

Normal Breast Tissue Implanted into Athymic Nude Mice Identifies Biomarkers of the Effects of Human Pregnancy Levels of Estrogen

Rognvald N. Blance,¹ Andrew H. Sims,⁴ Elizabeth Anderson,³ Anthony Howell² and Robert B. Clarke¹

Abstract

We have generated a novel model system for the study of estrogen intervention in normal breast tissue. Nulliparous human breast tissue was implanted into immunocompromised nude mice and treated with high-dose estrogen to simulate the effects of pregnancy. Treatment of mice with human mid-pregnancy levels of 17β -estradiol for a period of 4 weeks was followed by 4 weeks of withdrawal to mimic involution. Gene expression in the xenograft tissue was then analyzed by real-time reverse transcription-PCR to identify differences between treated and control tissues. Ten genes previously identified as altered by pregnancy in rodent models were found to be differentially expressed in human breast tissue with a ≥ 1.8 -fold up-regulation of *CDC42*, *TGF β ₃*, *DCN*, *KRT14*, *LTF*, and *AREG* and a ≥ 0.7 -fold down-regulation of *STAT1*, *CTGF*, *IGF1*, and *VAMP1*. Immunohistochemical analysis of archival paraffin-embedded adult premenopausal human breast tissue specimens identified a significantly lower level of expression of *STAT1* ($P < 0.05$, Mann-Whitney *U* test) in parous compared with age-matched nulliparous tissue (median of 24% compared with 42% epithelial cells positive). We conclude that many of the pregnancy-induced breast cancer-protective changes observed in rodent models also occur in human breast tissue following intervention using human pregnancy levels of estrogen and that *STAT1* expression is a potential biomarker of parity-induced breast cancer protection in the human breast.

A late first full-term pregnancy (FFTP) has been shown to increase the risk of breast cancer, whereas an early FFTP is known to be highly protective (1). Women who have their FFTP before the age of 18 have one third the breast cancer risk of those whose FFTP is delayed until age 35 or older (2). This protective effect of pregnancy can be reproduced in animal models where an early FFTP has been shown to confer resistance to the subsequent induction of tumors by chemical carcinogens (3). Mimicking the protective effects of pregnancy can be achieved in rodents by treatment with pregnancy levels of 17β -estradiol (E_2) and progesterone, although treatment with E_2 alone was shown to be sufficient to reduce suscepti-

bility to mammary carcinogenesis (4). It was found that the period of hormone treatment need not be that of full gestation (3 weeks), with a treatment period of only 1 week being sufficient to significantly reduce the incidence of mammary carcinogenesis (4). The mechanisms for this protective effect have not been fully elucidated although several hypotheses have been proposed to explain the effect including systemic changes in endocrine hormones, mammary differentiation, or other changes in the mammary epithelium and stroma.

Parity results in persistent alteration of the hormonal environment in the mammary gland. It has been found that parity in rats results in significant persistent reductions in circulating levels of growth hormone and also in mammary gland levels of both estrogen receptor and epidermal growth factor receptor (5). In humans, it has been shown that parous women have significantly lower levels of serum prolactin when compared with nulliparous women, but there is no association with age at first birth (6). Levels of prolactin in postmenopausal women are also known to be associated with mammographic density, which is itself is strongly correlated to a high risk of breast cancer (7). Changes in the responsiveness of the gland to hormonal stimulation have been proposed as a possible mechanism for parity-induced protection. In part, the changes in response to hormonal stimulation may be due to alterations in the stromal component of the gland. A recent report showed that transplantation of transgenic p53-null epithelium into the cleared mammary fat pad of hosts previously treated with E_2 and progesterone was as effective in preventing tumors as hormonal treatment of the p53-null mouse itself (8).

Authors' Affiliations: ¹Breast Biology Group and ²Medical Oncology, School of Cancer and Imaging Sciences, Paterson Institute for Cancer Research, University of Manchester, Manchester, United Kingdom; ³Cancer and Infection Research Area, AstraZeneca, Macclesfield, Cheshire, United Kingdom; and ⁴Breakthrough Research Unit, Edinburgh Cancer Research Centre, Western General Hospital, Edinburgh, United Kingdom
Received 8/14/2008; revised 12/1/2008; accepted 12/2/2008; published OnlineFirst 3/3/09.

Grant support: Christie Hospital Endowment Fund (R.N. Blance and E. Anderson), Breakthrough Breast Cancer (A.H. Sims), and Cancer Research UK and Breast Cancer Campaign (A. Howell and R.B. Clarke).

Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

Requests for reprints: Robert B. Clarke, Breast Biology Group, School of Cancer and Imaging Sciences, Paterson Institute for Cancer Research, University of Manchester, Wilmslow Road, Manchester M20 4B, United Kingdom. Phone: 44-161-446-3210; Fax: 44-161-446-3220; E-mail: robert.clarke@manchester.ac.uk.
©2009 American Association for Cancer Research.
doi:10.1158/1940-6207.CAPR-08-0161

Alternatively, the protective effect of parity may result from an increased state of differentiation and a reduction in proliferation within the mammary gland. It has been suggested that the protective effect is due to the complete differentiation in the mammary gland (9), although this is not supported by a study showing that near full lactational differentiation induced by increasing circulating prolactin levels in the absence of pregnancy does not protect against carcinogenic challenge (10). Several studies have identified molecular changes in parous mammary tissue compared with that from nulliparous animals. In rats, one group found that mimicking pregnancy through hormone treatment results in up-regulation of genes identified as markers of mammary differentiation, metabolism, extracellular matrix (ECM), cell contact, and regulatory factors including signaling molecules and transcription factors (11). Another group using microarray analysis of murine mammary glands showed that parity resulted in the persistent down-regulation of genes encoding for growth factors and up-regulation of genes encoding for the inhibitory growth factor transforming growth factor β_3 (*TGF β_3*) and several of its downstream targets. In addition, there was up-regulation of several markers of differentiation and genes involved with inflammatory response (12). A core signature of 70 differentially regulated, parity-induced genes were identified that are conserved across several rat strains. This signature included up-regulation of differentiation markers and immune response genes, whereas there was reduction in expression of growth factor-related genes and genes of the growth hormone/insulin-like growth factor axis (13). The lists of parity-induced genes from these studies are largely nonoverlapping.

