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Effect of Endocrine Therapy on Growth of T61 Human Breast Cancer Xenografts is Directly Correlated to a Specific Down-regulation of Insulin-like Growth Factor II (IGF-II)

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Insulin-like growth factors I and II (IGF-I and IGF-II) are potent mitogens for some human breast cancer cell lines, and expression of IGF-II mRNA in the oestrogen receptor-positive (ER+) and oestradiol (E2) stimulated human breast cancer cell line T47D is increased by E2, suggesting a role for IGF-II in the mitogenic response to E2. Very little information is available from the literature on the relation between growth inhibition by endocrine therapy and cellular production of IGF-II. Here we report on the effect of E2 and tamoxifen (TAM) on IGF-II mRNA and protein expression in the ER+T61 human breast cancer xenograft. Growth of the T61 tumour is inhibited by treatment with E2 and TAM. Ribonuclease (RNAse) protection assays with human- and mouse-specific IGF-II antisense probes were used to study the regulation of IGF-II mRNA by E2 and TAM in the tumour. IGF-II protein expression was studied by radioimmunoassay. Untreated T61 tumours have a high baseline expression of IGF-II mRNA. TAM treatment of T61 tumours, which results in inhibition of tumour growth without tumour regression, reduced IGF-II mRNA expression approximately 10-fold after 48 h of treatment. E2 treatment of T61 tumours, which results in tumour regression, was accompanied by a more pronounced decrease in IGF-II mRNA expression in the tumour cells; 96 h after initiation of E2 treatment, there was almost no detectable IGF-II mRNA. Analyses of IGF-II protein showed that both treatments significantly reduced the concentration of IGF-II protein in the tumours. This down-regulation was found to be specific for IGF-II, since analyses of the effect of E2 on the expression of IGF-I mRNA, 36B4 mRNA, transforming growth factor α(TGF-α) mRNA, and epidermal growth factor (EGF) receptor mRNA in T61 tumours did not reveal any down-regulation. To further study the relation between inhibition of tumour growth and down-regulation of IGF-II, we exposed T61 tumours to a monoclonal antibody, α-IR3, which abolishes the physiological effect of IGF-I and IGF-II by blocking the binding of both growth factors to the type I IGF receptor. Treatment with α-IR3 resulted in inhibition of tumour growth during treatment. Thus, blockade of the type I IGF receptor and down-regulation of IGF-II by E2 and TAM resulted in growth inhibition, suggesting that IGF-II expression is correlated to T61 tumour growth, and that specific down-regulation of IGF-II by E2 and TAM could be involved in inhibition of T61 breast tumour growth by these two types of endocrine therapy.
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INTRODUCTION

A NUMBER OF polypeptides which may regulate human breast cancer cell growth have been isolated [1, 2]. Many of these growth factors have mitogenic effects on epithelial cancer cells *in vitro* [3, 4], while only a few have growth inhibitory action [5, 6]. Experimental data suggest that steroids and steroid antagonists may regulate the growth of breast cancer cells by influencing the expression and secretion of such growth factors. Growth stimulation by steroids could result either from an increase in mitogenic growth factor secretion or a decrease in the secretion of inhibitory growth factors. Conversely, growth inhibition could result from a reversal of these processes.

To study the complex interaction between endocrine therapy and growth factor secretion, we established primary human breast cancers as xenotransplants in athymic nude mice. Compared with models relying on *in vitro* tissue culture systems, the nude mouse provides a model with a functioning endocrine system [7]. In addition, tumours growing in nude mice consist of different cell populations, including the human malignant cells, mouse fibroblasts, mouse endothelial cells and other mouse cells. Thus, in this model, important interactions between the tumour cells and the host, which are not addressed in a purely *in vitro* system, can be studied.

Insulin-like growth factor I (IGF-I) mRNA expression is found in many human breast cancer specimens, although data suggest that the stromal cells are the major source of the message [8]. This is in agreement with studies on experimental human breast cancers, demonstrating only one tumour line that expresses IGF-I mRNA [8, 9]. IGF-II mRNA is expressed in

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many clinical breast cancers [3], as well as in several experimental human breast cancer cell lines [3]. IGF-I and IGF-II can stimulate the growth of human breast cancer cell lines *in vitro* [3, 8]. In the oestradiol (E2)-stimulated T47D and MCF-7 human breast cancer cell lines, IGF-II mRNA is induced by E2 [3, 10] (also, Brünner *et al.*, *Breast Cancer Res Treat*, in press). Most clinical and experimental human breast cancers express IGF-I and IGF-II receptors [11, 12]. Taken as a whole, the experimental data suggest that IGF-I and IGF-II may be important regulators of breast cancer growth and that IGF-II may play an important role in E2-stimulated cell proliferation.

Very little is known about the relation between growth factors and inhibition of tumour growth by endocrine therapy. To further study the possible interactions between tumour growth factor production and endocrine therapy, we chose the T61 human breast xenograft model. Growth of this oestrogen and progesterone receptor-positive tumour is inhibited both by tamoxifen (TAM) and E2 [13, 14]. While TAM treatment results in a stabilisation of tumour size [13], E2 treatment induces tumour regression [14]. In the present study, we sought to determine the correlation between growth response to TAM and E2 treatment and expression of IGF-II and other growth factors in the T61 tumour.

RNAse protection assays using human-specific antisense RNA probes were used to measure the effect of TAM and E2 on growth factor mRNA expression. Cellular production of IGF-II protein was determined by radioimmunoassay (RIA) of total tumour extracts.

MATERIALS AND METHODS

Experimental design

Characterisation of the T61 tumour has been described previously [15]. Briefly, T61 is a human breast cancer xenograft which expresses oestrogen and progesterone receptors. Growth in nude mice is inhibited by E2 and TAM in a dose-dependent manner, but is unaffected by ovariectomy [13–15]. T61 tumours, which only grow *in vivo*, were serially transplanted by inoculating a tissue block of approximately 2 mm subcutaneously into each flank of ovariectomised NMRI/Bom nude mice.

