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# **MCF-10A-NeoST: A New Cell System for Studying Cell-ECM and Cell-Cell Interactions in Breast Cancer<sup>1</sup>**

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## Abstract

*Purpose:* There is a continuing need for genetically matched cell systems to model cellular behaviors that are frequently observed in aggressive breast cancers.

*Experimental Design:* We report here the isolation and initial characterization of a spontaneously arising variant of MCF-10A cells, NeoST, which provides a new model to study cell adhesion and signal transduction in breast cancer.

*Results:* NeoST cells recapitulate important biological and biochemical features of metastatic breast cancer, including anchorage-independent growth, invasiveness in threedimensional reconstituted membranes, loss of E-cadherin expression, and increased tyrosine kinase activity. A comprehensive analysis of tyrosine kinase expression revealed overexpression or functional activation of the Axl, FAK, and EphA2 tyrosine kinases in transformed MCF-10A cells.

*Conclusions:* MCF-10A and these new derivatives provide a genetically matched model to study defects in cell adhesion and signaling that are relevant to cellular behaviors that often typify aggressive breast cancer cells.

## Introduction

Some of the most dangerous forms of cancer arise when a subset of tumor cells breaks away from the primary tumor, translocates to distant sites in the body, and multiplies in a different microenvironment (1, 2). At the cellular level, metastasis is a disease of aberrant cell adhesion and signal transduction (3–5). Metastatic breast cells decrease their cell-cell adhesions to detach from the primary tumor and increase interactions with the surrounding ECM<sup>3</sup> to facilitate migration and invasion (3). Detachment from the primary tumor results in part from decreased expression of E-cadherin, and increased ECM interactions result from changes in the expression and localization of integrin or ECM proteins (3, 6). These coordinated changes in adhesion also promote dissemination of metastatic cells (2, 7, 8).

Many lines of investigation have revealed a complex interplay between cell adhesion and signal transduction (for reviews, see Refs. 9–11). On one hand, the formation and stability of cellular adhesions are controlled by intracellular signals. In particular, increased levels of protein tyrosine phosphorylation regulate the organization and interactions of adhesion molecules (12). At the same time, sites of cellular adhesions are specialized sites of signal transduction (13). A well-developed body of literature has established that cell attachments to ECM convey signals that govern cell growth and migration, whereas cell-cell contacts generally inhibit these behaviors (2, 3, 11, 14). Consequently, changes in the local microenvironment, such as those that rise after transplantation of normal cells into a foreign microenvironment, disrupt vital signals and thereby compromise the viability of normal cells. One hallmark of cancer is that malignant cells have overcome these constraints and thus can grow and survive in a foreign microenvironment (5, 15). Increased understanding of these changes in adhesion and signaling is thus critical in understanding the fundamental causes of metastasis and could translate into new approaches for therapeutic intervention.

Several studies have linked metastasis with elevated tyrosine kinase activity (16, 17). Tyrosine kinases control tumor growth, invasiveness, and survival (16). Because tyrosine phosphorylation is also vital for the function and survival of normal cells, inhibitors of protein tyrosine kinase activity often cause toxicity (18). Thus, there is a need to identify and target tyrosine kinases that are selectively overexpressed in cancer cells. Most studies to identify tyrosine kinases that are relevant to cancer have compared cells derived from unrelated individuals (19, 20). However, differences among the donors, origin, passage number, and culture conditions generate enormous variations that may be unrelated to normal and malignant behaviors. One way to deconvolute such analyses is to compare malignant and nonmalignant variants from the same progenitor. Matched models of human mammary epithelial cells include variants of HMT-3522, 184A1, and MCF-10A cells (21–25). Whereas these systems have provided valuable information about mammary cell differentiation and transformation, none of these system models important biochemical and biological behaviors of aggressive breast cancer cells, such as loss of E-cadherin (7, 21). Consequently, there remains a need for cell models that reflect more aggressive breast cancers.

Our laboratory has been studying tumor cell adhesion and signaling in normal and transformed variants of MCF-10A cells (7). We have shown that oncogene transformation of MCF-10A cells destabilizes cell-cell adhesions and increases ECM interactions at focal adhesions (26–28). We linked these changes to increased tyrosine kinase activity and showed that tyrosine kinase inhibitors restore a normal phenotype to oncogene-transformed cells (27). However, oncogene-transformed MCF-10A cells generally do not recapitulate important features of advanced breast cancer, such as loss of E-cadherin and Matrigel invasion (23, 27). Here, we report that Ras-transformed MCF-10A cells undergo a spontaneous transition to a more aggressive phenotype when grown at high cell density and that this yields a cell line that mimics aggressive breast cancers.

## Materials and Methods

### Cells and Antibodies

All cells were cultured as described previously (27). Antibodies specific for E-cadherin FAK and phosphotyrosine (PY20 and polyclonal sera) were purchased from Transduction Laboratories (Lexington, KY). Fluorescein-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR). Paxillin antibodies were generously provided by Dr. K. Burrige (University North Carolina). Antibodies specific for EphA2, phosphotyrosine (4G10), FAK, and Met were purchased from Upstate Biologicals, Inc (Lake Placid, NY). Antibodies specific for Axl were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Western Blot Analysis

Detergent extracts of cell monolayers were harvested in Tris-buffered saline containing 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 0.5% deoxycholate, and 0.01% SDS (27). After measuring protein concentrations using Coomassie assays (Bio-Rad, Hercules, CA), equal amounts of cell proteins were immunoprecipitated as described and resolved by SDS-PAGE and transferred to nitrocellulose (Protran; Schleicher & Schuell, Keene, NH) as described previously

(29). Western blot analyses were developed using enhanced chemiluminescence (Pierce) and detected by autoradiography (X-OMAT; Kodak, Rochester, NY). To confirm equal sample loading, the blots were stripped and re probed with antibodies specific for vinculin as described (27).

### **Immunofluorescence Microscopy**

Immunofluorescence staining of cell monolayers was performed as described (29). Images were visualized using an Olympus BX-60 epifluorescence microscope and recorded onto 35-mm film (T-Max 400; Kodak). Interference reflection microscopy was performed using appropriate filters as described (30).

### **Cell Growth Assays**

To measure the rates of cell growth in monolayer culture,  $5 \times 10^4$  cells were incubated in tissue culture-treated 24-well dishes. At 24-h intervals, duplicate samples were suspended using trypsin solution (Life Technologies, Inc.), and the number of cells was enumerated microscopically using a hemocytometer. To measure focus formation,  $1 \times 10^6$  cells were incubated in a 60-mm tissue culture dish for 10–14 days, changing the media every 3 days. Focus formation was determined microscopically using an Olympus IX-70 and recorded onto 35-mm film (T-Max 400; Kodak). Colony formation in soft agar was performed as described (26). Colony formation was scored microscopically, defining clusters of at least three cells as a positive.

