INTRODUCTION

Carcinomas of the breast can be classified by their intrinsic gene expression pattern, and the phenotype of the various tumor subtypes has been postulated to mimic specific differentiation stages within breast development.\(^1\,2\) It has been demonstrated that luminal cancers show a gene expression pattern consistent with the differentiated luminal compartment, while the HER2 (EGFR2, ErbB2) and triple negative subgroups show a more undifferentiated expression pattern. This is consistent with the tumor subgroups to arise from luminal and basal-like progenitor cells, respectively.\(^3\,4\)

How various critical oncogenic signals affect the differentiation state and thus affect the breast cancer subgrouping is still not well known. The reason for this is partly the lack of in vitro culture systems, modeling both normal breast stem cell biology and neoplastic transformation. D492 is a breast epithelial cell line with stem cell properties that can undergo epithelial to mesenchyme transition (EMT), generate luminal- and myoepithelial cells and form complex branching structures in three-dimensional (3D) culture. Here, we show that overexpression of HER2 in D492 (D492\(^{HER2}\)) resulted in EMT, loss of contact growth inhibition and increased oncogenic potential in vivo. HER2 overexpression, furthermore, inhibited endogenous EGFR expression. Re-introducing EGFR in D492\(^{HER2}\) (D492\(^{HER2}/\text{EGFR}\)) partially reversed the mesenchymal state of the cells, as an epithelial phenotype reappeared both in 3D cultures and in vivo. The D492\(^{HER2}/\text{EGFR}\) xenografts grow slower than the D492\(^{HER2}\) tumors, while overexpression of EGFR alone (D492\(^{EGFR}\)) was not oncogenic in vivo. Consistent with the EGFR-mediated epithelial phenotype, overexpression of EGFR drove the cells toward a myoepithelial phenotype in 3D culture. The effect of two clinically approved anti-HER2 and EGFR therapies, trastuzumab and cetuximab, was tested alone and in combination on D492\(^{HER2}\) xenografts. While trastuzumab had a growth inhibitory effect compared with untreated control, the effect of cetuximab was limited. When administered in combination, the growth inhibitory effect of trastuzumab was less pronounced. Collectively, our data indicate that in HER2-overexpressing D492 cells, EGFR can behave as a tumor suppressor, by pushing the cells towards epithelial differentiation.

EMT is a pivotal step during cancer progression where cells acquire motility by losing epithelial characteristics such as expression of cytokeratins and E-cadherin, and gain expression of mesenchymal markers, like vimentin, fibronectin and N-cadherin (reviewed by Moyret-Lalle\(^{14}\)). Changes in microRNA (miR) expression, such as the miR-200 members, have also been implicated with EMT in breast cancer.\(^8\,\ldots\,^{11}\) Given phenotypic flexibility and the stem cell nature of the D492 cells, they have a unique potential to model the effect of critical signaling molecules on cellular morphogenesis, differentiation, EMT and neoplastic transformation.

Dysregulation of the signaling pathways activated by the epidermal growth factor receptor (EGFR) family is oncogenic in many epithelial cells. Amplification of the HER2 (ErbB2) gene is especially injurious in breast epithelium where upregulation of HER2 is seen in approximately 20% of all breast cancers,\(^12\) leading to increased proliferation, cell motility and metastasis.\(^13\) These receptors are also key components in controlling polarization and cellular differentiation of the breast gland (reviewed by Sternlicht\(^{14}\)).

The clinical effects of HER2 and EGFR dysregulation are well known, and a range of options for inhibiting their adverse effects exists.\(^15\) New breast cancer treatment regimens have, however, actualized new clinical challenges. Development of resistance...
against EGFR family-targeted therapy is common, and in the HER2 breast cancer subgroup, systemic disease is now partially controlled, while metastatic disease in the central nervous system seems to escape both targeted and conventional therapy. Increased understanding how HER2 and EGFR signaling affects cellular behavior and differentiation in breast epithelial cells is therefore needed. The objective of this study was to address the functional consequences of HER2 and EGFR overexpression in breast epithelial progenitor cells focusing on both cellular differentiation and tumorigenicty. In vitro three-dimensional (3D) models and in vivo tumorigenicity assays were employed to measure changes in cellular phenotype, stemness and tumorigenicty.