A single intramuscular depot injection of 0.1 mg E2 valerate (Progynon-depot, Schering AG, Berlin) dissolved in peanut oil or an intramuscular depot injection of 1.0 mg TAM-citrate [13, 16] was used to study the effect of E2 and TAM. The type I IGF receptor antibody, α-IR3, which blocks the type I IGF receptor binding domain [17], was kindly provided by Dr Steven Jacobs, Wellcome Research Laboratories. Mice received 500 μg α-IR3 mouse monoclonal antibody intraperitoneally twice a week for 2 weeks. Only this arbitrarily selected schedule was used, since restricted amounts of α-IR3 were available. Control mice were treated with 500 μg mouse IgG (Sigma) on the same schedule as the α-IR3-treated mice.

The growth curve studies were performed in separate experiments. Tumour size was measured in two dimensions by sliding gauge twice a week. A computer program was used to fit the growth data to a transformed Gomperetz function [18]. Mean normalised tumour growth curves were generated and used for computing treatment-induced growth delay and tumour volume doubling time [15, 18]. The number of tumours included is shown in the legends to Figs 1 and 5.

After tumour transplantation, mice were injected with either TAM (day 51) or E2 (day 44). Antibody treatment was started on day 56 after transplantation and given twice a week for 2 weeks. For RNA studies, tumours were excised at time 0 and at 3, 6, 12, 24, 48 and 96 h after treatment with TAM or E2.

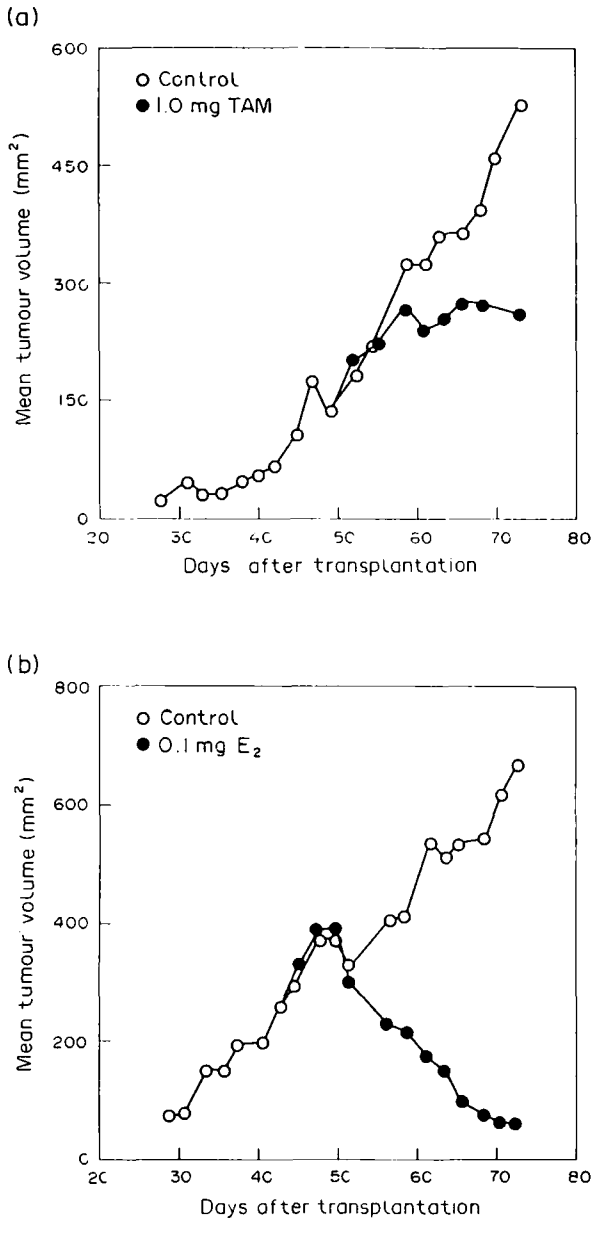

Fig. 1. Effect of TAM (a) and E2 (b) on growth of T61 human breast cancer grown in female athymic nude mice. The mice were treated with a single depot injection of either 1.0 mg TAM or 0.1 mg E2. 13 tumours were exposed to TAM and nine to E2. The number of untreated control tumours were 15 (TAM) and 12 (E2).

RNA preparation

Tumours excised at different time points after E2 treatment were immediately frozen in liquid nitrogen. Using a tissue homogeniser, RNAs were prepared using the guanidinium-isothiocyanate/cesium chloride method [19]. After ultracentrifugation, the RNAs were resuspended in RNA elution buffer, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and then precipitated with ethanol. RNAs were resuspended in water and the RNA concentration was determined by spectrophotometry.

Probes

The human IGF-II cDNA RNA probe used was kindly supplied by Dr Graeme Bell (University Chicago, H. Hughes Inst., Chicago, Illinois, U.S.A.) [20]. The probe is a 336–basepair RsaI-PstI fragment covering the coding portion of the preprohormone and a small portion of the 3′ untranslated region [3].

The mouse genomic IGF-II probe used was provided by Dr P. Rotwein (Washington University Medical School, St Louis, Missouri, U.S.A.). The probe comprised a 720 bp BamHI-PstI fragment that protects a 152 bp fragment. Human and mouse IGF-II nucleic acid sequences share an 86% homology in this 152 bp area.

The control probe 36B4 was derived from a subtraction hybridisation of untreated and oestrogen-treated MCF-7 human breast cancer cells and was shown not to be regulated by E2 [21]. The original cDNA was kindly provided by Dr P. Chambon (INSERM, Strassburg, France). The gene is cloned into a pGem4 vector and protects a 200 bp fragment in the RNAse protection assay.

cDNA for the type I IGF receptor was kindly provided by Dr Axel Ullrich (Max Planck, Marburg, F.R.G.) [22]. The probe is a 344 bp StuI–BamHI portion of the type I IGF receptor comprising the carboxy terminal end of the beta chain and a small portion of the 3′ untranslated region [23].

A 9 kb cDNA for the type II IGF receptor, provided by Dr William Sly (St Louis University, St Louis, Missouri, U.S.A.) [24], was digested with BamHI producing a 377 bp fragment including bases 2958–3335. This fragment was subcloned into a pGem 4 vector (Promega), which was subsequently linearised with Hind III and used as a template for the antisense RNA probe [23].

The IGF-1A probe was kindly supplied by K. Gabbay (Baylor College of Medicine, Houston, Texas, U.S.A.). The probe comprised a 540 bp fragment cloned into pGem4 [3].

The mouse IGF-I probe was kindly provided by Dr P. Rotwein. This probe comprises a 870 bp BamHI-EcoRI fragment in a pGEM Bluescript vector transcribed with T7 polymerase, and protects a 182 bp fragment.

