ETV6-NTRK3–Mediated Breast Epithelial Cell Transformation Is Blocked by Targeting the IGF1R Signaling Pathway

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Abstract

The insulin-like growth factor (IGF) 1 receptor (IGF1R) is an important therapeutic target under study in many cancers. Here, we describe a breast cancer model based on expression of the ETV6-NTRK3 (EN) chimeric tyrosine kinase that suggests novel therapeutic applications of IGF1R inhibitors in secretory breast cancers. Originally discovered in congenital fibrosarcomas with t(12;15) translocations, EN was identified subsequently in secretory breast carcinoma (SBC) which represent a variant of invasive ductal carcinoma. Because fibroblast transformation by EN requires the IGF1R axis, we hypothesized a similar dependency may exist in mammary cells and, if so, that IGF1R inhibitors might be useful to block EN-driven breast oncogenesis. In this study, we analyzed EN expressing murine and human mammary epithelial cell lines for transformation properties. Various IGF1R signaling inhibitors, including the dual specificity IGF1R/insulin receptor (INSR) inhibitor BMS-536924, were then tested for effects on three-dimensional Matrigel cell growth, migration, and tumor formation. We found that EN expression increased acinar size and luminal filling in Matrigel cultures and promoted orthotopic tumor growth in mice. Tumors were well differentiated and nonmetastatic, similar to human SBC. The known EN effector pathway, PI3K-Akt, was activated in an IGF1- or insulin-dependent manner. BMS-536924 blocked EN transformation *in vitro*, whereas BMS-754807, another IGIFR/INSR kinase inhibitor currently in clinical trials, significantly reduced tumor growth *in vivo*. Importantly, EN model systems mimic the clinical phenotype observed in human SBC. Moreover, EN has a strict requirement for IGF1R or INSR in breast cell transformation. Thus, our findings strongly encourage the evaluation of IGF1R/INSR inhibitors to treat EN-driven breast cancers.

Introduction

The IGF1R signaling axis is emerging as an essential pathway for many cancers, including breast carcinoma, and inhibitors of this pathway are in various stages of clinical development. IGF1R is normally activated by insulin-like growth factors 1 and 2 (IGF1 and IGF2), which trigger a myriad of intracellular signaling cascades including the PI3K-Akt and Ras-ERK pathways (reviewed in refs. 1, 2). Antireceptor (3) or anti-IGF antibodies (4), IGF1R or IGF antisense (5), or dominant-negative *IGF1R* mutants (6) block oncogenic transformation of tumor cell lines *in vitro* and *in vivo*. Perhaps, most compelling is the observation that almost all known dominantly acting oncogenes require a functional IGF1R axis to transform cells, including activated Ras, c-Src, SV40 large T, and oncogenic protein tyrosine kinases (PTK; refs. 7–9). As with EN, other translocation-associated chimeric oncoproteins, such as EWS-FLI1 and variant EWS fusions of Ewing family tumors and PAX3/7-FKHR of alveolar rhabdomyosarcoma, are incapable of transforming *Igfir*$^{-/-}$ murine embryo fibroblasts (MEF; ref. 10).

IGF1R overexpression in mammary epithelial cell lines disrupts acinar morphogenesis (11), and transgenic mice expressing constitutively active IGF1R develop mammary tumors at an early age (12). In human breast carcinoma, immunostaining for tyrosine phosphorylated IGF1R was recently identified in all subtypes and corresponds to poor survival (13). Therefore, IGF1R appears to represent an important therapeutic target for a wide variety of breast cancer

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subtypes. Several humanized blocking antibodies are currently in clinical trials for different tumor types including breast cancer (14–16). However, resistance to these agents is being reported, although IGF1R mutations have not been described. Other candidate mechanisms for resistance include receptor overexpression or increased ligand production (17). Moreover, IGF1R can synergize or physically interact with other receptors such as INSR (18), ER (19), and the EGFR family (20). In fact, it was recently shown that in murine IGF1R-dependent pancreatic β-cell neuroendocrine carcinogenesis, blocking IGF1R fails to reduce tumor burden unless INSR is simultaneously inactivated (21). Therefore, INSR activation may contribute to IGF1R oncogenesis and resistance to IGF1R inhibitors.

The *ETV6-NTRK3* gene fusion is present in virtually all cases of secretory breast carcinoma (SBC; ref. 22). SBC is a rare form of infiltrating ductal carcinoma accounting for less than 1% of all breast cancers and is classified within the basal-like category of breast cancers (23). To date, EN is the only known translocation-associated oncoprotein in human breast cancer. SBC tumors harbor t(12;15) translocations identical to the those originally described in congenital fibrosarcomas (24) and congenital mesoblastic nephromas with EN fusions (25). This fusion was also recently reported in a new subtype of salivary gland tumor known as mammary analogue secretory carcinoma of salivary glands (MASC; ref. 26). The t(12;15) translocation in all of these tumors fuses sequences encoding the sterile α motif (SAM) domain of the ETS transcription factor, ETV6 (TEL), to the PTK domain of the neurotrophin-3 receptor, NTRK3 (TRKC). The SAM domain promotes EN polymerization and subsequent ligand-independent PTK activation (27). EN binds to and phosphorylates the insulin receptor substrates 1 and 2 (IRS1 and IRS2; ref. 10), which bridge EN to the Ras-ERK and PI3K-Akt pathways (28), both of which are essential for fibroblast transformation (22). In EN-transformed fibroblasts, Ras-ERK activation results in cyclin D1 upregulation and proliferation whereas the PI3K-Akt pathway supports cell survival (22). EN requires IGF1R for fibroblast transformation, in particular for activating PI3K-Akt in cells to suppress *anoikis* under anchorage-independent conditions (29). *Igf1r*$^{-/-}$ MEFs are resistant to EN transformation but this is restored by reexpressing IGF1R or targeting EN to the membrane, suggesting that IGF1R may localize EN to the membrane (29).

Sustained EN expression in murine breast epithelial cells promotes tumor growth in immunodeficient mice, and breast tumors rapidly develop in EN knockin transgenic mice (30). However, the role of IGF1R in EN breast oncogenesis remains unknown. Therefore, we wished to determine if a similar IGF1R dependency exists for EN-mediated mammary epithelial cell transformation as observed in fibroblasts, and whether targeting IGF1R might be a tractable therapeutic strategy for EN-driven breast cancers. Moreover, as EN is a bona fide oncoprotein in human breast cancer, EN-transformed breast epithelial cells may provide novel insights into the role of IGF1R in breast oncogenesis. We, therefore, generated new *in vitro* and *in vivo* EN-based breast cancer model systems to further investigate the link between EN mammary transformation and IGF1R signaling. Our results indicate an essential role for IGF1R in EN breast cell oncogenesis. Moreover, we provide evidence that either IGFs or insulin can support this activity, consistent with a parallel or complimentary role for INSR in EN breast cell transformation.