RESULTS
HER2 and EGFR show distinct expression pattern in human breast epithelium
Initially, we analyzed HER2 and EGFR expression in the normal breast. CK19 and CK14 were used to identify luminal epithelial- and myoepithelial cells, respectively (Figure 1, top left). Co-staining of EGFR and HER2 with either CK19 or CK14 revealed distinct expression patterns with EGFR expression associated with the basal/myoepithelial compartment. HER2 expression was predominantly associated with the luminal epithelial cells (Figure 1, lower right). Co-staining of EGFR and HER2 revealed cells within the myoepithelial compartment being positive for both receptors (Figure 1, top right). Western blotting of isolated primary luminal- and myoepithelial cells from reduction mammoplasties confirmed a higher expression of HER2 in luminal epithelial cells compared with myoepithelial cells, and more EGFR in myoepithelial cells (Figure 1b) compared with luminal cells.

Overexpression of HER2 in D492 breast epithelial progenitor cell line leads to reduced EGFR expression and EGF-independent activation of EGFR and HER2
Corresponding to the basal-like phenotype of D4926,17 the cells express very low levels of HER2 (Figures 2a and b). To analyze the
Figure 2. HER2 overexpression reduces EGFR expression. (a) Expression of endogenous EGFR in D492HER2 is reduced compared with D492 ctrl. Confocal microscopy images of D492 cells expressing ctrl, HER2, EGFR and HER2/EGFR grown on culture flasks and analyzed by immunofluorescence staining for EGFR and HER2 expression. Bar = 50 μm. (b) HER2 overexpression leads to reduced EGFR transcription levels. Quantitative reverse transcriptase–PCR was performed using monolayer RNA isolates from all four cell lines. Transcription of HER2 and EGFR was analyzed, and normalized to GAPDH. (c) HER2 overexpression leads to ligand-independent EGFR and HER2 phosphorylation. Cells on monolayer were starved for 24 h in media without EGF. Cells were then given control media or EGF-containing culture media. Protein lysates were collected at +180 min and blotted for total and phosphorylated EGFR and HER2. Actin = loading control.
HER2-induced EMT in D492 cells

The effect of EGFR and HER2 overexpression on the cellular differentiation status was evaluated by measuring the expression of epithelial (CK14, CK19, E-cadherin, p63, P-cadherin) and mesenchymal (N-cadherin and Axl) markers in the transduced cell lines. D492HER2 cells showed a near complete loss of CK14 and CK19, E- and P-cadherin and p63 (Figure 3a, upper right), while partial re-expression of the four markers was seen in D492HER2/EGFR (Figure 3a, lower right). D492EGFR showed a heterogeneous expression pattern of CK19 and CK14, comparable to the control cell line, with most cells being either CK19 positive or CK14 positive while a smaller subpopulation was double positive (Figure 3a, lower left). In D492EGFR, no change in E-cadherin expression was seen, but expression of P-cadherin was increased, suggesting increased myoepithelial differentiation. The overall expression of luminal-, myoepithelial and mesenchymal markers in the cell lines was also compared using western blotting (Figure 3b). The loss of epithelial markers was confirmed in both D492HER2 and D492HER2/EGFR as evidenced by reduced expression of P63, CK14 and CK19 as well as E- and P-cadherins. Furthermore, these cells showed increased mesenchymal marker expression as shown by immunostaining with N-cadherin and Axl, indicating that HER2-overexpressing D492 cells have switched to a more mesenchymal phenotype (Figure 3b). D492EGFR, on the other hand, showed a more pronounced basal/myoepithelial differentiation, as evidenced by stronger CK14, P63 and P-cadherin expression and loss of CK19 (Figure 3b). It is well known that the miR-200 family regulates epithelial morphogenesis and EMT through a negative feedback loop with the ZEB1 and ZEB2 transcription factors.11 Compared with D492CTR, the miR-200c-miR141 cluster was greatly downregulated in D492HER2 cells (Figure 3c), accompanied with upregulation of ZEB1. Constitutive expression of EGFR, on the other hand, with or without HER2 resulted in increased miR-200-141 expression levels, and reduced ZEB1 (Figure 3c). Collectively, the results indicated that the tyrosine kinases EGFR and HER2 had distinct effects on differentiation in the breast epithelial progenitor cell line D492. Introduction of HER2 caused a loss of EGFR, cytokeratins and E-cadherin, and increased expression of mesenchymal markers indicating that the cells underwent EMT.

