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Hypersensitivity and Growth Adaptation of Oestrogen-deprived MCF-7 Human Breast Cancer Cells

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Abstract. *Background: Efficacy of endocrine therapy is compromised when human breast cancer cells circumvent imposed growth inhibition. The model of long-term oestrogen-deprived MCF-7 human breast cancer cells has suggested the mechanism results from hypersensitivity to low levels of residual oestrogen. Materials and Methods: MCF-7 cells were maintained for up to 30 weeks in phenol-red-free medium and charcoal-stripped serum with 10^{-8} M 17β-oestradiol and 10 μg/ml insulin (stock 1), 10^{-8} M 17β-oestradiol (stock 2), 10 μg/ml insulin (stock 3) or no addition (stock 4). Results: Loss of growth response to oestrogen was observed only in stock 4 cells. Long-term maintenance with insulin in the absence of oestradiol (stock 3) resulted in raised oestrogen receptor-alpha (ERα) levels (measured by western immunoblotting) and development of hypersensitivity (assayed by oestrogen-responsive reporter gene induction and dose response to oestradiol for proliferation under serum-free conditions), but with no loss of growth response to oestrogen. Conclusion: Hypersensitivity can develop without any growth adaptation and therefore is not a prerequisite for loss of growth response in MCF-7 cells.*

The ability to reduce the growth of oestrogen-responsive breast tumours by inhibiting oestrogen action (tamoxifen, fulvestrant) or oestrogen synthesis (aromatase inhibitors) has allowed for targeted endocrine therapy for breast cancer. However, long-term efficacy of this therapy is limited by the ability of human breast cancer cells to circumvent the imposed growth inhibition (1). Over the years, cell culture models have been developed in which this growth adaptation can be modelled *in vitro* through growing human breast cancer cell lines long-term in culture medium depleted of oestrogen. Under these conditions, cells which are initially dependent on oestrogen for their proliferation can adapt over time to grow at the same rate in the absence of oestrogen as they grew originally only in the presence of oestrogen, and thus become independent of oestrogen for their growth (2-9). In these models, there is no loss of oestrogen receptor (ER) number or function but rather ER levels increase. Oestrogen-regulated genes do not become constitutively activated, but remain expressed at low levels following oestrogen deprivation and their expression can be increased when oestrogen is supplemented, implying continued oestrogen sensitivity despite the adaptation to oestrogen-unresponsive growth (2-5, 9).

MCF-7 human breast cancer cells comprise the most extensively used model and one mechanism for growth adaptation in these cells has been suggested to result from increased ER levels causing cells to become hypersensitive to low levels of oestradiol remaining in oestrogen-depleted medium (5, 10). Whilst long-term oestrogen-deprived cells do demonstrate enhanced levels of oestrogen-inducible gene expression (5) and proliferation at lower concentrations of oestradiol when grown in serum-free medium (11), it remains unclear why this growth cannot be fully-inhibited by anti-oestrogens (tamoxifen or fulvestrant) (8, 9, 12), if this were the mechanism for the up-regulated growth. However, another equally plausible mechanism for this growth adaptation could be that the cells switch to dependence on one or more other non-ER-mediated growth signalling pathways. Many studies have shown this growth adaptation to be paralleled by alterations to growth factor signalling pathways and functionality has been demonstrated through altered sensitivity to specific inhibitors. In particular, increased signalling has been described through mitogen-activated protein kinase (MAPK) (13-16), phosphoinositide-3-kinase (PI3K) (17-19), mTOR (20, 21), the protein tyrosine kinase Src (22) and platelet-derived growth factor receptor (PDGFR)/ABL (23) pathways which offers potential therapeutic strategies using specific small-molecule inhibitors. Translational research, therefore, needs to clarify which of these two mechanisms are causal in the up-regulation of growth under oestrogen-deprived conditions in

