatory (85%). E_2, E_1 and EGF concentrations in BCF samples correlated significantly and positively with cell proliferation in MCF-7 cells, whereas a significant negative correlation was found for TGF-β2. In MCF-10A cells, only E_2-S and E_1-S exhibited significant positive correlation, whereas a significant negative correlation was found for TGF-β2. Progesterone (Pg), E_2 and EGF incubated under the same conditions had a stimulatory effect on [^{3}H]-thymidine incorporation into MCF-7 cells, whereas TGF-β2 inhibited this parameter. Pg, E_2, E_1 and EGF significantly stimulated this parameter in MCF-10A cells.

Conclusions The stimulatory action of BCF on cell proliferation in a model of human breast epithelial cells could partly explain the increased incidence of breast cancer in cyst-bearing women.

(Received 13 June 2005; returned for revision 11 July 2005; finally revised 9 September 2005; accepted 4 October 2005)

¶Present address: Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, USA.
Correspondence: Dr Isabel Alicia Lüthy, Obligado 2490, C1428ADN, Buenos Aires, Argentina. Tel.: 5411 4783 2869; Fax: 5411 4786 2564; E-mail: iluthy@dna.uba.ar

Introduction

Gross cystic disease (GCD) of the breast, defined on the basis of the presence of palpable cysts, was reported in 1981 to occur in 7% of women in the developed world.[1] Cysts are found mainly in women in their thirties to late forties/early fifties and are uncommon in post-menopausal women unless they are receiving hormone replacement therapy.[2] Higher risks of developing breast cancer, ranging from 1·7 to 7·5, have been reported in the literature for women with palpable cysts.[3] Breast cysts are not considered to be premalignant lesions but simply markers of an increased risk affecting the whole organ.[1] There are two clearly defined types of breast cysts.[4] Type I cysts are lined with apocrine epithelium and contain fluid with an electrolyte composition similar to that of intracellular fluid, high concentrations of potassium [K^+], low concentrations of sodium [Na^+], Na^+/K^+ ratio < 3, and high concentrations of steroid hormones, including oestrogen (both oestradiol and oestrone), androsterone, epiandrosterone, dehydroepiandrosterone, and their conjugates, mainly sulfates. Type II cysts are lined with flattened attenuated epithelium, contain fluid with an electrolyte composition similar to that of plasma (Na^+/K^+ ratio > 3) and have lower concentrations of steroid hormones and their conjugates than type I cysts.[4] Several groups have demonstrated that cyst fluid contains large amounts of steroid hormones,[5–7] peptides[8] and growth factors.[5,6,8–10] Some authors have suggested that oestrogen serum levels,[11,12] as well as growth factors, such as serum IGF levels[13,14] or intracystic epidermal growth factor (EGF) concentrations,[15] are correlated with an increased risk of breast cancer.

BCF has been reported to stimulate proliferation of NIH 3T3 mouse embryo fibroblasts.[16] When analysing the influence on breast cancer risk of the mitogenic activity in these cells, a stratification of this activity was found, with samples from the highest-risk group containing a several-fold increase in activity compared with samples from the lowest-risk women. Mitogenic activity was ascribed mainly to EGF concentration levels.[16] Other studies investigated the effect of BCF on oestrone-sulfatase activity in MCF-7 and MDA-MB-231 human breast cancer cells, counting cell number as a control. Most BCF samples analysed inhibited enzymatic activity but some samples inhibited cell proliferation.[17] Others reported that all the BCF samples tested inhibited cell growth in the MDA-MB-231 cell line while

 © 2005 Blackwell Publishing Ltd

the majority of the samples inhibited MCF-7 cell growth.[18] Again, cell proliferation was analysed only as a control for the activity of the same enzyme. Our group has attempted to analyse whether the increased risk of breast cancer in cyst-bearing patients could be mediated by a mitogenic activity of the BCF. As a model for the epithelium lining the cyst, two cell lines were chosen. MCF-7 cancer cells are well-differentiated, oestrogen-sensitive and metastatic, derived from pleural effusion.[19] MCF-10A, an immortal cell line that arose spontaneously from human diploid breast cells of extended lifespan, was obtained from a premenopausal woman with fibrocystic disease.[20]

Our group has recently suggested that the enhanced levels of IGF-I/IGFBP-3 found in patients with type I cysts might be associated with the increased risk of breast cancer described for these patients.[21]

In this study, the mitogenic activity of BCF was analysed in both MCF-7 and MCF-10A cells and correlated with hormone and growth factor concentration. Additionally, the cells were incubated under the same conditions with each hormone and growth factor to confirm their effect on [^{3}H]-thymidine incorporation.

Patients and methods

Experimental subjects

Fifty-five premenopausal women diagnosed with GCD of the breast, classified[1] according to Haagensen's criterion (cyst greater than 3 mm in diameter, as measured by ultrasound), were studied. Patients were studied because of a breast lump found by either auto-examination or clinical examination. All the patients underwent a physical examination, mammography, ultrasonic examination and fine-needle aspiration. Aspirated material was analysed cytologically. For therapeutic reasons, as indicated by clinical consensus,[22] the cysts, whether single or multiple, were aspirated only when their diameter measured by ultrasound was greater than 3 mm. All the cysts were aspirated during the early follicular phase, and blood samples were extracted simultaneously.

Of the 55 patients with GCD, 31 presented a single cyst, four presented double cysts and 10 presented more than three cysts simultaneously. All the patients showed cysts greater than 3 mm, which were aspirated at least once. Cysts recurred in only three patients and none developed breast cancer during the study. None of the patients presented with a family history of breast cancer. Postmenopausal women, patients with either endocrine pathologies or breast cancer, and pregnant women were excluded from the study. Therefore, all patients selected menstruated regularly and none of them had used oral contraceptives for 6 months prior to the study.

Patients were classified as possessing a type I cyst when the Na^+/K^+ ratio in the BCF was < 3, and type II when the ratio was > 3. BCF aspiration was performed during the early follicular phase of the menstrual cycle (between days 3 and 7).

Patients were examined in two Medical Centres by two well-trained ultrasound observers. The protocol was approved by two local ethics committees (Hospital Alemán and Instituto de Biología y Medicina Experimental) according to the Helsinki II Declaration.

