In vitro expansion of human breast cancer epithelial and mesenchymal stromal cells: optimization of a coculture model for personalized therapy approaches

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Abstract
Molecularly targeted, customized therapies are designed based on the molecular portraits of cancer tissue. The efficacy of targeted therapy in individual patients depends on the contribution of single individual cancer cells within the context of their microenvironment. We have developed an in vitro model of human mammary epithelial-stromal cocultures to answer specific clinical questions related to breast cancer, to provide a tool with which to identify a signature in each breast tumor, and to identify the metabolic molecular targets of therapy in an attempt to optimize the efficacy of targeted therapy in each patient. Fifty-five human breast cancer samples were obtained through surgery. Epithelial and stromal cells were isolated from tissue specimens by differential centrifugation, and cryopreserved. Western blot analysis and RT-PCR were used to identify the tissue-specific expression patterns of cancer cells. Dose-response curves were constructed for the aromatase inhibitor formestane and for herceptin, and a 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide assay was done for combined treatment. We collected and cryopreserved, for future use, viable living cells from 55 breast tumor specimens from which we derived short-term cocultures. The presence of cytokeratins and vimentin was evaluated in 20 samples, and pHER2/neu and aromatase were evaluated in 4 cocultures. Formestane and herceptin had a cumulative growth-inhibitory effect on cocultures expressing epidermal growth factor receptors and aromatase. The in vitro model of human mammary epithelial-stromal co-cultures reported herein can be used to examine, and to store, a patient’s tumor-derived, living cells that retain the characteristics of the mother-tissue and respond, in vitro, to therapy. [Mol Cancer Ther 2007;6(12):3091–100]

Introduction
Cancer development depends on changes in the heterotypic interactions between incipient tumor cells and their normal neighbors. Once formed, all types of human tumors continue to harbor complex mixtures of several cell types that collaborate to create malignant growth. This reconceptualization of the cancer cell biology has changed the way we study this disease experimentally. Continuing elucidation of cancer pathogenesis depends increasingly on heterotypic organ culture systems in vitro and evermore refined mouse models in vivo. These systems help us to chart elusive goals. However, cancer biology and treatment will depend on defining all the signals exchanged between the various cell types existing symbiotically within a tumor mass and knowing the effects they exert on the integrated circuits of each of those cell types (1).

In breast cancer, there is compelling evidence that fibroblasts surrounding cancer epithelial cells can influence epithelial transformation by producing paracrine factors. In addition, mutations in stromal fibroblasts can precede carcinoma development. The tissue specificity of stromal-epithelial interactions accounts for a tissue- and cell-type-specific role of the microenvironment in carcinoma development (2). Fibroblasts are prominent modifiers of cancer progression (3), but our knowledge of the role of resting and activated fibroblasts in cancer is still evolving, and evidence is accumulating that a subpopulation of fibroblasts—the so-called cancer-associated fibroblasts—are important promoters of tumor growth (4–6).

Overviews on breast cancer prognostic factors stress the importance of personalized treatment, and recommend a fundamental change in adjuvant systemic therapy towards endocrine responsiveness (7, 8). Tumors have been classified into three types: endocrine-responsive, in which the primary treatment should be endocrine; endocrine nonresponsive, in which endocrine therapy should not be used; and an intermediate group, for which both endocrine and other therapies should be offered. Several trials showed the efficacy of targeted therapy with trastuzumab in
appropriately selected patients (9) and emphasize how new information influences algorithms for selecting adjuvant therapy in a rapidly changing environment, and how clinical decisions must balance the potential harm of failing to tailor treatment against those of overinterpretation (10–12). However, the majority of patients who achieve an initial response to trastuzumab-based regimens in metastatic breast cancer generally acquire resistance within 1 year (9).