### **Cell Behavior in 3dRBM**

The behavior of cells in Matrigel was performed as described previously (31, 32). Briefly, tissue culture dishes were coated with Matrigel (Collaborative, Bedford, MA) at 37°C before adding  $1 \times 10^5$  cells. Cell behavior was assessed at 48-h intervals using an inverted light microscope (Olympus IX-70). All images were recorded onto 35-mm film (T-Max 400; Kodak).

### **Kinase Expression Profiling and Kinase Assays**

The expression levels of all known tyrosine kinases were evaluated in the MCF-10A variants as detailed (33). For *in vitro* kinase assays, immunoprecipitated EphA2 or FAK was washed in lysis buffer and incubated in 10 mM PIPES, 3 mM MnCl<sub>2</sub>, 5 mM PNPP (104 phosphatase substrate; Sigma Chemical Co.), 1 mM NaVO<sub>4</sub>, 1  $\mu$ M ATP, and 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]-ATP (New England Nuclear, Boston, MA) at 25°C for 5 min to facilitate autophosphorylation. The reactions were terminated by the addition of 5  $\times$  Laemmli sample buffer, and the samples were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose (Schleicher & Schuell) or Immobilon P (Pierce). To hydrolyze phosphoserine/threonine, the membranes were treated with 1N KOH, 65°C for 1 h, and reassessed by autoradiography. Incorporated radiolabel was detected by autoradiography. After five half-lives, the blot was subjected to Western blot analysis with appropriate antibodies to confirm equal sample loading.

## Results

### **Selection and Phenotype of NeoST Cells**

In the course of studying Ras-transformed MCF-10A cells (NeoT cells), we noted dramatic phenotypic changes after the cells reached confluence. Log phase cultures of NeoT cells adopted a characteristic “fried egg” morphology, forming numerous cell-cell contacts (Fig. 1). After reaching confluence, the entire population of NeoT cells transitioned to a spindle-like appearance and subsequently failed to organize into colonies. Once acquired, this phenotype was irreversible, even on introduction of fresh culture medium or subsequent passage of the cells at lower densities (data not shown). These changes did not represent the outgrowth of a contaminating population of cells because all of the NeoT cells converted to this phenotype in  $\leq 3$  days after reaching confluence. In the search for the causes of these changes, we were able to exclude that changes in the pH or composition of the culture medium (growth factor, serum depletion) were responsible for the transition (data not shown). However, the spontaneous transition to the NeoST phenotype did require prolonged ( $>2$  days) culture beyond confluence.

Our initial studies compared the two-dimensional and three-dimensional growth characteristics of nontransformed and transformed MCF-10A cells. In addition to the NeoT and NeoST cells, two nontransformed variants of MCF-10A cells were studied. Vector-transfected MCF-10A cells (Neo) provided a negative control for the transfection, whereas cells that overexpress wild-type Ras (NeoN) allowed for a comparison with nontransformed, but premalignant, MCF-10A cells. To compare the rates of anchorage-dependent growth,  $1 \times 10^5$  cells were incubated in monolayer culture, and samples were harvested and counted at 24-h intervals (Fig. 2A). The nontransformed and NeoT cells had doubling times of about 24 h, whereas the NeoST cells grew more slowly, with an average doubling time of 40 h.

Despite reduced growth rates in monolayer culture, the transformed character of NeoST cells became apparent when modeling anchorage-dependent constraints on cell growth. On reaching confluence, NeoT cells formed distinct foci of growth that consisted of light refractile cells. In contrast, NeoST cells did not form individual foci of growth, but instead, grew atop one another all throughout the culture. This multilayer growth caused cultures of NeoST cells to adopt a stratified-like morphology over time. Because focus formation, in part, requires anchorage-independent growth, we asked if NeoST cells would be capable of anchorage-independent growth in agar. To model this, MCF-10A Neo, NeoN, NeoT, or NeoST cells were suspended in soft agar, and colony formation was measured over the following 7–10 days (Fig. 2C). Although the nontransformed cells (Neo and NeoN) did not grow or survive under anchorage-independent constraints, NeoT and NeoST were able to colonize soft agar, with NeoST cells displaying the largest number of colonies. These results indicate that whereas NeoST cells have reduced levels of anchorage-dependent growth, they demonstrate a powerful growth advantage under three-dimensional, anchorage-independent constraints.

### **Altered Cell Adhesions**

Microscopic analysis revealed gross morphological differences between NeoT and NeoST cells. NeoT cells interacted with each other within stable colonies, whereas NeoST cells resisted cell-

cell contacts, even when cultured at high cell density (Fig. 1). The failure of NeoST cells to organize into colonies led us to measure their expression of E-cadherin, the primary cell-cell adhesion molecule in MCF-10A cells. Western blot analyses of whole cell lysates demonstrated that the levels of E-cadherin were greatly reduced in NeoST cells as compared with NeoT or nontransformed MCF-10A cells (Fig. 3A). Decreased E-cadherin expression in NeoST was confirmed by immunofluorescence microscopy and flow cytometry (data not shown).

The elongated morphology of NeoST cells also reflected increased cell interactions with the underlying substratum. Immunofluorescence staining with antibodies specific for paxillin, a cytoskeletal component of focal adhesions, demonstrated that NeoT and NeoST cells formed more numerous focal adhesions than nontransformed MCF-10A cells (Fig. 3B). The increased ECM attachments of transformed MCF-10A cells were also confirmed by interference reflection microscopy (data not shown). Consistent with increased focal adhesion formation, the actin cytoskeleton of NeoT and NeoST cells organized into stress fibers that spanned the basal cell surface (Fig. 4A, *top panels*). In addition, NeoST cells formed large lamellipodia, and actin failed to organize within sites of cell-cell contact, even when the NeoST cells were cultured at high cell density (Fig. 4 and data not shown).

The three-dimensional ECM microenvironment provides important physical linkages and signals that control mammary epithelial cell growth, differentiation, and survival (21). On the basis of the prominence of ECM attachments by transformed MCF-10A cells in two-dimensional (monolayer) culture, we asked how these cells would respond when cultured within a 3dRBM. To test this, the MCF-10A cell variants were embedded in Matrigel for 10 days (Fig. 5). Nontransformed MCF-10A cells organized into spherical colonies. NeoT cells also organized into spherical colonies, but these colonies were at least five times larger than the colonies formed by nontransformed cells (Neo or NeoN). Interestingly, NeoST cells failed to organize into spheres, but instead, formed tubular networks that radiated throughout the Matrigel. This phenotype resembled the behavior of aggressive breast cancer cells (*e.g.*, MDA-MB-435 and BT-549), which similarly formed tubular networks in Matrigel (data not shown).