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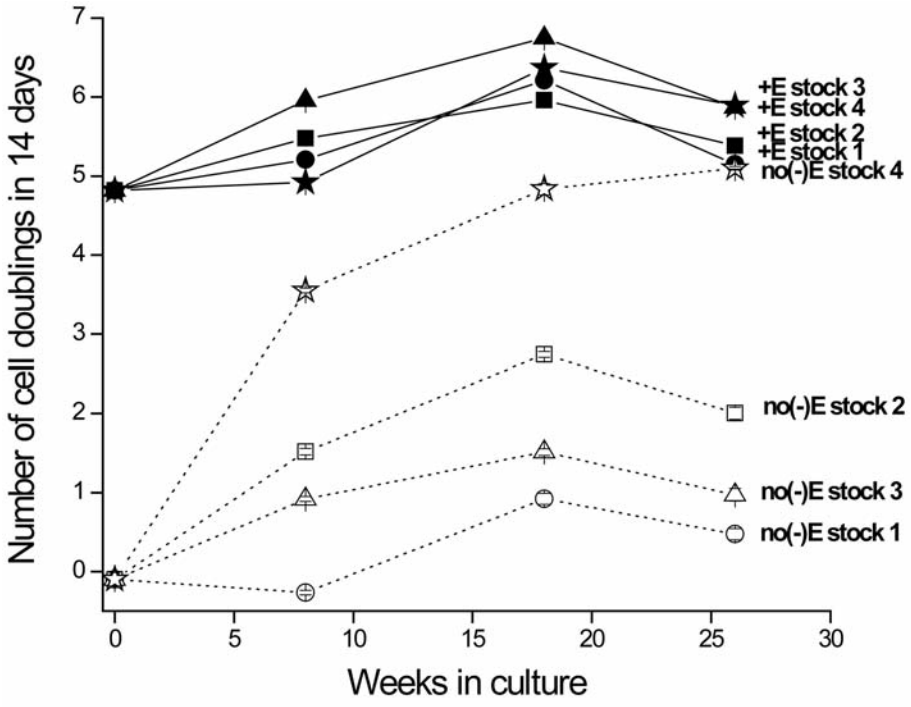

Figure 1. *Time course of the changes in proliferative response to 17β-oestradiol following long-term maintenance of MCF-7 human breast cancer cells with and without oestradiol or insulin. Cells were maintained for 26 weeks in phenol-red-free Dulbecco's modified Eagle's medium (DMEM) / 5% dextran charcoal-stripped foetal calf serum (DCFCS) with 10^{-8} M 17β-oestradiol and 10 μg/ml insulin (stock 1), with 10^{-8} M 17β-oestradiol only (stock 2), with 10 μg/ml insulin only (stock 3), or with no addition (stock 4). At increasing time points, proliferation of the cells was assayed as the number of cell doublings over 14 days in the same medium with no addition (no(-)E) or with 10^{-8} M 17β-oestradiol (+E). Error bars are the standard error of all nine values of triplicate estimates of cell numbers after 0 and 14 days, where no bar is seen, the error was too small for visual display.*

order for appropriate efforts to be directed towards the relevant therapeutic targets (10, 24).

The model of oestrogen deprivation of MCF-7 cells, however, involves not only non-addition of oestradiol but also removal of insulin and transfer to charcoal-stripped serum, which poses the question as to which of the manipulations are responsible for the growth adaptation observed (25). Despite the varied conditions used in different laboratories for the maintenance of MCF-7 cell stocks, the MCF-7 cell line was originally selected in the presence of insulin (26) and MCF-7 cell proliferation is increased by insulin, as well as by oestradiol (27). Whilst the purpose of stripping the serum with charcoal is to remove endogenous oestrogens (28), the procedure may also remove some other growth factors to which the cells are responsive. For these reasons, the effects of maintaining MCF-7 cells long-term in oestrogen-depleted medium with charcoal-stripped serum either alone or supplemented alone with oestradiol, alone with insulin, or with oestradiol and insulin together have been investigated. During the course of these studies, it has been have found that long-term maintenance with insulin, but no oestrogen, results in raised levels of ER and hypersensitivity to oestrogen but with no alteration to the growth response.

Materials and Methods

Culture of stock oestrogen-maintained MCF-7 cells. MCF-7 human breast cancer cells were provided from the laboratory of McGrath by Osborne in 1987 at passage number 390 (29). Stock MCF-7 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 5% (v/v) foetal calf serum (FCS) (Lonza, Slough, UK), 10 μg/ml insulin (Sigma, Poole, UK) and 10^{-8} M 17β-oestradiol (Steraloids, Croydon, UK) in a humidified atmosphere of 10% CO_2 in air at 37°C.

Long-term oestrogen deprivation of MCF-7 cells. Cells were maintained long-term in phenol-red-free DMEM containing 5% (v/v) dextran charcoal-stripped FCS (DCFCS) as described previously (9) with no further addition, with 10^{-8} M 17β-oestradiol, with 10 μg/ml insulin or with both 10^{-8} M 17β-oestradiol and 10 μg/ml insulin.

