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Original Article

Continuous requirement of ErbB2 kinase activity for loss of cell polarity and lumen formation in a novel ErbB2/Neu-driven murine cell line model of metastatic breast cancer

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Abstract

Background: Well over a quarter of human breast cancers are ErbB2-driven and constitute a distinct subtype with substantially poorer prognosis. Yet, there are substantial gaps in our understanding of how ErbB2 tyrosine kinase activity unleashes a coordinated program of cellular and extracellular alterations that culminate in aggressive breast cancers. Cellular models that exhibit ErbB2 kinase dependency and can induce metastatic breast cancer in immune competent hosts are likely to help bridge this gap. **Materials and Methods:** Here, we derived and characterized a cell line model obtained from a transgenic ErbB2/Neu-driven mouse mammary adenocarcinoma. **Results:** The MPPS1 cell line produces metastatic breast cancers when implanted in the mammary fat pads of immune-compromised as well as syngeneic immune-competent hosts. MPPS1 cells maintain high ErbB2 overexpression when propagated in DFCI-1 or related media, and their growth is ErbB2-dependent, as demonstrated by concentration-dependent inhibition of proliferation with the ErbB kinase inhibitor Lapatinib. When grown in 3-dimensional (3-D) culture on Matrigel, MPPS1 cells predominantly form large irregular cystic and solid structures. Remarkably, low concentrations of Lapatinib led to a switch to regular acinar growth on Matrigel. Immunofluorescence staining of control vs. Lapatinib-treated acini for markers of epithelial polarity revealed that inhibition of ErbB2 signaling led to rapid resumption of normal mammary epithelium-like cell polarity. **Conclusions:** The strict dependence of the MPPS1 cell system on ErbB2

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signals for proliferation and alterations in cell polarity should allow its use to dissect ErbB2 kinase-dependent signaling pathways that promote loss of cell polarity, a key component of the epithelial mesenchymal transition and aggressiveness of ErbB2-driven breast cancers.

Keywords: 3-D Matrigel, EMT, ErbB2/Her2/Neu, lapatinib, mouse models, syngeneic xenografts

BACKGROUND

ErbB2 (Her2/Neu), a member of the ErbB family of receptor tyrosine kinases (RTKs), is overexpressed and causally linked to oncogenesis in over a quarter of all cases of breast cancer; notably, ErbB2 overexpression defines a distinct molecular subtype of advanced breast cancers with an especially poor clinical prognosis.[1-3] The overexpression of ErbB2 in breast cancers has also provided an opportunity for targeted therapies that take advantage of the cell surface expression (humanized monoclonal antibodies, such as Trastuzumab) and kinase activity (kinase inhibitors, such as lapatinib) of ErbB2; both of these strategies are now in clinical use with a significant degree of success in patient management.[4-8] It is however clear that these elegant therapeutic strategies have been less successful than initially anticipated. Both primary and acquired resistance has emerged as a substantial barrier to success of targeted therapeutics.[9-12] These issues highlight the need for better understanding of how ErbB2 initiates and maintains the various hallmarks of oncogenesis, how targeted therapeutic agents produce their beneficial effects and what mechanisms contribute to their failure. Answers to these questions require appropriate experimental models that can be used for molecular, cellular and *in vivo* studies in a relatively seamless manner. Currently, few models with these attributes exist.

Much of our current understanding of signaling pathways downstream of ErbB2, mechanisms by which targeted therapeutic agents work and mechanism of resistance to these agents have been gleaned from studies of human ErbB2-overexpressing breast cancer cell lines analyzed *in vitro* and as xenograft tumors. A major weakness of these models is that *in vivo* analyses have to be carried out in immune-compromised murine hosts. It is now amply clear that components of the immune system play key negative as well as positive roles in oncogenesis.[13-16] Furthermore, immune mechanisms contribute prominently to the effectiveness of targeted therapy with humanized antibodies against ErbB2.[17-19] Thus, while human ErbB2-overexpressing tumor cell lines have provided critical *in vitro* systems to advance our understanding of ErbB2-mediated oncogenesis, these models lack key attributes relevant to oncogenesis and targeted therapy *in vivo*.

Transgenic mice that specifically overexpress ErbB2 in the mammary epithelium have provided an important complement to the xenograft-based models and have aided in analyses of the biology of ErbB2-driven oncogenesis as well as studies of targeted therapeutics.[20,21] The relatively long time to onset (many months), and variability in the onset and progression among individual animals in a cohort make these models challenging especially for studies investigating therapeutic strategies and mechanisms.

Thus, while human breast cancer cell lines together with transgenic mouse models have led to substantial improvement in our understanding of ErbB2-mediated oncogenesis, there is a substantial need for well-characterized syngeneic murine cell line models that eliminate the need for immune-compromised hosts and exhibit strict ErbB2 dependence. Models that incorporate ease of use of cell lines together with tumorigenesis in immune-competent mouse models are therefore highly desirable to help bridge gaps in our knowledge of ErbB2-driven oncogenesis and to improve ErbB2-targeted therapies. Cell line models derived from ErbB2 transgenic mouse mammary tumors are particularly well-suited to address these needs. Prior studies have described the establishment of cell lines from ErbB2 transgenic mice that could form tumors when inoculated in mammary fat pads of immune-compromised or immune-competent mice[22-24] and in certain cases could produce lung metastases either when injected intravenously or spontaneously from the primary site,[25] thus validating the approach. However, while these cell lines have proven useful in tumor vaccine and biological studies, it has not been firmly established with these cell line models whether these are strictly dependent on ErbB2 kinase, a very desirable trait. On the other hand, it is well established that ErbB2 overexpression in the mammary epithelium promotes invasive tumors that can metastasize.[3,26,27]

Here, we describe a cell line model derived from an MMTV-ErbB2/Neu transgenic mouse mammary tumor that is capable of forming primary as well as metastatic tumors following orthotopic mammary fat pad inoculation. Importantly, 2- and 3-dimensional culture studies establish that oncogenic attributes of the cell line established are strictly dependent on the ErbB2 kinase activity. In addition, we show that inhibition of the ErbB2 kinase activity promotes a normal epithelial-like polarity in 3-dimensional culture, suggesting that continued signaling downstream of the ErbB2 kinase maintains the oncogenic trait of loss of polarity in the model described here. Thus, the present model system should allow a molecular dissection of pathways that link ErbB2 to altered epithelial polarity and epithelial mesenchymal transition as part of the metastasis program of ErbB2-driven tumors.