In the case of postmenopausal breast cancer, clinical decisions should account for the power of local estrogen biosynthesis. In postmenopausal women, neither testosterone nor estradiol function to any extent as a circulating hormone. Both are mainly formed locally in target tissues, becoming active and metabolized therein (13). It has been determined that the concentration of estradiol present in the breast tumors of postmenopausal women is at least 20-fold greater than that present in the plasma. Aromatase inhibitor therapy causes a precipitous drop in the intratumoral concentrations of estradiol and estrone, and a corresponding loss of intratumoral aromatase activity. This indicates that aromatase acts within the tumor and the surrounding breast adipose tissue, and is responsible for the high tissue concentrations of estradiol. Hence, in the case of breast tumors, it is likely that circulating estrogen levels are minimally responsible for the relatively high endogenous tissue estrogen levels; rather, the circulating levels reflect the sum of local formation in its various sites. This is a fundamental concept for the interpretation of relationships between circulating estrogen levels in postmenopausal women and estrogen insufficiency or excess in specific tissues (14).

Isolated breast cells are a suitable in vitro model for the study of metabolism and cytotoxicity of xenobiotics. In breast cancer endocrine therapy, xenobiotic-metabolizing enzymes differ quantitatively and qualitatively between breast cancer patients, and this can result in differences in metabolic patterns, and in the efficacy and toxicity of inhibitors (15). Therefore, human breast epithelial and stromal cells represent an appropriate means with which to study the metabolism of agents such as selective estrogen receptor modulators and aromatase inhibitors.

Personalized therapy is based on molecular portraits of single individual cancer cells within their microenvironment. In this context, we developed an in vitro model of human mammary epithelial-stromal cocultures that can be used to examine and to store a patient’s tumor-derived, living cells.

Materials and Methods
Materials and Control Cells
MCF-7 and MDA-MB231 were from American Type Culture Collection. Human skin fibroblasts were donated by Drs. S. Coccozza and A. Monticelli (University of Naples “Federico II”). Fetal bovine serum (FBS) was purchased from Life Technologies (Invitrogen). Charcoal dextran–stripped FBS (DCC-FBS) was obtained with Norit A charcoal (Sigma) and dextran T70 (Sigma) according to the manufacturer’s instructions (Sigma-Aldrich). The final concentration of the 6H mixture was epidermal growth factor (10 ng/mL), hydrocortisone (1 μg/mL); insulin (5 μg/mL), estradiol (10 ng/mL), transferrin (5 μg/mL), T3 (10 μmol/L), all from Sigma-Aldrich. Trastuzumab (herceptin) from Genentech, estradiol, hydrocortisone, and testosterone (Sigma) were dissolved in 70% ethanol. The aromatase inhibitor formestane (Sigma) was dissolved in chloroform at 50 mg/mL.

Tumor Samples
After informed consent was obtained, breast cancer tissues were obtained from 55 patients affected by early breast cancer, median age 59.5 years (range, 36–84) undergoing surgery at the Azienda Ospedaliera Universitaria Federico II (Naples, Italy). Patients were 87% estrogen receptor-α-positive (≥10% by immunohistochemistry), 80% progesterone receptor-positive (≥10% by immunohistochemistry), 22% HER2 3+, 54% node-negative, and 42% node-positive, nodal status not assessed (Nx) in 4%. The Azienda Ospedaliera Universitaria Federico II Ethics Committee approved this study. In some cases of quadrantectomy (17 of 55), normal tissue from the same patient was also obtained and pathologically classified as healthy. Patients did not receive neoadjuvant or adjuvant endocrine therapy.