Cell proliferation experiments. Cells were added to the required volume of phenol-red-free DMEM containing 5% DCFCS at a density of 0.2×10^5 cells/ml and plated in monolayer in 0.5 ml aliquots into 24-well plastic tissue culture dishes (Nunc, Roskilde, Denmark). After 24 h, the medium was changed to phenol-red-free DMEM supplemented with 5% DCFCS with or without 10^{-8} M 17β-oestradiol or 10 μg/ml insuli, as required. Culture media were changed routinely every 3-4 days in all experiments. Cell counts were performed by counting released nuclei on a model ZBI Coulter

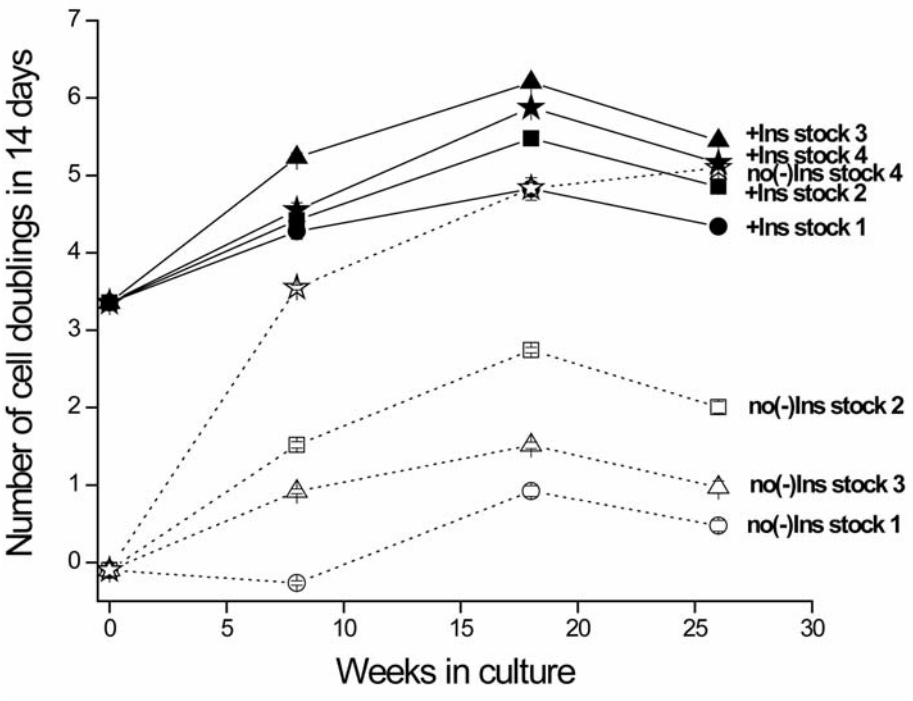

Figure 2. *Time course of the changes in proliferative response to insulin following long-term maintenance of MCF-7 human breast cancer cells with and without oestradiol or insulin. Cells were maintained for 26 weeks in phenol-red-free Dulbecco's modified Eagle's medium (DMEM) / 5% dextran charcoal-stripped foetal calf serum (DCFCS) with 10^{-8} M 17β-oestradiol and 10 μg/ml insulin (stock 1), with 10^{-8} M 17β-oestradiol only (stock 2), with 10 μg/ml insulin only (stock 3), or with no addition (stock 4). At increasing time points, proliferation of the cells was assayed as number of cell doublings over 14 days in the same medium with no addition (no(-)Ins) or with 10 μg/ml insulin (+Ins). Error bars are the standard error of all nine values of triplicate estimates of cell numbers after 0 and 14 days, where no bar is seen, the error was too small for visual display.*

Counter, as described previously (9). Doubling time of the cells was calculated from cell numbers at day 0 and the final time point as a function of the slope, m ($\log_{10}2$/m). The number of doublings was calculated as the mean±SE of all nine values for the triplicate estimates of cell numbers at day 0 and the final time point.

Assay of hypersensitivity to oestrogen for proliferation was carried out as above but under serum-free conditions. Cells were plated as above but using 0.25% DCFCS. After 24 h, the cells were washed free of serum using phenol-red-free DMEM, and then grown in phenol-red-free DMEM supplemented with 15 mM HEPES buffer, 2 μg/ml transferrin, 0.75 μg/ml fibronectin and 0.25% bovine serum albumin [resin-treated as described in (30)].

Assay of transiently-transfected oestrogen-responsive luciferase (ERE-LUC) reporter gene in MCF-7 cells. The oestrogen-inducible ERE-LUC vector consisted of the oestrogen response element (ERE)-containing nucleotide sequence (5'-CTAGAAAGTCAGG TCACAGTGACCTGATCAAT-3') cloned into the multiple cloning site of the pGL3 promoter vector containing the coding sequence for firefly luciferase (Promega, Southampton, UK). The control constitutive renilla luciferase vector pRL-TK was purchased from Promega. Dual luciferase assays were carried out as described previously (9).