Expression of HER2 leads to non-adherent growth

D492CT cells are growth inhibited when they reach confluence in two-dimensional culture. EGFR overexpression affected the growth pattern only to a limited degree (Figure 4a), while D492HER2 and D492HER2/EGFR cells had lost their contact inhibition and continued to proliferate after reaching confluence, piling up in culture (Figure 4a). Increased fraction of non-viable cells was not detected, and all sublines exhibited 95% and higher viability after 12 days in culture. Furthermore, as colonies of viable cells could be seen floating in the culture medium, we seeded cells into low-attachment plates and quantified survival based on colony formation (Figure 4b). In this environment, a subset of D492CT cells was able to survive and proliferate, forming small spheroid clusters (Figure 4b, GFP images). D492EGFR did not have significantly higher number of spheroids formed compared with D492CT, but the size of the spheres was significantly increased. D492HER2 and D492HER2/EGFR showed greatly increased sphere formation. In addition, they were not spherical with intact cell-cell adhesion as seen in D492CT and D492EGFR cells, instead the spheres appeared to form grape-like aggregates, perhaps indicative of changed cell-cell adhesion properties (Figure 4b, bottom GFP images).

HER2 maintained a mesenchymal phenotype in 3D culture that was partially restored by re-expression of EGFR

Considering the effects of HER2 overexpression on loss of contact inhibition and epithelial markers, it was interesting to compare the phenotypes of all four cell lines when cultured in rBM. The three cell lines that expressed EGFR (ctr, EGFR and HER2/EGFR) predominantly formed structures with a solid epithelial phenotype with robust cell-cell adherence and some budding/branching structures (Figure 5a). To test the differentiation status of the different 3D phenotypes, immunostaining showed that budding/branching and round structures expressed epithelial markers, while grape-like and spindle did not (Figure 5a). D492HER2 cells in rBM showed a diffuse phenotype containing almost only grape- or spindle-like structures. To see whether HER2 receptor signaling was crucial for formation of these mesenchymal colony-type structures, cultures were treated with the HER2 inhibitor CP724,714 (CP). Spindle colony formation was completely inhibited in all cultures when CP treated (Figure 5a, table panel). Furthermore, CP-treated D492HER2 cells formed more solid round spheres. These results indicated that HER2 expression is essential for the mesenchymal phenotype to appear, and that EGFR had a stronger effect on morphology in 3D rBM than in monolayer. Western blotting of lysates from 3D rBM cultures was performed to check whether the EGFR induced appearance of epithelial structures in 3D coincided with re-expression of epithelial markers. Cytokeratins 14 and 19 and E-cadherin were expressed in all EGFR-expressing cell lines in 3D rBM (Figure 5b). The observation that EGFR expression partially reverses HER2-induced EMT in 3D culture but not in monolayer (compare western blots in Figure 3b with Figure 5b) emphasizes how vastly different these culture conditions are. On the one hand, cells are grown on 2D collagen-coated plastic, while in the other, cells are embedded in 3D laminin-rich extracellular matrix. In 2D culture, N-cadherin staining is seen in control cells, while in 3D culture control cells express N-cadherin, indicating a partial switch in phenotype (Figure 5b). It is increasingly being recognized that signaling and gene expression is regulated differently in 3D
compared with 2D culture. These data indicate that EGFR partially inhibits the HER2-induced mesenchymal phenotype with reappearance of cytokeratins and E-cadherin, in addition to promoting epithelial morphology in 3D cultures.