Materials and Methods

Compounds

PI3K was inhibited using LY294002 (25 μmol/L; Calbiochem). MEK1/2 was inhibited using U0126 (25 μmol/L; Calbiochem). BMS-536924 is a benzimidazole compound with nanomolar potency against IGF1R and INSR (31, 32). BMS-754807 is a pyrrolotriazine compound that also inhibits both IGF1R and INSR kinase activity and is currently in clinical trials (33). BMS-536924 was used at 100 nmol/L concentrations for *in vitro* assays, and BMS-754807 as described in the section on *in vivo* tumor models.

Cell culture

MCF10A and MCF10ATk1 cell lines (from Dr. F. Miller) were cultured as described (34). EpH4 cells were obtained from Martin Oft (Schering-Plough Biopharma) and grown as described (22). All cell lines were tested routinely for their ability to respond to basement membrane by undergoing alveolar morphogenesis and to express differentiated protein products, such as the mouse milk protein β-casein, or epithelial markers such as E-cadherin. Cell lines were engineered via retroviral transduction to stably express control (MSCVpuro) and EN (ENpuro) as described (22). Lentiviral vectors carrying luciferase and a blasticidine selectable marker were used to infect MCF10A and MCF10A-EN cells.

Three-dimensional cultures

Monolayer EpH4 cells were serum and insulin starved for 24 hours prior to trypsinization and plating onto Matrigel (BD Biosciences). For overlay cultures, EpH4 cells were placed on polyHEMA-coated dishes for 8 hours and then clusters were collected, mixed in 1% Matrigel containing media, and plated. Cells were fed every 48 hours and collected at various time points and processed for Western blotting or immunofluorescence.

BrdU incorporation assays

Cells were serum and insulin starved for 24 hours and then plated on polyHEMA (4 μg/mL)-coated dishes for 5 hours and were allowed to form naked clusters. Cell clusters were mixed with media containing 1% Matrigel per 5 μg/mL insulin or 100 ng/mL IGF-1, plated, and grown for 3 to 5 days. Spheroids were incubated for 1 hour with BrdU labeling reagent and stained according to manufacturer's instructions (Roche).

Immunofluoresence microscopy

EpH4 cells were grown on glass coverslips in 3-dimensional (3D) cultures as described above, and fixed using standard procedures. The following antibodies were used: E-cadherin (1:500; Transduction Labs), β−catenin (1:250; Abcam), ZO-1 (1:250; Zymed), and Ki67 (1:200; Santa Cruz). Images were

collected using a BioRad Radiance plus confocal unit connected Zeiss Axiophot II microscope. Images were analyzed using NIH image software.

In vivo tumor models

Orthotopic mammary fat pad injections were performed as previously described (35). When EpH4-EN tumors reached a measurable size, 100 mg/kg/d of BMS-754807/PEG400/dH_2O (80:20) or vehicle alone was administered daily by oral gavage for 7 consecutive days. Measuring continued 15 days past the final treatment. Tumor mass calculation = $L \times W^2/2$. Error bars = SD of the mean for 8 tumors.

Tumor histology

Tumors were fixed and embedded using standard protocols. Three-dimensional spheroids were fixed in 4% paraformaldehyde, rinsed with 70% EtOH, embedded in agar and then in paraffin. Five-micrometer cut sections were stained with hematoxylin and eosin (H&E). A Zeiss Axioplan 2 microscope and Northern Eclipse (ver5.0) software was used for image capture.

Boyden chamber assays

Boyden chamber assays were performed as previously described (35). Experiments were performed in triplicate and percentages of migrated cells were calculated on the basis of standard curves generated for each cell line.

Time-lapse wound-healing migration assays

Wound-healing assays were performed as previously described (35).

Western blotting

Lysates were prepared and EN was immunoprecipiated as described previously (22). The following antibodies were used for Western blotting: β-casein, cyclin D1 (Clone 504; 1:2,000; Upstate), Grb2 (1:5,000; BD Biosciences), TrkC (1:500; Santa Cruz), phospho Akt (ser473; 1:1,000; Cell Signaling), phospho MEK1/2 (1:1,000; Cell Signaling), total Akt (1:1,000; Cell Signaling), INSRβ (1:1,000; Santa Cruz), IGF1Rβ (1:1,000; Santa Cruz), IRS-1 (1:1,000; Santa Cruz), and total actin (1:5,000; Santa Cruz).

Comparison of BMS-536924 with BMS-754807 using soft agar colony assays

Soft agar assays were performed as previously described (36). Increasing nanomolar concentrations of inhibitor were tested (see Supplementary Fig. S4A). ImageJ Analysis Software and a macro (Matthew Robertson) were used to calculate the percentage of colonies formed after 7 days. Three independent experiments were performed. Error bars = SEM.

siRNA-mediated gene knockdown

siRNAs to murine INSR and IGF1R (murine INSR Cat: J-043747; IGF1R Cat: J-056843) and human INSR (Cat: J-003014; Dharmacon) were transfected with Silentfect (BioRad), followed 72 hours later by evaluation for knockdown efficacy or plating in 3D Matrigel cultures.

Results

EN transforms murine EpH4 breast epithelial cells in 3D Matrigel cultures and renders them tumorigenic in immunodeficient mice

Stable expression of EN in EpH4 cells (EpH4-EN) was confirmed by Western blotting in 2 independently developed cell lines (Supplementary Fig. S1A). No clear difference in phenotype or downstream signaling pathways was apparent in control versus EN-expressing cells in conventional monolayer cultures (Supplementary Fig. S1B). However, in 3D Matrigel-containing cultures, striking phenotypic and signaling changes were observed. Multicellular spheroids generated by EpH4-EN cells were 2 to 3 times larger (Fig. 1A, top), exhibited increased Ki67 staining (Fig. 1A, bottom) and showed partially filled lumens (Fig. 1B, right and Fig. 1D, bottom left). Similar to nontransformed cells, EpH4-EN cells showed intact adherens junctions (AJ), as assessed by E-cadherin and β-catenin staining, and tight junctions (TJ) as assessed by ZO-1 staining (Fig. 1A and B). EpH4-EN cells responded to lactogenic hormones by expressing β-casein (Fig. 1C). Western blotting of lysates from 3D EpH4-EN cells revealed higher levels of phospho-Akt, phospho-MEK, and cyclin D1 (Fig. 1C), as described previously for EN-transformed fibroblasts (36). Together, these results indicate that although EN expression increases proliferation and affects luminal formation, it does not disrupt polarity or differentiation status of transformed cells *in vitro*.