The fact that the protective effects of pregnancy can be reproduced by a relatively short period of E_2 treatment offers the potential for developing new strategies for preventing breast cancer in humans. Previously, we have successfully used a mouse xenograft model to investigate the effect of serum concentrations of estradiol equivalent to those observed in the luteal phase of the menstrual cycle on normal human breast cell proliferation (14–16) and gene expression (17). Here, we develop a xenograft mouse model of human breast tissue where the effects of estrogen intervention can be tested by implanting nulliparous human tissue and treating with high-dose E_2 pellets to achieve human pregnancy levels of serum E_2 for a 1-month period.

Materials and Methods

Human tissues

Histologically normal human female breast tissues were obtained from 14 nulliparous women (mean age, 24 ± 2.4 y) undergoing operation for removal of benign lesions or reduction mammoplasty with the approval of South Manchester Research Ethics Committee. After obtaining patient consent, full details of reproductive histories were recorded. Tissue was collected and stored in DMEM (Life Technologies, Inc.) for a maximum period of 4 h until processed for mouse implantation or histology. In addition, an anonymized collection of age-matched paraffin-embedded archival specimens of normal breast tissue collected from both nulliparous ($n = 15$; mean age, 34 y) and parous ($n = 15$; mean age, 34 y) premenopausal women together with the appropriate details of their reproductive history was available (18). All samples used in the parous group came from women whose first full-term pregnancy occurred before the age of 25.

Animals

Animals used for the xenograft experiments were 6- to 10-wk-old female athymic nude BALB/c nu^{-}/nu^{-} mice from Harlan Ltd., whereas those for measurement of serum hormone levels were female BALB/c nu^{+}/nu^{+} littermates of the same age. All surgical procedures were done in accordance with the Animals (Scientific Procedures) Act 1986 and under isoflurane inhalation anesthesia (3–5% isoflurane, 50–70% O_2/NO_2 at 2–4 L/min; Fluovac Vapouriser, International Market Supplies). Subsequent to the surgical procedures, all animals received treatment with analgesic (Rimadyl, 0.1 mL/20 g of body weight).

Assessment of serum hormone levels

To measure serum levels of E_2 , female BALB/c nu^{+}/nu^{+} mice were assigned to one of three groups of 24 animals. Animals in each group received an implant of silastic pellets containing 6, 12, or 18 mg of E_2 , whereas control animals received sham pellets. All pellets were inserted s.c. on the dorsal surface close to the tail, and sites of incision were sealed with surgical wound clips. Four animals per group were anesthetized and exsanguinated via cardiac puncture at 14-d intervals to a total time of 84 d. To investigate the effects of the E_2 implants on levels of endogenous murine prolactin and growth hormone, three groups of eight female BALB/c nu^{+}/nu^{+} mice each received silastic implants of one of the three doses of estradiol as described above. These were removed after 28 d, at which point four animals from each dosing regimen were exsanguinated, with the remaining animals being sacrificed at 56 d. Any mice showing signs of E_2 toxicity during the course of any experiment were immediately sacrificed and excluded from any further analysis. E_2 toxicity was judged to be present by the appearance of red sores on the skin and problems with passing urine due to hypertrophy of the urogenital tract. The collected blood was centrifuged at 2,000 rpm for 10 min at $4^{\circ}C$, and serum was stored at $-70^{\circ}C$ for subsequent analysis of hormone levels done on a Bayer Immuno-One assay analyzer.

Simulating human pregnancy in a mouse xenograft model

Each normal human breast tissue sample ($n = 14$) was stripped of excess fat under sterile conditions and divided into pieces of $\sim 2 \times 1 \times 1$ mm. A total of eight mice were used per human sample (control, $n = 2$; treated, $n = 6$). Two small incisions were made across the mid-line dorsal skin through which eight tissue pieces were symmetrically placed as described previously (14). Fourteen days after xenograft implantation, implants equivalent to 6, 12, or 18 mg of E_2 were inserted as described above. These were maintained in the mice for a period of 28 d at which point the pellets were excised by sharp dissection. Control animals received the same treatment but using an equivalent number of sham pellets for each dosing regimen. The timing of xenograft harvest is illustrated in Fig. 1. Two xenografts were excised at 14, 21, 42, and 70 d after tissue implantation. At day 70, all animals were sacrificed by exsanguination and the collected blood was analyzed for E_2 content as described above. Xenografts collected at each time point were processed for histologic analysis ($n = 8$), whereas at day 70 xenografts were immediately snap-frozen in liquid nitrogen ($n = 6$) and stored at $-70^{\circ}C$ for subsequent extraction of RNA.

Morphometry

The cellular composition of xenograft tissue was examined by morphometry at each of the harvest time points ($n = 8$). Sections stained with H&E were viewed at $\times 250$ magnification through a 10×10 grid. Tissue was scored at the intersections of the grid as epithelium, fat, or stroma (including fibroblasts and endothelium). A minimum of three fields of view were scored from one E_2 -treated and one untreated section at each time point from a total of eight implanted human samples.

Immunohistochemistry

Xenograft tissue was fixed in 10% formalin overnight ($n = 8$), dehydrated through graded ethanol, and embedded in paraffin wax. Sections were cut at 3 μm and mounted on glass microscope slides precoated with 3-aminopropyltriethoxysilane (Sigma-Aldrich). Slides were dried in an oven at 40°C overnight followed by 60 min at 60°C. Dewaxing was achieved by immersing the slides in three changes of xylene for 10 min each, followed by rehydration through graded ethanol. Endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol for 10 min. Epitope retrieval was for 30 min at 98°C in 0.01 mol/L citric acid buffer (Sigma-Aldrich) using a Pre-Treatment Module (LabVision). Blocking for 3,3'-diaminobenzidine visualization was for 60 min at room temperature using a 10% solution of normal serum from the animal species of the secondary antibody with avidin/biotin block (Vector). Primary antibodies used were monoclonal rabbit anti-estrogen receptor (clone SP1, Labvision; 1:25) and anti-progesterone receptor (clone SP2, Labvision; 1:50), polyclonal rabbit anti-Ki67 (Vector; 1:1,000), and anti-STAT1 (Abcam; 1:50). For Ki67, the secondary antibody was biotinylated goat anti-rabbit (Vector) used at 1:200 for 60 min at room temperature. Visualization was achieved using Vectastain Elite ABC Kit (Vector) for 45 min at room temperature and 3,3'-diaminobenzidine solution (Sigma-Aldrich) for >5 min. For all other antibodies, visualization was done using ChemMate EnVision Detection Kits (DAKO) as per manufacturer's instructions. Slides were scored at $\times 400$ magnification. More than 1,000 cells selected across representative high-power fields were scored per sample.