Methods

Cell culture

MCF-7 and MCF-10A human breast cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were routinely cultured in phenol red free[23] Dulbecco's Modified Eagle's Medium (DMEM): Ham F12 (1 : 1) supplemented with 10% foetal calf serum (FCS) (South American FCS, Invitrogen Life Technologies, Carlsbad, CA, USA), 2 mM glutamine, 2 µg/ml bovine insulin, 100 UI/ml penicillin, 100 µg/ml streptomycin, and 15 mM HEPES. MCF-10A medium was supplemented with 20 ng/ml EGF. Cells were subcultured once a week after trypsinization [0·25% trypsin–0·025% ethylenediaminetetraacetic acid (EDTA)] and seeded at a concentration of 80 000 cells/25 cm^2 flask. The medium was changed twice a week.

Mitogenic activity of BCF

Cells were seeded at 7000–10 000 cells/well in 96-well plates and incubated at 37 °C in RPMI 1640 phenol-red free medium with 2% charcoal-stripped FCS (for MCF-7 cells) or 5% charcoal-stripped FCS (for MCF-10A cells), 2 mM glutamine, 100 UI/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B and 15 mM HEPES. After 24 h, the medium was changed and different amounts of BCF (from 2·5 to 15 µl in 200 µl of medium) and 0·2 µCi [6-^{3}H]-thymidine (Sp. Act: 20 Ci/mmol; Dupont-New England Nuclear, Boston, MA, USA) were added. After 20 h incubation at 37 °C, cells were harvested in a Nunc Cell Harvester 8, and filters were counted with scintillation liquid (Optiphase 'Hisafe' 3, Wallac, Turku, Finland) in a liquid scintillation counter. MCF-10A cells were trypsinized prior to harvesting.

Assays

Sodium and potassium determination. These electrolytes were determined with an EEL flame photometer, using 1 : 100 and 1 : 500 dilutions, respectively.

Radioimmunoassay (RIA) of E_2, E_2-S, E_1, E_1-S and progesterone (Pg) in BCF. To measure the different steroids, several purification steps were undertaken. For extraction, 2 ml diethylether was added to 500 µl BCF. The organic phase was used to measure the nonconjugated steroids (E_2, E_1 and Pg). The aqueous phase containing E_2-S and E_1-S was deproteinized with ethanol (1 : 4), and enzymatically hydrolysed,[24] with modifications to the enzyme concentration and incubation time as described by Sheckleton *et al.*[25] These steroids were then measured as E_2 and E_1.

E_2 was determined by RIA with a Diagnostic System Laboratories Inc. kit (Webster, TX, USA). The antibody has a very low cross-reaction with E_1 (3·4%), E_1-S (0·21%) and dehydroepiandrosterone-sulfate (DHEA-S, nondetectable). The intra-assay coefficient of variation (CV) was 5·3%, the interassay CV was 9·1% and the functional sensitivity of E_2 was 5 pg/ml. E_1 was determined with a commercial RIA kit (Diagnostic System Laboratories Inc.) containing an antibody of high specificity. Cross-reactions were low: 1·2% with E_2,

2·0% with E_1-S, and nondetectable for DHEA-S. Intra-assay and interassay CVs were 4·6% and 5·8%, respectively, and the functional sensitivity was 5 pg/ml. Pg was determined with an RIA kit from Diagnostic System Laboratories Inc. with an intra-assay CV of 7·6%, an interassay CV of 9·8% and a functional sensitivity of 1 ng/ml.

EGF determination. RIA determination of EGF was performed using the double antibody method, developed by Molinolo et al.[26] and adapted for BCF. In brief, the BCF samples were diluted (1 : 20) in phosphate buffer, and 100 µl was incubated with 100 µl of the anti-EGF rabbit antibody (Peninsula Laboratory, USA, final dilution 1 : 100 000), 100 µl of 3% normal rabbit anti-serum, 100 µl phosphate-buffered saline and 100 µl of ^{125}I-EGF (20 000 counts per minute with specific activity of 20 µCi/µg) for 24 h at room temperature, after which 100 µl of a second antibody [goat anti-rabbit immunoglobulin G (IgG) diluted 1 : 10] was added and the tubes incubated for 24 h at room temperature. The range of the standard curve was 0·006–6·25 ng/ml. The detection limit of the assay was 25 pg.

Transforming growth factor-beta 2 (TGF-β2 determination. A commercial enzyme-linked immunosorbent assay (ELISA) was used (R&D System, Quantikine, Minneapolis, MN, USA). Following the manufacturer's instructions, latent TGF-β2 was activated by incubation with 1 N HCl. The dilutions used were 1 : 20 for type I BCF and 1 : 50 for type II BCF. The standard curve was in the range 31·5–2000 pg/ml. Intra-assay CV was 7·1% for low-concentration samples and 2·7% for high-concentration samples and the typical minimum detectable dose was 7·0 pg/ml (according to the manufacturer). All the samples were included in the same determination.

Serum IGF determination. IGF-I was determined in serum by an immunoradiometric assay (IRMA) after separation of IGFs from IGFBPs by acid–ethanol extraction (Diagnostic System Laboratories, Inc.). The detection limit was 0·80 ng/ml, the intra-assay CV (measured by us) was 4·4% and the interassay CV was 6·5%.

Statistical analysis

A nonparametric Kruskall–Wallis test, followed by a *posteriori* Dunn's test, was used to compare concentrations between groups. Correlation analysis was performed by GraphPad Software calculating Spearman's correlation.

Results

BCF concentration levels of several hormones and growth factors in type I and II cysts were measured in order to correlate them with their mitogenic activity in tumour (MCF-7) or nontumour (MCF-10A) cells. Figure 1 depicts the concentration levels found for these compounds. When the oestrogen concentration level was analysed, nearly half the BCF samples showed nondetectable concentrations of E_2 (Fig. 1a) and/or E_1 (Fig. 1b). However, as reported previously,[7] oestrogen concentration was significantly higher in type I cysts than in type II. When considering the concentration levels of oestrogen-sulfate, both E_2-S (Fig. 1c) and E_1-S (Fig. 1d) concentrations were significantly higher in type I cysts than in type II, as reported previously.[7] These higher hormone concentrations in type I cysts were also evident for other conjugated hormones such as DHEA-S and 5-androstene-3β,17β-adiol-sulfate (Adiol-S) (data not shown). Pg (Fig. 1e) concentration levels did not differ significantly between type I and type II cysts, although a tendency to higher levels in type II cysts was evident, in contrast to lower serum levels of this hormone found in type II cyst bearers. The levels of EGF and TGF-β2 were significantly different between both types, with higher levels of EGF (Fig. 1f) in type I cysts, and higher TGF-β2 levels in type II cysts, as described previously.[27,28]