Western immunoblotting. Cells were grown and lysates prepared as described previously (9) but using lysis buffer [50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.3% (v/v) Triton-X-100, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF 0.3 mM), leupeptin (10 μg/ml) and aprotonin (2 μg/ml)]. Lysates were run on 10% polyacrylamide-sodium dodecyl sulphate (SDS) gels (100 μg protein per track), proteins transferred onto Hybond-ECL membranes (GE-Healthcare, Amersham, UK) and immunoblotted with antibodies ERα Ab-1D5 (Dako, Copenhagen, Denmark) or β-actin Ab-6276 (AbCam, Cambridge, UK) as published elsewhere (9).

Results

Experimental model. These MCF-7 human breast cancer cells (29) are dependent on oestrogen for growth in that when grown in phenol-red-free medium with 5% DCFCS their proliferation is low (less than one doubling in 14 days) unless oestrogen is added to the medium (when they can go through 5-6 doublings over 14 days) (31) (Figure 1). However, after long-term oestrogen deprivation in phenol-red-free medium with 5% DCFCS, the cells gradually increase their proliferation rate, in such way that eventually after between 20-25 weeks, their growth rate rises to equal that of the previous growth rate in the presence of oestrogen alone and further addition of oestradiol does not alter the growth rate. At this point, the cells are described as being independent of oestrogen for growth (9) (Figure 1).

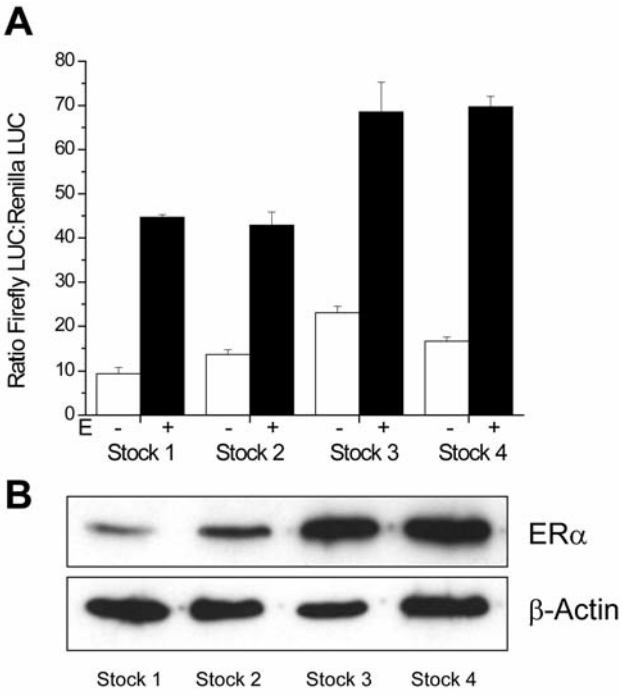

Figure 3. *Regulation of a transiently-transfected oestrogen-inducible luciferase (ERE-LUC) reporter gene (A) and levels of oestrogen receptor alpha (ERα) protein (B) following long-term maintenance of MCF-7 human breast cancer cells with and without oestradiol or insulin. Cells were maintained for 28 weeks in phenol-red-free Dulbecco's modified Eagle's medium (DMEM)/5% dextran charcoal-stripped foetal calf serum (DCFCS) with 10^{-8} M 17β-oestradiol and 10 μg/ml insulin (stock 1), with 10^{-8} M 17β-oestradiol only (stock 2), with 10 μg/ml insulin only (stock 3), or with no addition (stock 4). A: Induction of ERE-LUC was assayed in the same medium with no addition (-E) or with 10^{-8} M 17β-oestradiol (+E) and results presented as the ratio of firefly luciferase:renilla luciferase. Error bars are the standard error of triplicate wells. B: ERα was measured by western immunoblotting.*

Effect of oestradiol and insulin on preservation of the growth response. Time course of the proliferative responses to oestradiol (Figure 1) and insulin (Figure 2) are shown for MCF-7 cells after up to 26 weeks of maintenance in phenol-red-free DMEM/5% DCFCS with both 10^{-8} M 17β-oestradiol and 10 μg/ml insulin added (stock 1), with 10^{-8} M 17β-oestradiol only (stock 2), with 10 μg/ml insulin only (stock 3) or with no addition (stock 4). In this way, the cells were maintained in parallel in the same batch of DCFCS and the effects of oestradiol or insulin could be monitored alone and combined. Long-term maintenance with both oestradiol and insulin (stock 1) allowed for a preservation of the growth response to either oestradiol (Figure 1) or insulin (Figure 2). Long-term maintenance without oestradiol or insulin (stock 4) resulted in a loss of growth response to either oestradiol (Figure 1) or insulin (Figure 2). The growth rates with oestradiol (Figure 1) or insulin (Figure 2) were not affected, but the basal growth increased in such way that addition of oestradiol or insulin had no further effect. Long-term maintenance with either oestradiol-alone (stock 2) or insulin-alone (stock 3) was sufficient to allow for a preservation of the growth response to both oestradiol (Figure 1) and insulin (Figure 2), without any increase in the basal growth without oestradiol or insulin.