Extraction of RNA and generation of cDNA

Frozen xenografts ($n = 6$) were crushed into fine powder under liquid nitrogen, and RNA was isolated using TRIzol reagent (Invitrogen Ltd.) according to the manufacturer's instructions. RNA was purified with RNeasy columns (Qiagen Ltd.), and contaminating DNA removed using the DNA-free kit (Ambion Ltd.). The molecular integrity of RNA samples was measured on an Agilent Bioanalyser (Agilent Technologies); any samples with an RNA integrity number lower than 5 were considered to be degraded and excluded from further processing. To produce sufficient quantities of RNA for reverse-transcription to cDNA for use in quantitative reverse transcription-PCR, cRNA was produced using the MessageAmp II aRNA Amplification Kit (Qiagen). Two 14-h amplification steps were followed according to the manufacturer's instructions. Reverse transcription of cRNA to cDNA was achieved using the TaqMan Reverse Transcription Kit (Applied Biosystems) as per manufacturer's instructions.

Quantitative real-time PCR

Genes for analysis by quantitative reverse transcription-PCR were selected for being differentially expressed in more than one published study on the protective effects of early parity in animal models (11, 12, 19–21). Primers were designed using the Human Assay Design Center⁵ based on the gene accession numbers and purchased from MWG Biotech. Probes used were from the Exiqon universal human probe library (Roche; Supplementary Table S1). Primer efficiencies were tested using serial dilutions of cDNA templates, and reactions (total volume of 10 μL) were carried out in 384-well reaction plates for 40 cycles on a 7900HT Real-Time PCR System (Applied Biosystems). Reaction volumes were plated using an Eppendorf EPMotion robot and consisted of 5 μL of 2 ng/ μL cDNA template with 4.25 μL of TaqMan Universal PCR Master Mix (Applied Biosystems) and 0.25 μL each of probes and forward and reverse primers. Relative gene expression levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method (22) comparing cDNA generated from treated and control tissues collected at day 70 of the *in vivo* experiments. The expression levels of each gene were

normalized against the average expression levels of three housekeeping genes: *B2M*, *L14*, and *PGK1* (Supplementary Table S2).

Statistical analysis

Statistical tests used were the unpaired *t* test for differences in tissue composition. One-way ANOVA was used to test differences detected by immunohistochemistry in xenograft tissues, and Mann-Whitney *U* test was used to test differences by immunohistochemistry in the human samples.

Results

Pregnancy serum steroid hormone levels

Three doses of E_2 (6, 12, and 18 mg) in formulated slow-release silastic pellets were implanted s.c. into three groups of BALB/c *nu*⁺/*nu*⁺ female mice. Serum was collected from groups of four animals for assay of serum E_2 levels. For all three treatments, serum E_2 levels reached a maximum by day 14 and gradually decreased over the period of the experiment (Fig. 2A). Maximum levels (mean \pm SE) were $42,380 \pm 9,754$, $29,397 \pm 978$, and $14,262 \pm 1,835$ pmol/L for the 18, 12, and 6 mg treatments respectively. The two higher doses (12 and 18 mg) yielded serum levels equivalent to human mid-pregnancy serum concentrations when assayed at 14 and 28 days of treatment. Serum was collected from mice with implanted xenografts at day 70, four weeks after removal of E_2 pellets (see Fig. 1 for experimental time scale). No significant difference in serum E_2 was observed between the control and treated groups after removal at any of the three doses (Fig. 2B), indicating that serum E_2 levels had returned to baseline in treated mice. Serum prolactin and growth hormone were undetectable when assayed on the Bayer Immuno-One assay analyzer at any of the time points sampled.

Tissue composition

Tissue sections stained with H&E from control and treated xenografts of eight implanted human samples (6 mg E_2 , $n = 4$; 12 mg E_2 , $n = 2$; 18 mg E_2 , $n = 2$) were examined for cellular content to determine variability in breast tissue heterogeneity throughout the time course of the experiments (Fig. 1). Over the period of xenograft tissue implantation, the stromal content significantly increased whereas adipose content significantly decreased in both control and treated samples (Fig. 3A and C), suggesting an effect of s.c. transplantation into the mouse that was unrelated to E_2 treatment. Nevertheless, we were able to detect an increase in the epithelial compartment over the period of E_2 treatment, which was accompanied by a reduction in the stromal component (Fig. 3A and B), suggesting that E_2 -induced proliferation increases the proportion of epithelial cells.

Steroid receptor expression and proliferation

We next determined whether simulating human pregnancy serum E_2 influenced the proliferation marker Ki67 or estrogen and progesterone receptor expression in eight implanted human samples (6 mg E_2 , $n = 4$; 12 mg E_2 , $n = 2$; 18 mg E_2 , $n = 2$). Receptors and proliferation were expressed as a percentage of the total number of epithelial cells counted and intensity was not determined. Estrogen receptor expression did not differ significantly between the treatment and control groups at any of the time points examined. In contrast, progesterone receptor was significantly higher after administration

⁵ <https://www.roche-applied-science.com/sis/rtPCR/upl/index.jsp>

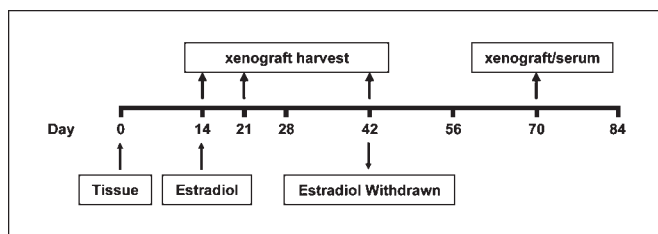


Fig. 1. Experimental design. Eight human breast xenografts were s.c. implanted in immunocompromised BALB/c *nu⁺/nu⁻* mice. The xenografts were maintained in the mice for 14 d before implantation of silastic pellets containing either 12 or 18 mg of E₂. E₂ treatment was withdrawn after 28 d. Two xenografts were harvested at 14, 21, 42, and 70 d after tissue implantation, and sera were taken at 70 d.