MATERIALS AND METHODS

Establishment of mouse mammary tumor cell lines from MMTV-ErbB2 transgenic mice

All animal-related procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines. MMTV-Neu Tg [FVB/N-Tg(MMTV-Neu)202Mul/J] mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice were followed for appearance of palpable mammary tumors. Once established, tumors were aseptically resected, minced, and digested in F12 medium with 5 % FCS supplemented with 3 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN) and 1.5 mg/ml trypsin (Invitrogen, Carlsbad, CA) for 2 hours at 37° C. Dissociated cells were centrifuged at 200 *g*, washed twice, resuspended in DFCI-1 medium and cultured in a humidified incubator with 5% CO2 at 37° C. Cells were released with trypsin/EDTA when confluent. After serial passaging, we obtained three independent stable cell lines named MPPS1, MPPS2 and MPPS3 (please see footnote⋆) from individual mammary tumors arising in a female MMTV-Neu transgenic female mouse. MPPS1 tumor cell line was used in the present study. The MPPS1LA sub-line was developed by adapting MPPS1 cells to grow on ultra low attachment plates (Corning, Lowell, MA) in D2 medium; D2 is a derivative of DFCI-1 medium that lacks fetal bovine serum and bovine pituitary extract.[28]

Antibodies and other reagents

The following primary antibodies were obtained, from the indicated commercial sources: rabbit anti-cNeu (ErbB2) (C-18) from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit antibody broadly reactive with cytokeratins (pan-keratin) from DAKO (Carpinteria, CA); mouse monoclonal anti-vimentin (clone RV202) from BD Pharmingen (San Jose, CA); mouse monoclonal anti-zona occludens 1 (ZO-1) (clone ZO-1-1A12) from Invitrogen Inc (Carlsbad, CA); mouse monoclonal anti-heat shock cognate protein of 70 kDa (Hsc70) (B-6) from Santa Cruz Biotechnology; and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (clone 6C5) from Chemicon Int. (Temecula, CA). The mouse monoclonal anti-phosphotyrosine (anti-pY; 4G10)[29] was kindly provided by Dr. Brian Druker (Oregon Health and Science University, Portland, OR). The ErbB receptor tyrosine kinase inhibitor lapatinib[30,31] was from LC laboratories (Woburn, MA) and was stored as a 1mM solution in dimethyl sulfoxide (DMSO).

Assessment of cell proliferation

To quantify the effects of lapatinib on cell proliferation, cells were seeded in triplicate at 0.5 x 10^4/well in 96-well plates and incubated in the presence of the indicated concentrations of lapatinib. Seventy-two hours later, the culture medium was removed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 0.5 mg/ml for 4 hours. The cells were lysed by adding SDS to 1 % and absorbance was read at 570 nm. The % of cells alive at the end of the drug treatment, based on the absorbance of the formazan was calculated as $[(O.D_{570})^{treated}/(O.D_{570})^{untreated}] \times 100$.[32]

Three-dimensional (3-D) Matrigel culture

Laminin-rich basement membrane matrix (Matrigel®) for 3-D cultures was obtained from BD Biosciences (San Jose, CA). Trypsin/EDTA-released and washed cells were suspended in 2% (v/v) Matrigel in DFCI-1 medium and seeded over a layer of polymerized 100% Matrigel at 3 x 10^4 per cm^2 in 6- or 24-well plates or in 8-well chamber slides (for Confocal microscopy), essentially as described previously.[33] For growth on Ultra Low Attachment culture ware (Corning), cells were suspended in 4% (v/v) Matrigel/DFCI-1 medium mix.

Immunofluorescence microscopy

Immunofluorescence staining of cells grown in 3-D cultures was performed essentially as described.[33] In brief, the cells were fixed in 4% paraformaldehyde in PBS, washed with PBS/100 mM glycine and permeabilized in 0.5% Triton X-100 for 5 minutes. After washing, cells were blocked with 10% goat serum and then incubated with anti-ErbB2 (1/500), pan-anti-cytokeratin (1/5000), anti-vimentin (1/200) or anti-ZO-1 (1/200) for 1 hour at room temperature. After 3 washes, species-appropriate secondary antibodies conjugated with Alexa 488 or Alexa 546 (Invitrogen) was used to visualize the bound primary antibody. Alexa Fluor 594 phalloidin (Invitrogen) was mixed with the secondary antibody for visualization of polymerized actin. Vectashield Hard Set mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was used for mounting. Images were captured under a 63x oil immersion lens using LSM510 or LSM410 fluorescence confocal microscopes (Carl Zeiss) and processed with Zeiss LSM Image Browser version 4.2.0.121. Experiments were repeated at least thrice and an isotype-matched IgG was used as a negative control.

In vivo tumorigenicity assay

Athymic nude mice (Nu/Nu) were from Charles River Laboratories (Wilmington, MA). FVB/NJ mice were from Jackson Laboratory (Bar Harbor, ME). Seven-week old female mice were anesthetized and kept sedated using Ketamine along with Xylazine to directly visualize the fourth mammary

⋆ The author CFO-C named the cell lines as MPPS as a tribute to the life of his late wife Merlyn Patricia Pozada Sanchez.

gland through a small skin incision. 10^6 cells in 20 μl of DFCI medium were injected into the mammary fat pad using a Hamilton syringe. The tumor size was measured weekly using vernier calipers. Tumor volumes were calculated as: ½ larger diameter x (smaller diameter)^2.[34] Animals were euthanized and necropsies were performed when tumors reached a size of approximately 1,000 mm^3. Primary tumors and lungs were harvested for histopathology. At least five animals were used per experimental group.