We previously showed that when injected subcutaneously into the flanks of immunodeficient NOD/SCID mice, EpH4-EN cells form stable xenografts (22). We next compared the histology of these EpH4-EN xenografts as well as of EpH4-EN 3D spheroids with those of tumors from EN knockin transgenic mice (11) and human SBC cases, as shown in Figure 1D. This demonstrated a number of morphologic similarities among the 4 specimens. Each showed characteristic lumens lined by multicellular epithelial surfaces, with varying degrees of luminal filling. The similarity to human SBC was particularly evident in the EpH4-EN 3D spheroids. These results support the use of EpH4-EN *in vitro* and *in vivo* model systems to study EN breast oncogenesis.

EN transforms human MCF10A breast epithelial cells and promotes orthotopic tumor formation *in vivo*

To establish a human model system for EN transformation, we utilized the MCF10A cell line model (37). MCF10A cells are derived through spontaneous immortalization of breast epithelial cells from a patient with fibrocystic disease and do not form persistent xenografts in immunocompromised mice. A second cell line, MCF10ATk1, expresses activated *H-ras* and produces hyperproliferative lesions but is nonmetastatic in immunodeficient mice (34, 38). EN was retrovirally transduced into both cell lines and expression was confirmed by Western blotting (Fig. 2A). Although indistinguishable from control MCF10A and MCF10ATk1 cells in monolayer cultures, EN-expressing MCF10A (MCF10A-EN) cells formed large acini similar in size to MCF10ATk1 cells in 3D cultures (Fig. 2B, bottom). In contrast, enlarged acini were not observed in 3D

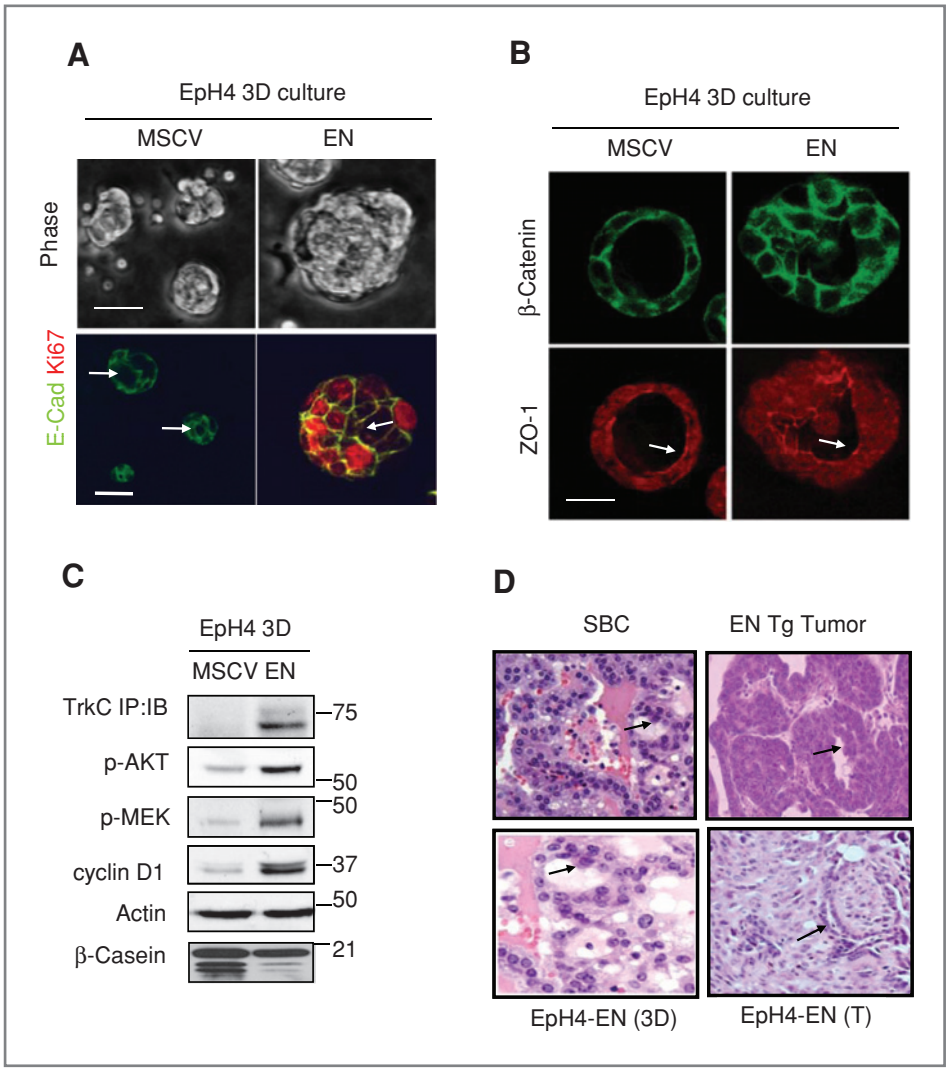

Figure 1. EN transforms murine mammary epithelial cells in 3D Matrigel cultures. A, top, phase contrast photomicrographs of spheroids after 3 days in Matrigel. Scale: 50 μm. Bottom, E-cadherin (green) and Ki67 (red) immunofluorescence staining. Scale: 50 μm. B, confocal microscopy images of control and EN EpH4 spheroids stained for β-catenin (green) and ZO-1 (red). Arrows indicate apical staining. Scale: 30 μm. C, Western blot analysis of EpH4-MSCV and EpH4-EN cells grown in 3D cultures. D, H&E-stained sections of human SBC, EN knockin transgenic tumor (Tg), EpH4-EN cells grown in 3D culture (3D), and EpH4-EN subcutaneous tumor (T). Arrows highlight lumen formation.

cultures in EN-expressing MCF10ATk1 cells (MCF10ATk1-EN), which instead generated distinctive phenotypic changes in both monolayer and 3D cultures similar to what is observed when the Y-box protein-1 (YB-1) protein is overexpressed in MCF10ATk1 cells (see Fig. 2B and ref. 35, and the next section). We next injected MCF10A-EN cells into mammary fat pads of immunodeficient NOD/SCID mice. This resulted in the formation of stable xenografts that grew slowly and were non-metastatic over the 4-month monitoring period (Fig. 2C). Therefore, MCF10A-EN cells also provide useful in *vitro* and *in vivo* model systems to study EN breast oncogenesis.