Regulation of ERE-LUC reporter gene expression and levels of ERα protein. After 28 weeks, dual luciferase assays showed that oestrogen-regulated ERE-LUC reporter gene expression was increased by 10^{-8} M oestradiol in all four stock lines but an enhanced level of oestrogen-stimulated expression was observed in stocks 3 and 4 compared to stocks 1 and 2 (Figure 3A). Western immunoblotting of cell lysates after 28 weeks showed that the increased oestrogen sensitivity of gene expression in stocks 3 and 4 correlated with increased ERα levels in these stock cells compared with ERα levels in stocks 1 and 2.

Hypersensitivity to oestrogen for proliferation. After 30 weeks, dose response curves of effects of oestradiol on proliferation rate were performed on each stock line in serum-free medium (Figure 4). Whilst the oestradiol concentrations for half-maximal proliferation (EC_{50}) were 10^{-11} M for both cells of stocks 1 and 2, a hypersensitivity to oestradiol for growth was observed in cells of stocks 3 and 4, reducing the EC_{50} to 2×10^{-12} M for stock 3 and to 4×10^{-13} M for stock 4 (Figure 4). Stocks 1 and 2 had a significant effect on growth at 10^{-12} M oestradiol but not at 10^{-13} M oestradiol. However, stock 3 had a significant effect on growth at 10^{-13} M oestradiol and stock 4 even at 10^{-14} M oestradiol.

Discussion

The results presented here demonstrate that the growth adaptation to oestrogen-unresponsive growth reported in the model of long-term oestrogen deprivation of MCF-7 cells (2-9) can be prevented not only by supplementation with oestradiol but also with insulin. The increase in basal growth is not a function of transfer to the charcoal-stripped serum because supplementation with oestradiol preserved the growth response. However, the fact that the growth response was also preserved by long-term maintenance with insulin in the absence of any added oestradiol demonstrates that the mechanism of loss of growth response is not simply a function of oestrogen deprivation. The interdependence of oestradiol and insulin in growth regulation of these cells was demonstrated by the fact that growth response to oestradiol was preserved by long-term maintenance with insulin, and *vice versa*.