Isolation of Primary Epithelial and Stromal Cells from Breast Specimens
Tumor specimens were processed within 1 to 2 h of surgery. Each sample was minced in small fragments (<1 mm³), in a sterile environment. Tissue fragment were then digested at 37°C, for 2 to 4 h in a solution of type IV collagenase, 1 mg/mL (Sigma-Aldrich), containing 40 mg/mL of bovine serum albumin, 2 mg/mL of glucose, 100 units/mL of penicillin and 100 mg/mL of streptomycin (P+S; Sigma-Aldrich), 50 mg/L of gentamicin (Sigma-Aldrich), and 1.25 mg/L of Fungizone (Life Technologies). After incubation, the samples were extensively rinsed with PBS and suspended in culture media. Each sample was divided into two aliquots, one to be frozen and one to be cultured. Cells underwent three cycles of centrifugation and were differentially separated by centrifugation as reported elsewhere (16) using the algorithm depicted in Fig. 1. Briefly, increasing speeds of centrifugation (40–100–200 × g) generated three cell populations: epithelial breast–enriched (EB), stromal breast–enriched (SB), and “organoid substance” (OS). The cells were seeded separately in 24-wells at a density of 10⁵ cells/well and cultured in standard medium: minimal essential Dulbecco/Ham’s F12 (1:1; DMEM/F12 medium; Sigma-Aldrich) without phenol red, supplemented with 2 mmol/L of glutamine (Sigma-Aldrich), P+S, 15 mmol/L HEPES (Sigma-Aldrich), and with different percentages of FBS and hormones and growth factors. EB and OS cells were cultured in 0.5% FBS and a mixture of six supplements (6H) for at least 15 days. SB were cultured in 10% FBS medium. Because after 15 days, the growth rate of EB cells was very low or null, we enzymatically [trypsin-EDTA solution (Cambrex) for 2 min at 37°C] detached and seeded the cells on top of the
sister semiconfluent SB dish. Thus, we obtained a coculture (K no.) in which SB cells served as a nongrowing substrate for EB cells, in a medium supplemented with 0.5 FBS + 6H. This method, previously used for MCF7 cells (17, 18), favors the growth of epithelial cells in culture. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2. The medium was renewed twice weekly. The two cell types were easily identified by their morphology.

**Western Blot Analysis**

Western blots were done with extracts of cocultures (K ID no.) as indicated. MCF7 cells, MDA-MB-231, and human skin fibroblasts served as controls. Protein preparations from tissues and cells were obtained by lysing samples in 50 mmol/L of TRIS (pH 7.5), 100 mmol/L of NaCl, 1% NP40, 0.1% Triton, 2 mmol/L of EDTA, 10 μg/mL of aprotinin, and 100 μg/mL of phenylmethylsulfonyl-fluoride. Protein concentration was measured by the Bio-Rad protein assay (19). Polyacrylamide gels (8–15%) were prepared as described by Laemmli (20). Prestained molecular weight standards were from Bio-Rad (Milan). Proteins separated on the polyacrylamide gels were blotted on a nitrocellulose membrane (Hybond-C pure; Amersham Italia). The membrane was stained with Ponceau S (Sigma) to enable us to evaluate the success of transfer, and to locate the molecular weight markers. Free protein binding sites on the nitrocellulose were blocked with nonfat dry milk and a Tween 20/TBS solution. The membranes were washed and stained with specific primary antibodies and with secondary antisera, and then conjugated with horseradish peroxidase (Sigma-Aldrich) diluted 1:2,000. The monoclonal anti-pancytokeratin, vimentin, was purchased from Sigma-Aldrich, and anti-pNeu was from Cell Signaling Technology. The luminescent signal was visualized with the ECL Western blotting detection reagent kit (Amersham) and quantified by scanning with a Discover Pharmacia scanner equipped with a Sun Spark Classic Workstation.

**Selection of Cocultures and Doses for Growth Inhibition Experiments**

Growth inhibition experiments were done on 4 of 55 selected cocultures derived from the corresponding specimen (K ID no.). For growth experiments with drugs, FBS was substituted with DCC-FBS supplemented with testosterone at 10^-7 mol/L as a substrate for aromatase. To experimentally ascertain the dose of drugs required to attain a submaximal effect, dose-response curves were constructed according to a "fixed dose method", as previously described (21, 22). The growth curve responses of the control cell lines and of cocultured cells were evaluated after treatment with different concentrations of formestane or herceptin alone. The fraction corresponding to 50% (ED50) was found to be suitable for combined experiments. Cells were thus assigned for treatment with the ED50 dose.