### **Tyrosine Kinase Profile**

The mesenchymal behavior of transformed MCF-10A cells could be reversed using tyrosine kinase inhibitors (*e.g.*, herbimycin A). Treatment of NeoT or NeoST cells with herbimycin A restored a phenotype that more closely resembled nontransformed controls (Fig. 4, *bottom panels*). Whereas vehicle-treated NeoST cells resisted cell-cell contacts, herbimycin A-treated cells organized into cell colonies, even when cultured at low density. Herbimycin also altered the organization of the actin cytoskeleton, increasing the prominence of circumferential adhesion belts between cells while eliminating the lamellipodia that had been observed in NeoST cells.

On the basis of the restored epithelial morphology in response to herbimycin treatment, the levels of protein tyrosine phosphorylation were assessed in the MCF-10A variants. Western blot analysis of whole cell lysates with phosphotyrosine specific antibodies revealed that transformed MCF-10A cells (NeoT and NeoST) had elevated levels of protein tyrosine (Fig. 6A), with specific increases in the tyrosine phosphorylation of proteins with apparent molecular masses of 116 and 130 kDa. Identical results obtained multiple phosphotyrosine-specific

antibodies (PY20, 4G10, and polyclonal antiphosphotyrosine), thus confirming that the differential phosphotyrosine did not represent the unique antigenicity of any single antibody.

The elevated levels of tyrosine phosphorylation in NeoT and NeoST cells led us to conduct a comprehensive analysis of tyrosine kinases in MCF-10A cells (Table 1). The expression levels of all known tyrosine kinases were evaluated as described previously (33), revealing three groups of overexpressed tyrosine kinases. The first group consisted of Axl and c-Abl, which were detected at equivalent levels in NeoT and NeoST cells (*i.e.*, Neo < NeoT = NeoST). The overexpression of Axl was confirmed by Western blot analyses (Fig. 6B), and phosphorylated Axl accounted for much of the tyrosine phosphorylated band at 130 kDa (See Fig. 6A). The second group consisted of NK116B and MAKB, which were found at the highest levels in NeoST cells (*i.e.*, Neo < NeoT < NeoST). The third group reflected tyrosine kinases that were expressed at equivalent levels in nontransformed and transformed MCF-10A cells (Table 1). Unfortunately, a lack of specific antibodies for most of these kinases prevented confirmation of high protein levels in the transformed MCF-10A cells.

Tyrosine phosphorylated proteins are largely found within sites of cell-cell and cell-substratum adhesion (34, 35). Because tyrosine kinase activity was necessary for the mesenchymal-like adhesions of transformed cells, we focused on two adhesion-associated tyrosine kinases, FAK and EphA2 (36, 37). These kinases were emphasized because FAK is regulated by ECM adhesions, whereas EphA2 function is regulated by cell-cell adhesion (29, 36, 38–41). Although not overexpressed, the FAK in transformed MCF-10A cells was more activated (Fig. 6, B and C). The increased phosphotyrosine content of FAK in NeoT and NeoST cells was consistent with their increased substratum adhesions, and phosphorylated FAK accounted for much of the tyrosine phosphorylated material with an electrophoretic mobility of 116 kDa (See Fig. 6A).

EphA2 was overexpressed and functionally altered in transformed MCF-10A cells. Although EphA2 mRNA levels were equivalent in nontransformed and transformed MCF-10A cells (data not shown), Western blot analyses revealed more EphA2 protein in NeoT and NeoST cells (Fig. 7A). *In vitro* analyses confirmed that transformed MCF-10A cells had higher levels of EphA2 enzymatic activity than nontransformed cells (Fig. 7B). Notably, EphA2 enzymatic activity did not relate to its phosphotyrosine content. Despite high levels of enzymatic activity, the phosphotyrosine content of EphA2 was decreased in NeoT and NeoST cells. Decreased phosphotyrosine content corresponded with changes in EphA2 subcellular localization. Whereas EphA2 was enriched within sites of cell-cell contact in nontransformed cells, EphA2 was diffusely distributed in NeoT and NeoST cells. It is well established that EphA2 becomes tyrosine phosphorylated in response to ligand (Ephrin-A1) binding, which is notable because EphrinA1 is anchored to the surface of adjoining cells (40, 41). Thus, we postulated that decreased cell-cell contacts prevented EphA2 from binding its ligands. To test this, monolayers of NeoST cells were treated with EphrinA1-Fc, a recombinant and soluble form of ligand. EphrinA1-Fc treatment of NeoST cells induced vigorous tyrosine phosphorylation of EphA2, indicating that soluble ligand could correct for defective ligand binding. Thus, high levels of EphA2 contribute to the increased tyrosine kinase activity in NeoST cells despite its inability to bind endogenous ligands.



## Discussion

The major finding of this study is the development of a new cell model to study cell adhesion and signal transduction in breast cancer. We have shown that Ras-transformed MCF-10A cells reliably and irreversibly transition to a more invasive and mesenchymal phenotype on reaching confluence. The resulting NeoST cells display important hallmarks of aggressive breast cancer cells, including cell growth in soft agar, invasiveness in 3dRBM, and loss of E-cadherin expression. We have also identified tyrosine kinases that are overexpressed or functionally altered in transformed MCF-10A cells.

A number of human mammary epithelial cell models have been developed to study breast cancer (22, 24, 42). Nonmalignant, premalignant, and malignant variants of MCF-10A, 184A1, and HMT-3522 mammary epithelial cells have been successfully used to model mammary cell differentiation and oncogenesis (21, 23, 25). These cell lines provide genetically matched models to deconvolute problems that often plague comparisons of nonmalignant and tumor cells from unrelated donors. Whereas these have provided complementary models of mammary epithelial cells, some differences distinguish among the different systems. Until the isolation of NeoST cells, transformed variants of these cell systems did not display biochemical (*e.g.*, loss of E-cadherin) or cellular characteristics (*e.g.*, tubular network formation in 3dRBM) that were consistent with advanced stages of breast cancer. On the other hand, aggressive and metastatic human breast cell lines, such as MDA-MB-231 and MDA-MB-435, do not have matching normal cells. Thus, NeoST cells may provide an important, additional tool to model certain cellular behaviors that typify aggressive breast cancers.