BCF samples were tested for their capacity to influence cell proliferation of two breast cell lines, the cancer MCF-7 cells (55 BCF) and the spontaneously immortalized nontransformed MCF-10A cells (27 BCF). When analysing the effect of the direct incubation of different concentrations of BCF on [^{3}H]-thymidine incorporation into both cells lines, the effect was usually seen even at very low amounts of BCF (2 µl). However, at 10 µl (in 200 µl total volume) the effect was always evident. For this reason, further determinations used this amount of BCF. As can be seen in Table 1, half of the BCF samples were able to stimulate [^{3}H]-thymidine incorporation into cancer cells MCF-7, while 34·5% were inhibitory, and the rest did not produce any effect. The stimulation or inhibition caused by type I and type II cysts differed, with a 2·3-fold main stimulation by type I cysts and a marginal stimulation (1·2-fold) by type II cysts ($P < 0·0001$, not shown). However, on nontransformed MCF-10A, most BCF samples were stimulatory (85%). Again, the 3·35-fold main stimulation by type I cysts was significantly greater than the 1·9-fold stimulation by type II cysts ($P < 0·005$, not shown).

Mitogenic activity was correlated with the concentration levels of these hormones and growth factors in BCF. As seen in Fig. 2, both E_2 and E_1 correlated significantly with [^{3}H]-thymidine incorporation into MCF-7 cells. E_2-S, E_1-S and Pg did not correlate significantly with this parameter, and consequently were not included in the figure. A significant positive correlation was found between mitogenic activity and EGF, whereas a significant negative correlation was found between mitogenic activity and TGF-β2 concentration. When

Table 1. Effect of different breast cyst fluids (BCF) on [^{3}H]-thymidine incorporation into breast cancer cells MCF-7 and nontransformed MCF-10A in culture

		Stimulation *n* (%)	Without effect *n* (%)	Inhibition *n* (%)
MCF-7: type I and II	*n* = 55	28 (51)	8 (14·5)	19 (34·5)
MCF-7: type I	*n* = 32	25 (75)	5 (15·6)	3 (9·3)
MCF-7: type II	*n* = 23	4 (17·4)	3 (13)	16 (69·6)
MCF-10A: type I and II	*n* = 27	23 (85)	2 (7·5)	2 (7·5)
MCF-10A: type I	*n* = 14	14 (100)	0	0
MCF-10A: type II	*n* = 13	9 (70)	2 (15)	2 (15)

Effect of the incubation of 10 µl of different BCF on 200 µl of medium with 7000–10 000 human breast MCF-7 (tumoral) or MCF-10A cells (immortalized nontumoral). The number of BCF samples that evidenced either stimulation or inhibition or had no effect and the percentage obtained by comparing those numbers to the total number of samples (in parentheses) discriminated by type are shown.

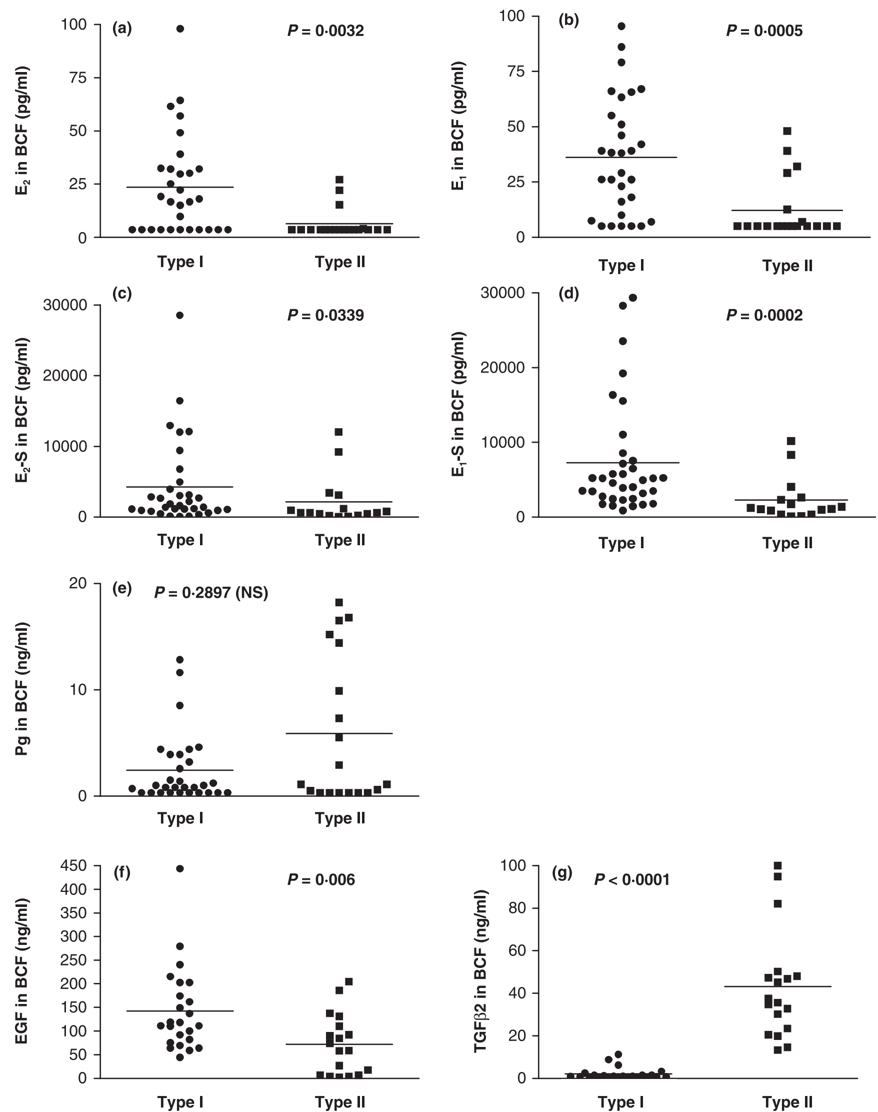


Fig. 1 (a) Oestradiol (E_2), (b) oestrone (E_1), (c) oestradiol-sulfate (E_2-S), (d) oestrone-sulfate (E_1-S), (e) progesterone (Pg), (f) epidermal growth factor (EGF) and (g) transforming growth factor-β (TGF-β) concentration levels in breast cyst fluid (BCF) from patients with type I and type II cysts. The points represent individual measurements whereas the bars are the median for each group. Statistical analysis was performed by the nonparametric Kruskall–Wallis test followed by Dunn's analysis.

these hormone and growth factor concentration levels in BCF were correlated with the mitogenic activity in MCF-10A cells, only E_2-S and E_1-S exhibited a significant positive correlation while TGF-β2 showed a negative correlation (Fig. 3). Moreover, a positive correlation was found (Fig. 4) between the stimulation of MCF-7 and MCF-10A, suggesting that some of the compounds present in BCF were able to influence proliferation in both cell lines. A very striking correlation was found between serum IGF-I concentration and BCF mitogenic activity in MCF-7 cells (Fig. 5a). However, as serum IGF-I correlated with EGF concentration in BCF, this could be the mediator of serum IGF-I (Fig. 5b). When combinations of hormones or growth factors were assessed, no correlation was found for $E_2 + E_1/\text{Pg}$, whereas the combination $E_2 + E_1 + \text{Pg}$ correlated significantly ($P < 0{\cdot}0001$).