of E₂ at both days 21 and 42 but returned to baseline at day 70 (Fig. 4A). There were no significant differences between control groups at any of the time points. Proliferation of epithelial cells, as assessed with the antibody marker Ki67, was significantly higher following 7 and 28 days of E₂ administration (days 21 and 42; Fig. 4B). Following E₂ withdrawal by removal of the pellet, the proliferation of treated samples decreased to baseline levels. There were no significant differences between the control untreated tissues at any of the time points. These data suggest that serum levels of E₂ that mimic those seen during human pregnancy induce progesterone receptor expression and elicit a proliferative response in the epithelial cells leading to increases in epithelial content of the tissue. Following E₂ withdrawal, proliferation and E₂-induced progesterone receptor expression decrease to baseline levels. The increase in epithelial content seen during the induced pregnancy-like changes is also lost at this time.

Expression analysis reveals overlap between the human and rodent genes regulated by pregnancy

Quantitative reverse transcription-PCR was used to analyze the expression of genes previously identified as pregnancy-induced in rodents (11, 12, 19–21). Expression was compared in xenografted human tissue from animals treated with E₂ versus tissue from untreated animals (Fig. 5). The numbers of samples retrieved at 72 days posttransplantation ranged from three to six xenografts for each of the six patients (Supplementary Table S3). Gene expression was measured in RNA obtained from individual xenografts and no tissue was pooled for analysis. Data were pooled from the two higher serum E₂ doses and showed that 10 of 19 selected genes were found to be differentially regulated by real-time PCR. Up-regulated genes included *TGFβ₃* (3.34-fold), decorin (*DCN*; 2.56-fold), cell division cycle 42 (*CDC42*; 2.06-fold), amphiregulin (*AREG*; 1.95-fold), keratin 14 (*KRT14*; 1.94-fold), and lactotransferrin (*LTF*; 1.90-fold), suggesting increases in growth factors, differentiation markers, and ECM components. Down-regulated genes included connective tissue growth factor (*CTGF*; 0.52-fold), signal transducer and activator of transcription 1 (*STAT1*; 0.61-fold), and vesicle-associated membrane protein 1 (*VAMP1*; 0.69-fold). On exclusion of the sample from the oldest nulliparous patient (age 34), insulin-like growth factor I (*IGF1*) was also differentially down-regulated (0.53-fold). Thus, these data indicated decreases in cytokine/growth factor signaling following exposure of nulliparous breast tissue to human pregnancy serum E₂ levels.

Epithelial STAT1 protein levels are reduced in breast tissues of parous women

To validate gene expression data derived from our *in vivo* model of human breast tissue, we performed *STAT1* immunohistochemistry on 30 age-matched parous and nonparous specimens (Fig. 6). The median percentage of cells scored positive for *STAT1* in the parous group was 23.9% (interquartile range, 16.6–33%), which was significantly lower than the median percentage for *STAT1* in the nulliparous group of 41.8% (interquartile range, 21.7–55.4%; *P* < 0.05, Mann-Whitney *U* test). This suggests that reduction in epithelial *STAT1* protein levels is a good marker of parity-induced changes.

Discussion

The protective effect of an early full-term pregnancy has been shown in both human epidemiologic studies and *in vivo* experiments in rodents. This protective effect can be mimicked in rodent models by treatment with pregnancy levels of exogenous E₂. Previous work from this laboratory successfully used a mouse xenograft model to investigate menstrual cycle levels of E₂ on cell proliferation and gene expression (14–17). Here, we have used this xenograft model to simulate parity by implanting nulliparous human breast tissue in immunocompromised nude mice and treating them with high-dose E₂. Treatment of mice with human 2nd trimester pregnancy levels of E₂ for a period of 4 weeks was followed by 4 weeks of withdrawal to mimic involution. We identified genes

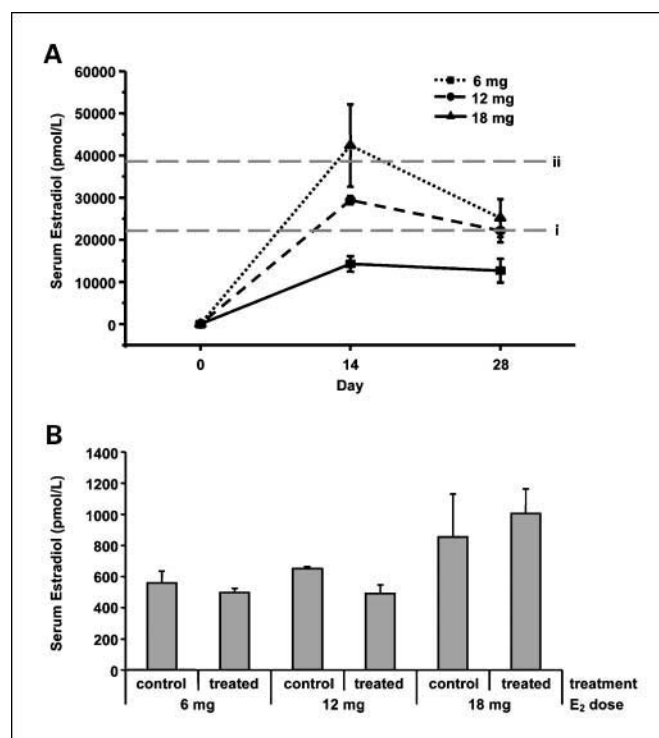


Fig. 2. Assessment of serum E₂ levels in experimental mice. Three groups of 24 BALB/c *nu⁺/nu⁻* mice received 6-, 12-, or 18-mg doses of E₂. Four animals from each group were sacrificed at 14-d intervals and sera were collected. A, serum estradiol levels for the different doses of silastic implant. Hatched horizontal lines, E₂ levels attained during human pregnancy at the end of first trimester (i) and second trimester (ii). B, serum E₂ levels in xenograft experimental animals at day 70 subsequent to 4 wk of hormone withdrawal.