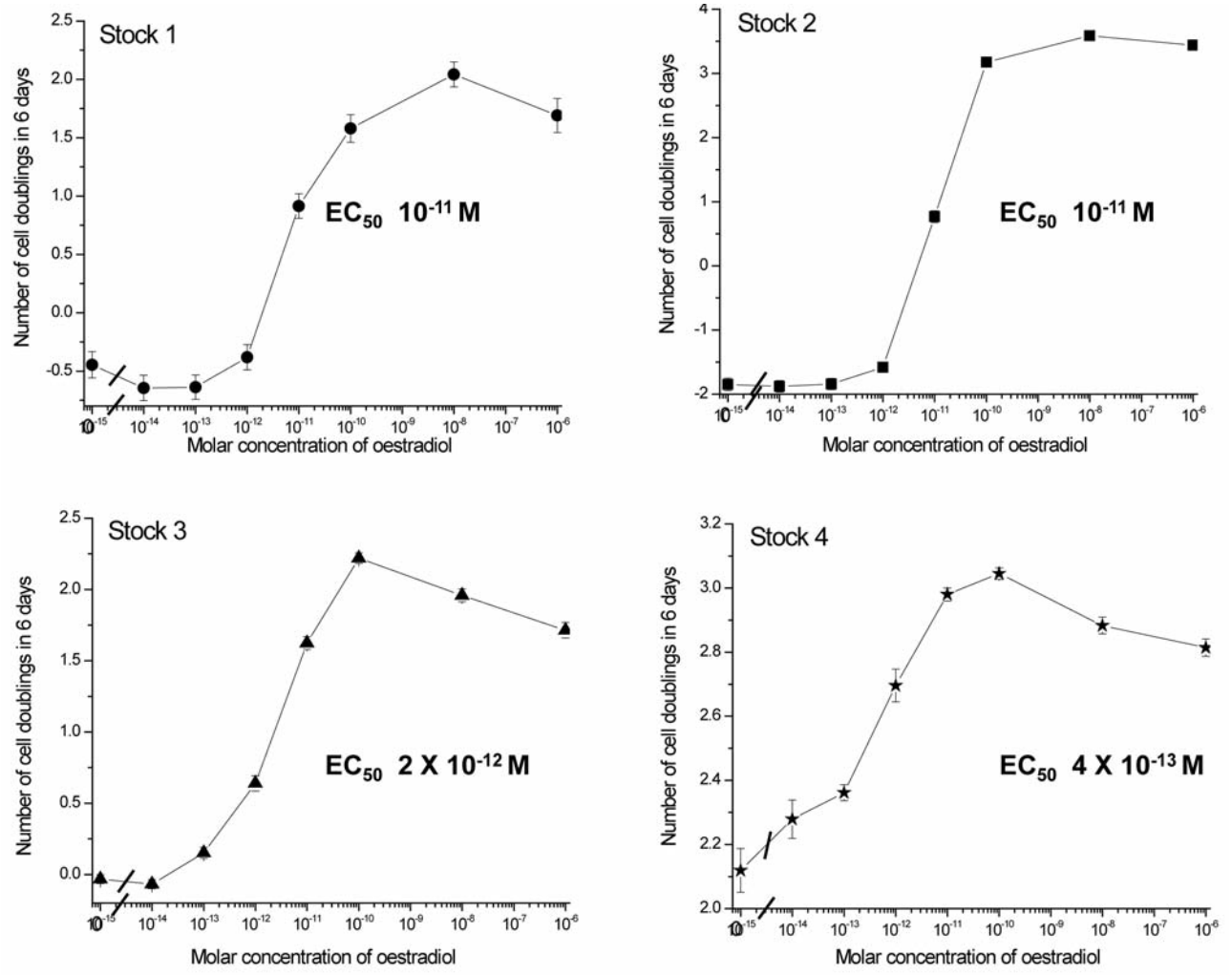

Figure 4. *Development of hypersensitivity to oestradiol for proliferation in serum-free conditions following long-term maintenance of MCF-7 human breast cancer cells with and without oestradiol or insulin. Cells were maintained for 30 weeks in phenol-red-free Dulbecco's modified Eagle's medium (DMEM) / 5% dextran charcoal-stripped foetal calf serum (DCFCS) with 10^{-8} M 17β-oestradiol and 10 μg/ml insulin (stock 1), with 10^{-8} M 17β-oestradiol only (stock 2), with 10 μg/ml insulin only (stock 3), or with no addition (stock 4). Proliferation was assayed under serum-free conditions and results are presented as number of cell doublings in 6 days at the indicated concentrations of 17β-oestradiol. Error bars are the standard error of all nine values of triplicate estimates of cell numbers after 0 and 6 days, where no bar is seen, the error was too small for visual display.*

Interestingly, it was noted that long-term maintenance with insulin but no added oestradiol gave rise to hypersensitivity to oestradiol in assays for both gene expression and for proliferation under serum-free conditions, but the basal unstimulated growth rate remained low and the growth responses to both oestrogen and insulin were preserved. This implies that hypersensitivity can develop under conditions where there is no growth adaptation and therefore this alone is not sufficient to drive the loss of growth response. The hypersensitivity, which was observed in both stocks 3 and 4 but not in stocks 1 and 2, did correlate, however, with increased ERα levels in the cells. Since ERα is known to be down-regulated by oestradiol (32), it could be expected that in the presence of oestradiol (stocks 1 and 2) ERα levels would be lower than in cells in the stocks maintained without oestradiol (stocks 3 and 4). There was, therefore, a clear correlation between hypersensitivity and raised levels of ERα, but hypersensitivity did not correlate with loss of growth response.

These results have implications for understanding mechanisms underlying development of endocrine resistance in human breast cancer. Firstly, since insulin preserved the growth response to oestradiol, loss of endocrine-responsive growth should be considered a function of more than oestrogen deprivation-alone. The insulin concentrations used in this study would not be reached in the human body and can be considered