To determine whether the concentration levels of each of these compounds found in BCF could stimulate or inhibit cell proliferation, [^{3}H]-thymidine incorporation was measured after incubation under the same conditions as when incubating with BCF samples. As can be seen in Fig. 6a, Pg, E_2 and EGF had a stimulatory effect on MCF-7 cell proliferation, whereas TGF-β2 inhibited this parameter with no significant effect of 1 μM E_1. When [^{3}H]-thymidine incorporation into MCF-10A cells was studied, Pg, E_2, E_1 (marginally) and EGF (even at concentrations as low as 1 ng/ml) significantly stimulated this parameter (Fig. 6b).

Discussion

The aim of this study was to determine whether BCF samples are able to stimulate the proliferation of human breast cell lines, as a model for the epithelial cells lining the cyst. A nontumoral cell line (MCF-10A) obtained from a premenopausal woman with fibrocystic disease[20] was chosen as a model of the nonmalignant epithelium. However, the human breast cancer cells MCF-7 were also used to determine whether BCF could influence transformed cells. We were unable to produce primary cultures from the BCF samples we obtained, so could not undertake the experiments on proliferation of the epithelial cells lining the cyst. However, most of the BCF samples studied significantly stimulated [^{3}H]-thymidine incorporation into the nontransformed cells, whereas only half of them stimulated the tumour cells. This result can be attributed to the fact that nontumoral cells are normally more subject to hormone and growth

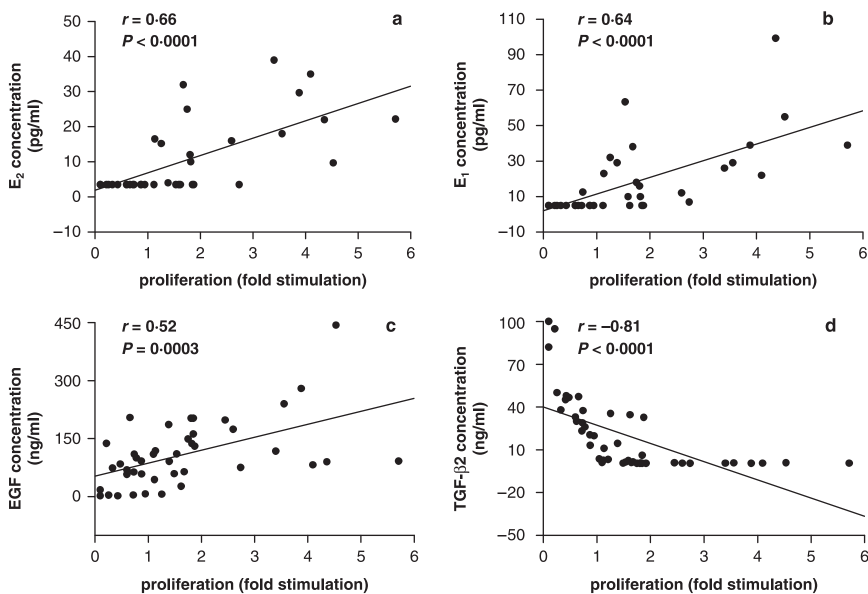


Fig. 2 Correlation between (a) oestradiol (E_2), (b) oestrone (E_1), (c) epidermal growth factor (EGF) and (d) transforming growth factor-β2 (TGF-β2) in breast cyst fluid (BCF) and their mitogenic activity of the in MCF-7 cells. The points are the measurement of individual BCFs and the Spearman correlation coefficient was obtained, as indicated in Patients and Methods, by GraphPadPrism software.

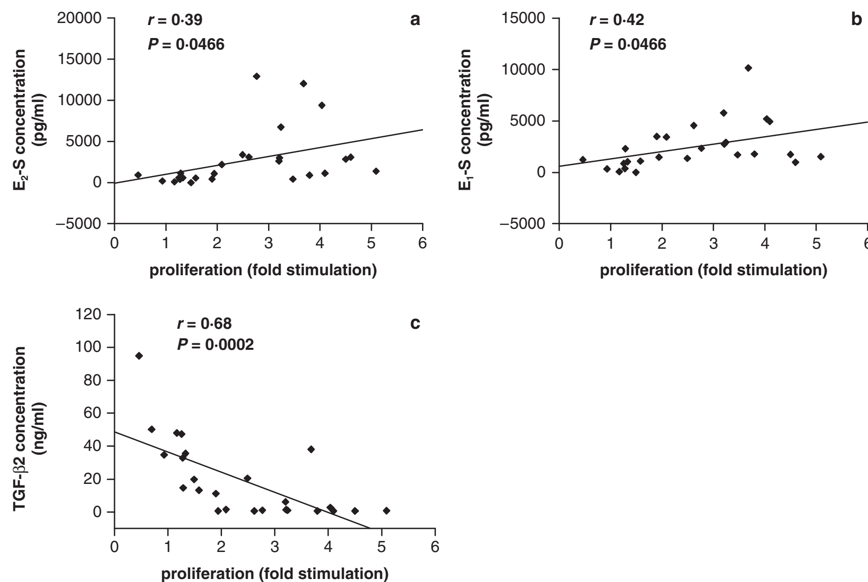


Fig. 3 Correlation between (a) oestradiol-sulfate (E_2-S), (b) oestrone-sulfate (E_1-S) and (c) transforming growth factor-β2 (TGF-β2) concentration levels in breast cyst fluid (BCF) and their proliferative effect on MCF-10A cells. The points are the measurement of individual BCFs and the Spearman correlation coefficient was obtained, as indicated in Patients and Methods, by GraphPadPrism software.