RESULTS

Derivation and characterization of an ErbB2-driven mouse mammary tumor cell line MPPS1

Three separate primary mammary tumors (PT1, 2 and 3) were resected from an eleven-month old virgin female MMTV-Neu Tg mouse that developed multiple mammary tumors. Independent cell lines established from these three tumors were designated MPPS1, MPPS2 and MPPS3; MPPS1 line was characterized in detail. These cells exhibit robust and continuous growth as monolayers in DFCI-1 medium, and maintain high levels of ErbB2/Neu comparable to those in the primary tumors, as demonstrated using Western blotting [Figure 1a]. We did not notice any significant loss of ErbB2/Neu expression as has been reported by Guo *et al*. 2006 with primary tumor cells derived from MMTV-Neu(YD) mice upon prolonged *in vitro* propagation;[35] in fact, the levels of ErbB2 in MPPS1 cells remained unchanged when these were maintained continuously over 20 passages under our culture conditions [Figure 1a]. As expected for an epithelial cell-derived tumor cell line, MPPS1 cells stained positive with a pan-cytokeratin antibody while they did not stain significantly with an anti-vimentin antibody [Figure 1b]. In addition, smooth muscle actin was not detectable by immunostaining (data not shown). The cells stained prominently with an anti-ErbB2 antibody with the ErbB2 staining predominantly localized at the plasma membrane, giving a honey-comb appearance in confluent cultures [Figure 1c]. Addition of the EGFR/ErbB2 dual kinase inhibitor lapatinib in the culture medium led to a dose-dependent inhibition of cell proliferation, with an IC_{50} of 0.125 ± 0.001 μM [Figure 1d], indicating that MPPS1 cells are fully dependent on ErbB2 activity for cell proliferation. The ability of lapatinib to inhibit the ErbB2 kinase activity was shown by a concentration-dependent reduction in auto-phosphorylated ErbB2 levels [Figure 1e]. As reported by Scaltriti *et al*[36] in human ErbB2-overexpressing breast cancer cell lines, we also observed that lapatinib treatment led to accumulation of ErbB2 proteins [Figure 1 e; note the dose-dependent increase in the

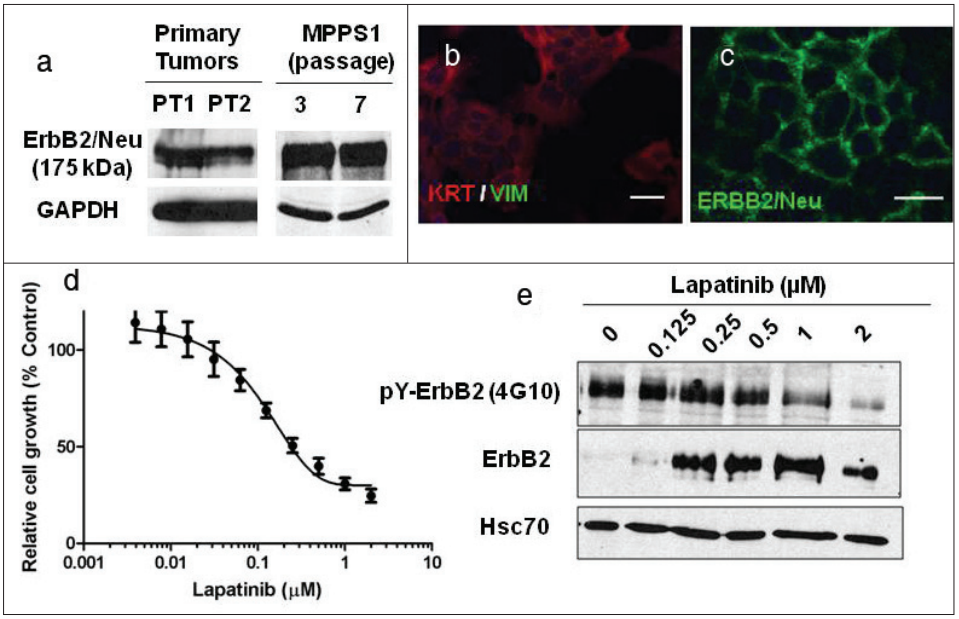


Figure 1: Characterization of ErbB2/Neu-driven mouse mammary tumor cell line (MPPS1): (a) Western blots comparing the expression of ErbB2/Neu in primary mammary tumors (PT1, PT2) from MMTV-Neu Tg mouse to the levels at the indicated passages of MPPS1 cell line (derived from PT1). 100 µg of total protein was loaded per lane. GAPDH is used as a loading control. (b, c) MPPS1 cells grown on glass coverslips were fixed and stained with pan-keratin (KRT), vimentin (VIM) and anti-ErbB2 (ErbB2/Neu) antibodies and detected with fluorochrome-conjugated secondary antibodies. The confocal image shows pan-keratin stained in red and vimentin in green; Scale bars in B and C are 20 µm in length. (d) Sensitivity of MPPS1 to growth inhibition by lapatinib - MPPS1 cells plated in 96-well plates as described in the methods section were treated with an increasing concentrations of lapatinib for 72 hours. The % of viable cells was estimated based on MTT assay. The calculated IC50 value (using Graphpad Prizm software) was 0.125 ± 0.001µM. (e) Concentration-dependent decrease in pY-ErbB2 levels in MPPS1 cells following treatment with lapatinib at the indicated concentrations. Cells were treated with the indicated concentrations of lapatinib for 48 hours. The reduction in pY-ErbB2 levels were assessed by SDS-PAGE/ Western blotting analysis from equal amount of total protein lysates (100 µg). Hsc70 was used as loading control.

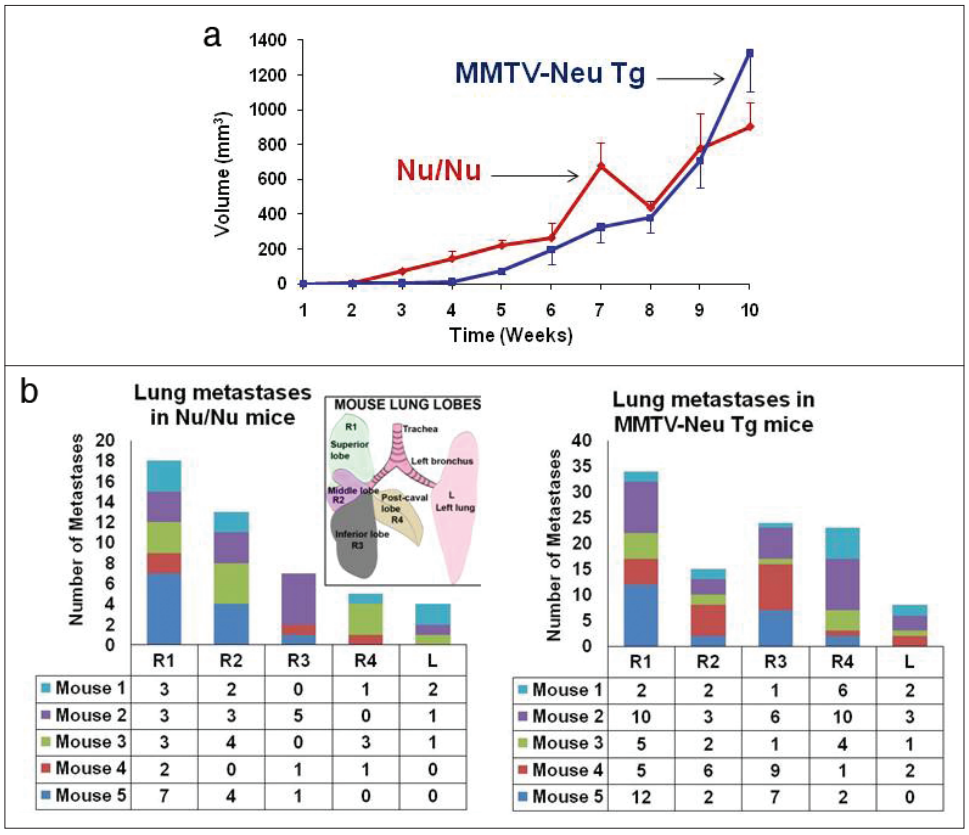
intensity of ErbB2 signal following lapatinib treatment]; this is likely due to reduced degradation of ErbB2 upon inhibition of its kinase activity.