The transforming growth factor α (TGF-α) probe was a 152 bp SphI–ApaI fragment in pGEM7zf linearised with EcoRI and transcribed with T7 polymerase.

The epidermal growth factor (EGF) receptor probe (J. Schlesinger, NIH, Bethesda, Maryland, U.S.A.) was a 141 bp BamHI–PstI fragment in pGEM4 linearised with HindIII and transcribed with SP6 polymerase.

RNAse protection assay

60 μg of total RNA was hybridised with 5×10^4 cpm of the probe of interest for 12–16 h at 50°C. 60 μg of yeast tRNA was always included as a control for non-specific hybridisation and for the possible presence of undigested probe. As positive controls, RNA from the human cell line CHP100 was used for the IGF-I mRNA assay, and RNA from the human cancer cell line HepG2 was used for the IGF-II assay. The antisense RNA probes were synthesised from the cDNA templates described above, using a riboprobe kit (Promega). The hybridisation buffer consisted of 800 μl 80% formamide, 0.4mol/l NaCl, 0.1 mol/l EDTA, and 40 mmol/l piperazine-N, N′-bis (2-ethanesulphonic acid). Samples were digested with RNAse A and RNAse T1 for 30 min at 30°C followed by proteinase K treatment for 15 min at 37°C. The samples were then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), following which they were precipitated with 2 μg tRNA and 2 volumes of absolute ethanol. The pellets were washed once in 70% ethanol and then lyophilised. The lyophilised pellets were resuspended in a 80% formamide loading buffer, boiled for 5 min and run for 2.5 h on a 6% polyacrylamide sequencing gel containing 8 mol/l urea. Size markers were prepared by end-labelling MspI digested fragments of pBR322 (New England Biolabs, Beverly, Massachusetts, U.S.A.). The dried gels were exposed to X-ray film in the presence of a Quanta III (Dupont, Wilmington, Delaware, U.S.A.) intensifying screen. The intensity of the protected fragments was determined by densitometry using a Beckman DU-8 spectrophotometer.

Tumour extraction procedure

Approximately 1 g frozen tumour tissue was pulverised in a mortar and pestle on dry ice. The frozen tumour powder was transferred to a 15-ml dounce homogeniser, and suspended in a 2.5 ml 1 mol/l acetic acid with 100 μg/ml polymethyl sulphoxide (PMSF) and 2 μg/ml aprotinin. The powder was dounced at least 25 times and then the homogenate was sonicated for 5 s at full power to disrupt any remaining intact cells and membranes. The membrane fraction was removed by centrifugation in an Eppendorf tube for 10 min at 15 000 rpm, and the supernatant was collected. Protein levels in the supernatant were determined using a Biorad protein assay and the values were used to equalise total protein content in all samples.

Binding protein extraction procedure

After an overnight incubation in 1 mol/l acetic acid at 4°C, supernatant containing approximately 10 mg of total protein was processed with a Sep-Pak C-18 column (Millipore) as follows: prior to sample loading, the column was washed with 10 ml isopropyl alcohol, then loaded with 1 ml 1 mol/l acetic acid with 5% bovine serum albumin (BSA). Next the column was washed with 10 ml 1 mol/l acetic acid. After the column was prepared, the 1 ml tumour sample was loaded on it. The column was then washed with 10 ml 4% acetic acid. The sample was eluted with a 1:1 mix of acetonitrile and 4% acetic acid. Approximately 90% of the sample elutes were in the first 2 ml. The eluate is dried in a speed vac and then resuspended in 1 ml 1 mol/l acetic acid. 80–90% of binding protein was removed in the first extraction, with recovery of about 80% of IGF-II. The extraction process was repeated with the final eluate containing about 60% of the starting IGF-II, and less than 5% of the original binding protein.

IGF-II RIA

Samples for RIA were dried, and resuspended in 0.1 mol/l sodium phosphate, pH 7.4 and 1% BSA and 0.02% sodium azide. Recombinant human IGF-II (Bachem, Torrence, California, U.S.A.) was used as the standard. [^{125}I] IGF-II was obtained from Amersham. Monoclonal mouse anti IGF-II (Amano, Troy, VA), 0.3 ng per sample, was added to give a total incubation volume of 400 μl. The samples were incubated overnight, precipiated with 500 μl 25% polyethylene glycol and 100 μl 5% gamma globulin, and then counted on a gamma counter. All samples were run in duplicate.

Binding protein assay

Since IGF binding protein can produce a false positive result in a standard RIA, a separate assay for binding protein was performed on all samples after extraction, but prior to RIA, to ensure removal of all binding protein. Samples were dried and

then resuspended in 300 μl phosphate buffer containing 20 000 cpm of radiolabelled IGF, and the mixture was incubated overnight at 4°C. Unbound labelled IGF was precipitated with 1 ml of phosphate buffer containing 5 mg charcoal and 0.2 mg protamine sulphate. PBS was used as a negative control and serum was used as a positive control. Labelled IGF, which was associated with binding protein in the sample, remained in the supernatant and was counted on a gamma counter.

RESULTS

Effect of TAM and E2 on T61 tumour growth

T61 human breast cancer xenografts form tumours in untreated female nude mice [13, 14]. Previous investigations showed, that treatment with a single dose of 1.0 mg TAM halted tumour growth without tumour regression [13], and treatment with a single dose of 0.1 mg 17β-E2 caused tumour shrinkage with almost complete tumour disappearance 30 days after treatment [14]. The response to TAM and E2 was reproduced in the present experimental series (Fig. 1a and b).

IGF-II mRNA expression in the T61 tumour

Human tumours grown in nude mice contain cells of human and mouse origin. Thus, RNA obtained from the xenotransplanted tumours consists of a mixture of human and mouse RNA. Therefore, a highly specific method is necessary to distinguish between transcripts of human and mouse origin. We demonstrate here that human and mouse IGF-II mRNA can be distinguished from one another by RNAse protection assay with human- and mouse-specific antisense RNA probes. The RNAse protection assay is very specific and only small mismatches in the nucleotide sequence between the mouse and the human mRNA result in digestion of the probe at the point of mismatch. As seen in Fig. 2, an antisense RNA probe synthesised from a mouse IGF-II genomic probe detected IGF-II mRNA expression in adult mouse brain (lane 4), but no protected fragment was seen in T61 (lane 3). Conversely, applying a human specific antisense IGF-II RNA probe, a protected mRNA sequence was found in T61 (lane 1), whereas no protection was seen in mouse brain (lane 2). Whether mouse fibroblasts do not express IGF-II mRNA, or whether they indeed express IGF-II but have too low a fraction in the tumour to allow detection of the IGF-II mRNA, cannot be answered by this study. However, our results strongly indicate that the IGF-II mRNA detected in the T61 tumour originates from the human cancer cells.