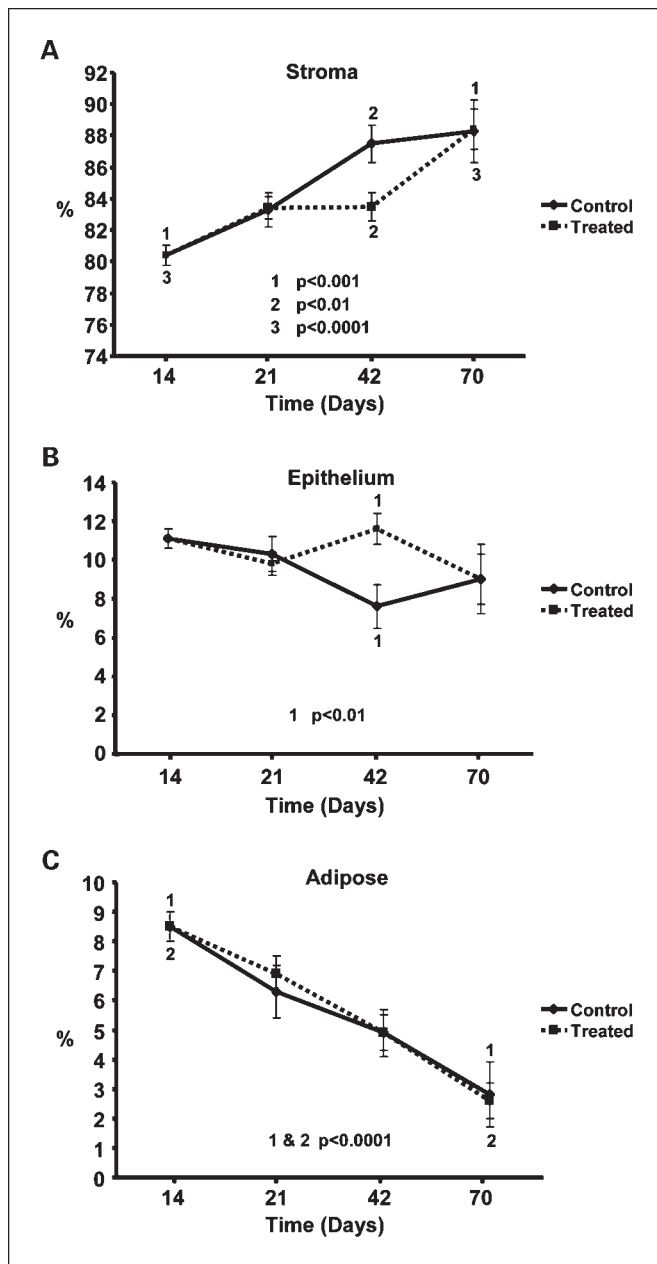


Fig. 3. Tissue composition of implanted human breast xenografts. Changes in composition of implanted tissues over the time of the experiment. The numbers by the error bars represent significance values for differences between time points. A, stroma. 1, differences between day 14 and day 70 within the control group ($P < 0.001$). 2, differences between the control group and the treated group at day 42 ($P < 0.01$). 3, differences within the treated group between day 14 and day 70 ($P < 0.0001$). B, epithelium. 1, difference between the control and treated groups at day 42 ($P < 0.01$). C, adipose. Difference between day 14 and day 70 in both the control (1, $P < 0.0001$) and treated (2, $P < 0.0001$) groups.

with differential expression following treatment with and withdrawal from pregnancy E_2 levels. Our experimental approach differs from that of Russo and coworkers (23) who compared gene expression in postmenopausal breast tissue from parous and nonparous women. In contrast, we show gene expression differences induced in premenopausal nulliparous breast tissue by intervention with pregnancy levels of E_2 . These genes were previously identified in rodents,

suggesting that similar changes are taking place in the two species. Furthermore, we showed that protein expression for one of these genes, *STAT1*, could be measured by immunohistochemistry in tissue sections of normal breast epithelium and that differences between age-matched parous and nonparous women could be detected.

All 10 genes identified as being differentially expressed by real-time PCR in this study have been previously identified as differentially expressed in animal models (11–13). The direction of change in expression, either up or down, was also the same with the exception of *AREG*. *AREG* has a major role in the promotion of breast cell proliferation and is known to be induced by estrogen in the human mammary gland (17). It is thought to act as a paracrine regulator of