Orthotopic implantation of MPPS1 cells in the mouse mammary gland results in tumors at the implant site as well as distant metastases

To assess whether ErbB2-dependent MPPS1 cells retain their oncogenic potential, we assessed their ability to form tumors at the site of orthotopic implantation in the mouse mammary gland as well as their ability to produce distant metastases. While implantation in syngeneic murine hosts is expected to allow tumor growth, studies of implanted MMTV-ErbB2/Neu transgenic tumors have shown that the "take rate" is better in ErbB2 transgene-carrying host mice (implanted prior to tumor development) than in naïve syngeneic hosts; this trait is likely related to induced immune tolerance mechanisms that are not fully elucidated[37,38] Therefore, we tested the tumor-forming capabilities of MPPS1 cells in female mice of three host backgrounds: immune-compromised nude mice; young isogenic MMTV-Neu Tg mice before any evidence of endogenous tumors; the FVB/NJ strain mice (same background as the tumor-bearing mice from which MPPS1 line was derived). Table 1 lists the various conditions used for

Table 1: Host mouse strain-dependent tumor development following implantation of MPPS1 cell line in the mammary fat pad

Mouse strain	Number of implanted cells	Mice (n)	Mice with tumors	% Mice with Tumors
FVB/NJ (parental)	10 x 10^6	6	2	33.3
FVB/NJ (parental)	1 x 10^6	5	0	0
FVB/N-Tg (MMTVneu)	1 x 10^6	5	5	100
Nu/Nu	1 x 10^6	5	5	100
Nu/Nu	0.5 x 10^6	5	4	80
Nu/Nu	50,000	5	2	40



	R1	R2	R3	R4	L
Mouse 1	3	2	0	1	2
Mouse 2	3	3	5	0	1
Mouse 3	3	4	0	3	1
Mouse 4	2	0	1	1	0
Mouse 5	7	4	1	0	0

	R1	R2	R3	R4	L
Mouse 1	2	2	1	6	2
Mouse 2	10	3	6	10	3
Mouse 3	5	2	1	4	1
Mouse 4	5	6	9	1	2
Mouse 5	12	2	7	2	0

Figure 2: Tumor growth and metastasis of orthotopically implanted MPPS1 cells in MMTV-Neu Tg and Nu/Nu mice: (a) MPPS1 cells were orthotopically implanted into immune-competent ErbB2/Neu transgenic (MMTV-Neu Tg) or immune-compromised Nu/Nu mice (n = 5 per group) and tumor growth monitored as described in the methods section. Shown here is a comparison of tumor growth in MMTV-Neu Tg vs. Nu/Nu background mice. (b) Lungs from the mice used in A were analyzed for metastatic tumor nodules. Shown is a comparison of the numbers of metastatic lesions recorded in various parts of the lung (see illustration in the inset).

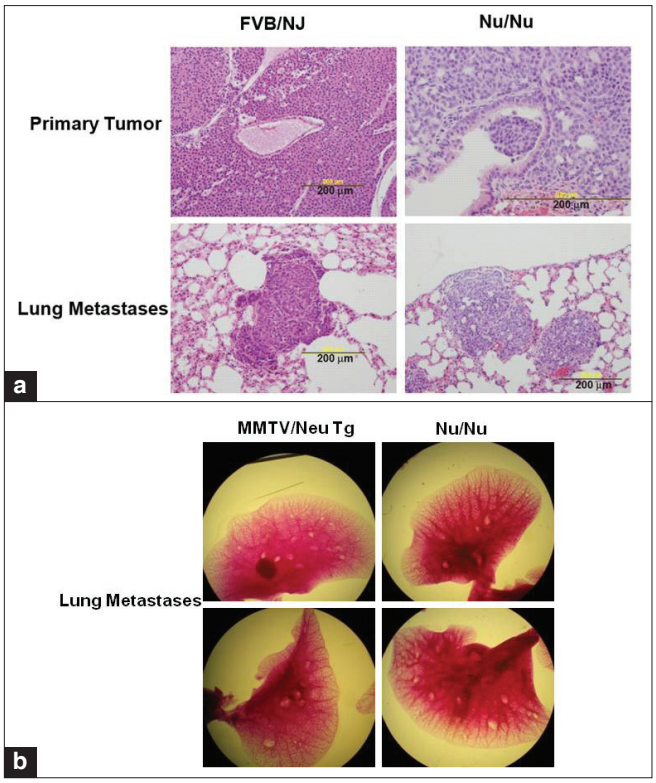


Figure 3: Comparative histopathological analysis of primary tumors and lung metastasis of MPPS1 cells orthotopically implanted into mammary fat pads of syngeneic immune-competent mice (FVB/NJ or MMTV-Neu Tg) or immune-compromised Nu/Nu mice: (a) Primary tumors from mammary fat pads and lungs with metastatic lesions developing in FVB/NJ and Nu/Nu mice injected with MPPS1 cells were fixed in formalin and processed for histopathology. Images of primary tumor and lung are from the same animal. Scale bar represents 200 µm. (b) Carmine staining of lungs isolated from MMTV-Neu Tg and Nu/Nu mice that were used in the tumor growth and metastasis study described in the legend to Figure 2. Sites of metastatic lesions are seen as clear areas within carmine-stained lungs. Scale bar represents 1 mm length.

orthotopic implantation into the mammary fat pad. Consistent with the reported facilitation of tumor formation in MMTV-Neu transgenic mice, 100 % of the nude and MMTV-Neu Tg recipients developed tumors around the site of injection [Table 1]; the tumors reached a volume of approximately 1,000 mm^3 in 9 to 10 weeks in both recipient strains [Figure 2a]. Tumors also formed when MPPS1 cells were orthotopically implanted in the mammary fat pad of FVB/NJ mice; however, 10 times more cells were required for orthotopic tumor growth compared to the other two recipient strains as shown in Table 1. Upon necropsy, multiple tumor nodules were observed in various parts of the lungs of tumor-bearing animals in all three recipient strains [Figure 2b].