factor control than are tumoral cells.[29] In both cases, type I cysts were found to be more efficient in inducing proliferation than type II cysts, probably because of their higher concentrations of steroids and growth factors. Some authors have reported[6,30] similar E_1 and E_2 concentrations in cysts of both types. However, another group[5] reported a significantly higher E_2 concentration level in type I than in type II cysts, in accordance with our results. Several groups have also reported a higher concentration level of sulfated oestrogen[7,31,32] on type I cysts as compared with type II cysts. In addition, the E_1-S concentration was very high in BCF, much higher than that found in serum.[33] The importance of these concentration levels of sulfated hormones is that breast tissue expresses oestrone sulfatase,[34,35] then allowing the hydrolysation of this hormone and acting as a reservoir for active oestrogens. It has been shown that breast cysts, namely type I cysts, contain large amounts of EGF, which is probably secreted locally by breast epithelium under the control of steroids,[5] and that the risk of breast cancer is strongly related to the intracystic level of EGF.[15] Another study indicates that EGF levels are higher in cysts aspirated from breasts with an associated proliferative pathology, either benign or neoplastic.[36] Thus, the association between EGF concentration level and type I cysts could reflect a proliferative stimulus on the mammary tissue around the cyst. The concentration of TGF-β2 is considerably higher in type II than in type I cysts. Similar results have been reported previously,[28,37] for TGF-β2 as well as for TGF-β1, although the former has levels 10–100 times higher. It should be noted that, contrary to the other growth factors, TGF-β is an inhibitor of proliferation, a promoter of differentiation, and an inductor of apoptosis in the majority of human epithelial cells.[38] The question of whether the human breast cyst is an active endocrine gland, rather than an inactive reservoir in which hormones, growth

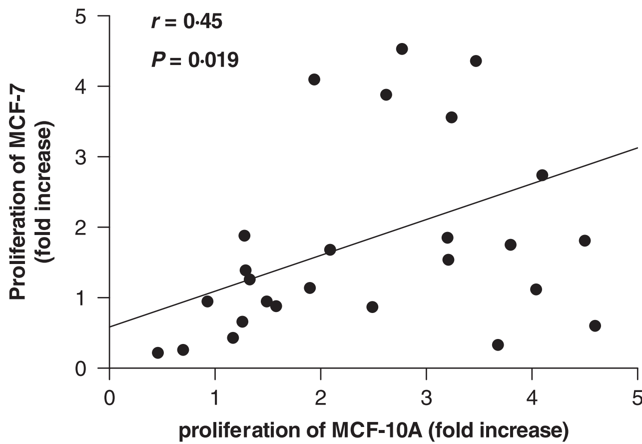


Fig. 4 Correlation between mitogenic activity of breast cyst fluid (BCF) in both cell lines (MCF-7 and MCF-10A). The points are the measurement of individual BCFs and the Spearman correlation coefficient was obtained, as indicated in Patients and Methods, by GraphPadPrism software.

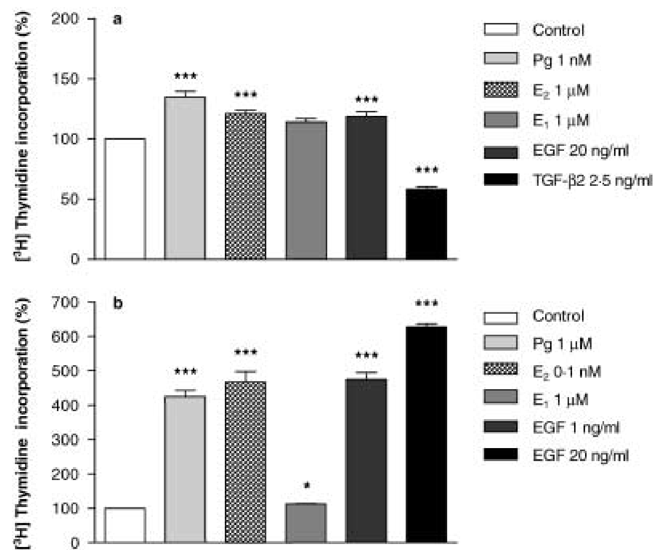


Fig. 6 The minimal concentration of progesterone (Pg), oestradiol (E_2), oestrone (E_1), epidermal growth factor (EGF) and transforming growth factor-β2 (TGF-β2) that exhibited a mitogenic effect on (a) MCF-7 and (b) MCF-10A cells. MCF-7 and MCF-10A cells were incubated as indicated in Patients and Methods. The minimal concentration level that increased or inhibited proliferation was obtained from a dose–response curve for each of the compounds tested. In the case of MCF-10A, two equally stimulatory concentrations of EGF are shown. Statistical analysis was performed by a nonparametric Kruskall–Wallis test, followed by a *posteriori* Dunn's test.

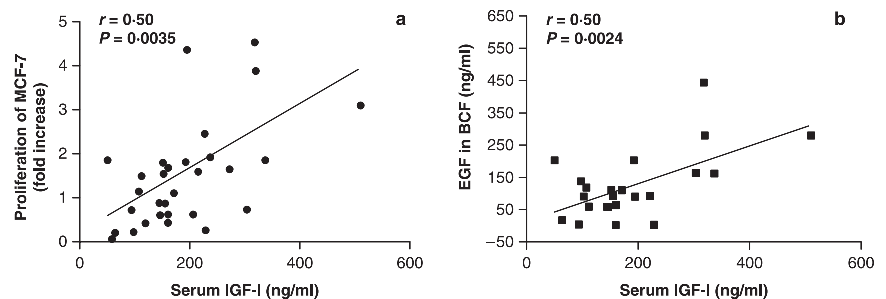
factors and other compounds are accumulated in unusual concentrations, is still unresolved. It is possible that fibrocystic disease may result from an inappropriate histological response to changing hormonal levels.[39]

The different hormones and growth factors described in BCF were incubated with both cell lines to determine their action under the same conditions of incubation as the BCF. MCF-7 cell proliferation is modulated by (but not dependent on) a series of hormones, such as oestrogen and Pg.[40,41] The effect of EGF/TGF-α on cell proliferation has been known for a long time.[42] Although MCF-7 cells were not considered to respond to TGF-β,[43] a growth-inhibiting action is mediated by membrane-bound type II receptors.[44] An indirect action by stimulation of sulfatase activity has also been described in these cells.[45] MCF-10A cells are nontransformed with several characteristics of normal epithelium, rendering it a good model for human normal epithelium. Even if no oestrogen receptor α has been described in these cells,[46] we, as well as others, have found that E_2 was mitogenic at 10 nM.[47] Oestrogen receptor β expression was recently described in these cells.[48] MCF-10A cell development is arrested in the absence of EGF in the culture medium.[49]

Two previous studies on the sulfatase activity of the enzyme converting E_1-S into E_1 analysed the effect of BCF on cell proliferation of MCF-7 cells as a control. In the first study, 7/21 (33%) BCF samples were shown to be inhibitory on MCF-7 cell proliferation,[17] and in the second study, 8/10 were inhibitory.[18] These studies were performed for longer periods than those used in the present study, and these differences could account for the prevalence of a stimulatory effect on MCF-7 cells. To discard any possible nonspecific effect, parallel incubations were performed with charcoal-stripped FCS at the same concentration levels (data not shown). The effect of FCS was inhibitory in both cell lines.