EN fails to induce a full EMT and only minimally increases cell migration

As mentioned, MCF10ATk1-EN cells produced a refractile and spindle-shaped appearance in monolayer cultures. When plated in Matrigel, these cells possessed a mesenchymal-like spindle-shaped phenotype and did not form multicellular spheroids. This is reminiscent of cells undergoing an epithelial-to-mesenchymal transition (EMT), a well-described process that facilitates epithelial cell migration and metastasis (reviewed in ref. 39). EMTs are characterized by decreased epithelial markers including E-cadherin, ZO-1, cytokeratin 18 (CK18), and mucin 1 (MUC1); and increased expression of mesenchymal proteins such as vimentin, N-cadherin, fibronectin, α-smooth muscle actin (SMA), CK14, and p63. We, therefore, assessed expression of selected EMT markers in MCF10A-EN and MCF10ATk1-EN cells by Western blotting (Fig. 3A) and immunofluorescence (Fig. 3B). As a positive control, we used MCF10ATk1 cells overexpressing the cold shock domain RNA/DNA binding protein, YB-1, which induces a robust EMT in MCF10ATk1 cells and renders them highly metastatic (35). MCF10ATk1-YB-1 cells are invasive in 3D cultures and possess very low levels of E-cadherin and increased expression of vimentin, N-cadherin, and other EMT proteins (ref. 35 and Fig. 3A and B). In contrast, MCF10A-EN and MCF10ATk1-EN cells showed only slight to moderate decreases in E-cadherin and minor changes in ZO-1 and no vimentin induction (Fig. 3A and B). Boyden chamber assays were next used to determine migration rates

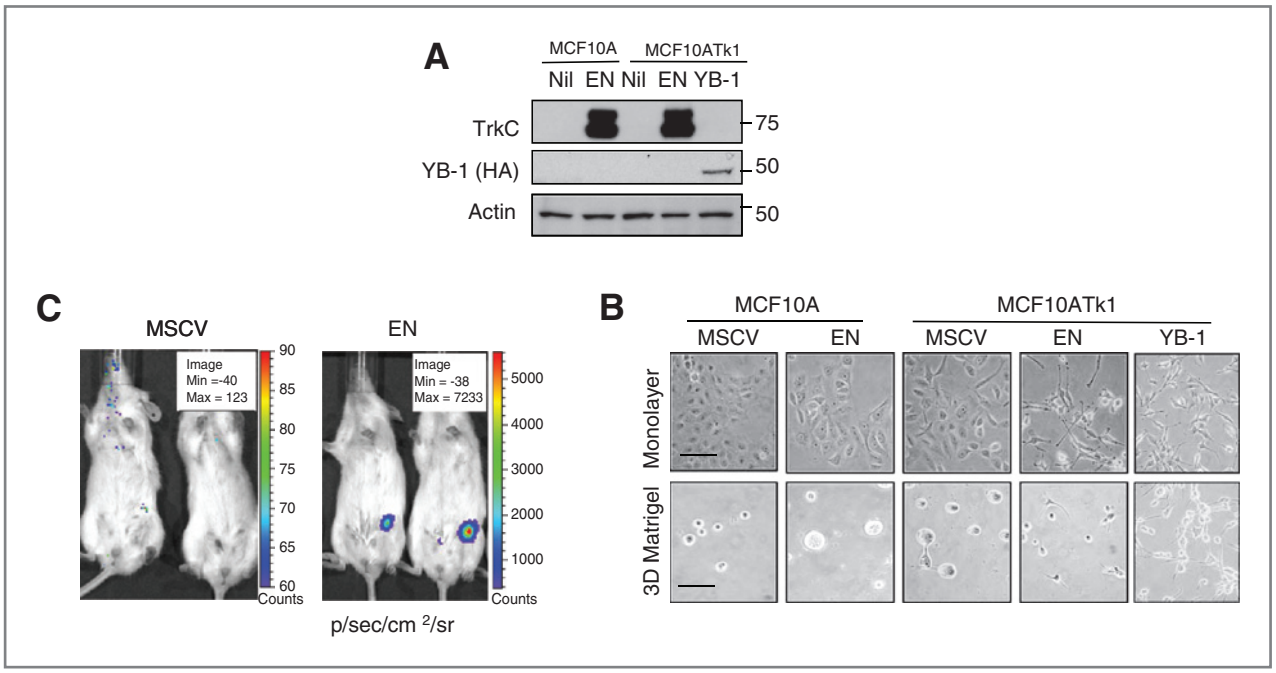

Figure 2. EN transforms human mammary epithelial cells in 3D cultures. A, Western blot analysis of MCF10 and MCF10ATk1 cells with or without EN expression. Positive control = HA-tagged YB-1 MCF10ATk1. B, top, phase contrast monolayer photomicrographs. Scale: 100 μm. Bottom, spheroid morphology after 7 days in 3D Matrigel culture. Scale: 200 μm. C, orthotopic mammary fat pad tumor growth. MCF10A-control cells (left) and EN-transformed cells (right). Representative IVIS scans of the mice at 3 months postinjection are shown. Scale = p/sec/cm^2/sr.

of serum-starved MCF10-EN and MCF10ATk1-EN cells in response to serum or serum-free media containing IGF1 or insulin as chemoattractants. As shown in Figure 3C, although there was a slight increase in MCF10-EN or MCF10ATk1-EN cells versus parental cell lines, effects were dramatically less than for MCF10ATk1-YB-1 cells. Taken together, these results suggest that despite certain morphologic changes, EN does not induce a full EMT in MCF10A or MCF10ATk1 cells. Time-lapse photomicroscopy was then used to track the movement of control EpH4 versus EpH4-EN cells. No significant migration differences were observed in the absence of serum or growth factors (Supplementary Fig. S2). In the presence of serum or serum-free media plus IGF1, there were slight increases in track length (distance traveled) and random movement for EpH4-EN cells compared with control cells (Fig. 3D, top and middle). Taken together, these results suggest that although EN induces minor migratory increases in response to serum or IGF1, it fails to induce a highly invasive phenotype, in keeping with the low metastatic behavior of SBC.