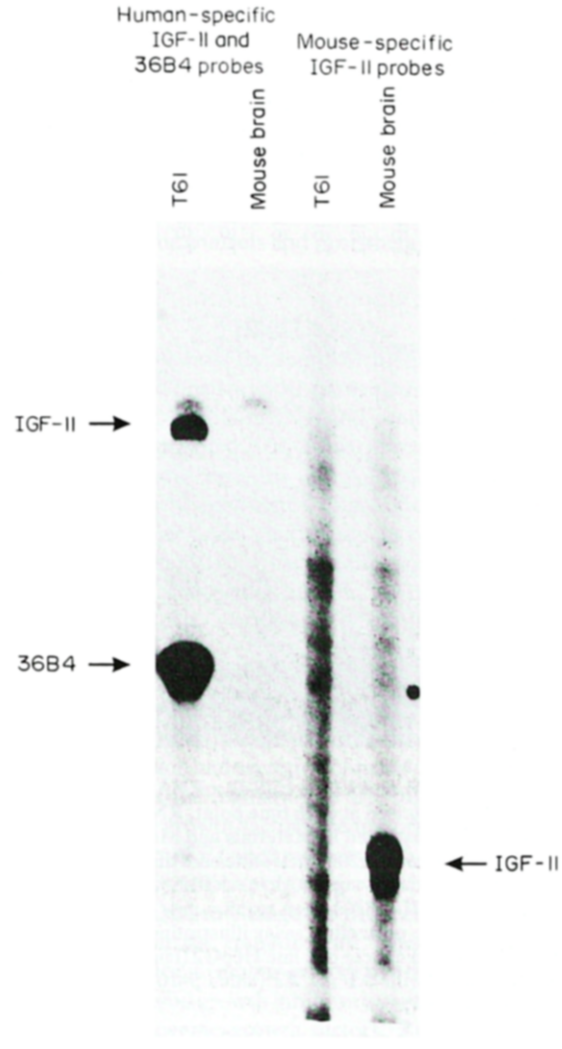

Fig. 2. RNAse protection assays of 60 μg of total RNA from T61 tumours and of 60 μg of total RNA from mouse brain using a ^{32}P-labelled human-specific IGF-II cDNA probe and a ^{32}P-labelled mouse-specific IGF-II genomic probe, respectively. The 36B4 probe was included in the hybridisation with the human-specific IGF-II probe.

The human-specific IGF-II riboprobe only protected mRNA fragments in the T61 tumour (lane 1) whereas the mouse-specific IGF-II riboprobe only protected IGF-II mRNA in mouse brain (lane 4). The weak band in the lane with mouse brain RNA hybridised to the human specific IGF-II probe is due to a small amount of undigested probe. The 36B4 probe only protected a fragment in the sample containing human cells.

Effect of TAM and E2 on IGF-II mRNA expression in the T61 tumour

The effect of TAM and E2 treatment on IGF-II mRNA expression in the T61 tumour was studied using the human IGF-II RNA probe in the RNAse protection assay. As seen in Fig. 3(a), TAM treatment resulted in a gradual decrease in IGF-II mRNA expression, reaching a lower steady-state level at approximately 12 h after initiation of TAM treatment. As measured by densitometry, the new level of IGF-II mRNA expression was 10-fold lower than that in untreated tumours. Following E2 treatment a significant decrease in IGF-II mRNA was seen 12 h after treatment, and the message was almost undetectable at 96 h [Fig. 3(b)]. The down-regulation from time 0 to 12 h after E2 treatment is ≥ 10-fold.

Effect of TAM and E2 on expression of 36B4 mRNA in the T61 tumour

A probe for 36B4 [21], which is not regulated by E2 in MCF-7, another ER+ human breast cancer cell line, was used as a standard in order to control for equal loading of RNA. In the experiment shown in Fig. 2, the 36B4 probe was only included in the hybridisation with the human-specific IGF-II probe. Our data suggest that the 36B4 antisense RNA probe is human-specific in the RNAse protection assay. No protected fragments were seen in the mouse tissues investigated, as opposed to the human tumour tissue (Fig. 2, lanes 1 and 2). The same features appear in Figs 3(b) and 4, demonstrating lack of 36B4 mRNA expression in mouse liver. Thus, 36B4 could be used as a specific control for the human RNA. No regulation of this mRNA species by TAM or E2 was observed in the T61 tumour (Figs 3a and b), confirming previous observations with cells grown *in vitro* [21]. Furthermore, the 36B4 data exclude the possibility