Histopathological comparison of primary tumors and lung metastasis between one of the two mice that developed tumors in the FVB/NJ background with that from Nu/Nu [Figure 3a] showed similar features with ductal/glandular structures consistent with a well-differentiated adenocarcinoma. No significant differences were observed among MMTV-Neu Tg and Nu/Nu in the extent of metastatic lesions within the lungs [Figures 2b and 3b]. Together, these analyses demonstrate that transgenic ErbB2-overexpressing murine mammary tumor-derived cell line MPPS1 retains its oncogenic ability with a unique feature that orthotopic implantation in the mammary gland is associated with rapid metastasis to distant sites.

MPPS1 cell line as a model of persistent ErbB2 kinase activity-dependent alterations in epithelial cell polarity and lumen-filling hyper-proliferation phenotypes

Studies of ErbB2-driven human breast cancers as well as transgenic mouse models have shown that loss of epithelial cell polarity is a key event in oncogenic progression and a harbinger of epithelial-mesenchymal transition (EMT)[39-42] that has been linked to more aggressive tumor formation and more recently to cancer stem cell phenotype.[43] Given the ability of MPPS1 cells to metastasize from orthotopic primary tumors in the mammary gland, we wished to further characterize the alterations in the epithelial cell-cell junctions and polarity of these cells as well as the relationship of these traits with activity of the driver oncogene ErbB2. We and others have previously demonstrated that overexpression of ErbB family receptors in non-tumorigenic human mammary epithelial cells leads to alterations in cellular polarity which allows hyper-proliferation when cells are cultured under polarity-inducing 3-D Matrigel culture system.[33,39] Therefore, we carried out the characterization of cellular structures formed by MPPS1 cells when grown in 3-D culture. Many of these analyses were done with a low attachment-adapted and morphologically more homogenous subline (MPPS1LA) which was more suitable for immunofluorescence studies; The MPPS1LA cells expressed ErbB2 at levels comparable to that of ErbB2 in the parental MPPS1 cell line (data not shown) and were ErbB2-dependent as seen by the effect of lapatinib treatment on their growth [Figures 1d and 5c].

When seeded in Matrigel, MPPS1LA cells formed irregular spherical structures without any evident lumen [Figure 4a, left panel]; however, branched structures which are seen with *in vitro* ErbB2-transformed immortal human mammary epithelial cells[33,39] were rare. Immunofluorescence staining using established markers demonstrated a complete lack of epithelial cell polarity in 3D-cultured MPPS1LA cells. For example, the tight junction marker ZO-1 that is normally concentrated at the sub-apical cell-cell junctions (of a single layered epithelium of acini with a lumen)[33,44] is instead seen diffusely localized throughout the disorganized cellular masses that lack any semblance of a lumen [Figure 4b]. Staining for F-actin (phalloidin stained), normally localized to the apical

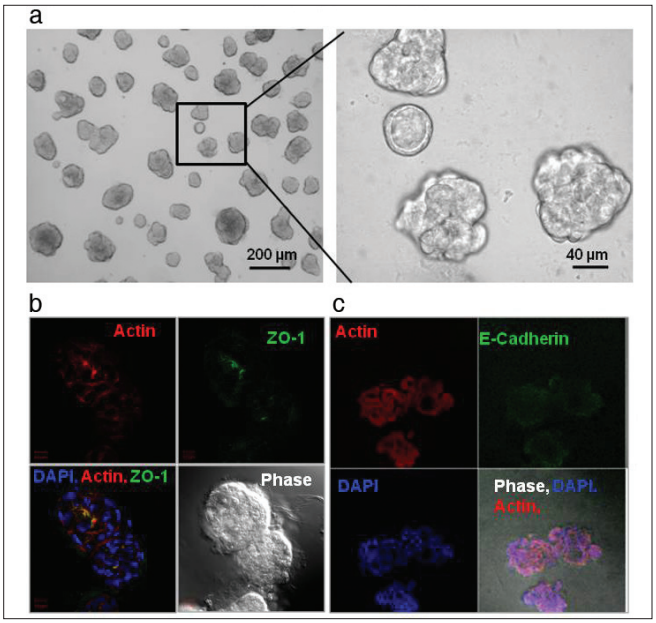


Figure 4: Characterization of the cellular structures formed by MPPS1 cells when grown in 3-D Matrigel culture: (a) Single cell suspensions of MPPS1LA cells were grown on 2% Matrigel as described in the methods section. Growth and morphology of 3-D structures were visualized using phase-contrast microscopy and photographed. Shown here are pictures of 3-D structures at day 8 after seeding. A higher magnification of the boxed area shown in the left panel is depicted in the panel on the right. (b) MPPS1LA cells grown in 2% Matrigel in glass chamber slides for 8 days were fixed and stained with Alexa594-conjugated phalloidin for sub-apical cortical actin, anti-ZO-1 (tight junction marker) using anti-ZO-1 antibody followed by Alexa488-conjugated secondary antibody against anti-ZO-1. The slides were mounted with a medium containing DAPI (to visualize nuclei) and imaged. Shown is a confocal microscopy picture of a 3-D cluster of cells visualizing the organization of actin filaments (red), ZO-1 (green). Also shown is a merged image including the nuclei (blue) and the phase contrast image; (c) MPPS1LA cells similarly grown in 2% Matrigel in glass chamber slides were fixed and stained with Alexa594-conjugated phalloidin or E-Cadherin (basolateral marker) using the anti-E-Cadherin antibodies followed by Alexa488-conjugated secondary antibody (green) against anti-E-Cadherin antibody. The slides were mounted with a medium containing DAPI (to visualize nuclei) and imaged. Images of a Matrigel colony of cells, depicting the disorganized actin filaments (red), and diffuse E-Cadherin (green) distribution. Also shown are the nuclei (in blue) and a merged image showing actin plus E-Cadherin along with the phase contrast.