The mitogenic effect of the BCF was correlated with the different hormone and growth factor concentrations measured. When correlating [^{3}H]-thymidine incorporation into MCF-7 cells with hormone and growth factor concentration levels, a significant positive correlation was found for E_1, E_2 and EGF, whereas the correlation with TGF-β2 was negative. Although a very high oestrone-sulfatase activity has been described in MCF-7 cells,[50] no correlation was found between oestrogen-sulfate concentration and [^{3}H]-thymidine incorporation into MCF-7 cells. A possible explanation could be that

Fig. 5 Correlation between mitogenic activity of (a) breast cyst fluid (BCF) in MCF-7 cells or (b) epidermal growth factor (EGF) concentration in BCF and serum IGF-I concentration. The points are the measurement of individual patients and the Spearman correlation coefficient was obtained, as indicated in Patients and Methods, by GraphPadPrism software.



these cells are not very sensitive to oestrogen, showing only 30–40% stimulation with micromolar concentrations of free oestrogen.

For MCF-10A, [^{3}H]-thymidine incorporation correlated positively with E_1-S and E_2-S concentration whereas TGF-β2 concentration correlated negatively. E_2 was found to stimulate nearly fourfold [^{3}H]-thymidine incorporation into MCF-10A cells. Although oestrogen concentration did not correlate with this parameter, oestrogen sulfates correlate significantly. Considering that MCF-10A cells express oestrone sulfatase, these results can be understood considering the huge concentrations of oestrogen sulfates in BCF, nearly three orders of magnitude higher than the corresponding nonconjugated oestrogens. Although, to our knowledge, oestrone sulfatase activity has not been evaluated in MCF-10A cells, it has been reported that this activity is higher in tumours than in regions considered normal.[34] However, this activity is 130–300 times higher than aromatase activity in both tumour and normal breast tissue.[34] Even if it has been reported that in other cell lines sulfatase activity is inhibited by BCF,[17,18] the levels of nonconjugated oestrogens probably attained by incubation of the MCF-10A cells with BCF could justify the correlation of these parameters. MCF-10A was shown to be more sensitive to stimulation with several steroid or growth factors than MCF-7 tumour cells.

There was a notable absence of correlation between [^{3}H]-thymidine incorporation into MCF-10A cells and EGF concentration in BCF. These cells are dependent on the presence of this factor for their growth, with cell cycle arrest on its deprivation.[51] EGF is also an inhibitor of apoptosis in this cell line.[52] Nevertheless, concentration levels of this growth factor as low as 1 ng/ml are able to maximally stimulate [^{3}H]-thymidine incorporation into MCF-10A cells, as shown in this study. As each BCF sample tested had a higher EGF concentration level, maximal cell proliferation by this factor was attained and no further increase could be observed. With respect to TGF-β2 negative correlation, this factor has been described previously as being inhibitory in this cell line.[44,53]

Additionally, a significant correlation was found between [^{3}H]-thymidine incorporation into MCF-10A and into MCF-7 cells. Although this correlation was significant, the low correlation coefficient suggests that while some of the growth factors and hormones may be influencing both cell lines, some of them may be acting differently. Both growth factors could account for the similarities while steroids could account for the differences. Finally, serum IGF-I showed a significant correlation with BCF mitogenic activity in MCF-7. This finding can be understood considering the correlation found between serum IGF-I concentration levels and EGF concentration levels in BCF. Consequently, the serum IGF-I effect could be ascribed to an enhanced EGF concentration level within the cyst. This finding is of considerable importance as we have recently reported[21] that IGF-I concentration levels were significantly higher in sera from patients with type I cysts than in patients with type II cysts. A highly significant decrease in IGFBP-3, the major IGFBP, was found in patients with type I cysts with respect to healthy women, whereas no significant difference was evident between the different cyst types. The ratio IGF-I/IGFBP-3, an estimate of biologically active IGF-I, was significantly higher in patients with type I cysts than in both type II patients and healthy women. IGFBP-1 levels were significantly lower in patients with type I cysts than in controls or type II cysts. IGF-I/IGFBP-1 was significantly higher in patients with type I cysts than in type II bearers or in healthy women. Oestrogen levels correlated with IGF-I in both patients and controls.

Could EGF concentration in BCF be the missing link between serum IGF-I concentration levels and the increased cancer risk described for type I cyst bearers?

The results presented here suggest that BCF is able to stimulate [^{3}H]-thymidine incorporation into both human breast cancer MCF-7 cells and human breast noncancer MCF-10A cells. This stimulation correlates with E_1, E_2 and EGF in MCF-7 and E_1-S and E_2-S in MCF-10A cells and could consequently be mediated by a combination of them. In the latter cells, EGF concentration in BCF was always higher than the concentration needed for maximal stimulation, and thus it does not correlate with [^{3}H]-thymidine incorporation. In both cases TGF-β2 appears to contribute to inhibiting this parameter. The presence of these hormones and growth factors in correlation with mitogenic activity of the BCF could eventually explain, at least in part, the increased breast cancer risk in cyst-bearing women.

Acknowledgements

This work was supported by the 'Consejo Nacional de Investigaciones Científicas y Técnicas' (CONICET), and the 'Agencia Nacional de Promoción Científica y Tecnológica' (FONCYT), Argentina.

References

1 Haagensen, C.D., Bodian, C. & Haagensen, E. (1981) *Breast Carcinoma: Risk and Detection*. W.B. Saunders, Philadelphia.

2 Cheung, K.L. & Lam, T.P. (2005) Approach to a lump in the breast: a regional perspective. *Asian Journal of Surgery*, **28**, 65–70.

3 Boccardo, F., Lunardi, G., Guglielmini, P., Parodi, M., Murialdo, R., Schettini, G. & Rubagotti, A. (2004) Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. *European Journal of Cancer*, **40**, 84–89.