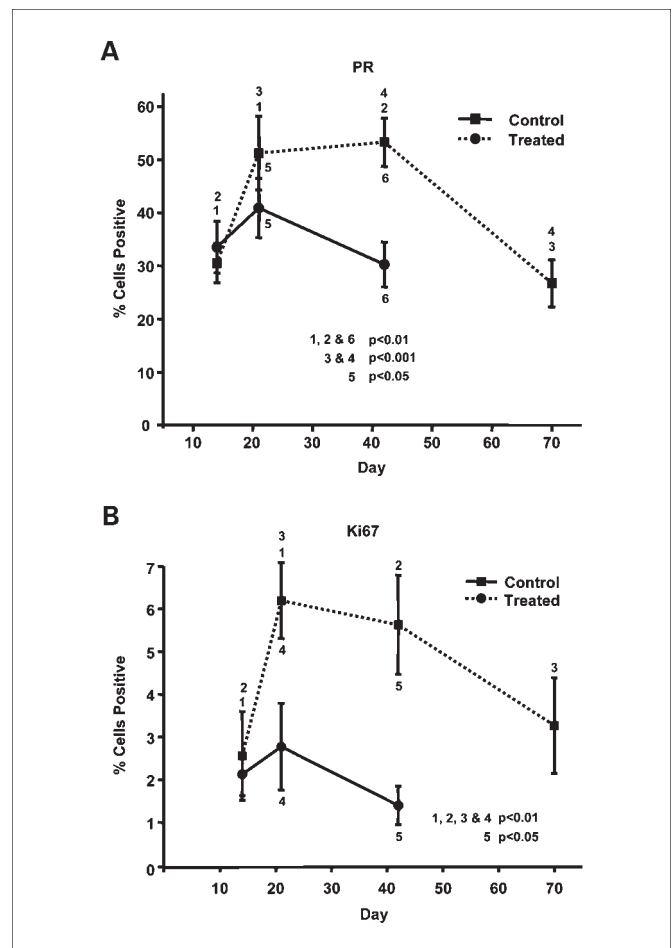


Fig. 4. Expression of progesterone receptor and Ki67 (proliferation) in human breast tissue xenografts removed from nude mice. Xenografts harvested at days 14, 21, 42, and 70 were processed for standard immunohistochemistry (IHC). Scoring is expressed as percentage of total epithelial cells positive for the respective marker. Numbers by the error bars represent significance values for the differences between time points. A, progesterone receptor (PR). Differences within the treated group: 1, between day 14 and day 21 ($P < 0.01$); 2, between day 14 and day 42 ($P < 0.01$); 3, between day 21 and day 70 ($P < 0.001$); 4, between day 42 and day 70 ($P < 0.05$). Differences between the treated and control groups: 5, day 21 ($P < 0.05$); 6, day 42 ($P < 0.01$). B, the proliferation marker Ki67. Differences within the treated group: 1, between day 14 and day 21 ($P < 0.01$); 2, between day 14 and day 42 ($P < 0.01$); 3, between day 21 and day 70 ($P < 0.01$). Differences between the treated and control groups: 4, day 21 ($P < 0.01$); 5, day 42 ($P < 0.05$).

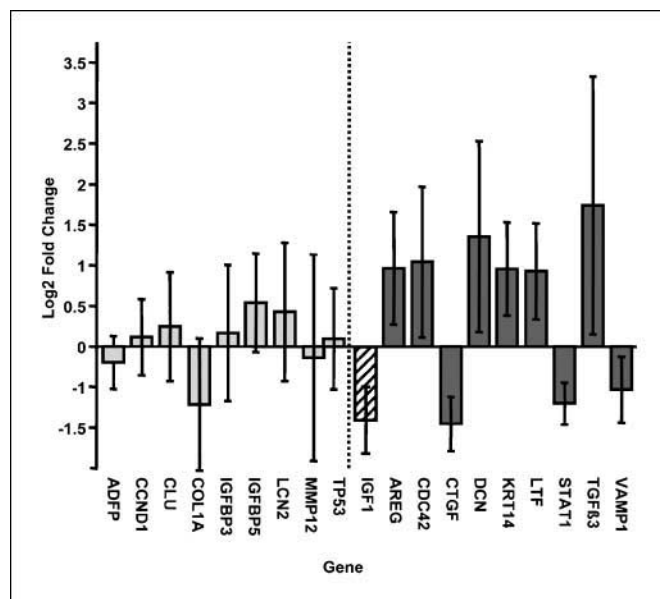


Fig. 5. Quantitative real-time PCR. RNA was extracted from xenografts harvested at day 70 from both treated and control animals, amplified, and then reverse transcribed to cDNA. Columns, mean fold changes in gene expression between control and treated samples, calculated as described in Materials and Methods. Columns to the right of the dotted line represent the genes considered to be differentially regulated, which was judged to be when the mean less the SE remained >1-fold. The hatched column was found to be differentially regulated in all but one of the implanted tissues, whereas the genes represented by dark gray columns were differentially regulated in all samples.

estrogen signaling (24). Expression of *AREG* has been shown to increase during pubertal development and to decline during late pregnancy and lactation in rodents (25). Previous animal models of the protective effect of parity have indicated down-regulation of *AREG*, and the authors have speculated that the suppression of its growth-promoting properties may be a contributory factor in the protective effect (12, 13). Our finding of up-regulated *AREG* may indicate that its expression diminishes more slowly in humans than in rodents following decreases in E_2 levels. However, we show that the growth of epithelial cells has returned to baseline, nonpregnant levels at this time point, suggesting that the sustained *AREG* levels are no longer stimulating pregnancy levels of epithelial cell proliferation. One explanation for this may be that expression of growth inhibitory growth factors such *TGFβ₃* are increased after treatment with E_2 . *TGFβ₃* is a local apoptosis inducing or growth inhibiting factor, which is expressed in all stages of mammary development with the exception of lactation (26). In addition to the increase of a growth inhibitory factor, the expression of two growth-stimulatory factors was decreased by pregnancy levels of E_2 . *CTGF* is known to be estrogen inducible and is overexpressed in steroid-dependent breast and uterine tumors (27). It is thought to be an important downstream mediator of estrogen and progesterone-regulated cell growth although it may affect other growth regulatory pathways in breast cancer cells (27). The other down-regulated gene was *IGF1*, which functions along with growth hormone and estrogen in postpubertal breast development and has been shown to be a survival factor in breast (28). Thus,

our results suggest that down-regulation of growth factors and up-regulation of growth inhibitory factors by parity may contribute toward its protective effect.

In addition to growth factors, we found that genes involved in ECM, tissue remodeling, and differentiation were modulated by pregnancy levels of E_2 . For example, we found increased levels of *DCN* expression after treatment with E_2 . There is some evidence to suggest that ECM components such as collagen and proteoglycans may be the primary determinants of mammographic density, which is, in turn, related to breast cancer risk (7, 19, 29). Therefore, we might speculate that pregnancy-associated changes in ECM components such as *DCN* may reduce breast density.

We found increased expression of *CDC42*, which is a member of the Rho GTPase family of proteins and performs important functions in cell migration (30). Migration is likely to be increased during remodeling of the breast tissue, which takes place following pregnancy. We also found increased expression of two differentiation markers, *LTF* and *KRT14*.