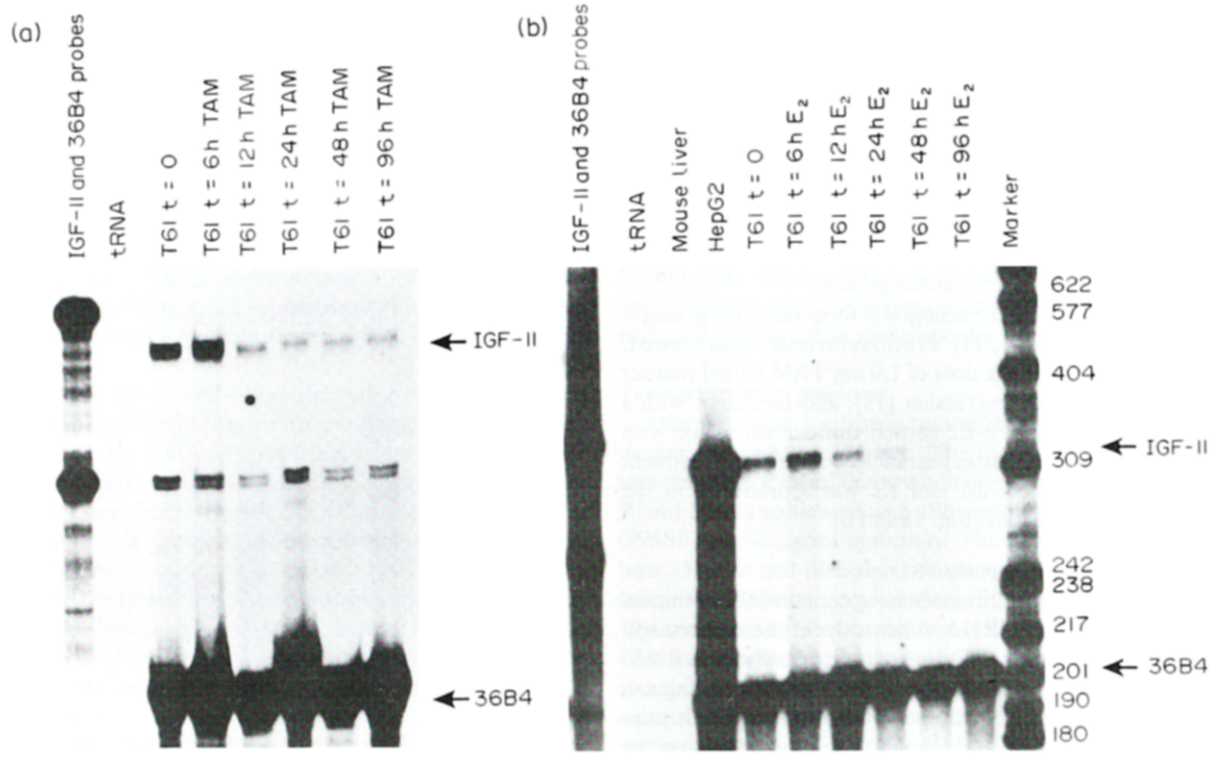

Fig. 3. Effect of TAM and E2 on IGF-II mRNA expression in the T61 tumour. Representative RNAse protection assay of 60 μg of total RNA from tumours excised at each time point. RNA was hybridised to the human-specific IGF-II cDNA riboprobe and processed for RNAse protection assay as described in Materials and Methods. The bands at approximately 312 nucleotides represent the protected portion of the IGF-II probe. The bands at 200 nucleotides are due to protection of the 36B4 probe. The band in between these two bands is undigested 36B4 probe. Treatment with 1.0 mg TAM resulted in a down-regulation of IGF-II mRNA detectable at 12 h after the initiation of treatment (a, lanes 3–8). The IGF-II mRNA then reach a new steady state level, approximately 10-fold lower than the level of untreated tumours. (b) A representative RNAse protection assay illustrating the effect of E2 on IGF-II mRNA expression. 60 μg of total RNA from mouse liver (lane 3) and from the human cancer cell line HepG2 (lane 4) were included as controls. A dramatic down-regulation of IGF-II mRNA was observed following treatment with 0.1 mg E2 (lanes 5–10). The decreasing in IGF-II mRNA was seen from 12 h after treatment, being almost undetectable at 96 h (b).

that the IGF-II down-regulation simply reflected a general down-regulation of cellular mRNA.

Effect of E2 on expression of IGF-I, TGF-α, and EGF receptor mRNAs in the T61 tumour

In order to clarify whether the observed down-regulation of IGF-II mRNA was a specific effect, i.e. whether or not potentially relevant growth factor mRNAs were also down-regulated by the applied endocrine treatment, total RNA from T61 tumours, representing different time points after E2 exposure, were hybridised to IGF-IA, TGF-α, and EGF receptor antisense RNA probes using the RNAse protection assay. None of these mRNA species were down-regulated by E2 treatment (Fig. 4 and data not shown).

The two bands protected by the IGF-IA probe in T61 represent IGF-IA and IGF-IB transcripts [9], respectively. Furthermore, the protected IGF-I fragments in T61 are smaller than the protected fragment seen in the CHP100 cell line due to a difference in the 5'-end of the IGF-I gene in these cell lines [9].

Type I and type II IGF receptor mRNA expression in the T61 tumour

Specific receptors for IGF-I and IGF-II have been identified and cloned. The mitogenic effects of both IGF-I and IGF-II are probably mediated by the IGF-I receptor [23]. Previous studies have demonstrated that both IGF-I and IGF-II receptors are expressed ubiquitously in breast cancer cell lines and their xenotransplanted counterparts. Using the RNAse protection assay and antisense RNA probes encoding the type I and type II IGF receptors, the T61 tumour was shown to express messages for both receptors (data not shown).

TAM and E2 regulation of IGF-II protein in the T61 tumour

After acid chromatographic extraction of IGF binding proteins from T61 tumour extracts, a charcoal exclusion binding assay demonstrated that less than 5% of the original binding protein remained, and was equal in all samples. RIA showed 0.52 ng IGF-II per mg total protein in untreated controls. 24 h after TAM treatment, the concentration of IGF-II had decreased to 31% of untreated tumours; at 72 h after TAM treatment, the IGF-II concentration was 42% of untreated tumours. In tumours treated with E2, IGF-II concentration fell to 63, 46 and 38% at 12, 48 and 96 h after treatment, respectively, suggesting a long half-life for the IGF-II protein.

Effect of α-IR3 on T61 tumour growth

To further characterise the relations between endocrine therapy and IGF-II expression in the T61 tumour, we exposed the tumour to the IGF-I receptor-blocking monoclonal antibody α-IR3. The 500 μg of antibody was injected twice a week for 2 weeks in animals with established tumours. Figure 5 shows the