cortical region of polarized mammary epithelial cells to form a continuous circular collar around the acini,[33,44] is also diffusely present throughout the disorganized cellular structures [Figure 4b and c]. Staining for E-cadherin [Figure 4c], which in polarized mammary epithelial cells is basolaterally localized to adherens junctions with little intracellular staining,[33,44] showed weak and relatively diffuse staining throughout the cells with many intracellular punctae and no discernible adherens junction-like staining. These analyses revealed that MPPS1 cells fail to polarize when grown in 3-D Matrigel culture, a feature consistent with their transformed phenotype.[39,45]

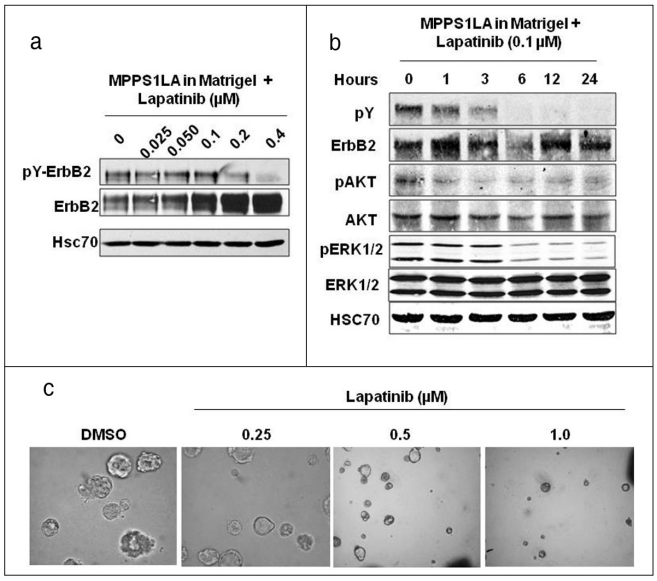


Figure 5: Analysis of the requirement of ErbB2 kinase activity and downstream signaling pathways in abnormal polarity and associated hyper-proliferation of MPPS1 cells in 3-D culture: (a) Concentration-dependent decrease in pY-ErbB2 in MPPS1LA cells, grown in 3-D Matrigel following treatment with Lapatinib. Cells were treated with the indicated concentrations of Lapatinib for 48 hours. The reduction in pY-ErbB2 levels was assessed by SDS-PAGE/Western blotting analysis from equal amounts of total lysate protein (100 µg). Hsc70 was used as a loading control. (b) Kinetics of decrease in pY-ErbB2 in MPPS1LA cells grown in 3-D. 50 µg total lysate protein was analyzed for each indicated time point. Blot strips were probed first with anti-pY ErbB2, anti-pAKT and anti pERK1/2, and then stripped and reprobed for ERBB2/Neu, AKT and ERK, respectively. (c) MPPS1LA grown on Matrigel for 8 days, were treated with the indicated concentrations of Lapatinib for another 6 days. The changes in colony morphology were recorded as described in the legend to Figure 4a. Representative images are shown to illustrate the concentration-dependent effect of Lapatinib on the morphology and size of 3-D structures.

Given the dependence of MPPS1 cell proliferation on ErbB2 when analyzed in 2-D culture [Figure 1d], we utilized lapatinib treatment to assess if the abnormal polarity and the associated hyper-proliferation of MPPS1 cells in 3-D culture are also causally linked to continued kinase activity-dependent signaling of the driver oncogene ErbB2. First, we looked at the dose-response and kinetics of lapatinib-induced reduction in pY-ErbB2 levels in cells grown on 3-D Matrigel cultures. Immunoblotting of cell lysates obtained from 3-D cultures demonstrated that lapatinib treatment indeed induced a dose-dependent reduction in phosphorylated (active) ErbB2 [Figure 5a] with a concomitant increase in the total level of ErbB2 when examined after 48 h of treatment, as seen in cells treated with lapatinib in 2-D culture [Figure 1e]. Notably, the effects of lapatinib were relatively quick, as seen by an essentially complete loss of pY-ErbB2 signals within 6h of treating 3-D cultures with 100 nM lapatinib [Figure 5b]. Importantly, lapatinib treatment was associated with expected reduction in phosphorylated (active) pools of AKT and ERK1/2 without a

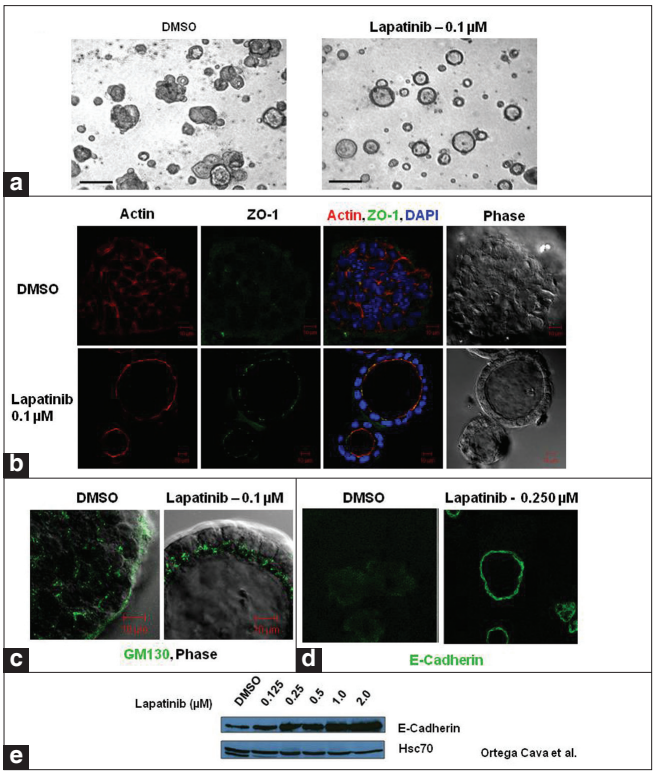


Figure 6: Characterization of Lapatinib-induced normalization of epithelial cell polarity in 3-D Matrigel cultures of MPPS1 cell line. (a) MPPS1 LA cells were grown on Matrigel for 5 days and then were treated with 0.1 μM Lapatinib for 3 days, visualized using phase-contrast microscopy and photographed. Shown is a comparison of the morphology for 3-D structures in cultures treated with DMSO (left panel) or 0.1 μM Lapatinib (right panel)., (b-d) Cells grown on Matrigel (similar to 6A) in 8-well chamber slides were treated with DMSO or 0.1 μM Lapatinib. Following treatment, the cells were paraformaldehyde fixed and stained with anti-ZO1 (B) or anti-GM1 (c; apical marker) or anti-E-cadherin (D) followed by Alexa488-conjugated secondary antibody and Alexa594-phalloidin (for actin). The slides were mounted with a medium containing DAPI and imaged; (e) Lapatinib treatment-induced accumulation of E-cadherin protein levels in MPPS1LA cells; shown is a western blot analysis of 25 μg total proteins from lysates of MPPS1LA cells treated with the indicated concentrations of Lapatinib for 24h. Hsc70 is shown as a loading control.

change in total AKT or ERK1/2 levels [Figure 5b], indicating that lapatinib treatment at relatively low concentrations indeed abrogated ErbB2 kinase-dependent downstream signaling.