An interesting feature of our present study is that the transition to the NeoST phenotype can be rapidly and reliably reproduced in culture. These changes could be useful for determining the causes of such transitions. HMT-3522 undergo a similar epithelial-to-mesenchymal transition in response to stromelysin-1 production, and it is notable that these cells have irreversibly lost E-cadherin (43–45). Future investigations could examine whether matrix metalloproteinases are up-regulated or contribute to the phenotypic alterations of NeoST cells. It is also important to note that the rapid conversion of NeoT cells to the NeoST phenotype could alter data interpretation if left unrecognized. Similarly, nontransformed MCF-10A cells can also convert to a distinct phenotype when cultured at high cell density or deprived of growth factors (epidermal growth factor or insulin).<sup>4</sup> The sensitivity of these changes also emphasizes the need for caution in designing and interpreting studies with MCF-10A cells.

Our studies also emphasize important differences that distinguish the growth of nonmalignant and malignant epithelial cells. Monolayer (two-dimensional) assays demonstrated reduced growth of NeoST cells relative to controls. A similar reduction in monolayer growth has been noted in MCF-10A cells transformed by either EphA2 or oncogenic PKC- $\alpha$  (46, 47). However, NeoST cells demonstrated a distinct growth advantage in soft agar. This finding is important because one hallmark of cancer is that metastatic cells can uniquely grow and survive independent of anchorage and in a foreign microenvironment (1, 15). The aggressive behavior of NeoST cells similarly was also more apparent in studies using 3dRBM. The tubular networks formed by NeoST cells in Matrigel were indistinguishable from structures formed by aggressive breast cancer cell lines (*e.g.*, MDA-MB-435 and BT-549; Ref. 19). This behavior is interesting

because tubular network formation in 3dRBM (collagen gels) is predictive of vasculogenic mimicry, a process by which metastatic cells can undergo angiogenesis independent of endothelial cells (48, 49). Together, our results with soft agar and 3dRBM indicate that three-dimensional measurements of tumor cell growth and invasiveness (*e.g.*, soft agar or 3dRBM) can be more predictive of malignant behavior than more standard two-dimensional analyses.

The NeoST cells reorganize their interactions with one another and with their local microenvironment in a manner that resembles aggressive, tumor-derived breast cancer cells, *e.g.*, NeoST cells have lost expression of E-cadherin, which is similarly lost in many aggressive breast cancers (6). On the basis of evidence that E-cadherin suppresses metastasis, there has been much interest in understanding the regulation of E-cadherin gene expression (50). The basis for decreased E-cadherin expression in NeoST cells remains unknown, but the rapid and reliable conversion of NeoT to NeoST may provide a model to study these events in the laboratory. Future studies should also begin comprehensive analysis of MCF-10A-NeoST cells using xenograft analyses to assess if these variants are capable of spontaneous metastasis *in vivo*.

Because elevated tyrosine kinase activity was necessary for the altered adhesions of NeoST cells, we identified tyrosine kinases that were overexpressed or functionally altered in transformed MCF-10A cells. The FAK kinase is activated in response to cell-ECM adhesion, which is consistent with the fact that FAK activation related to the substratum attachments of transformed MCF-10A cells. Although FAK is overexpressed in some breast cancers (51), FAK activation was increased in transformed MCF-10A cells independent of increased expression. FAK activation has been linked to a variety of cellular behaviors that are relevant to tumorigenesis and metastasis, including cell growth, survival, and migration (13, 52–54). Thus, it will be interesting to determine whether FAK contributes to the malignant behavior of NeoST cells.

EphA2 is another adhesion-associated tyrosine kinase that was overexpressed and enzymatically active in NeoT and NeoST cells. A recent study by one of us showed that EphA2 is overexpressed in a large number of breast cancers, where it functions as a powerful oncoprotein (47). The fact that NeoST cells overexpress EphA2 and form tubular networks in 3dRBM is also interesting in light of recent evidence that EphA2 overexpression is necessary for vasculogenic mimicry (55). Thus, future investigation should determine whether EphA2 contributes to the phenotypic alterations of NeoST cells and if NeoST cells might provide a cell system to study vasculogenic mimicry in breast cancer.

EphA2 is also functionally altered in transformed MCF-10A cells because unstable cell-cell contacts prevent interactions with its cell-attached ligands. Defective ligand binding has been observed in tumor-derived breast cancer cells, and EphA2 similarly retained enzymatic activity in these cells despite decreased ligand binding and phosphotyrosine content (29). Decreased ligand binding likely contributes to the malignant behavior of NeoT and NeoST cells because restoration of ligand binding blocks the growth and migration of metastatic cells (29, 47, 56). One possibility is that ligand binding changes EphA2 subcellular localization and thereby redirects EphA2 toward different substrates in nontransformed and transformed cells. Alternatively, changes in the phosphotyrosine content of EphA2 might link it to different downstream signaling pathways via interactions with signaling proteins with SH2 or PTB domains.

Axl expression is also significantly increased in NeoST cells. Axl is the founding member of a family of tyrosine kinases characterized by an N-CAM like extracellular domain (57, 58). As such, they may modulate the adhesive properties of a cell. This family of kinases can be activated by soluble ligand Gas6 or through homotypic binding (59). All three members of this family, including Axl, have been shown to transform fibroblasts (57, 60). We found elevated Axl levels after Ras transformation, which correlated with anchorage-independent growth, invasiveness, and decreased cell-cell adhesions of Ras-transformed epithelial cells (27). A role for Axl in mammary tumorigenesis is supported by evidence that Axl is overexpressed in ~25% of human breast cancers (61). Moreover, a recent study showed that Gas6, the ligand for Axl, induces growth of contact-inhibited C57 mammary cells (62). This induction is accomplished through activation of Axl, phosphatidylinositol 3'-kinase, Akt, and down-regulation of GSK3, resulting in the phosphorylation and stabilization of  $\beta$ -catenin. This report additionally implicates Axl with a role in tumor cell growth and cell adhesion. We (63) *et al.* (59, 62) showed that Axl protects cells from apoptosis. The potential role of Axl in invasiveness is underscored by a report that Axl mediates Gas6-directed chemotaxis (64). All these features make Axl a potential progression factor for breast cancer.

Our present study characterizes a variant of Ras-transformed MCF-10A cells, NeoST, and demonstrates that these cells can be used to model important biochemical and biological hallmarks of aggressive breast cancer. We have detailed defects in cell adhesion and signal transduction in malignant NeoST cells and have used new technologies to contrast tyrosine kinase expression in nontransformed and malignant cells. These findings indicate that NeoST cells may provide an important new model to understand the causes and treatment of aggressive breast cancers.