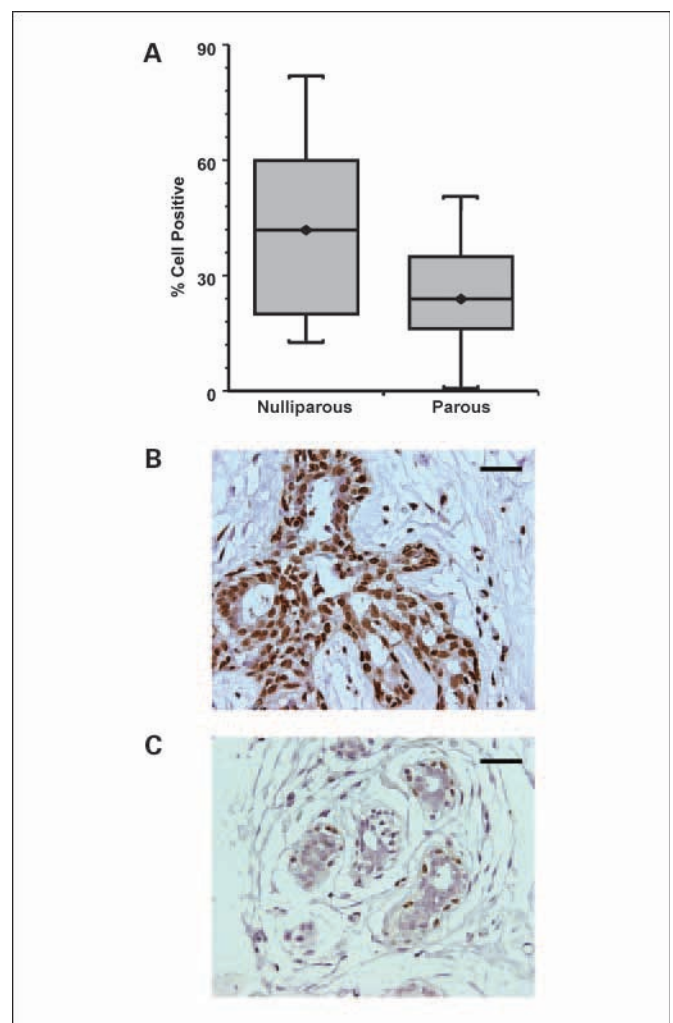


Fig. 6. Comparison of staining for STAT1 by immunohistochemistry between age-matched samples of archival human breast tissue. A, percentage of cells positive was significantly higher in nulliparous tissue as compared with parous tissue (Mann-Whitney *U* test, $P = 0.042$). Photomicrographs illustrating differing staining patterns observed in nulliparous (B) and parous (C) samples (bar, 50 μ m). Cells with brown staining nuclei were scored as positive.

Finally, we identified *STAT1*, the intracellular signaling component downstream of IFNs, to be highly down-regulated by pregnancy E_2 levels in our model. Importantly, we confirmed that a down-regulation in *STAT1* protein occurs in the breast epithelial cells of parous compared with nonparous women of the same age (Fig. 6). *STAT1* reduction in parous breast tissue suggests a decrease in the proportion of cells that could respond to IFN. Interestingly, IFN- α has recently been shown to induce hematopoietic stem cell activity (31), and one could speculate that epithelial stem cell activity in breast tissue is altered and reflected in *STAT1* expression. This finding of reduced *STAT1* expression in parous compared with nonparous women suggests that our xenograft model is a good simulation of hormonal effects during human pregnancy and reveals *STAT1* as a potential biomarker of pregnancy-induced breast cancer protection.

It has been suggested that possible mechanisms of pregnancy-induced protection include changes to the hormonal profile of parous women, changes in epithelial responses to hormones, a more differentiated and thus less susceptible mammary gland, or changes within specific epithelial cell subpopulations (32). Our results do not test whether there are sustained systemic hormonal changes, although serum E_2 levels were found to return to baseline after treatment was withdrawn for 4 weeks. At this time, we also determined whether the number of cells expressing estrogen receptor or progesterone receptor had changed and found no evidence for this, although changes in the quantity of receptors per cell was not measured and could influence response to hormones. We did see an increase in *KRT14*, perhaps indicating an increase in the proportion of basal cells, but we have not used any markers to identify specific changes in stem, basal, myoepithelial, or luminal cell number in our study.

It is also well founded epidemiologically that there is an increase in risk of breast cancer shortly after pregnancy (up to 10 years) and that it is after this period that the protective effect is observed (33). This suggests that the genes that change in the short term, which our model measures, may be associated with an increased, rather than a decreased, risk of breast cancer. This paradoxical finding may confound our search for markers of decreased risk. However, the identification of *STAT1* as down-regulated both in our model shortly after withdrawal from pregnancy E_2 levels and in parous women some years after pregnancy indicates that valuable markers of pregnancy-induced protection can be identified.

In conclusion, we have identified changes in gene and protein expression in nulliparous, normal human breast tissue treated with pregnancy levels of E_2 and correlated these with the effects of parity in premenopausal women. Our results show that many of the pregnancy-induced breast cancer-protective changes observed in rodent models also occur in human breast tissue following estrogen intervention and that *STAT1* expression is a potential biomarker of parity-induced breast cancer protection in the human breast.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank all the women who took part in this study for their participation and willingness to donate tissue for laboratory studies; the Department of Surgery at South Manchester University Hospitals for tissue collection; and Gail Bruder of the Biological Resources Unit and Emma Saunders of the Molecular Biology Core Facility (both at Paterson Institute for Cancer Research) for their excellent technical assistance.