only as the necessary functional concentrations for this model system. However, *in vivo*, it would be unlikely that growth factor activity would ever be lacking completely and it may be that combinations of low levels of growth factors might work together to preserve growth response, maybe even in combination with low levels of physiological oestrogens. Recent work has shown that low levels of oestradiol (50 pM) can indeed preserve against loss of the growth response in MCF-7 cells but reduction to 1 pM was too low a concentration for preservation of response (33). It remains a possibility that even 1 pM oestradiol might act to preserve the growth response if added in combination with low levels of growth factors. Furthermore, given that hundreds of environmental chemicals with oestrogenic activity have now been measured in the human breast (34), the oestrogenic environment of the human breast can no longer be assessed simply from measuring concentrations of physiological oestrogens. Measurements of environmental oestrogens in different breast tissue samples have shown considerable variation (34), demonstrating the potential for differing oestrogenic environments to differentially-influence loss of growth response when using endocrine therapy (35). Secondly, it can no longer be assumed that hypersensitivity to low levels of oestradiol is a mechanism by which the cells escape from oestrogen regulation of cell growth. The hypersensitivity and increase in ERα levels reported in previous studies of MCF-7 cells (5, 11, 24, 36) and observed also here are consequential to oestrogen deprivation rather than causal in the loss of growth response. Given the many studies showing alterations in sensitivity to other non-oestrogen-regulated growth signalling pathways during the growth adaptation (see introduction), it would seem more appropriate to now concentrate translational research on developing inhibitors of those therapeutic targets for use following failure of endocrine therapy.

Acknowledgements

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References

1 Lonning PE (editor): Endocrinology and treatment of breast cancer. Clin Endocrinol Metab *18*: 1-130, 2004.

2 Katzenellenbogen BS , Kendra KL, Norman MJ and Berthois Y: Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res *47*: 4355-4360, 1987.

3 Welshons WV and Jordan VC: Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer Clin Oncol *23*: 1935-1939, 1987.

4 Daly RJ and Darbre PD: Cellular and molecular events in loss of estrogen sensitivity in ZR-75-1 and T-47-D human breast cancer cells. Cancer Res *50*: 5868-5875, 1990.

5 Jeng MH, Shupnik MA, Bender TP, Westin EH, Bandyopadhyay D, Kumar R, Masamura S and Santen RJ: Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. Endocrinol *139*: 4164-4174, 1998.

6 Stephen RL, Shaw LE, Larsen C, Corcoran D and Darbre PD: Insulin-like growth factor receptor levels are regulated by cell density and by long-term estrogen deprivation in MCF7 human breast cancer cells. J Biol Chem *276*: 40080-40086, 2001.

7 Chan CMW, Martin LA, Johnston SRD, Ali S and Dowsett M: Molecular changes associated with the acquisition of oestrogen hypersensitivity in MCF-7 breast cancer cells on long-term oestrogen deprivation. J Steroid Biochem Mol Biol *81*: 333-341, 2002.

8 Staka CM, Nicholson RI and Gee JMW: Acquired resistance to oestrogen deprivation: role for growth factor signaling kinases/oestrogen receptor cross-talk revealed in new MCF-7X model. Endocr Relat Cancer *12*: S85-S97, 2005.

9 Shaw LE, Sadler AJ, Pugazhendhi D and Darbre PD: Changes in oestrogen receptor-α and –β during progression to acquired resistance to tamoxifen and fulvestrant (Faslodex, ICI 182,780) in MCF7 human breast cancer cells. J Steroid Biochem Mol Biol *99*: 19-32, 2006.

10 Santen RJ, Song RX, Masamura S, Yue W, Fan P, Sogon T, Hayashi S, Nakachi K and Eguchi H: Adaptation to estradiol deprivation causes up-regulation of growth factor pathways and hypersensitivity to estradiol in breast cancer cells. Adv Exp Med *630*: 19-34, 2008.

11 Masamura S, Santner SJ, Heitjan DF and Santen RJ: Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab *80*: 2918-2925, 1995.

12 Martin LA, Pancholi S, Chan CMW, Farmer I, Kimberley C, Dowsett M and Johnston SRD: The anti-oestrogen ICI 182,780, but not tamoxifen, inhibits the growth of MCF-7 breast cancer cells refractory to long-term oestrogen deprivation through down-regulation of oestrogen receptor and IGF signaling. Endocr Rel Cancer *12*: 1017-1036, 2005.

13 Shim WS, Conaway M and Masamura S: Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells *in vivo*. Endocrinol *141*: 396-405, 2000.

14 Santen R, Jeng MH and Wang JP: Adaptive hypersensitivity to estradiol: Potential mechanism for secondary hormonal responses in breast cancer patients. J Steroid Biochem Mol Biol *79*: 115-125, 2001.

15 Yue W, Wang JP, Conaway M, Masamura S, Li Y and Santen RJ: Activation of the MAPK pathway enhances sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol. Endocrinol *143*: 3221-3229, 2002.

16 Martin LA, Farmer I, Johnston SRD, Ali S, Marshall C and Dowsett M: Enhanced estrogen receptor (ER) α, ERBB2, and MAPK signal transduction pathways operate during the adaptation of MCF-7 cells to long term estrogen deprivation. J Biol Chem *278*: 30458-30468, 2003.