Ectopic expression of EGFR attenuates HER2-induced tumorigenicity in D492
To test whether EGFR, HER2 or double overexpression of these two receptors affected growth in vivo, the four cell lines were

Figure 3. HER2 overexpression induces mesenchymal transition. (a) D492HER2 cells lose epithelial phenotype in monolayer culture. Confocal microscopy images of D492 cells expressing ctrl, HER2, EGFR and HER2/EGFR grown on culture flasks and analyzed by immunofluorescence staining for CK14, CK19, E-, N- and P-cadherin expression. Bar = 50 μm. (b) D492HER2 and D492HER2/EGFR gain mesenchymal phenotype in monolayer culture. Western blotting performed using lysates from monolayer cultures. Membranes were blotted for CK14, CK19, p63, E-, P-, N-cadherin and Axl expression. Actin = Loading control. (c) In D492HER2, miR-200c and miR141 are strongly downregulated. Quantitative reverse transcriptase–PCR was performed using monolayer RNA isolates from all four cell lines. Transcription of miR-200c, miR-141 and ZEB1 was analyzed and normalized to GAPDH.
injected into mammary fat pads of NSG mice. With variable efficiency, all cell lines gave rise to small but palpable tumors 1 week post injection (Figure 6a). HER2 overexpression gave rise to fast growing tumors, while tumors from D492ctrl and D492EGFR ceased to grow (Figure 6b). Interestingly, and in good concordance with our 3D data, D492HER2/EGFR tumors had a longer lag period, and did not grow as fast as D492HER2, indicating that EGFR expression suppressed the growth-promoting effect of HER2 (Figure 6b and Supplementary Figure S2A). Based on these findings it was interesting to test whether inhibition of EGFR signaling in HER2-expressing cells would affect tumor growth. D492HER2 tumor-bearing animals were treated with trastuzumab (HER2 mAb/Herceptin), cetuximab (EGFR mAb/Erbitux) or a combination of the two, and changes in tumor volume were monitored. Upon trastuzumab treatment, tumors decreased significantly in size compared with control, and although some of the individual tumors seemed to regain growth after a lag period, trastuzumab had a clear growth inhibitory effect. D492HER2/EGFR tumor-bearing animals were treated with trastuzumab (HER2 mAb/Herceptin), cetuximab (EGFR mAb/Erbitux) or a combination of the two, and changes in tumor volume were monitored. Upon trastuzumab treatment, tumors decreased significantly in size compared with control, and although some of the individual tumors seemed to regain growth after a lag period, trastuzumab had a clear growth inhibitory effect. D492HER2/EGFR tumors showed that the in vivo expression of epithelial markers correlated well to the 3D in vitro phenotype (Figure 7). D492HER2 cells expressed HER2 but had low EGFR expression. Furthermore, they were CK14 negative demonstrating lack of myoepithelial differentiation and were also E-cadherin and CK19 negative (Figure 7b and Supplementary Figure S3B). In contrast, D492HER2/EGFR formed tumors that were heterogeneous in terms of marker expression. These tumors were positive for both HER2 and EGFR with HER2 expressed in all tumor cells, whereas EGFR was partially lost. CK14 expression was restricted to EGFR-expressing cells, thus indicating retention of epithelial characteristics within these tumors (Figure 7b). Additionally, E-cadherin and CK19 staining were seen in EGFR-expressing cells (Figure 7b and Supplementary Figures S3 and S5). Ki67 staining in HER2 and HER2/EGFR tumors was comparable, but markedly higher than in control or EGFR tumors (Figure 7c and Supplementary Figure S3B, respectively). Interestingly, staining for cleaved caspase-3 indicated that apoptosis levels were considerably higher in HER2/EGFR tumors compared with HER2 alone. Incidentally, active caspase-3 staining was found to be strongest in close proximity to tumor cells with an epithelial phenotype (CK14-positive cells; Figure 7c). Staining for cleaved caspase-3 was comparable to the control tumors. The observed in vivo effects of HER2 overexpression and inhibition made it interesting to investigate the patterns of receptor and marker expression within the xenograft tumors. Sections of immunofluorescently-stained D492HER2 and D492HER2/EGFR tumors showed that the
Collectively, these data showed that coexpression of EGFR and HER2 resulted in slower tumor growth than HER2 alone and suggested that EGFR might suppress the oncogenic properties of HER2, through maintenance of the epithelial phenotype.