Addition of lapatinib to 3-D cultures led to a concentration-dependent reduction in the number of irregular acini and their overall size. At high concentrations (0.5 to 1 μM), the cell structures tended to disintegrate consistent with cytotoxic effects [Figure 5c]. Notably however, cultures treated with intermediate concentrations (0.1 to 0.25 μM) of lapatinib showed a predominance of regular acinus-like structures with lumens [Figure 5c and 6a], suggesting a reversal of abnormal polarity. Staining with markers of polarity demonstrated that this was indeed the case: as is evident in Figure 6b, a lapatinib-treated 3-D

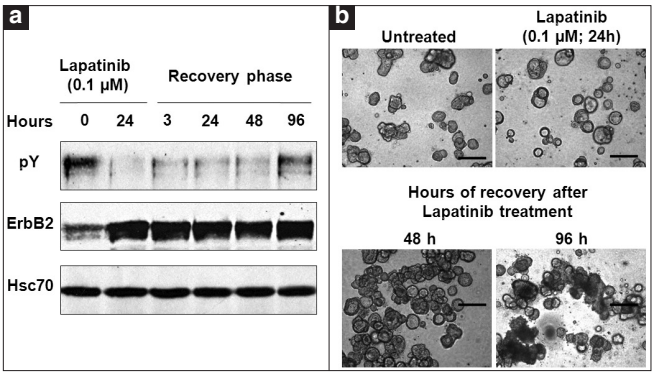


Figure 7: Reversal of Lapatinib-induced normalization of epithelial cell polarization in 3-D Matrigel upon removal of inhibitor: MPPS1LA cell grown in 6-well plates on Matrigel were treated with 0.1 μM Lapatinib or DMSO vehicle for the indicated times. For evaluating the reversibility of Lapatinib-induced normalization of epithelial architecture and lumen formation, control and Lapatinib-treated 3-D structures were harvested from Matrigel cultures, spun, washed with PBS and replated in fresh Matrigel. The cultures were imaged at the indicated times. The results are representative of three experiments. a, Western blotting of 50 μg protein confirming the effects of Lapatinib-treatment on pY-ErbB2 and total ErbB2 levels and a time-dependent recovery of pY-ErbB2 following Lapatinib withdrawal. b, Digital images of cell structures, left untreated, Lapatinib treated (24 h), or recovery (48 or 96 h) after Lapatinib withdrawal, are shown. Scale bars are 100 μm.

cultures showed a predominance of single epithelial layer-lined acinar structures with a clear lumen (as seen with DAPI staining near the periphery and lack of nuclei in the middle). Importantly, these acini showed clear and well-formed tight junctions (ZO-1 staining), sub-apical actin bands (phalloidin staining), apical GM130 and E-cadherin localization in basolateral adherens junctions [Figures 6b-d]. Biochemically, lapatinib treatment led to a concentration-dependent accumulation of E-cadherin protein levels [Figure 6e]. These results suggest that continuous signaling downstream of an active ErbB2 kinase produces the abnormal polarity and associated lumen-filling hyper-proliferation phenotypes of MPPS1 cells.

The ability of MPPS1 cells to attain normal epithelial-like polarity and growth characteristics upon ErbB2 kinase inhibition, as early as 12-24 hours after initiating treatment [Figure 5b], prompted us to ask if the lapatinib effect was terminal or could be reversed upon washout of the drug. Treatment of 3-D cultures with 0.1 lapatinib for 24 h was associated with a reduction in pY-ErbB2 levels as expected, as well as the appearance of regular acinar structures [Figures 7a and b]. Washout of lapatinib led to a slow but almost complete recovery of pY-ErbB2 signals [Figure 7a] and the cultures concomitantly resumed proliferation regaining a predominance of abnormal cellular structures that lacked lumens [Figure 7b]. These results indicate that MPPS1 cells offer a reversible system to link ErbB2 signals with specific oncogenic traits.

DISCUSSION

Cellular models have provided important clues into mechanisms by which the human cancer oncogene ErbB2, which is causally linked to over a quarter of invasive breast cancers, unleashes the program of oncogenic transformation of mammary epithelial cells. Yet, there is a lack of facile cellular models that allow biochemical and molecular analyses over a wide range of oncogenic transitions from relatively normal cellular behavior to metastasis. Furthermore, full dependence of oncogenic progression on ErbB2 kinase-dependent signaling would help enhance the appeal of such cellular models. Recent studies have focused on either human cancer-derived ErbB2-overexpressing cell lines or on deliberate transformation of non-tumorigenic immortal human mammary epithelial cell lines; however, the former often exhibit variable dependence on ErbB2 signals while the latter typically show only subtle oncogenic transformation discernible only using sensitive systems such as 3-D culture in Matrigel; such transformed cells often fail to make tumors in xenotransplanted mice and typically do not exhibit metastatic behavior.[33,46,47] As ErbB2 transgenes reproducibly lead to mammary tumors that metastasize,[25,48] cellular models from such mice could bridge the current gap in our understanding of mechanisms of ErbB2-driven oncogenesis. While several previous attempts have been made to establish cell line models from ErbB2 transgenic mice, these cell systems have not been well characterized as suitable models of critical oncogenic traits associated with ErbB2-mediated oncogenic transformation. Here, we have generated and characterized a murine ErbB2 transgene (rat ErbB2/Neu)-induced mammary tumor cell line system whose attributes make it a desirable cellular model to help bridge gaps in our knowledge of mechanisms by which ErbB2 signals unleash the oncogenic program of mammary epithelial cells.