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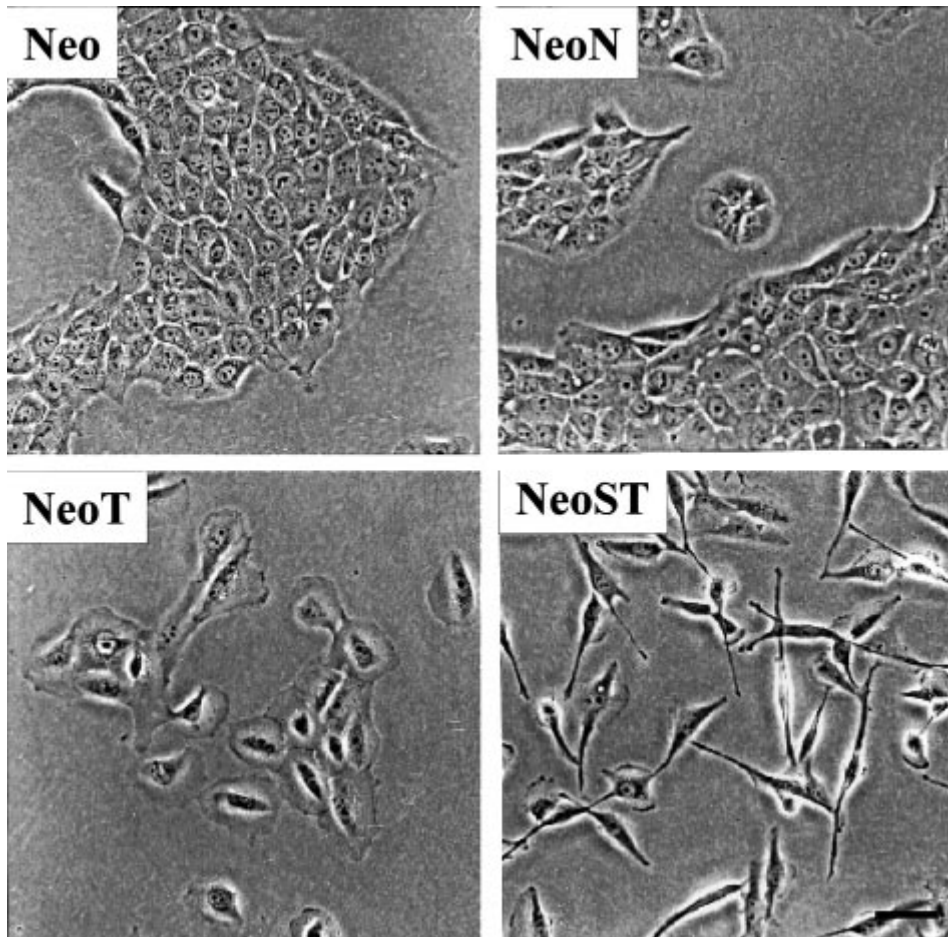
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## Figures and Tables

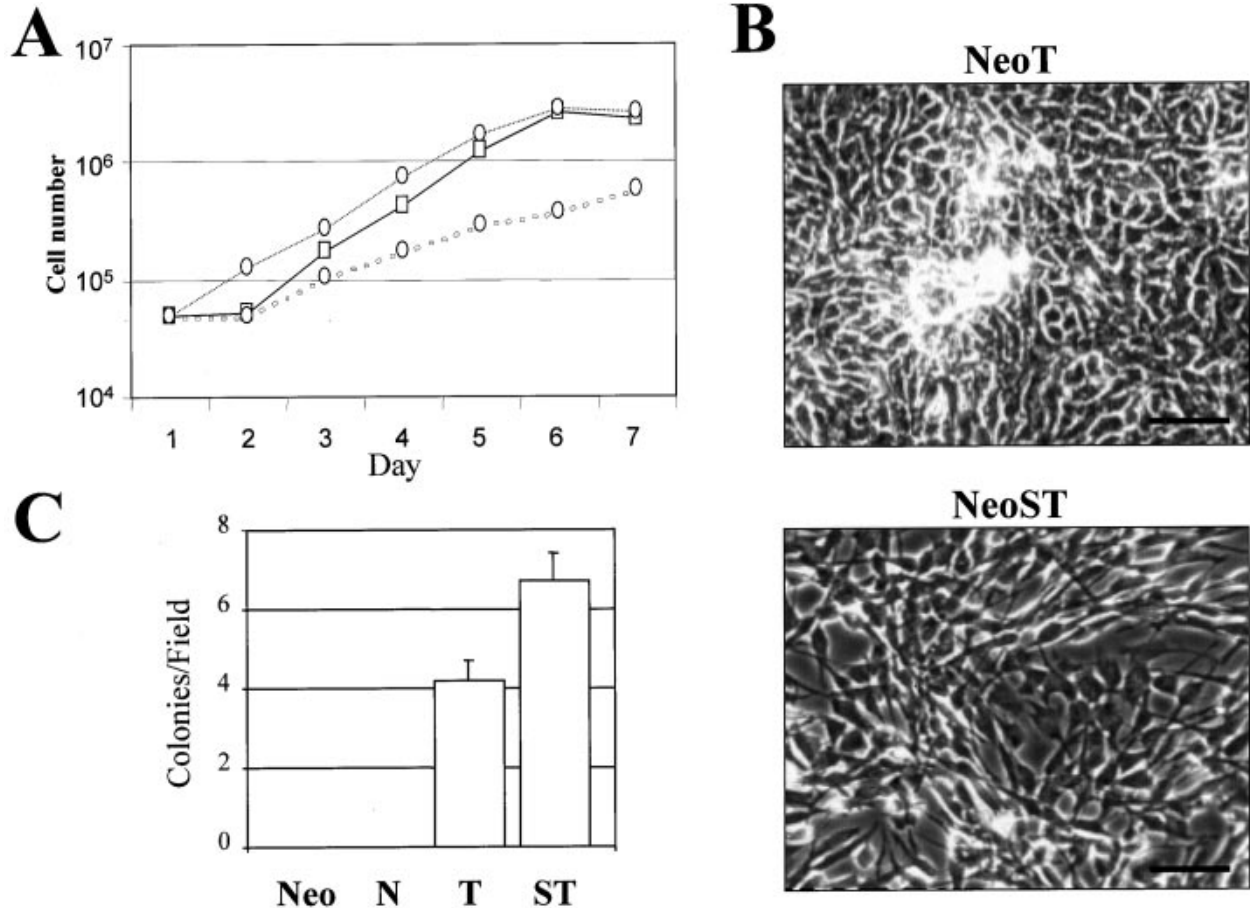
FIGURE 1



Morphology of MCF-10A-NeoST cells. MCF-10A cells, grown on coverslips in culture media overnight, were viewed using phasecontrast microscopy. Whereas nontransformed (*Neo* and *NeoN*) cells displayed an epithelial morphology, the morphology of Ras-transformed cells (*NeoT* and *NeoST*) were more stromal like. Note the spindle-like morphology of NeoST cells and their decreased cell-cell interactions within colonies. *Bar* = 50  $\mu\text{m}$ .