**Isolation and storage procedure**

![Flow-chart (algorithm) of cell isolation, selection, and storage and phase-contrast microscopy of cocultures showing the coexistence of the two cell types (bar, 50 μm).](image)

**Figure 1.** Flow-chart (algorithm) of cell isolation, selection, and storage and phase-contrast microscopy of cocultures showing the coexistence of the two cell types (bar, 50 μm).
of formestane combined with increasing doses of herceptin and vice versa. The ED\textsubscript{50} computed by the individual dose-response curves, was plotted on the vertical and horizontal axes, respectively. The theoretical dose-effect line, including its 95% fiducial limits, was obtained to calculate the dose to be used, and resulted in a dose of 70 nmol/L of formestane, and 0.7 \( \mu \)g/mL of herceptin.

**Growth Inhibition Experiments**

Cell viability after treatment was determined by the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide assays as previously described (23), and growth curves. For the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide assay, SB cells (1 \( \times 10^5 \) cells/0.2 mL) were plated in 96-well plates and kept in the incubator at 37\(^\circ\)C. After 24 h, 2 \( \times 10^5 \) EB cells were seeded on the top of sister SB cells in 5% FBS. Forty-eight hours later, cocultures were washed with PBS and starved for a further 24 h in 0.5% FBS. Cells were then treated with the same medium supplemented with ED\textsubscript{50} (70 nmol/L) of formestane alone, and ED\textsubscript{50} (0.7 \( \mu \)g/mL) of herceptin alone, or formestane plus herceptin and incubated for a further 48 h. Cells were then washed, collected in tubes, and centrifuged at 1,000 rpm for 5 min. Supernatants were removed and the cells were resuspended in medium. Control cells were processed in the same way. A 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide stock solution was made by dissolving the crystals in phenol red–free DMEM/F12 medium to a final concentration of 1 mg/mL, and 25 \( \mu \)L of this solution was added to each well containing suspended cells in 200 \( \mu \)L of medium. After an additional 4-h incubation at 37\(^\circ\)C, cells were collected by aspiration and centrifuged at 1,500 rpm for 7 min. The converted dye was extracted from the supernatant obtained by adding acidic isopropanol (0.1 mol/L HCl) to the pelleted cells. absorbance ratio at 260:280 nm. RNA was stored at -70\(^\circ\)C in aliquots of 50 ng/mL. Total RNA (1 \( \mu \)g) from the cells was converted to first-stranded cDNA primed with a random hexamer in a 50 \( \mu \)L reaction volume using a RNA PCR kit (ImProm-II Reverse Transcription System; Promega). The primer pair and RT-PCR conditions used for aromatase mRNA amplification has been described by others (24). The sense and antisense primers used in PCR amplification for aromatase were GAATATTGGAAG-ACATCGCTCAGACACCA- and GGTTAAAGATCATTTCCAGCATGT, respectively. For the semiquantitative analysis, the PCR reactions consisted of 35 cycles (52\(^\circ\)C for 1 min). Amplification cycles were preceded by a denaturation step (95\(^\circ\)C for 5 min) followed by an elongation step (72\(^\circ\)C for 10 min). PCR products measured 293 bp and were analyzed using 2% polyacrylamide gels and ethidium bromide staining. Staining intensity was quantified using a Spectroline Transilluminator Model TR365. The expression levels of individual mRNA bands were normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA. The sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase were ACATCGCTCAGACACCA-TGG and GTAGTTGAGGTGCAAGGG, respectively, and PCR was done for 29 cycles. RT-PCR was done under conditions of linearity to obtain semi quantitative amplification responses.

**Data Analysis**

Each experiment was repeated two to four times and found to be reproducible. Human tissue samples were not pooled, therefore, each sample served as its own control. We used the Prism 3.0 software (GraphPad Software Inc.) to generate graphs and for statistical analyses. Error bars are presented as SE. We used ANOVA to identify statistically significant differences among means. Data were analyzed according to Tallarida (25).