4 Dixon, J.M., McDonald, C., Elton, R.A. & Miller, W.R. (1999) Risk of breast cancer in women with palpable breast cysts: a prospective study. Edinburgh Breast Group. *Lancet*, **353**, 1742–1745.

5 Boccardo, F., Torrisi, R., Zanardi, S., Valenti, G., Pensa, F., de Franchis, V., Barreca, A., Ferraro, P. & Minuto, F. (1991) EGF in breast cyst fluid: relationships with intracystic androgens, estradiol and progesterone. *International Journal of Cancer*, **47**, 523–526.

6 Belanger, A., Caron, S., Labrie, F., Naldoni, C., Dogliotti, L. & Angeli, A. (1990) Levels of eighteen non-conjugated and conjugated steroids in human breast cyst fluid: relationships with cyst type. *European Journal of Cancer*, **26**, 277–281.

7 Bradlow, H.L., Rosenfeld, R.S., Kream, J., Fleisher, M., O'Connor, J. & Schwartz, M.K. (1981) Steroid hormone accumulation in human breast cyst fluid. *Cancer Research*, **41**, 105–107.

8 Lai, L.C., Ghatei, M.A., Takahashi, K., Patel, K.V., Schrey, M.P., Ghilchik, M.W., Bloom, S.R. & James, V.H. (1990) Mitogenic peptides in breast cyst fluid: relationship with intracystic electrolyte ratios. *International Journal of Cancer*, **46**, 1014–1016.

9 Zanardi, S., Pensa, F., Torrisi, R., de Franchis, V., Barreca, A., Minuto, F. & Boccardo, F. (1996) Presence and distribution of growth factors in breast cyst fluid. *Annals of the New York Academy of Sciences*, **784**, 542–549.

10 Ness, J.C., Sedghinasab, M., Moe, R.E. & Tapper, D. (1993) Identification of multiple proliferative growth factors in breast cyst fluid. *American Journal of Surgery*, **166**, 237–243.

11 Li, J.J., Weroha, S.J., Lingle, W.L., Papa, D., Salisbury, J.L. & Li, S.A. (2004) Estrogen mediates Aurora-A overexpression, centrosome amplification, chromosomal instability, and breast cancer in female ACI rats. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 18123–18128.

12 Liehr, J.G. (2000) Is estradiol a genotoxic mutagenic carcinogen? *Endocrine Reviews*, **21**, 40–54.

13 Carboni, J.M., Lee, A.V., Hadsell, D.L., Rowley, B.R., Lee, F.Y., Bol, D.K., Camuso, A.E., Gottardis, M., Greer, A.F., Ho, C.P., Hurlburt, W., Li, A., Saulnier, M., Velaparthi, U., Wang, C., Wen, M.L., Westhouse, R.A., Wittman, M., Zimmermann, K., Rupnow, B.A. & Wong, T.W. (2005) Tumor development by transgenic expression of a constitutively active insulin-like growth factor I receptor. *Cancer Research*, **65**, 3781–3787.

14 Peyrat, J.P., Bonneterre, J., Hecquet, B., Vennin, P., Louchez, M.M., Fournier, C., Lefebvre, J. & Demaille, A. (1993) Plasma insulin-like growth factor-1 (IGF-1) concentrations in human breast cancer. *European Journal of Cancer*, **29A**, 492–497.

15 Boccardo, F., Lunardi, G.L., Petti, A.R. & Rubagotti, A. (2003) Enterolactone in breast cyst fluid: correlation with EGF and breast cancer risk. *Breast Cancer Research and Treatment*, **79**, 17–23.

16 Tapper, D., Gajdusek, C., Moe, R. & Ness, J. (1990) Identification of a unique biological tumor marker in human breast cyst fluid and breast cancer tissue. *American Journal of Surgery*, **159**, 473–478.

17 Erbas, H., Lennard, T.W. & Lai, L.C. (1996) Effect of breast cyst fluid on oestrone sulphatase activity in MCF-7 breast cancer cells. *Anticancer Research*, **16**, 833–836.

18 Elsadig, R.E., Reimann, K., Yip, C.H. & Lai, L.C. (2001) Inhibition of oestrone sulphatase activity in the MDA-MB-231 breast cancer cell line by breast cyst fluid from Malaysian women. *Anticancer Research*, **21**, 2693–2696.

19 Soule, H.D., Vazguez, J., Long, A., Albert, S. & Brennan, M. (1973) A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute*, **51**, 1409–1416.

20 Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D. Jr, Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F. & Brooks, S.C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Research*, **50**, 6075–6086.

21 Enriori, P.J., Fischer, C.R., Gori, J.R., Etkin, A.E., Calandra, R.S. & Luthy, I.A. (2003) Augmented serum levels of the IGF-I/IGF-binding protein-3 ratio in pre-menopausal patients with type I breast cysts. *European Journal of Endocrinology*, **148**, 177–184.

22 Leis, H.P. Jr (1993) Gross breast cysts: significance of apocrine type, identification by cyst fluid analysis, and management. *Breast Disease*, **6**, 185–194.

23 Berthois, Y., Katzenellenbogen, J.A. & Katzenellenbogen, B.S. (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 2496–2500.

24 Raju, U., Levitz, M., Jacobowitz, G., Fleisher, M., Bencsath, F.A., Field, F.H., Breed, C.N., Naganuma, H. & Bradlow, H.L. (1987) Quantification of the sulfates of 16 alpha-hydroxy androgens that are possible precursors of estriol-3-sulfate in human breast cyst fluid. *Steroids*, **50**, 559–574.

25 Shackleton, C.H., Phillips, A., Chang, T. & Li, Y. (1997) Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstanediols. *Steroids*, **62**, 379–387.

26 Molinolo, A.A., Lanari, C., Charreau, E.H., Sanjuan, N. & Pasqualini, C.D. (1987) Mouse mammary tumors induced by medroxyprogesterone acetate: immunohistochemistry and hormonal receptors. *Journal of the National Cancer Institute*, **79**, 1341–1350.

27 Boccardo, F., Marenghi, C., Ghione, G., Pepe, A., Parodi, S. & Rubagotti, A. (2001) Intracystic epidermal growth factor level is predictive of breast-cancer risk in women with gross cystic disease of the breast. *International Journal of Cancer*, **95**, 260–265.

28 Lai, L.C., Siraj, A.K., Erbas, H. & Lennard, T.W. (1994) Transforming growth factor alpha, beta 1 and beta 2 in breast cyst fluid. *Anticancer Research*, **14**, 2805–2809.