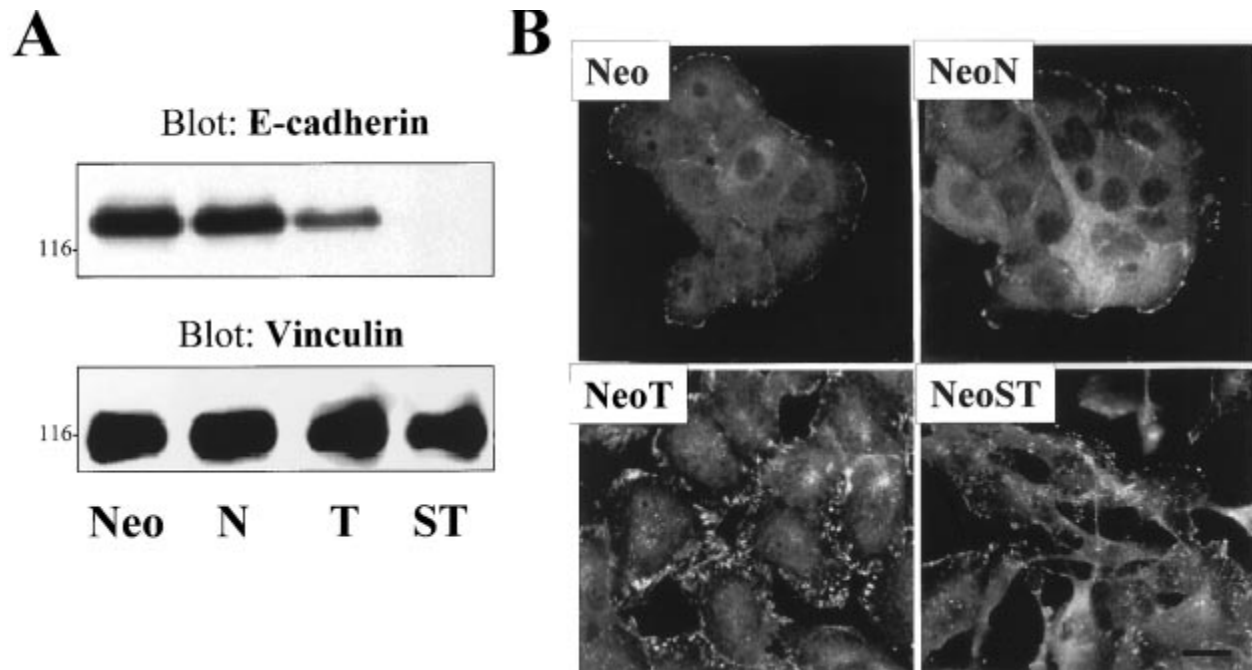


FIGURE 2



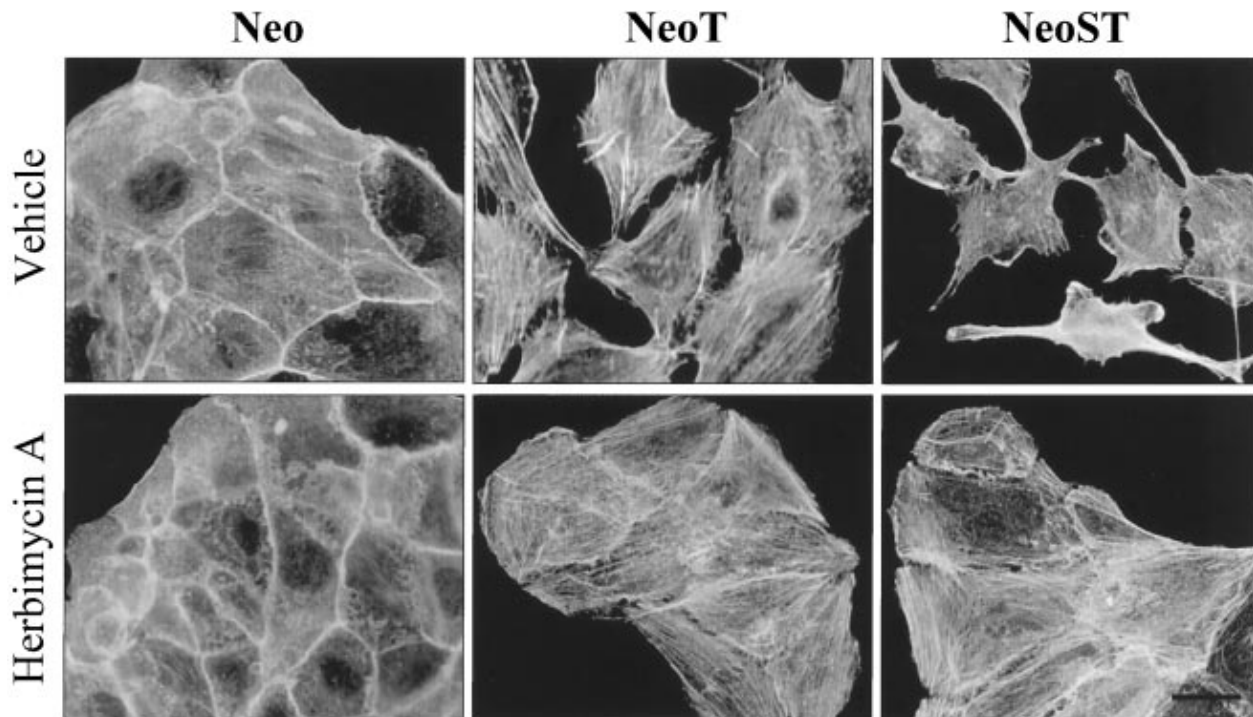
Growth characteristics of NeoST cells. In A, the anchorage-dependent (monolayer) growth of MCF-10A cells was measured at 24-h intervals after incubation of  $5 \times 10^4$  cells at day 0. Whereas the growth rates of nontransformed (*Neo*, OOO) and NeoT cells (*black ●●●●*) were comparable, NeoST cells (*gray ●●●●*) grew more slowly in monolayer culture. In B, the morphology of foci formed by NeoT and NeoST cells was compared 7 days after the cultures had reached confluence. Whereas NeoT cells formed distinct foci, the NeoST cells grew atop one another all across the culture dish. In C, anchorage-independent growth was assessed by incubating cells in soft agar for 7 days, followed by microscopic evaluation of colony formation. *Bar* = 50  $\mu\text{m}$ .