**Results**

**Isolation of Breast Stromal and Epithelial Cells**

The cell types were separated according to size (see algorithm in Fig. 1). Tumor specimens were divided into two aliquots, one to be frozen and stored and one to be processed. The latter was minced in small fragments and then enzymatically digested, and suspended in culture media. Each digested sample was divided into two aliquots, one to be frozen and one to be cultured. Cells were differentially separated by three cycles of centrifugation. This technique separates three kinds of samples on the basis of the size of the suspended material: epithelial breast cells, stromal breast cells, and organoid substance constituted by tissue fragments containing cells but not completely digested. Because clonal dilution affects the efficiency of plating of primary cultures, we did not clone isolated cells after centrifugation. Consequently, we obtained EB and SB cells, which we seeded separately at a high density. We used this procedure to obtain cells from cancerous tissues and, when available after quadrantectomy or mastectomy, from normal mammary tissue of the same patient. To generalize the culture conditions on 10 of 55 randomly selected specimens, we tested the efficacy on growth rate (measured as cell number) of different media supplemented with different percentages of FBS and various combinations of hormones and growth factors (0.5% FBS, 5% FBS, 5% DCC-FBS, 10% FBS, 10% DCC-FBS, each with or without 6H or various combinations of E2, T3, epidermal growth factor, transferrin, cortisol, and insulin, respectively). We found that EB grew better in

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5% of FBS and 6H, whereas SB grew better in 10% of FBS medium. After 15 days, we detached the cells and seeded them on the top of the sister semiconfluent SB dish, thereby resulting in a coculture in which the SB served as a substrate for the EB cells in 5% FBS + 6H combinations. The two cell types were easily identified by their morphology (shown in Fig. 1). After being cocultured for a further 15 days, the cells were collected for Western blot analysis. During this period, one may carry out growth inhibition tests with drugs in a defined medium, with stripped serum or 0.1% to 0.5% of serum, or with 0.1% bovine serum albumin, supplemented with a single or multiple growth factors and hormones. In preliminary experiments, we tested whether the media conditioned by epithelial cells affected the growth of stromal cells and vice versa (data not shown), but we were not able to stimulate cell proliferation. This reinforces the concept that cell-to-cell contact between epithelial and stromal cells is needed for growth.

Cocultures Can Be Cryopreserved

Storage of Breast-Derived, Normal and Cancer Viable Living Cells. Cryopreservation of tissue does not always yield viable cells that have a high rate of adherence to culture dishes after freezing/thawing, and the success rate also depends on ATP status, whereas cryopreservation of isolated cells is routinely carried out and almost 100% of cells are viable after thawing. Because our procedure entails the immediate separation of epithelial from stromal cells, different culture media can be used, each of which is optimal for the maintenance of each cell type. Moreover, living isolated cells can be frozen. With our protocol, cells can also be isolated from the very small amount of tumor remaining after clinical tests. Depending on specimen size, five samples of the specimen per patient could be stored: nondigested tissue, digested fragments, organoid substance, stromal breast, and epithelial breast (Fig. 1, 1–5, respectively). To evaluate the viability of frozen samples, we tested 20 randomly selected samples after freezing for a

![Figure 2](image-url)
period ranging from 1 week to 1 year. We also tested the viability of cells after freezing digested fragments and submitting them to differential centrifugation after thawing. The percentage of viable cells per thawed sample ranged from 70 to 100 cells. All the thawed samples gave rise to viable cells.

Cocultures Maintain the Characteristics of the Tissue. Stromal and epithelial cells were easily identified in coculture by their morphology (Fig. 1). Western blot analysis of the 20 thawed samples confirmed the expression of vimentin, CK8, CK18, and CK19 expression in cocultured cells, which indicates that both cell types have preserved their original cytoskeletal scaffolds. All cells were identified using a pancytokeratin monoclonal antibody. Differential expression of vimentin and CK8, CK18, and CK19 in cocultured cells as compared with fibroblasts, MDA-MB231, and MCF-7 reference cell lines is shown in Fig. 2A and B. Table 1 summarizes the cytokeratins and vimentin expression of all cocultured cells.