29 Basolo, F., Elliott, J., Tait, L., Chen, X.Q., Maloney, T., Russo, I.H., Pauley, R., Momiki, S., Caamano, J. & Klein-Szanto, A.J. (1991) Transformation of human breast epithelial cells by c-Ha-ras oncogene. *Molecular Carcinogenesis*, **4**, 25–35.

30 Lai, L.C., Dunkley, S.A., Reed, M.J., Ghilchik, M.W., Shaikh, N.A. & James, V.H. (1990) Epidermal growth factor and oestradiol in human breast cyst fluid. *European Journal of Cancer*, **26**, 481–484.

31 Orlandi, F., Caraci, P., Puligheddu, B., Torta, M., Dogliotti, L., Del Monte, I. & Angeli, A. (1990) Estrone-3-sulfate in human breast cyst fluid. Relationship to cation-related cyst subpopulations. *Annals of the New York Academy of Sciences*, **586**, 79–82.

32 Miller, W.R., Scott, W.N., Kelly, R.W. & Hawkins, R.A. (1990) Steroid hormones in breast cyst fluids. *Annals of the New York Academy of Sciences*, **586**, 60–69.

33 Raju, U., Sepkovic, D.W., Miller, W.R., Dixon, J.M., Bradlow, H.L. & Levitz, M. (2000) Estrone and estradiol metabolism in vivo in human breast cysts. *Steroids*, **65**, 883–888.

34 Chetrite, G.S., Cortes-Prieto, J., Philippe, J.C., Wright, F. & Pasqualini, J.R. (2000) Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. *Journal of Steroid Biochemistry and Molecular Biology*, **72**, 23–27.

35 Pasqualini, J.R. & Chetrite, G.S. (1999) Estrone sulfatase versus estrone sulfotransferase in human breast cancer: potential clinical applications. *Journal of Steroid Biochemistry and Molecular Biology*, **69**, 287–292.

36 Torrisi, R., Zanardi, S., Pensa, F., Valenti, G., de Franchis, V., Nicolo, G., Barreca, A., Minuto, F. & Boccardo, F. (1995) Epidermal growth factor content of breast cyst fluids from women with breast cancer or proliferative disease of the breast. *Breast Cancer Research and Treatment*, **33**, 219–224.

37 Erbas, H., Lennard, T.W. & Lai, L.C. (1999) Latent forms of transforming growth factor-beta 2 (TGF-beta 2) in breast cyst fluid. *Anticancer Research*, **19**, 3077–3082.

38 Rosfjord, E.C. & Dickson, R.B. (1999) Growth factors, apoptosis, and survival of mammary epithelial cells. *Journal of Mammary Gland Biology and Neoplasia*, **4**, 229–237.

39 Tsung, J.S., Wang, T.Y., Wang, S.M. & Yang, P.S. (2005) Cytological and biochemical studies of breast cyst fluid. *Breast*, **14**, 37–41.

40 Lippman, M., Bolan, G. & Huff, K. (1976) The effects of glucocorticoids and progesterone on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Research*, **36**, 4602–4609.

41 Lippman, M., Bolan, G. & Huff, K. (1976) The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Research*, **36**, 4595–4601.

42 Dickson, R.B., Huff, K.K., Spencer, E.M. & Lippman, M.E. (1986) Induction of epidermal growth factor-related polypeptides by 17 beta-estradiol in MCF-7 human breast cancer cells. *Endocrinology*, **118**, 138–142.

43 Brattain, M.G., Ko, Y., Banerji, S.S., Wu, G. & Willson, J.K. (1996) Defects of TGF-beta receptor signaling in mammary cell tumorigenesis. *Journal of Mammary Gland Biology and Neoplasia*, **1**, 365–372.

44 Lynch, M.A., Petrel, T.A., Song, H., Knobloch, T.J., Casto, B.C., Ramljak, D., Anderson, L.M., DeGroff, V., Stoner, G.D., Brueggemeier, R.W. & Weghorst, C.M. (2001) Responsiveness to transforming growth factor-beta (TGF-beta)-mediated growth inhibition is a function of membrane-bound TGF-beta type II receptor in human breast cancer cells. *Gene Expression*, **9**, 157–171.

45 Erbas, H. & Lai, L.C. (2000) Effect of transforming growth factor beta 2 on oestrogen metabolism in the MCF-7 breast cancer cell line. *Journal of Clinical Pathology*, **53**, 322–324.

46 Pilat, M.J., Christman, J.K. & Brooks, S.C. (1996) Characterization of the estrogen receptor transfected MCF10A breast cell line 139B6. *Breast Cancer Research and Treatment*, **37**, 253–266.

47 Raju, U., Katz, J. & Levitz, M. (1997) Effect of bile acids and estradiol on thymidine incorporation into DNA in MCF-7 and MCF-10A breast cell lines. *Steroids*, **62**, 643–646.

48 Liu, S. & Lin, Y.C. (2004) Transformation of MCF-10A human breast epithelial cells by zeranol and estradiol-17beta. *Breast Journal*, **10**, 514–521.

49 Blagosklonny, M.V., Bishop, P.C., Robey, R., Fojo, T. & Bates, S.E. (2000) Loss of cell cycle control allows selective microtubule-active drug-induced Bcl-2 phosphorylation and cytotoxicity in autonomous cancer cells. *Cancer Research*, **60**, 3425–3428.

50 Pasqualini, J.R. & Chetrite, G.S. (2005) Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer. *Journal of Steroid Biochemistry and Molecular Biology*, **93**, 221–236.

51 Chou, J.L., Fan, Z., DeBlasio, T., Koff, A., Rosen, N. & Mendelsohn, J. (1999) Constitutive overexpression of cyclin D1 in human breast epithelial cells does not prevent G1 arrest induced by deprivation of epidermal growth factor. *Breast Cancer Research and Treatment*, **55**, 267–283.

52 Davis, J.W., Melendez, K., Salas, V.M., Lauer, F.T. & Burchiel, S.W. (2000) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits growth factor withdrawal-induced apoptosis in the human mammary epithelial cell line, MCF-10A. *Carcinogenesis*, **21**, 881–886.

53 McCaig, C., Fowler, C.A., Laurence, N.J., Lai, T., Savage, P.B., Holly, J.M. & Perks, C.M. (2002) Differential interactions between IGFBP-3 and transforming growth factor-beta (TGF-beta) in normal vs. cancerous breast epithelial cells. *British Journal of Cancer*, **86**, 1963–1969.