The MMTV-Neu Tg mouse-derived mammary tumor cell line MPPS1 not only produces primary tumors when orthotopically implanted in the mammary gland but it also exhibits rapid metastatic ability as shown by the presence of lung metastasis in essentially all primary tumor-bearing animals. While rapid tumor growth and metastatic behavior by itself is not unique, it is rather notable that both the primary and metastatic tumors maintained relatively well-differentiated tumor histology [Figure 3a]. More importantly, *in vitro* characterization demonstrated that MPPS1 cells proliferate continuously but unlike previous reports[35] the levels of ErbB2 are not lost in this cell line during culture [Figure 1a]. This in itself suggested the potential dependence of oncogenic traits of MPPS1 cells on continued ErbB2 expression and its signaling. Indeed inhibition of ErbB2 kinase activity with lapatinib demonstrated that cell proliferations in 2-D and 3-D culture is fully dependent on continued activity of ErbB2. Thus, MPPS cells should provide a useful model to investigate pathways that provide an essential link between ErbB2 signals and deregulation of cell cycle and associated programs such as cancer cell metabolism, especially under 3-D culture conditions that mimic tissue architecture.

As previous studies have demonstrated, the pathways of normal cellular proliferation and differentiation as well as their aberrations during oncogenesis are dramatically influenced by the interaction of cells with each other and with the microenvironment as well as the nature of matrix.[49,50] Thus, a tumor cell line model that exhibits complete abrogation of cell polarity and lumen formation, important attributes of normal mammary epithelial cells in tissues as well as in 3-D culture, when the driver oncogene (ErbB2) is active but rapidly reverses these attributes towards a normal cell behavior [Figures 6a-e] when oncogenic signals are turned off should provide a valuable system to mechanistically trace upstream ErbB2 signals to regulation of mammary epithelial morphogenesis and its alterations in breast cancer. Our initial studies indicating that MPPS1 cell system allows reversion of oncogenic traits upon lapatinib inhibition as well as resumption of oncogenic traits upon lapatinib washout make the current system particularly attractive.

Complementing the *in vitro* attributes, it is notable that metastatic tumors developed with a short latency after orthotopic implantation in host mice, and this could be achieved in immune competent hosts as well albeit more tumor cells were needed to develop tumors in naïve parental strain. As antibody-dependent targeted therapy has become an essential component of therapy for ErbB2-driven breast cancers, models in which metastatic disease develops reproducibly and quickly are needed to investigate mechanisms of therapeutic response as well as resistance using *in vivo* settings with an intact immune system, something lacking with xenograft models. The current system should be very useful in this regard.

It is of note that tumor growth and metastasis in syngeneic (MMTV-Neu Tg) females were rapid, highly reproducible and comparable to tumors seen in immune-compromised nude mice. However these attributes were somewhat slower and less robust in parental FVB/NJ mice. This observation is consistent with previous reports that rat ErbB2/Neu oncogene is immunogenic in non-transgenic FVB mice.[37,38] However, the ability of MPPS1 cells to reproducibly grow and metastasize spontaneously from the primary tumor in immune competent MMTV-Neu Tg mice should still provide a practical immune-competent model to study tumor progression and therapeutic mechanisms. As the latency of spontaneous tumors in the MMTV-Neu Tg mice was as reported previously (about 6 months),[27,51] yet the implanted MPPS1 tumor grew and metastasized before these mice had attained this age (about 7

weeks of age at implant plus 10-11 weeks of tumorigenesis for a total of 17-18 weeks of age at the completion of the experiment), it is unlikely that the tumors at the implant site or metastases in the lung represent spontaneous tumors. This interpretation is supported by lack of any tumors in non-implanted mammary glands of recipient mice. We have recently developed GFP-expressing MPPS1 cells which should allow future studies to unequivocally distinguish implanted tumors and their metastases from endogenous tumors.

Recent years have seen an expansion of high throughput technologies that have begun to yield important mechanistic clues relevant to biology of cancer as well as mechanisms of therapeutic response and resistance. In initial studies, we have observed that MPPS1 cell system is easily adaptable for gene transfer experiments and cell-based assays. Together with the ErbB2-dependent 3-D culture traits and the ability to rapidly induce primary and metastatic tumors in syngeneic hosts, the MPPS1 cell line should be easily adaptable for linked *in vitro* and *in vivo* high throughput studies within a single cell model.

CONCLUSIONS

Collectively, the MPPS1 cell model of ErbB2-driven breast cancer described here demonstrates that loss of cellular polarity and lumen filling in 3-D environment requires continuous ErbB2 signals. Our results suggest that the relatively unique cellular model described here should help in molecular dissection of oncogenesis as well as mechanisms of therapeutic response and resistance in ErbB2-driven primary and metastatic breast cancer using *in vivo* studies in immune-competent animals that can be completed in a short time and in a cost effective manner. This cellular model should also provide a tool to dissect signaling pathways linking upstream ErbB2 signaling with early as well as late oncogenic traits using high throughput approaches that exploit the ability to carry out *in vitro* and *in vivo* tumor progression studies in a single cellular system.

List of Abbreviations used

DAPI - 4',6-diamidino-2-phenylindole; DFCI-1 – mammary epithelial cell culture media named after Dana Farber Cancer Institute; D2 - a derivative of DFCI-1 medium that lacks fetal bovine serum and bovine pituitary extract; DMSO – Dimethyl Sulfoxide; EMT – Epithelial to Mesenchymal Transition; ErbB2 (Her2/Neu) – Epidermal Growth Factor Receptor 2; EDTA – Ethylene Diamine Tetra Acetic acid; FCS – Fetal Calf Serum; GAPDH – Glyceraldehyde phosphate dehydrogenase; Hsc70 – Heat shock cognate protein 70; MPPS1 (and MPPS2 and 3) – ErbB2/Neu driven mammary epithelial cell line derived from primary tumors from MMTV-Neu Tg mice; MPPS1LA – MPPS1 derived cell line enriched for growth in low attachment cell culture plates; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PT1, 2 and 3 – Primary Tumor 1, 2 and 3; SDS – Sodium dodecyl sulfate; Tg – Transgenic; ZO-1 – Zona Occludens 1

AUTHOR CONTRIBUTIONS

The study was conceived and directed by HB, VB, MN, SMR and CFO-C. The experimental work was executed by CFO-C with contributions from ZL, RG, SHW, MD, LD, BM, HL, TAB, MN and SMR. ACE helped with the histopathology-related studies. CFO-C wrote the first draft that was edited, into final manuscript by HB, SMR and MN. TAB, HL and BM also helped organizing the manuscript figures and literature for citations.

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