To evaluate whether cell viability and attachment could depend on tissue features, we chose four samples with different clinicopathologic features for further characterization. We chose both premenopausal and postmenopausal cases which differed in histology, ductal, lobular, and mixed morphology, variegate tumor-node-metastasis (T1–T2, N0–N1, all M0) and grade (G1–G2), all estrogen receptor–positive, and from 0% to 90% progesterone receptor expression, and both c-erbB positive and negative. In agreement with the clinicopathologic features, whereas only specimen no. 54 was positive for the c-erbB receptor (Table 2), Western blot analysis showed that sample no. 54 was positive for the phosphorylated form of the receptor, pNeu (Fig. 2C). Semiquantitative RT-PCR showed aromatase mRNA in all the selected cocultures, thereby confirming the presence of viable stromal cells (Fig. 2D).

Aromatase Inhibitor Formestane and Herceptin Inhibit Cell Growth. To determine the combined effect of formestane and herceptin, we carried out a preliminary assay to establish the dose of drugs that resulted in a submaximal effect. To this aim, we constructed dose-effect graphs on the control cell lines MDA-MB231 (Fig. 3), MCF7, and fibroblasts (data not shown). For growth curves, we carried out parallel triplicate experiments by plating 1 × 10^5 SB cells/well in six-well plates. After 24 h, 2 × 10^5 EB cells were seeded on the top of sister SB cells in 5% FBS. Forty-eight hours later, cocultures were washed with PBS and starved for a further 24 h in 0.5% FBS. Cells were then washed with PBS and treated with DCC-FBS supplemented with formestane alone, herceptin alone, or formestane plus herceptin, and were incubated for the times indicated. Cells were then detached and counted in a hemocytometer chamber. For growth experiments with drugs, culture medium was supplemented with testosterone as a substrate for aromatase, because higher levels of testosterone were required to provide an adequate substrate for estrogen biosynthesis. The growth inhibition responses were evaluated after treatment with different concentrations of formestane (50–150 nmol/L; Fig. 3, top left) or herceptin (0.1–10 μg/mL) alone (Fig. 3, top right). The ED_{50} was found to be suitable for combined

![Table 1. Cytokeratins (CK8, CK18, and CK19) and vimentin expression in cocultured and control cells](image-url)
experiments. Cells were thus assigned to receive treatment consisting of the ED_{50} dose of formestane combined with increasing doses of herceptin and vice versa. The ED_{50}, computed by the individual dose-response curves, was plotted on the vertical and horizontal axes, respectively. From the theoretical dose-effect line, we calculated the dose to be used, which was 70 nmol/L of formestane, and 0.7 μg/mL of herceptin (Fig. 3, bottom left). Consequently, we used these doses for combined experiments with drugs.

Cells were treated, as in the growth experiments, with formestane alone, herceptin alone, or formestane plus herceptin, and analyzed by the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide assay. The percentage of absorbance of treated samples versus untreated controls is reported as a percentage of viable cells/controls. Figure 3 (bottom right) shows the effect of combined treatment on control cells MDA-MB231. The treatment with single agent formestane or herceptin and the combination of both had no effect on fibroblast control cells and MCF7 (data not shown). Figure 4 shows the graphs of combined treatment

### Table 2. Clinicopathologic features of tumor specimens selected

<table>
<thead>
<tr>
<th>ID</th>
<th>Age of surgery</th>
<th>Menopause age</th>
<th>Histologic type</th>
<th>Tumor size, histologic grade, lymph</th>
<th>Ki-67 (%)</th>
<th>ER (%)</th>
<th>PgR (%)</th>
<th>c-erbB</th>
</tr>
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<tr>
<td>K34</td>
<td>51</td>
<td>no</td>
<td>IDC</td>
<td>pT_{1a}, G_{2}, N_{0}</td>
<td>7</td>
<td>80</td>
<td>90</td>
<td>Negative</td>
</tr>
<tr>
<td>K40</td>
<td>57</td>
<td>52</td>
<td>ILC</td>
<td>pT_{1a}, G_{3}, N_{0}</td>
<td>25</td>
<td>80</td>
<td>20</td>
<td>Negative</td>
</tr>
<tr>
<td>K43</td>
<td>66</td>
<td>46</td>
<td>IDC + mucin Ca</td>
<td>pT_{2}, G_{3}, N_{0}</td>
<td>10</td>
<td>80</td>
<td>80</td>
<td>Negative</td>
</tr>
<tr>
<td>K54</td>
<td>81</td>
<td>56</td>
<td>IDC + tub-diff</td>
<td>pT_{1a}, G_{2}, N_{1}</td>
<td>25</td>
<td>80</td>
<td></td>
<td>Negative+++</td>
</tr>
</tbody>
</table>

Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; mucin Ca, mucinose carcinoma; tub-diff, focal tubular differentiation; Ki-67 index; ER, estrogen receptor; PgR, progesterone receptor; C-erbB2, C-erbB2 receptor.

**Figure 3.** Growth-inhibitory effects of single-agent treatment with formestane (A) and herceptin (B) on the growth of MDA-MB231 cell line. Cells were treated with the indicated concentration of each agent alone. Cells were counted on days 4 and 7. Triplicate data are expressed as the number of cells/well. The ED_{50} of each agent (C) was calculated; points, percentage of growth inhibition as compared with the growth of untreated control cells. Inhibitory effects of combined treatment with formestane, herceptin, and formestane plus herceptin (D) on the viability of control cells MDA-MB231. ED_{50} of each drug (formestane = 70 nmol/L; herceptin = 0.7 μg/mL) was chosen for combined experiments. Quadruplicate data are expressed as the percentage of growth inhibition as compared with the growth of untreated control cells. Columns, average of three different experiments; bars, SD. Formestane (F), herceptin (H), and formestane plus herceptin (F + H).
on the selected cocultures and reports how, with the same submaximal doses of drugs, the percentage of growth inhibition varied among samples depending on their individual molecular characteristics.

Discussion
Although the stages of human mammary epithelial cell differentiation and markers uniquely identifying differentiated and progenitor cells are not well defined, extensive studies confirm molecular breast tumor heterogeneity (26). We designed and developed an in vitro model of short-term cocultures of breast cancer cells to address specific clinical questions, to provide a tool with which to identify a signature in individual breast tumors, and to identify the metabolic molecular targets of therapy in the attempt to optimize the efficacy of targeted therapy in each patient. With the protocol reported herein, one can examine and store tumor-derived living cells that retain the characteristics of the mother-tissue and respond, in vitro, to therapy.

Tissue banking is an essential tool for future potential clinical applications, i.e., the identification of prognostic and predictive markers and testing new therapeutic agents (27, 28). In some model systems, a preincubation step in culture medium at 37°C before cryopreservation was efficient in increasing plating efficiency of cells after thawing (29, 30). The advantage of our model system is that one may store living viable cells from both tumor and nontumor tissues from the same individual even when the tumor tissue remaining after routine clinical tests is very small.

Primary cultures have a mixed cell composition and the relative percentage of each cell type in the culture is unknown. Clonal dilution affects the efficiency of plating of primary cultures and thus, it does not allow the separation of stromal cells from epithelial cells. On the contrary, differential centrifugation of digested fragments yields EB and SB cells that can be seeded separately at a high density. EB and SB cells proliferate slowly even in culture medium that is optimal for the maintenance of each cell type. In addition, our finding that medium conditioned by epithelial cells does not affect stromal cell growth and vice versa suggests that cell-to-cell interactions between epithelial and stromal cells are needed for growth. The two cell types were easily identified by their morphology, and by the expression of specific tissue markers. In fact, the cocultures expressed both the “epithelial” markers cytokeratins 8, 18, 19, and the “stromal” marker vimentin. During the short coculture, exponential growth of epithelial cells allows growth inhibition tests with drugs in a defined medium.

The growth inhibition experiments we performed on four selected cocultures suggest that some expression markers can be identified and could predict response to treatment.