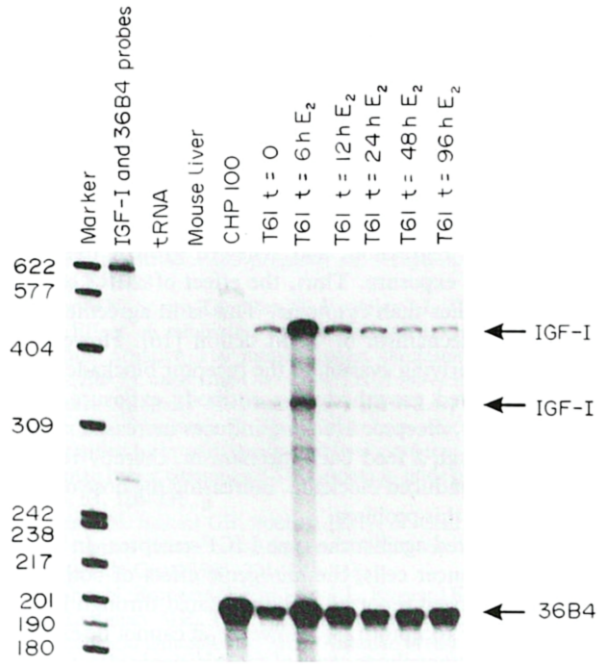

Fig. 4. Effect of E2 on IGF-I mRNA expression in T61. Representative RNAse protection assay of 60 μg total RNA from T61 tumours excised at each time point after E2 exposure. RNA was hybridised to the IGF-IA cDNA riboprobe and processed for RNAse protection as described in Materials and Methods. 60 μg of total RNA from mouse liver (lane 4) and from the human cell line CHP100 (lane 5) were included as controls. The IGF-I cDNA probe used appeared to be human-specific since no protected fragment was seen with mouse liver (lane 4). Subsequent RNAse protection using the mouse-specific IGF-I genomic probe protected IGF-I mRNA in mouse liver but not in T61 (not shown). The bands at approximately 450 and 320 nucleotides represent the protected portion of the IGF-I probe [9]. The bands at 200 nucleotides are due to protection of the 36B4 probe. The 6 h lane appears to be slightly overloaded. E2 treatment had no effect on IGF-I or 36B4 mRNA expression (lanes 6–11).

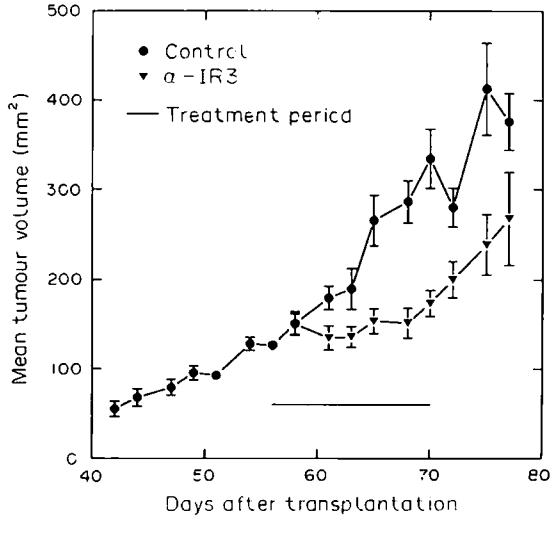

Fig. 5. Effect of α-IR3 on growth of T61. Tumour-bearing mice were given 500 μg of α-IR3 monoclonal antibody twice weekly for 2 weeks. Control mice received treatment with 500 μg of a mouse monoclonal antibody directed against human IgG twice weekly for 2 weeks (solid bar). The calculated mean tumour volume data were normalised to the first day of treatment. Bars = S.E.M. The number of tumours included were seven control and eight α-IR3 treated.

effect of the monoclonal antibody on T61 tumour growth. In animals treated with control antibody, no change in tumour growth rate was found, indicating that the irrelevant mouse IgG had no effect onT61 tumour growth. In contrast, α-IR3 slowed tumour growth during the treatment period with a calculated growth delay of 7 days. Following termination of therapy, the tumours again began to grow, regaining the growth rate of control tumours. The tumour volume doubling time was 14.08 and 13.69 days for controls and post-therapy tumours, respectively.

DISCUSSION

To better understand the complex interaction between endocrine therapy and breast cancer growth, we studied the effect of TAM and E2 on IGF-II mRNA and protein expression in the TAM- and E2-inhibited T61 human breast cancer xenograft. The data indicate that TAM- and E2-induced tumour growth inhibition is directly correlated to specific down-regulation of IGF-II mRNA and protein in the tumour cells.

A large number of studies have been conducted which examine the mechanisms underlying E2-induced stimulation of human breast cancer growth. In most studies, human breast cancer cells grown *in vitro* have been used. Results from these studies indicate that human breast cancer cells can express and secrete polypeptide hormones that via autocrine pathways can influence the growth of the cells. In hormone-dependent breast cancer cells, the expression and secretion of many of the peptide growth factors are under the regulation of E2, suggesting that cellular secreted peptide growth factors are important mediators of the stimulatory effect of E2 on these cells [1, 3, 4, 8, 25].

Inhibition of breast cancer cell growth by endocrine therapy has also been linked to changes in the secretion of polypeptide growth factors in the tumour cells. Theoretically, endocrine therapy could inhibit tumour growth by stimulating production of cellularly secreted growth inhibitory peptides or by decreasing secretion of mitogenic growth factors. Recent studies on the effect of the anti-oestrogen TAM indicate that growth inhibition by TAM is closely related to a concommitant increase in the production of the growth inhibitory peptide TGF-β_1 [5]. These observations have led to the hypothesis that TGF-β_1 is the mediator of the growth inhibitory effect of TAM. However, this hypothesis is still unconfirmed [26].

Little attention has been given to how E2 inhibits breast cancer growth, although high-dose E2 treatment has proven to be as effective as anti-oestrogens in breast cancer treatment [27, 28]. Investigation of the relation between E2-induced growth inhibition and regulation of cellular peptide growth factor production might provide further information into the molecular mechanisms involved in E2 regulation of tumour cell growth.

IGF-I and IGF-II have been implicated as important growth factors in the regulation of breast cancer growth [3, 8, 10, 16, 29–31]. Most primary human breast cancer specimens express IGF-I and IGF-II mRNA [3, 8], but in the case of IGF-I the message mainly originates from the stromal elements of the tumours. T61 is the only experimental human breast cancer which has been demonstrated to express IGF-I mRNA [9]. Like T61, other human breast cancer cell lines express IGF-II mRNA [3].

Most breast cancer cell lines express both type I and type II IGF receptors [11, 12. 23]. IGF-I and IGF-II are potent mitogens for some breast cancer cell lines [3, 8, 10], and the mitogenic effects of both IGF-I and IGF-II appear to be mediated through binding to the type I IGF receptor [23]. Antibodies

directed against the type I IGF receptor can inhibit breast cancer growth *in vitro* [30] and *in vivo* [31], suggesting that IGFs may play an important role in the growth regulation of breast cancer.