FIGURE 3



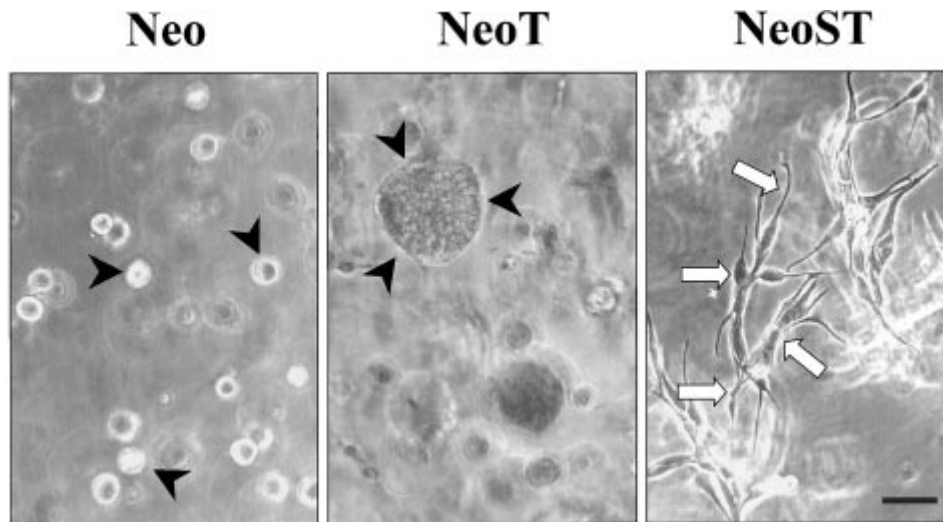
Altered cellular adhesions of NeoST cells. In *A*, the protein levels of E-cadherin were assessed by immunoblotting. Monolayers of the MCF-10A cells were extracted with a radioimmunoprecipitation assay buffer and blotted with Ecadherin antibodies. The blots were stripped and reprobed with vinculin antibodies as a loading control. In *B*, MCF-10A cells, grown on coverslips in culture media overnight, were stained with antibodies specific for paxillin, a cytoskeletal component of focal adhesions. Note the increased size and number of focal adhesions in transformed relative to nontransformed cells. *Bar* = 25  $\mu$ m.

FIGURE 4



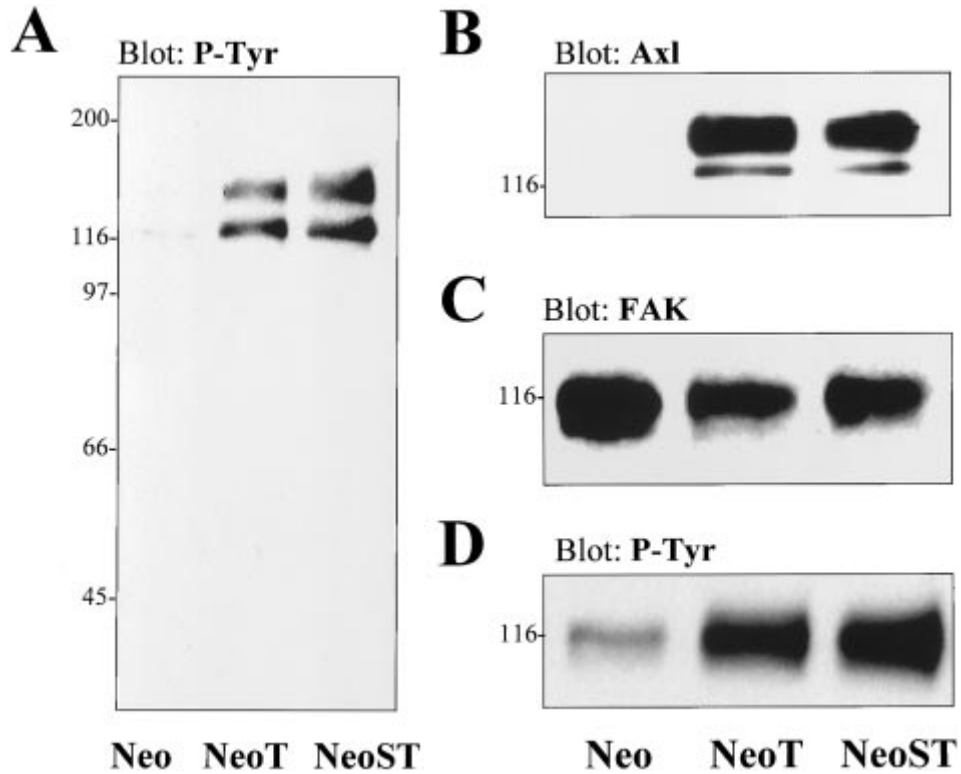
Elevated tyrosine kinase activity alters the organization of the actin cytoskeleton in transformed MCF-10A cells. Monolayers of the indicated variants of MCF-10A cells were incubated with a vehicle control (*top panels*) or with the tyrosine kinase inhibitor herbimycin A (875 nM). The actin cytoskeleton was stained with fluorescein-conjugated phalloidin, revealing that transformed cells have more prominent actin stress fibers and lamellipodia. Note that herbimycin-treated cells adopt a more epithelial morphology, with increased actin adhesion belts between cells and decreased lamellipodia. *Bar* = 25  $\mu\text{m}$ .