References

- Clavel-Chapelon F. Differential effects of reproductive factors on the risk of pre- and postmenopausal breast cancer. Results from a large cohort of French women. *Br J Cancer* 2002;86:723–7.
- MacMahon B, Cole P, Brown J. Etiology of human breast cancer: a review. *J Natl Cancer Inst* 1973;50:21–42.
- Grubbs CJ, Peckham JC, Cato KD. Mammary carcinogenesis in rats in relation to age at time of *N*-nitroso-*N*-methylurea administration. *J Natl Cancer Inst* 1983;70:209–12.
- Rajkumar L, Guzman RC, Yang J, Thordarson G, Talamantes F, Nandi S. Short-term exposure to pregnancy levels of estrogen prevents mammary carcinogenesis. *Proc Natl Acad Sci U S A* 2001;98:11755–9.
- Thordarson G, Jin E, Guzman RC, Swanson SM, Nandi S, Talamantes F. Refractoriness to mammary tumorigenesis in parous rats: is it caused by persistent changes in the hormonal environment or permanent biochemical alterations in the mammary epithelia? *Carcinogenesis* 1995;16:2847–53.
- Eliassen AH, Tworoger SS, Hankinson SE. Reproductive factors and family history of breast cancer in relation to plasma prolactin levels in premenopausal and postmenopausal women. *Int J Cancer* 2007;120:1536–1.
- Greendale GA, Huang MH, Ursin G, et al. Serum prolactin levels are positively associated with mammographic density in postmenopausal women. *Breast Cancer Res Treat* 2007;105:337–46.
- Rajkumar L, Kittrell FS, Guzman RC, Brown PH, Nandi S, Medina D. Hormone-induced protection of mammary tumorigenesis in genetically engineered mouse models. *Breast Cancer Res* 2007;9:R12.
- Russo J, Balogh GA, Heulings R, et al. Molecular basis of pregnancy-induced breast cancer protection. *Eur J Cancer Prev* 2006;15:306–42.
- Guzman RC, Yang J, Rajkumar L, Thordarson G, Chen X, Nandi S. Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy. *Proc Natl Acad Sci U S A* 1999;96:2520–5.
- Ginger MR, Gonzalez-Rimbau MF, Gay JP, Rosen JM. Persistent changes in gene expression induced by estrogen and progesterone in the rat mammary gland. *Mol Endocrinol* 2001;15:1993–2009.
- D'Cruz CM, Moody SE, Master SR, et al. Persistent parity-induced changes in growth factors, TGF- β 3, and differentiation in the rodent mammary gland. *Mol Endocrinol* 2002;16:2034–51.
- Blakely CM, Stoddard AJ, Belka GK, et al. Hormone-induced protection against mammary tumorigenesis is conserved in multiple rat strains and identifies a core gene expression signature induced by pregnancy. *Cancer Res* 2006;66:6421–31.
- Clarke RB, Howell A, Anderson E. Estrogen sensitivity of normal human breast tissue *in vivo* and implanted into athymic nude mice: analysis of the relationship between estrogen-induced proliferation and progesterone receptor expression. *Breast Cancer Res Treat* 1997;45:121–33.
- Laidlaw IJ, Clarke RB, Howell A, Owen AW, Potten CS, Anderson E. The proliferation of normal human breast tissue implanted into athymic nude mice is stimulated by estrogen but not progesterone. *Endocrinology* 1995;136:164–71.
- Bramley M, Clarke RB, Howell A, et al. Effects of oestrogens and anti-oestrogens on normal breast tissue from women bearing BRCA1 and BRCA2 mutations. *Br J Cancer* 2006;94:1021–8.
- Wilson CL, Sims AH, Howell A, Miller CJ, Clarke RB. Effects of oestrogen on gene expression in epithelium and stroma of normal human breast tissue. *Endocr Relat Cancer* 2006;13:617–28.
- Potten CS, Watson RJ, Williams GT, et al. The effect of age and menstrual cycle upon proliferative activity of the normal human breast. *Br J Cancer* 1988;58:163–70.
- Guo S, Russo IH, Russo J. Difference in gene expression profile in breast epithelial cells from women with different reproductive history. *Int J Oncol* 2003;23:933–41.
- Srivastava P, Russo J, Russo IH. Chorionic gonadotropin inhibits rat mammary carcinogenesis through activation of programmed cell death. *Carcinogenesis* 1997;18:1799–808.
- Verlinden I, Gungor N, Wouters K, Janssens J, Raus J, Michiels L. Parity-induced changes in global gene expression in the human mammary gland. *Eur J Cancer Prev* 2005;14:129–37.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta CT)$ method. *Methods* 2001;25:402–8.

23. Russo J, Balogh GA, Russo IH. Full-term pregnancy induces a specific genomic signature in the human breast. *Cancer Epidemiol Biomarkers Prev* 2008;17:51–66.
24. LaMarca HL, Rosen JM. Estrogen regulation of mammary gland development and breast cancer: amphiregulin takes center stage. *Breast Cancer Res* 2007;9:304.
25. Schroeder JA, Lee DC. Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. *Cell Growth Differ* 1998; 9:451–64.
26. Robinson SD, Silberstein GB, Roberts AB, Flanders KC, Daniel CW. Regulated expression and growth inhibitory effects of transforming growth factor- β isoforms in mouse mammary gland development. *Development* 1991;113:867–78.
27. Brigstock DR. The CCN family: a new stimulus package. *J Endocrinol* 2003;178:169–75.
28. Lamote I, Meyer E, Massart-Leen AM, Burvenich C. Sex steroids and growth factors in the regulation of mammary gland proliferation, differentiation, and involution. *Steroids* 2004;69:145–59.
29. Li T, Sun L, Miller N, et al. The association of measured breast tissue characteristics with mammographic density and other risk factors for breast cancer. *Cancer Epidemiol Biomarkers Prev* 2005; 14:343–9.
30. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature* 2002;420:629–35.
31. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 2008; 135:1118–29.
32. Britt K, Ashworth A, Smalley M. Pregnancy and the risk of breast cancer. *Endocr Relat Cancer* 2007;14:907–33.
33. Schedin P. Pregnancy-associated breast cancer and metastasis. *Nat Rev Cancer* 2006;6:281–91.

Cancer Prevention Research

Normal Breast Tissue Implanted into Athymic Nude Mice Identifies Biomarkers of the Effects of Human Pregnancy Levels of Estrogen

Rognvald N. Blance, Andrew H. Sims, Elizabeth Anderson, et al.

Cancer Prev Res 2009;2:257-264. Published OnlineFirst March 3, 2009.

Updated version Access the most recent version of this article at:
doi:[10.1158/1940-6207.CAPR-08-0161](https://doi.org/10.1158/1940-6207.CAPR-08-0161)

Cited articles This article cites 33 articles, 10 of which you can access for free at:
<http://cancerpreventionresearch.aacrjournals.org/content/2/3/257.full#ref-list-1>

Citing articles This article has been cited by 4 HighWire-hosted articles. Access the articles at:
<http://cancerpreventionresearch.aacrjournals.org/content/2/3/257.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerpreventionresearch.aacrjournals.org/content/2/3/257>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.