EN requires IGF1R/INSR signaling and Akt activation to transform breast epithelial cells

We next wished to determine if any of the observed EN effects on breast epithelial cell transformation require IGF1R signaling, as this axis is critical for EN fibroblast transformation. We first analyzed growth of EpH4-EN cells in 3D Matrigel cultures with or without IGF1 (100 ng/mL) or insulin (5 μg/mL), given that IGF1R and INSR share numerous downstream signaling elements (40). EN expression markedly increased spheroid sizes and luminal filling, but only when IGF1 or insulin was present (see Fig. 4A). In fact, in the absence of growth factors, EpH4-EN cells failed to grow and could not be further assessed experimentally (data not shown). We next performed Western blotting of EpH4 cells from 3D cultures grown in the presence of IGF1 or insulin. At early time points, there was no obvious correlation between EN expression and PI3K-Akt or Ras-ERK activation (shown for the 2-hour time point in Fig. 4B, left). However, by 96 hours, EN-expressing cells began to exhibit higher levels of phospho-Akt and cyclin D1 compared with parental cells (Fig. 4B, right). A similar pattern was previously observed in MCF10A-EN cells (ref. 30 and data not shown). To further explore effects of IGF1R/INSR signaling on EN mammary cell transformation, spheroid cultures were labeled for 1 hour with BrdU, fixed, stained, and visualized by confocal microscopy. The percentage of BrdU-positive spheroids was calculated by counting spheroids possessing greater than 4 BrdU-positive cells (Fig. 4C). Similarly, the percentage of spheroids possessing hollow lumens was calculated by scoring DAPI (nuclear)-stained spheroids using confocal microscopy (Fig. 4D). Consistent with the above results, EpH4-EN spheroids were larger than EpH4 controls (Fig. 1A and Supplementary Table S1), and the increased size and BrdU staining was only observed in the presence of IGF1 or insulin. Approximately, 40% of EpH4-EN spheroids possessed greater than 4 BrdU-positive cells under full serum, compared with only 8% when cultured in serum-free media. Addition of IGF1 or insulin alone to serum-free medium increased the latter percentage to 21% and 31%, respectively. Significantly less

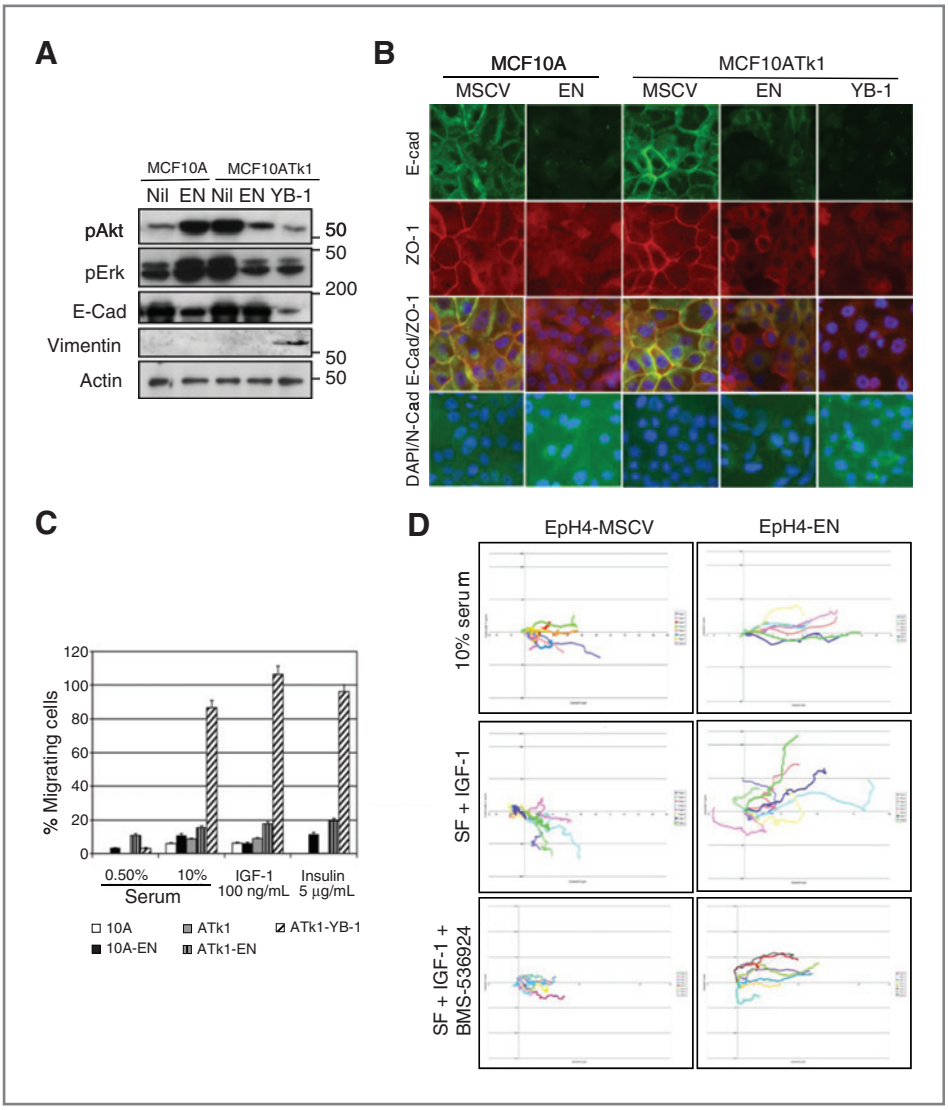

Figure 3. EN expression in MCF10A and MCF10ATK1 cells does not promote a full EMT. A, Western blot analysis for control and EN expressing MCF10A and MCF10ATk1 cells grown in 3D cultures. B, immunofluorescence staining of monolayer cells. Scale: 30 µm. C, Boyden chamber assays assessing response to serum (0.5% and 10%), IGF-1 (100 ng/mL) and insulin (5 µg/mL). Experiment was repeated 3 times and a representative experiment is displayed. Error bars = SD of triplicate wells. D, EpH4-EN cells respond to serum and IGF-1 (100 ng/mL) stimulation in wound-healing assays using time-lapse photomicroscopy. Each line represents the track per movement of a single cell. Coincubation with the IGF1R/IR inhibitor BMS-536924(100 nmol/L) inhibits movement.

BrdU staining was observed for control spheroids under all conditions.

We next assessed effects of specific siRNAs to IGF1R or INSR. Knockdown of IGF1R and INSR was confirmed in monolayer cultures (Fig. 5A, left). In 3D cultures, knockdown of either receptor blocked luminal filling (Fig. 5A, right), and when used in combination, dramatically decreased EpH4-EN spheroid sizes (see Fig. 5B). By Western blotting, there was no effect of knockdown on EN effector pathways in monolayer cultures (data not shown). However, in 3D lysates, knockdown of either or both receptors decreased Akt phosphorylation and cyclin D1, (Fig. 5A, right). Knockdown with siRNAs to INSR also decreased spheroid size, decreased pAkt and cyclin D1 levels in MCF10A-EN cells in 3D cultures (Supplementary Fig. S4A and S4B). Because insulin was also able to support growth of EN-transformed cells in 3D cultures, we wondered whether the insulin effects were mediated through INSR or IGF1R. Interestingly, we found that insulin could activate IGF1R in both EN-transformed and parental fibroblasts (Supplementary Fig. S3A). Moreover, at concentrations of 250 ng/mL or higher, insulin was able to activate both INSR and IGF1R in MCF10A cells (Supplementary Fig. S3B). More studies are required to elucidate whether insulin functions through IGF1R or INSR, or indeed through both receptors, such as via cross-talk between IGF1R and INSR (ref. 40; see the Discussion section).