We have previously shown [3] that IGF-II mRNA expression was induced by E2 in the E2-stimulated T47D human breast cancer cell line, the growth of which is also stimulated by IGF-II [3, 32, 33]. Furthermore, treatment with anti-oestrogen, which is inhibitory to the T47D cells, abolished the E2-induced increase in IGF-II mRNA expression, indicating that IGF-II may be involved in both the stimulatory effect of E2 and in the inhibitory effect of anti-oestrogen in this human breast cancer cell line. In clinical studies, TAM treatment of breast cancer significantly reduces IGF-I plasma concentrations [34], suggesting a correlation between the anti-tumour effect of TAM and suppression of IGF-I.

We have done a series of studies to reveal the molecular mechanisms involved in the responses to TAM and E2 in the T61 human breast cancer xenograft. In this tumour, TAM slows tumour growth but does not induce tumour regression [13], whereas E2 both inhibits growth and induces significant tumour regression [14]. The present study shows that both TAM and E2 treatment of T61 tumours result in a specific down-regulation of IGF-II mRNA expression and protein production. In agreement with the growth responses induced by these two types of endocrine therapy, TAM induced an approximately 10-fold reduction to a new steady state level of IGF-II mRNA expression, while E2 treatment resulted in an almost total loss of IGF-II mRNA expression.

The down-regulation of IGF-II mRNA by TAM and E2 in T61 tumours may simply reflect an overall decrease in the total quantity of mRNA produced per cell. However, the lack of 36B4 mRNA down-regulation by TAM and E2 in the tumours argues against this possibility. To further substantiate the specificity of the IGF-II down-regulation, we used antisense RNA probes for IGF-I, TGF-α and EGF receptor mRNA in the RNAse protection assay. None of these mRNA species were down-regulated by the endocrine treatment, indicating that the IGF-II down-regulation by TAM and E2 was specific.

The present study does not rule out the possibility that endocrine or paracrine sources (murine) of IGF-I are also important; however, in other hormone-dependent human breast cancer model systems (MCF-7), these sources of IGF-I are not sufficient to cause oestrogen-independent growth. It would thus seem unlikely that these sources of IGF-I would be important to T61 growth. In fact, oestrogen inhibits T61 growth while probably increasing murine IGF-I production, which argues against murine IGF-I playing a role in T61 growth regulation.

As mentioned above, the monoclonal antibody α-IR3 blocks the mitogenic effects of IGF-I and IGF-II by inhibiting binding of both ligands to the type I IGF receptor [23]. Thus, to substantiate the growth regulatory role of IGF-II, animals with T61 tumours were treated with α-IR3. The antibody-induced growth inhibition during the treatment, providing further evidence that the observed IGF-II down-regulation plays an important role in the responses to TAM and E2 in the T61 tumour.

The α-IR3 treatment inhibited T61 tumour growth during the treatment period, but growth recommenced when treatment stopped. Similar results were obtained when T61 tumours were exposed to TAM [13]. In contrast, E2 treatment induced a dose-related growth inhibition with total tumour regression at the higher doses [14]. The modest tumour growth delay observed following α-IR3 treatment may indicate that the effect of E2 on T61 includes additional mechanisms which induce pronounced tumour regression. Alternatively, either increasing doses of α-IR3 or extending the treatment period might have improved the effect. This would correspond to the study by Arteaga *et al.* [31] in which a dose–response relation between α-IR3 and tumour growth inhibition was found. Interestingly, in two other studies exposing human tumour xenografts to α-IR3 [31, 35], blocking the type I IGF receptor resulted in inhibition of tumour growth without tumour regression, and renewed growth took place during antibody exposure. Thus, the effect of α-IR3 seems to be cytostatic rather than cytotoxic. This is in agreement with the proposed mechanism of TAM action [16]. However,the mechanism underlying evasion of the receptor blockade, which results in renewed growth during antibody exposure, is not known. Possibly, receptor blocking induces increased receptor expression through a feed back mechanism, thereby reducing the effect of the induced blockade. Increasing the dose of α-IR3 might overcome this problem.

α-IR3 is directed against the type I IGF-receptor. In MCF-7 human breast cancer cells, the mitogenic effect of both IGF-I and IGF-II has been reported to be mediated through binding to the type I IGF receptor [23]. However, it cannot be excluded that in T61 xenotransplants some of the mitogenic effect of IGF-II is mediated through binding to the type II IGF receptor [32, 33]. Blocking of the type I IGF receptor would then not totally inhibit tumour proliferation, and slowed tumour growth instead of tumour regression could be the result of IGF-II binding to its own receptor.

In conclusion, the data presented here suggest that growth inhibition by TAM and E2 of the T61 human breast cancer xenograft is mediated through a specific down-regulation of IGF-II. In E2-dependent human breast cancer cell lines such as T47-D, IGF-II is induced by E2, and it has been suggested that IGF-II might mediate the mitogenic effect of E2 in these cells. Although E2 has varying growth effects in these systems, the hormonal regulation of IGF-II correlates to the growth state of the tumour cells in both cases, suggesting that IGF-II expression is critical in the overall regulation of tumour growth.

1. Dickson RB, Lippman ME. Estrogen regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocr Rev* 1986, 8, 29–43.
2. Brünner N, Zugmaier G, Bano M, *et al.* Endocrine therapy of human breast cancer cells: the role of secreted polypeptide growth factors. *Cancer Cells* 1989, **1**, 81–86.
3. Yee D, Cullen KJ, Paik S, *et al.* Insulin-like growth factor II mRNA expression in human breast cancer. *Cancer Res* 1988, **48**, 6691–6696.
4. Bates SE, Davidson NE, Valverius EM, *et al.* Expression of transforming growth factor alpha and its mRNA in human breast cancer; its regulation by estrogen and its possible functional significance. *Mol Endocrinol* 1988, **2**, 543–555.
5. Knabbe C, Lippman ME, Wakefield LM, *et al.* Evidence that transforming growth factor beta is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* 1987, **48**, 417–428.
6. Ervin PR, Kaminski MS, Cody RL, Wicha MS. Production of mammastatin, a tissue-specific growth inhibitor, by normal human mammary cells. *Science* 1989, **244**, 1585–1587.
7. Brünner N, Svenstrup B, Spang-Thomsen M, Bennett P, Nielsen A, Nielsen J. Serum steroid levels in intact and endocrine ablated BALB/c nude mice and their intact littermates. *J Steroid Biochem* 1986, 25, 429–432.
8. Yee D, Paik S, Lebovic GS, *et al.* Analysis of IGF-I gene expression in malignancy—evidence for a paracrine role in human breast cancer. *Mol Endocrinol* 1989, **3**, 509–517.
9. Tobin G, Yee D, Brünner N, Rotwein P. A novel human insulin-like growth factor I messenger RNA is expressed in normal and tumour cells. *Mol Endocrinol* 1990, **4**, 1914–1920.