![Figure 4](image)

**Figure 4.** Inhibitory effects of combined treatment with formestane (F), herceptin (H), and formestane plus herceptin (F + H) on the viability of cocultures K34, K40, K43, and K54. Quadruplicate data are expressed as the percentage of growth inhibition as compared with the growth of untreated control cells. Columns, average of three different experiments; bars, SD.
Amplification of Her-2/Neu in tumor tissue is now an established prognostic factor in breast cancer. In order to initiate signal transduction, erbB-receptor monomers need to form homodimers or heterodimers. It has been shown that Her-2/Neu is the preferred coexpression partner in node-positive tumors and thus the most likely dimerization candidate in malignant breast tumors. Moreover, no direct ligand has been identified for Her-2/Neu, but several studies suggest that Her-2/Neu receptor protein acts as a coreceptor that leads to the formation of homodimeric or heterodimeric complexes with other family members (31). Burden and Yarden reported at least 10 possible homodimeric and heterodimeric receptor combinations that elicit distinct biological responses to ligands, thus demonstrating the combinatorial complexity of signal transduction by erbB family members (32). Tyr1248 and Tyr1221/1222 are the major autophosphorylation sites in erbB2, and phosphorylation of these sites couples erbB2 to the Ras-Raf-MAP kinase signal transduction pathway (33). In our model, experiments with control cells MDA-MB231, which express the phosphorylated form of the pNeu/Her-2 receptor, showed that herceptin had a dose- and time-dependent inhibitory effect. This effect was absent when herceptin was added to coculture no. 54, although Western blot analysis showed phosphorylation of the pNeu/Her-2 receptor. These results reinforce the hypothesis that the extracellular blockade of the epidermal growth factor receptor domain is not sufficient to inhibit cell growth and that other factors such as gene mutations, activation of downstream signaling pathways or receptor polymorphisms may govern the response to treatment.

On the other hand, in postmenopausal breast cancer, activation of the estrogen receptor proliferative pathway of the epithelial breast cell depends on the aromatase activity of the surrounding adipose tissue that is responsible for high intratumoral concentrations of estradiol and estrone (34). Aromatase inhibitor therapy results in a precipitous drop in the intratumoral concentrations of estradiol and estrone together with a corresponding loss of intratumoral aromatase activity (26). We found aromatase mRNA in all our cocultures, thereby confirming the presence and activity of stromal cells, although growth inhibition experiments showed that the response varied among samples. The premenopausal state of patient no. 34 could be responsible for the meager effect of the drug whether alone or in combination.

In this scenario, although several predictive markers of response to breast cancer treatment are well established, this is not always the case, and the possibility of identifying additional molecular markers in a viable system derived directly from the patient is a better surrogate than nonviable systems, and are therefore more predictive of response to treatment.

The in vitro model of human mammary epithelial-stromal cocultures we developed can be used to examine, and store living normal and tumor cells derived from the same individual. The short period of culture ensures similarity in terms of expression markers, receptor status, and responsiveness with the tissue of origin. Cocultures maintain the characteristics of the tissue in expressing cytokeratins and vimentin, which confirms that these cells have preserved their original cytoskeletal scaffolds. The ability of this coculture system to surrogate the response to drugs used in the treatment of hormone-responsive breast cancer is shown by the experiments of growth inhibition in cocultures with agents able to interfere with the growth signals based on tumor-stromal interactions.

We have collected cancer specimens and isolated cancer cells from the primary breast tumors of patients undergoing clinical follow-up. The cocultures seem to be well-suited to the identification of molecular features that predict resistance/sensitivity to therapeutic agents. We found that with the same submaximal doses of drugs, the percentage of growth inhibition varied among samples depending on their individual molecular characteristics. This supports the concept that in a complex cellular function such as proliferation, the identification of some molecular features does not necessarily correspond to the real, at least in vitro, cellular response. The clinical follow-up, a more detailed characterization of these in vitro cells, the development of cell lines and the assessment of combinations of multiple targeted agents should lead to an understanding of breast cancer physiopathology and of the mechanisms of tumorigenesis, which in turn, should result in new therapies targeted to interfere selectively with one or more of these processes.

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In vitro expansion of human breast cancer epithelial and mesenchymal stromal cells: optimization of a coculture model for personalized therapy approaches

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