We also tested effects of BMS-536924, a dual, specific IGF1R/INSR kinase inhibitor, or the PI3K inhibitor LY294002. Both inhibitors markedly decreased EpH4-EN spheroid sizes in 3D cultures (Fig. 5C, left; Supplementary Fig. S6A). LY294002 treatment decreased BrdU incorporation and reduced cyclin D1 levels whereas inhibition of MEK activity using U0126 had only a small effect (Supplementary Fig. S6 and Table S1). Moreover, BMS-536924 also decreased the length of EpH4-EN cell tracks in response to IGF1 stimulation (Fig. 3D, bottom), suggesting that IGF1R and/or INSR activation may influence migration of EN-transformed breast epithelial cells. Finally, when EpH4-EN cells were injected subcutaneously into immunodeficient mice, BMS-785807, a

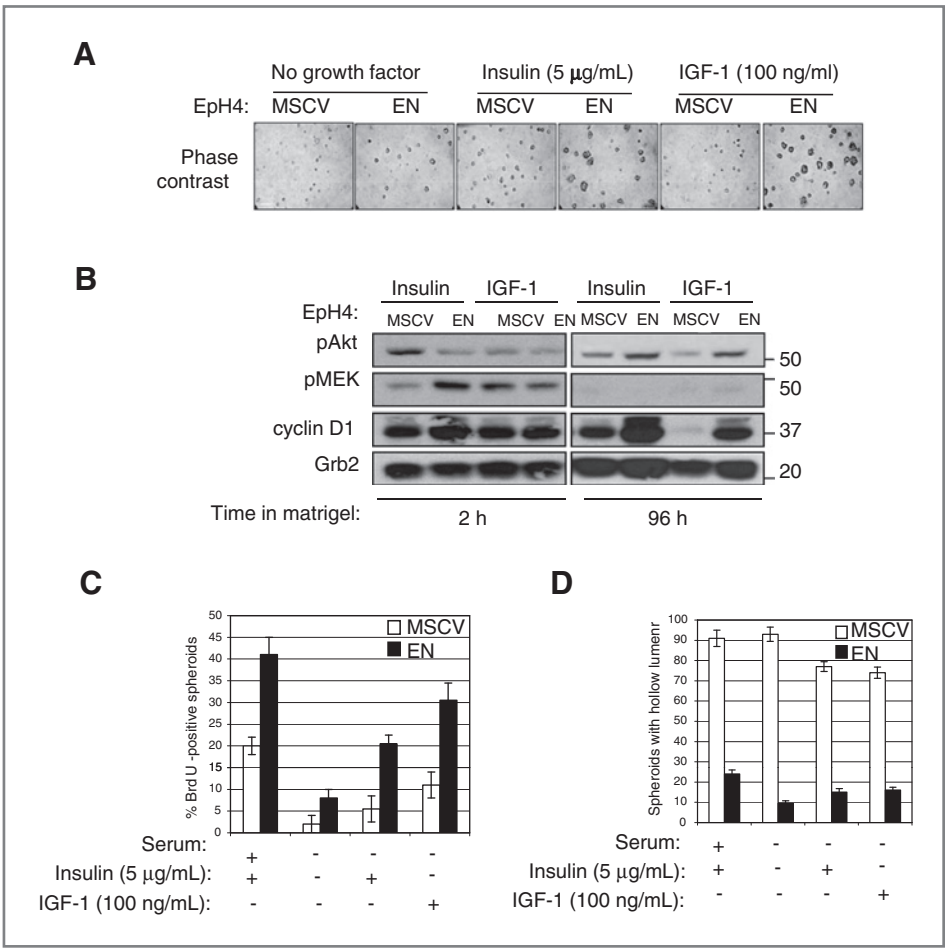

Figure 4. EN requires IGF-1 or insulin to transform EpH4 cells. A, photomicrographs of spheroids formed in 3D Matrigel cultures in the presence of insulin (5 μg/mL) or IGF-1 (100 ng/mL). Scale: 500 μm. B, Western blot of 3D cultures. C, average number of spheroids found in EpH4-MSCV or EpH4-EN 3D cultures with 4 or more BrdU-positive cells. D, percentage of spheroids possessing hollow lumen in 3D cultures in each of the 3 conditions.

dual specificity IGF1R/INSR kinase inhibitor currently being tested in clinical trials with solid tumors (ref. 33; see Supplementary Fig. S5 for *in vitro* comparison with BMS-536924), significantly decreased tumor volumes when administered for 7 days by oral gavage (Fig. 5D). Together, these findings strongly indicate that growth factor stimulation of the IGFR/INSR axis stimulates proliferation of EpH4-EN spheroids via an Akt-dependent mechanism, and that blocking these receptors inhibits growth of EpH4-EN cells *in vitro* and *in vivo*.

IRS-1 interacts with ETV6-NTRK3 *in vivo*

We previously demonstrated an essential role for the IGF1R adapter protein, insulin-like growth factor substrate-1 (IRS-1), in EN-mediated transformation of fibroblasts (28). To determine whether IRS-1 may also interact with EN in breast epithelial cells, we immunoprecipitated EN from tumor tissues collected from 2 primary SBC cases, as well as breast tumors from ETV6-NTRK3 knockin transgenic mice (30). In all cases, IRS-1 was found to coimmunoprecipitate with the EN fusion protein (Fig. 6A and B). This indicates a role for IRS-1 in EN transformation and may also provide the link between the IGF1R and downstream PI3K-Akt activation in SBC.

Discussion

Although, our understanding of the role of oncogenic fusions in epithelial tumors has increased in recent years (41), identification of the *ETV6-NTRK3* gene fusion as a primary genetic event in SBC (22) remains the only known example of a dominantly acting oncogene in human breast carcinoma. Using 3D cell culture systems as well as orthotopic tumor models, we demonstrate here that EN expression transforms both murine and human breast epithelial cell lines. Further characterization revealed that although EN expression leads to higher proliferation rates with luminal filling and increased acinar sizes, EN spheroid cells remain polarized, are relatively well differentiated, and do not display prominent migratory/invasive capacity in keeping with the clinical behavior of human SBC. We confirmed an essential role for the IGF1R/INSR axis in EN breast cell transformation and demonstrated a critical requirement for this axis in activation of the PI3K-Akt cascade and upregulation of cyclin D1. Moreover, we show that, similar to EN-transformed fibroblasts, EN binds IRS-1 in SBC tumors. These findings demonstrate the utility of *in vitro* and *in vivo* model systems of EN transformation to study breast cell