10. Myal Y, Shiu RPC, Bhaumick B, Bala M. Receptor binding and growth-promoting activity of insulin-like growth factors in human breast cancer cells (T47D) in culture. *Cancer Res* 1984, **44**, 5486–5490.
11. De Leon DD, Bakker B, Wilson DM, Hintz RL, Rosenfeld RG. Demonstration of insulin-like growth factor (IGF-I and -II) receptors and binding protein in human breast cancer cell lines. *Biochem Biophys Res Commun* 1988, **152**, 398–405.
12. Peyrat JP, Bonneterre J, Beuscart R, Djiane J, Demaille A. Insulin-like growth factor I receptors in human breast cancer and their relation to estradiol and progesterone receptors. *Cancer Res* 1988, **48**, 6429–6433.
13. Brünner N, Spang-Thomsen M, Vindeløv LL, Wolff J, Engelholm SA. Effect of tamoxifen on the receptor-positive T61 and the receptor negative T60 human breast carcinimas grown in nude mice. *Eur J Cancer Clin Oncol* 1985, **11**, 1349–1354.
14. Brünner N, Spang-Thomsen M, Vindeløv LL, Nielsen A, Engelholm SA, Svenstrup B. Dose-dependent effect of 17β-estradiol determined by growth curves and flow cytometric DNA analysis of a human breast carcinoma (T61) grown in nude mice. *Exp Cell Biol* 1985, **53**, 320–332.
15. Brünner N. Bastert GB, Poulsen HS, *et al.* Characterization of the T61 human breast carcinoma established in nude mice. *Eur J Cancer Clin Oncol* 1985, **221**, 833–843.
16. Brünner N, Bronzert D, Vindeløv LL, Rygaard K, Spang-Thomsen M, Lippman ME. Effect on growth and cell cycle kinetics of estradiol and tamoxifen on MCF-7 human breast cancer cells grown *in vitro* and in nude mice. *Cancer Res* 1989, **49**, 1515–1520.
17. Kull FC, Jacobs S, Su Y-F, Svoboda ME, Van Kyk JJ, Cuatrecasas V. Monoclonal antibodies to receptor for insulin and somatomedin-C. *J Biol Chem* 1983, **258**, 6561–6566.
18. Rygaard K, Spang-Thomsen M. Growth—a computer program for determination of mean growth curves and calculation of response to therapy of solid tumour xenografts. In Wu, *et al.*, eds. *Immune-deficient Animals in Experimental Medicine*. Basel, Karger, 1989, 301–306.
19. Chirgwin JM, Przybla AE, McDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry* 1979, **18**, 5294–5299.
20. Bell GI, Merryweather JP, Sanches-Pescador R, *et al.* Sequence of a cDNA clone encoding human preproinsulin-like growth factor II. *Nature* 1984, **310**, 775–777.
21. Masiakowski P, Breathnachb R, Block J, Gannon F, Krust A, Chambon P. Cloning of cDNA sequences of hormone-regulated genes from MCF-7 human breast cancer cell line. *Nucl Acids Res* 1982, **10**, 7895–7903.
22. Ullrich A, Gray A, Tam AW, *et al.* Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J* 1986, **5**, 2503–2512.
23. Cullen KJ, Yee D, Sly WS, *et al.* Insulin-like growth factor receptor expression and function in human breast cancer. *Cancer Res* 1990, **50**, 48–53.
24. Oshima A, Nolan CM, Kule JW, Grubb JH, Sly WS. The human cation-independent mannose 6-phosphate receptor: cloning and sequence of the full length cDNA and expression of functional receptor in COS cells. *J Biol Chem* 1988, **263**, 2553–2563.
25. Dickson RB, McManaway ME, Lippman ME. Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. *Science* 1987, **232**, 1540–1543.
26. Arteaga CL, Tandon AK, Von Hoff DD, Osborne CK. Transforming growth factor β: potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells. *Cancer Res* 1988, **48**, 3898–3904.
27. Ingle JN, Ahmann DL, Green SJ, *et al.* Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *New Engl J Med* 1988, **304**, 16–21.
28. Beex L, Pieters G, Smals A, *et al.* Tamoxifen versus ethinyl estradiol in the treatment of postmenopausal women with advanced breast cancer. *Cancer Treat Rep* 1981, **65**, 179–185.
29. Huff KK, Knabbe C, Lindsey R, *et al.* Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. *Mol Endocrinol* 1988, **2**, 200–208.
30. Rohlik QT, Adams D, Kull FC, Jacobs S. An antibody to the receptor for insulin-like growth factor I inhibits the growth of MCF-7 cells in tissue culture. *Biochem Biophys Res Commun* 1987, **149**, 276–281.
31. Arteaga CL, Kitten L, Coronado E, *et al.* Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. *J Clin Invest* 1989, **84**, 1418–1423.
32. Osborne CK, Coronado EB, Kitten LJ, Arteaga CI, Fuqua SAW, Ramasharma K, Marshall M, Li CH. Insulin-like growth factor-II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Mol Endocrinol* 1989, **3**, 1701–1709.
33. Mathieu M, Rochefort H, Barenton B, Prebois C, Vignon F. Interactions of cathepsin- D and insulin-like growth factor II (IGF-II) on the IGFII/mannose-6-phosphate receptor in human breast cancer cells and possible consequences on mitogenic activity of IGF-II. *Mol Endocrinol* 1990, **4**, 1327–1335.
34. Colletti RB, Roberts JD, Devlin JT, Copeland KC. Effect of tamoxifen on plasma insulin-like growth factor I in patients with breast cancer. *Cancer Res* 1989, **49**, 1885–1889.
35. Gansler T T, Furlanetto R, Gramling TS, *et al.* Antibody to type I insulin-like growth factor receptor inhibits growth of Wilm's tumor in culture and in athymic nude mice. *Am J Pathol* 1989, **135**, 961–966.

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