**DISCUSSION**

Overexpressing two oncogenes, HER2 and EGFR, in the D492 breast epithelial progenitor cell line resulted in changed cellular phenotypes. D492HER2 underwent EMT as evidenced by loss of epithelial markers, and gain of mesenchymal markers accompanied by formation of spindle-like colonies in 3D rBM culture. Furthermore, D492HER2 cells showed tumorigenic properties in vivo. These findings are in concordance with other studies where loss of cytokeratins and epithelial cadherins following HER2 limited in control and EGFR tumors (Supplementary Figure S3B), indicating low levels of apoptosis.

Collectively, these data showed that coexpression of EGFR and HER2 resulted in slower tumor growth than HER2 alone and suggested that EGFR might suppress the oncogenic properties of HER2, through maintenance of the epithelial phenotype.
overexpression has been described. HER2 has previously also been overexpressed in non-tumorigenic mammary epithelial cells. In the present study we confirmed that HER2 activity was necessary for the tumor-initiating ability and that simultaneous expression of EGFR suppresses the oncogenic activity of HER2 by maintaining epithelial integrity.

There are a number of studies showing that myoepithelial cells can act as tumor suppressors in the breast gland (reviewed by Barsky et al.). Myoepithelial cells maintain the correct polarity of luminal epithelial cells by providing basement membrane proteins such as laminins. In cancer, the transition from ductal carcinoma in situ to invasive carcinoma is among other things characterized by the progressive loss of myoepithelial cells. Forster et al. have recently shown that p63 positive myoepithelial cells are important regulators of epithelial integrity in the breast gland. Loss of p63 resulted in failure of luminal epithelial cells to lactate, due to a failure of myoepithelial cells to express the EGF family member neuregulin. Interestingly, HER2 overexpression in our model

Figure 7. EGFR expression reverses HER2-induced loss of cytokeratin expression in xenografts tumors. (a) Mosaic picture of sections from H&E staining of D492HER2 and D492HER2/EGFR tumors. Tumors were paraffin embedded, sliced and stained with H&E. Squares denote areas represented in immunofluorescence in (b). Bar = 1 mm. (b) EGFR reverses HER2-induced loss of cytokeratin expression. Confocal images showing xenograft slices from D492HER2 and D492HER2/EGFR tumors stained with HER2, EGFR, CK14 and E-cadherin. Bar = 100 μm. (c) Apoptosis levels are higher in close proximity to epithelial, CK14-expressing tumor areas. Confocal images showing xenograft slices from D492HER2 and D492HER2/EGFR tumors stained with CK14, Ki67 and cleaved caspase-3 antibodies. Bar = 100 μm.
prompted a clear decline in EGFR transcription, coinciding with loss of all epithelial markers. One can speculate if the loss of EGFR, which is a driver of myoepithelial differentiation, allowed the more aggressive HER2-expressing cells to proliferate. Supporting this, a loss of EGFR expression accompanied by gain of other EGFR family members during breast cancer progression has been previously described, and predicted to be a response to changes in HER2 receptor family downstream signaling, such as estrogen receptor (ER). Indeed, ER status negatively correlates with EGFR expression, as EGFR is often highly expressed in TNBC, while HER2 is positively correlated with ER. Our data might indicate that HER2, but not ER, is affecting EGFR expression and phosphorylation. In a recent paper, Wheeler et al. analyzed the correlation between total EGFR, pY1068EGFR expression/phosphorylation and prognosis in head and neck cancer. They demonstrated that total EGFR and pY1068EGFR were prognostic markers in both early stage detected cancers and cetuximab-treated cancers. Furthermore, they also demonstrated that pY1068 was an independent negative factor even when the total EGFR expression was low. This is partially in line with our data, where D492-overexpressing HER2 or HER2/EGFR showed low expression of total EGFR but increased phosphorylation of Y1068EGFR. The mechanisms connecting increased Y1068 phosphorylation and reduced tumorigenicity in EGFR/HER2-expressing D492 cells, however, remain to be elucidated.