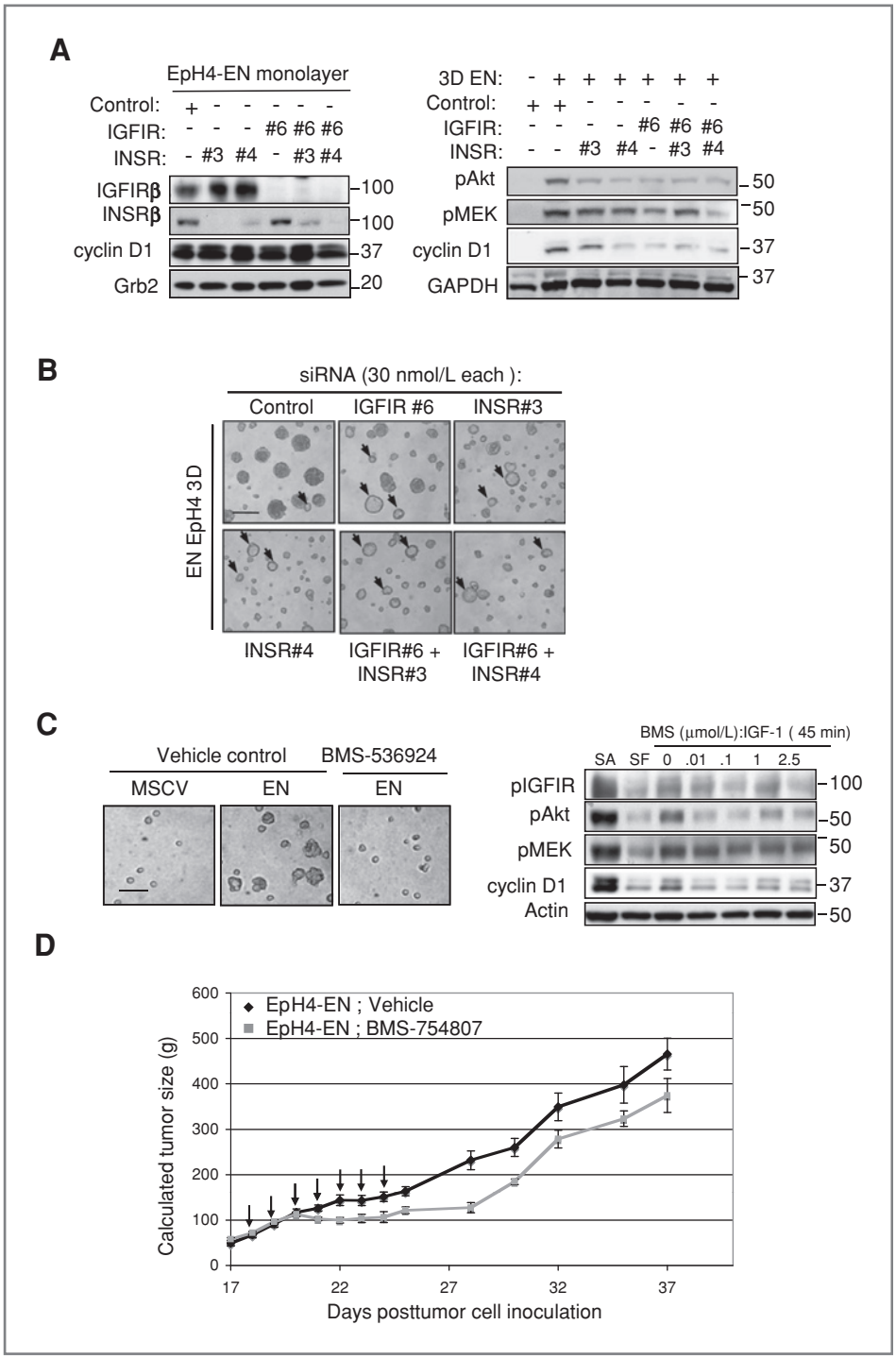

Figure 5. Inhibiting IGF1R/INSR blocks EpH4-EN transformation. A, monolayer (left) and 3D (right) Western blots. B, effects of siRNAs to IGF1R (#6) and INSR (#3 and #4) on EpH4-EN spheroid growth. Arrows indicate presence of hollow lumens. Pictures taken after 5 days in Matrigel. Scale: 200 μm. C, effects of BMS-536924 on EpH4-EN 3D spheroid size (left) and signaling pathways (right). Pictures = 2 days in 3D Matrigel cultures. Scale: 200 μm. Western blots performed on monolayer EpH4-EN cells. BMS-536924 was added 2 hours prior to stimulation with 100 ng/mL IGF-1 (45 minutes). D, effects of inhibiting IGF1R/IR kinase activity by BMS-754807 on EpH4-EN subcutaneous tumor growth *in vivo*. Arrows indicate a 1× oral dose of the inhibitor or control carrier. SA, serum always; SF, serum free.

oncogenesis, and for investigating the role of IGF1R/INSR signaling in this process. Moreover, our models recapitulate many aspects of the SBC phenotype and highlight the potential for therapeutically targeting IGF1R/INSR pathways in SBC.

We found that induction of cyclin D1 and consequent cell proliferation in EpH4-EN and MCF10A-EN cells requires the IGF1R/INSR axis. D-type cyclins have well-documented roles in breast carcinogenesis, and are overexpressed in approximately 40% of breast cancer cases (42). Furthermore, the role of cyclin D1 in rapid breast cell growth is underscored by the observation that in cyclin D1 knockout mice, alveolar lobular cells fail to expand during pregnancy (43–45). Activation of the IGF1R/INSR axis is associated with recruitment of members of the IRS family of adaptor proteins, and subsequently, proteins such as Shc, Grb10, 14-3-3, and the PI3K p85 subunit (reviewed in refs. 1, 46). In particular, IRS1 and IRS2 contain binding sites

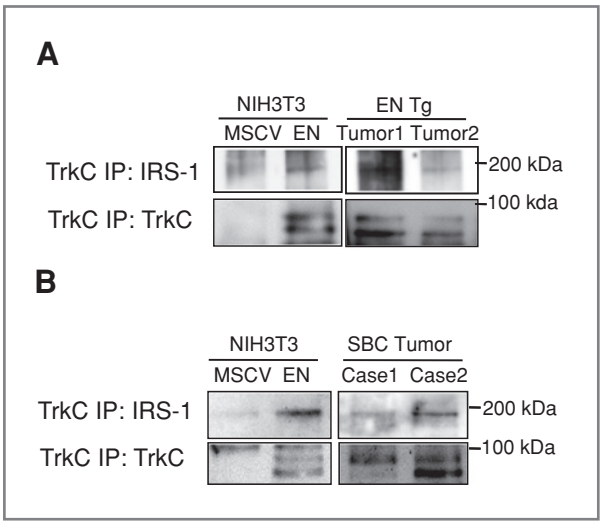

Figure 6. EN interacts with IRS-1 in murine and human EN-positive tumors. IRS-1 coimmunoprecipitated with the EN oncoprotein in lysates derived from EN-positive murine knockin transgenic tumors and 2 independent cases of human SBC. Wild-type and EN-expressing NIH3T3 cells were used as negative and positive controls, respectively.

for Grb2 and PI3K, and function as links to key downstream signaling pathways including Ras-Erk and PI3K-Akt cascades. EN interacts physically with IRS-1 in EN-positive murine breast tumors and human SBC, as it does in EN-transformed fibroblasts, indicating an important role for this adaptor in EN transformation of breast epithelial cells. How exactly EN utilizes its interaction with IRS-1 to link to Ras-Erk and PI3K-Akt cascades, and how this might involve IGF1R, remains to be determined. It is tempting to speculate that EN may physically associate with IGF1R through IRS-1, but this requires experimental validation.