FIGURE 5



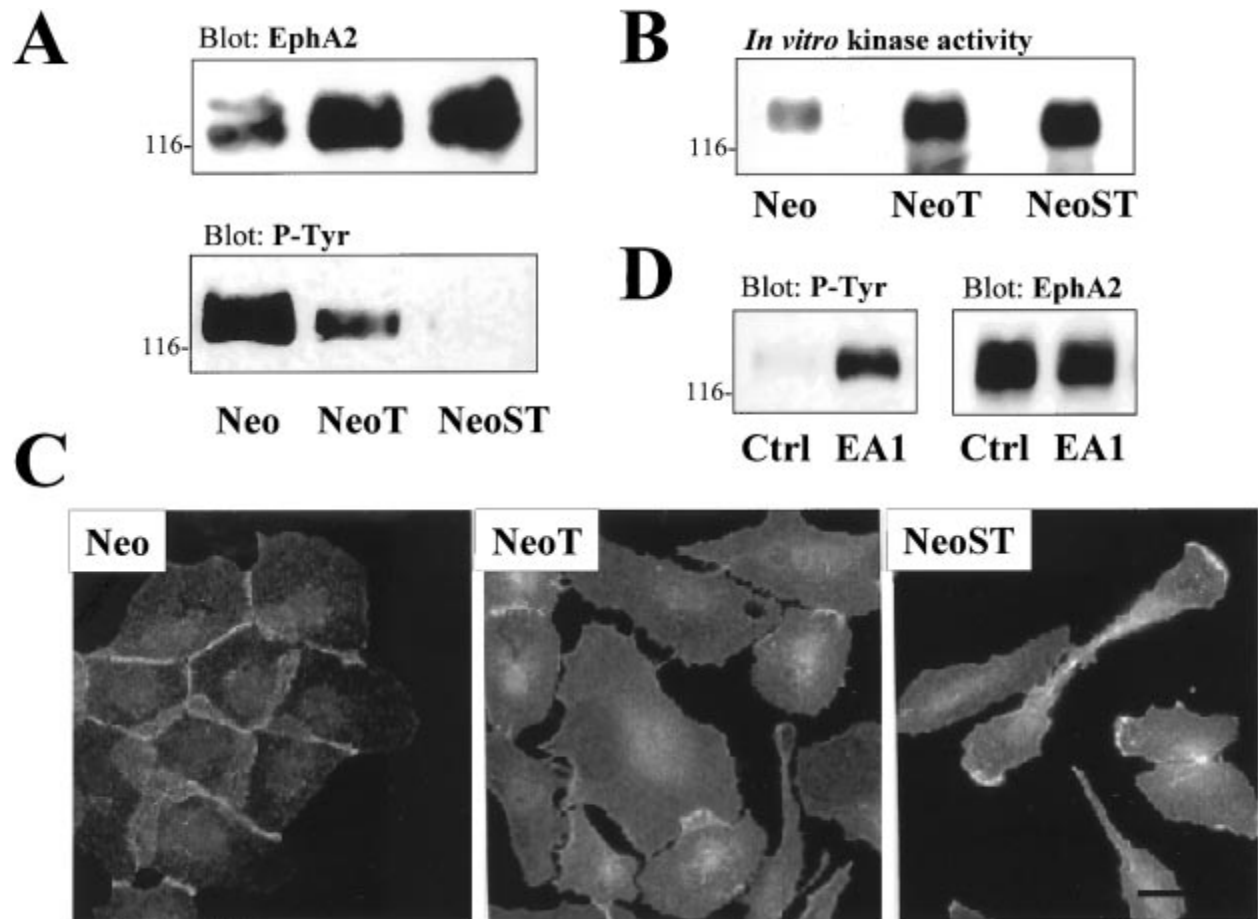
Differential behavior of NeoST cells suspended in Matrigel. The variants of MCF-10A cells were cultured within a 3dRBM (Matrigel) at 37°C for 7 days. The morphology of cell aggregates was evaluated using phase-contrast microscopy. Note that whereas NeoT cells form larger cell colonies than nontransformed (*Neo*) cells (denoted by *arrowheads*), the NeoST cells formed tubular networks (*open arrows*) that dispersed throughout the Matrigel. *Bar* = 50  $\mu\text{m}$ .

FIGURE 6



Increased tyrosine kinase expression transformed MCF-10A cells. Monolayers of MCF-10A cell variants were extracted in radioimmunoprecipitation assay buffer and resolved by SDS-PAGE. The samples were Western blotted with antibodies specific for phosphotyrosine (A) or Axl (B). The protein levels (C) and phosphotyrosine content (D) of immunoprecipitated FAK were assessed by Western blot analysis with specific antibodies.

FIGURE 7



Altered EphA2 expression and function in NeoST cells. In *A*, the protein levels and phosphotyrosine content of the EphA2 receptor tyrosine kinase were measured by Western blot analyses of immunoprecipitated material. In *B*, the enzymatic activity of immunoprecipitated EphA2 in nontransformed and transformed MCF-10A cells was assessed using *in vitro* autophosphorylation assays. In *C*, the localization of EphA2 and actin in nontransformed (*Neo*) and transformed (*NeoT* and *NeoST*) cells was assessed by immunofluorescence microscopy. In *D*, monolayers of NeoST cells were treated with a vehicle control (*Ctrl*) or EphrinA1-Fc (*EA1*) for 5 min before evaluation of EphA2 phosphotyrosine content, as described above. The membranes were stripped and reprobbed with EphA2 antibodies as a loading control (*right side*). Note that the EphA2 in transformed cells has greater enzymatic activity, but that EphA2 itself is not phosphorylated because of decreased ligand binding. *Bar* = 25  $\mu$ m.

TABLE 1

*Table 1* Identification of tyrosine kinases that are overexpressed in transformed MCF-10A cells

Shown are the results of a comprehensive analysis of the expression levels of 36 known tyrosine kinases to identify those tyrosine kinases that are selectively overexpressed in transformed MCF-10A cells. The mRNA levels of all known tyrosine kinases were profiled as described in Ref. 33, and the results indicate at least a two-fold difference in kinase expression. Kinases with no detectable expression include: CHED, EphA3, EphB1, EphB3, FGFR1, FGFR3, FGFR4, LCK, LYN, MER, NK116C, and SKY.

Overexpressed in transformed MCF-10A (Neo < NeoT = NeoST)	Selective overexpression in NeoST (Neo < NeoT < NeoST)	No change (Neo = NeoT = NeoST)	Decreased in transformed MCF-10A (Neo > NeoT > NeoST)
c-Abl	MAKB	ARG	DDR1
Axl	MLK	CSK	EphA1
EphA2 <sup>a</sup>	NK116B	EGFR	FMS
		EphA2 <sup>a</sup>	HER2
		EphA7	HER3
		EphB2	MET
		GSK3	
		JAK1	
		MEK5	
		PLK1	
		PYK2	
		RET	
		SAK	
		YES	

<sup>a</sup> The elevated levels of EphA2 protein, relative to EphA2 mRNA expression, reflected increased protein stability in transformed MCF-10A cells.