17 Yue W, Wang JP, Conaway MR, Li Y and Santen RJ: Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways. J Steroid Biochem Mol Biol *86*: 265-274, 2003.

18 Sabnis GJ, Jelovac D, Long B and Brodie A: The role of growth factor receptor pathways in human breast cancer cells adapted to long-term estrogen deprivation. Cancer Res *65*: 3903-3910, 2005.

19 Fox EM, Arteaga CL and Miller TW: Abrogating endocrine resistance by targeting ERα and PI3K in breast cancer. Front Oncol *2*: article 145, 1-6, 2012.
20 Ray S and Darbre PD: Crosstalk with insulin and dependence on PI3K/AKT/mTOR rather than MAPK pathways in up-regulation of basal growth following long-term oestrogen deprivation in three human breast cancer cell lines. Horm Mol Biol Clin Invest *5*: 53-65, 2011.
21 Ray S, Fry MJ and Darbre PD: Enhanced sensitivity to rapamycin following long-term oestrogen deprivation in MCF-7, T-47-D and ZR-75-1 human breast cancer cells. J Endocrinol *208*: 21-29, 2011.
22 Fan P, Griffith OL, Agboke FA, Anur P, Zou X, McDaniel RE, Creswell K, Kim SH, Katzenellenbogen JA, Gray JW and Jordan VC: c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. Cancer Res *73*: 4510-4520, 2013.
23 Weigel MT, Banerjee S, Arnedos M, Salter J, A'Hern R, Dowsett M and Martin LA: Enhanced expression of the PDGFR/ABL signaling pathway in aromatase inhibitor-resistant breast cancer. Ann Oncol *24*: 126-133, 2013.
24 Dunbier AK, Martin LA and Dowsett M: New and translational perspectives of oestrogen deprivation in breast cancer. Mol Cell Endocrinol *340*: 137-141, 2011.
25 Sadler AJ, Pugazhendhi D and Darbre PD: Use of global gene expression patterns in mechanistic studies of oestrogen action in MCF-7 human breast cancer cells. J Steroid Biochem Mol Biol *114*: 21-32, 2009.
26 Soule HD, Vazquez J, Long A, Albert S and Brennan M: A human cell line from a pleural effusion derived from a breast carcinoma. J Nat Cancer Inst *51*: 1409-1416, 1973.
27 Osborne CK, Bolan G, Monaco ME and Lippman ME: Hormone responsive human breast cancer in long-term tissue culture: Effect of insulin. Proc Natl Acad Sci USA *73*: 4536-4540, 1976.
28 Darbre P, Yates J, Curtis SA and King RJB: Effect of estradiol on human breast cancer cells in culture. Cancer Res *43*: 349-354, 1983.
29 Osborne CK, Hobbs K and Trent JM: Biological differences among MCF-7 human breast cancer cell lines from different laboratories. Breast Cancer Res Treat *9*: 111-121, 1987.
30 Worton RG, McCulloch EA and Till JE: Physical separation of hemopoietic stem cells from cells forming colonies in culture. J Cell Physiol *74*: 171-182, 1969.
31 Darbre PD and Daly RJ: Effects of oestrogen on human breast cancer cells in culture. Proc Roy Soc Edinb *95B*: 119-132, 1989.
32 Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M and Martin MB: Regulation of the estrogen receptor in MCF-7 cells by estradiol. Mol Endocrinol *2*: 1157-1162, 1988.
33 Sikora MJ, Strumba V, Lippman ME, Johnson MD and Rae JM: Mechanisms of estrogen-independent breast cancer growth driven by low estrogen concentrations are unique *versus* complete estrogen deprivation. Breast Cancer Res Treat *134*: 1027-1039, 2012.
34 Darbre PD and Fernandez MF: Environmental oestrogens and breast cancer: Long-term low-dose effects of mixtures of various chemical combinations. J Epidemiol Commun Health *67*: 203-205, 2013.
35 Darbre PD and Charles AK: Environmental oestrogens and breast cancer: Evidence for combined involvement of dietary, household and cosmetic xenoestrogens. Anticancer Res *30*: 815-828, 2010.
36 Santen RJ, Song RX, Zhang Z, Kumar R, Jeng MH, Masamura S, Lawrence Jr J, MacMahon LP, Yue W and Berstein L: Adaptive hypersensitivity to estrogen: Mechanisms and clinical relevance to aromatase inhibitor therapy in breast cancer treatment. J Steroid Biochem Mol Biol *95*: 155-165, 2005.

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