The ability of EN to transform fibroblasts requires a functional IGF1R axis, as exemplified by EN's inability to transform *Igf1r*$^{-/-}$ MEFs (10). However, in EN-transformed fibroblasts, IGF1R is not required for cyclin D1 induction or cell progression, as the latter are induced through the Ras-Erk pathway independently of IGF1R (29). Instead, IGF1R activation correlates with increased Akt-dependent cell survival in EN-transformed fibroblasts, particularly under anchorage-independent conditions (29). In contrast, when mammary EpH4-EN spheroid cultures were treated with the PI3K inhibitor, LY294002, cell proliferation was dramatically inhibited, correlating with complete loss of cyclin D1 expression. These results are consistent with other studies demonstrating that expression of constitutively active Akt in mammary epithelial cells leads to translational upregulation of cyclin D1/2 (47). Hence, there may be fundamental differences in the role of IGF1R in supporting EN transformation of mesenchymal cells versus breast epithelial cells, which may have important implications for targeting IGF1R in different tumor types. Further studies of such differences may also reveal key insights into the overall role of IGF1R/INSR signaling in oncogenesis.

We found that both IGF1 and insulin could support 3D growth of EN-transformed breast cells and induced cyclin D1 expression and cell proliferation. There are several possible explanations for this finding. One is that either IGF1R or INSR activation can support EN breast cell transformation. For example, promiscuity may exist in the activation of signaling cascades downstream of either receptor, such as PI3K-Akt, which may allow either IGF1R or INSR to induce cyclin D1 expression in EN-transformed cells. In a recent mouse model of IGF1R-dependent pancreatic β-cell neuroendocrine carcinogenesis, inactivation of both IGF1R and INSR was required to reduce tumor burden (21). This was interpreted as indicating either a role for IGF2-mediated activation of INSR or, conversely, insulin-mediated activation of INSR itself, in IGF1R oncogenesis, and that INSR signaling may functionally underlie resistance to IGF1R inhibition. On the other hand, accumulating evidence highlights the existence of heterodimers between IGF1R, and INSR, particularly the isoform A form of the latter that is known to be overexpressed in certain cancers such as breast carcinoma (40, 48). These hybrid receptors can be activated by insulin as well as by IGFs. Therefore, heterodimer formation may occur in EN-transformed cells as a second explanation for the observed insulin effects on EN transformation. Thirdly, because insulin can activate IGF1R at high doses such as those used in our studies, the insulin effects may be occurring through IGF1R activation. In fact, we observed that insulin induced IGF1R tyrosine phosphorylation in EN-transformed fibroblasts and parental MCF10A breast epithelial cells (Supplementary Fig. S3A and B). This effect is consistent with our observation that EN fails to transform *Igf1r*$^{-/-}$ MEFs (10), which still express INSR. More studies are required to elucidate the basis of insulin effects on EN transformation.

Disruption of cell–cell contacts during EMT is a well-established feature of invasive epithelial tumor cells (reviewed in ref. 49). In the current study, we found that EN expression alone is not sufficient to disrupt cell–cell junctions, reduce E-cadherin, or to induce a full EMT. In fact, a polarized acinar cell phenotype is maintained in 3D cultures. Activation of ErbB2 in differentiated mammary acini also leads to the reinitiation of cell proliferation to form noninvasive multilayered spheroids, generating a phenotype similar to EN-expressing cells. However, unlike EN, ErbB2 activation also leads to loss of acinar polarization (50), and this lack of organization appears to be a precursor to subsequent invasive capacity and cancer progression. In SBC, tumor cells retain polarized multilayered glandular structures, and these tumors only very rarely metastasize (23). EN expression in EpH4 or MCF10A cells did not induce an invasive phenotype in 3D cultures, and IGF1 induced limited migration of EN-expressing cells only in wound-healing assays but not in Boyden chambers. Therefore, EN expression alone is likely insufficient for inducing a highly migratory phenotype, in keeping with the predominantly nonaggressive clinical behavior of SBC. This lack of appreciative migratory capacity in EN-transformed breast cells is somewhat surprising given that deregulation of growth factor and PI3K signaling pathways are both known to disrupt cell–cell junctions and to induce an invasive phenotype in many cell types (51–54). Why this does not occur in EN-transformed breast cells is completely unknown, but argues that additional genetic events must occur to execute a full

EMT and to confer a metastatic phenotype. Alternatively, EN may itself repress such pathways through an unidentified mechanism. It will be of great interest to assess rare metastatic SBC tumors for genetic events that might underlie aggressive clinical behavior. Early stages of breast cancer, such as hyperplasia and ductal carcinoma *in situ,* are characterized by an increase in luminal cell proliferation, loss of acinar organization, and luminal filling as observed for EN-transformed mammary epithelial cells. Therefore, EN breast tumors may model early breast cell transformation as opposed to progression, and the model systems described here may be particularly useful for determining genetic or epigenetic steps necessary for the transition to invasive carcinomas.

In summary, our studies provide evidence that although EN robustly initiates cell proliferation in breast epithelial cells, it does so without blocking differentiation or inducing an invasive phenotype, thus mimicking the morphologic features of SBC. Our findings confirm the importance of IGF1R/INSR activation in EN breast cell transformation, as well as the importance of PI3K in this process. However, in contrast to what is observed in EN-transformed mesenchymal cells, IGF1R/INSR signaling is required for PI3K-AKT–mediated cyclin D1 induction and proliferative effects of EN on breast cells. Finally, our *in vitro* and *in vivo* results using BMS-536924 and BMS-754807 suggest that it may be possible to treat EN-expressing breast cancers with IGF1R/INSR kinase inhibitors. Such tumors typically occur in very young patients in which invasive surgery can have dramatic long-term cosmetic side effects, highlighting the need for alternative modes of therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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