Although total EGFR expression is lowered we see increased relative phosphorylation levels of Y1068EGFR. EGFR is frequently ubiquitinated and sorted for intracellular degradation. Unlike EGFR, HER2 largely evades ubiquitination, and undergoes internalization and recycling, which prolongs signals generated by HER2-containing heterodimers, and another mechanism that enables HER2 to enhance signaling, despite its inability to directly bind a ligand growth factor, includes inhibition of EGFR dissociation from HER2-containing heterodimers. The importance of EGFR expression for preserving the epithelial phenotype was underscored in the experiments where EGFR was re-introduced in D492HER2 (D492HER2/EGFR). The in vivo experiments showed that D492HER2/EGFR tumors grew slower than D492HER2 and the resulting tumors were heterogeneous with a mixed epithelial and mesenchymal phenotype, data that were well supported in the resulting tumors were heterogeneous with a mixed epithelial and mesenchymal phenotype. Indeed, ER status negatively correlates with EGFR expression, as EGFR is often highly expressed in TNBC, while HER2 is positively correlated with ER. Our data might indicate that HER2, but not ER, is affecting EGFR expression and phosphorylation. In a recent paper, Wheeler et al. analyzed the correlation between total EGFR, pY1068EGFR expression/phosphorylation and prognosis in head and neck cancer. They demonstrated that total EGFR and pY1068EGFR were prognostic markers in both early stage detected cancers and cetuximab-treated cancers. Furthermore, they also demonstrated that pY1068 was an independent negative factor even when the total EGFR expression was low. This is partially in line with our data, where D492-overexpressing HER2 or HER2/EGFR showed low expression of total EGFR but increased phosphorylation of Y1068EGFR. The mechanisms connecting increased Y1068 phosphorylation and reduced tumorigenicity in EGFR/HER2-expressing D492 cells, however, remain to be elucidated.

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**MATERIALS AND METHODS**

**Cell culture**

D492 cells were maintained in H14 media as described previously. In tissue culture treated, collagen I (Advanced Biomatrix, San Diego, CA, USA)-coated T25 Falcon flasks (BD Biosciences, Franklin Lakes, NJ, USA). The cells were subcultured weekly in a 1:20 ratio in new flasks. Cells were fed three times per week and routinely checked for mycoplasma contamination.

**Immunohistochemistry**

Cryosections from fresh-frozen tissue samples from reduction mammoplasties (Icelandic National Bioethics Committee No. VSN-13-057) or 3D experiments were fixed in 3.7% formaldehyde for 15 min and permeabilized with 0.1% Triton-X for 10 min. Samples were blocked using 5% normal goat serum (Life Technologies, Carlsbad, CA, USA)-containing T25 Falcon flasks (BD Biosciences (BD), Franklin Lakes, NJ, USA). The cells were subcultured weekly in a 1:20 ratio in new flasks. Cells were fed three times per week and routinely checked for mycoplasma contamination.

**Diagnosis**

EGFR inhibits HER2 tumorigenicity

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Sections from paraffin-embedded mouse tissue were processed in xylene and ethanol to remove paraffin, and then rehydrated in dH2O. After rehydration, high-temperature antigen retrieval was performed. Samples were stained as described above.

Antibodies used for immunostaining/western blotting
The following antibodies were used in this study: CK14 (Leica Microsystems, Wetzlar, Germany, LL-002 and Abcam, Cambridge, UK, ab 15461), CK19 (Abcam, ab7754), Actin (Abcam ab8229), glyceraldehyde 3-phosphate dehydrogenase (Abcam, ab9484), p63 (Abcam, ab3239), E-cadherin (BD, BD010590; N-cadherin (BD, BD610921), P-cadherin (Cell Signaling (CS), Danvers, MA, USA, #2130), EGFR (CS#4267), EGFR pY1068 (CS#3777), EGFR pY1173 (CS#4407), HER2 (CS#2165) HER2 pY1121/1122 (CS#2243), Axl (CS#8661), Ki67 (Abcam, ab15580), Cleaved Caspase-3 (Abcam, ab2302) and Phalloidin (Life Technologies, A22283, A12379).

Viral transduction
All vectors used for viral production or cloning were acquired from Addgene and are shown in Supplementary Figure S1. pBABE-EGFR and empty backbone (#11011, #1764, respectively)25 were used as provided. HER2 coding sequence (#1625747) was cloned into the lentiviral vector pWPI (#12254, gift from gift from Dr. Trono). Retroviral (EGRF) virus production was performed in Phoenix HEK293 cells, using Arrestin transfection (Life Technologies). D492 cells were infected overnight with viral supernatant containing 8 μg/ml Polybrene (Sigma-Aldrich). EGFR-transduced cells were selected using 2 μg/ml puromycin (Sigma-Aldrich). HER2-containing lentiviral plasmids were transfected into HEK293T cells using Arrestin, and D492 cells were infected with viral supernatant containing 8 μg/ml polybrene. HER2-overexpressing cells were selected based on eGFP expression in FACSAria (BD). Control cells were transduced with both empty vector backbones for EGFR and HER2.

Primary cultures
For western blotting we isolated normal primary mammary cells from reduction mammaplasties. Donor material was given after acquiring informed consent from the donor. Approved by the Icelandic National Bioethics Committee VSN-13-057.

Primary normal human mammary tissue was processed as previously described.26,27 Luminal epithelial cells were sorted by positive selection or MUC1 (Life Technologies) and EpCAM (Leica) using magnetic cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany), whereas myoepithelial cells were selected for using Thy-1 (Dianova, Hamburg, Germany) and subsequently with beta 4 integrin (Millipore, Billerica, MA, USA) selection.

3D assays
For 3D assays, 10⁴ cells were seeded into 300 μl Matrigel (BD) and maintained over 15 days. Culture media (H14) was changed three times per week. After the culture period, cultures were processed for protein extraction or immunostaining of isolated colonies as described by Lee et al.28 For experiments when cells were treated with the HER2 inhibitor CP724,714, media was supplemented with a final concentration of 100 nM inhibitor dissolved in dimethyl sulfoxide.

Proliferation assay
Cells were seeded in triplicate into collagen-coated six-well trays at a density of 50,000 cells per well in H14 media. Every other day, cells were detached and combined with the floating fraction from the spent media. Cells were then stained with Acridine Orange and DAPI (both from Chemometec A/S, Allerod, Denmark): staining all cells and dead cells, respectively. Viable and dead cells were counted using NucleoCounter-3000 (Chemometec).

Western blotting
Protein lysates were acquired using RIPA lysis buffer supplemented with both phosphatase and protease inhibitor cocktails (Life Technologies). For western blots, 5 μg protein was used per lane, unless otherwise stated. Samples were denatured using 10% mercaptoethanol at 95 °C for 5 min and run on NuPage 10% Bis-Tris gels (Life Technologies) in 2-(N-morpholino)ethanesulfonic acid (MES) running buffer. Samples were then transferred to Immobilon FL PVDF membranes (Millipore). Membranes were blocked in Li-cor blocking buffer and primary antibodies were incubated overnight at 4 °C. Near-infrared fluorescence visualization was measured using Odyssey CLx scanner (Li-Cor, Cambridge, UK).

Low attachment assay
For low-attachment assays, six-well trays were coated with 1.2 μg/ml poly-HEMA (Sigma-Aldrich) overnight at 37 °C. Cells were seeded in triplicate at a density of 10⁴ per well, and cultured for 2 weeks. After the culture period, colonies were imaged and counted.

Quantitative reverse transcriptase–PCR
Total RNA was extracted with Trizol (Life Technologies), DNAase treated and reverse transcribed with hexanucleotides using ReverAid (#K1622, Life Technologies). Resulting cDNA was used for quantitative reverse transcriptase–PCR, in master mix (Life Technologies) with primer pairs and probes for EGFR (Hs00540086_m1, Life Technologies), HER2 (Hs01076902_m1), ZEB1 (Hs00232783_m1) and glyceraldehyde 3-phosphate dehydrogenase (Hs99999905_m1). Experiments were carried out in triplicate on 7500 Real Time PCR System (Life Technologies). EGFR, HER2 and ZEB1 mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase and relative mRNA differences were calculated with the 2⁻ΔΔCT method.

Quantitative reverse transcriptase–PCR analysis of miRNAs was performed using miRCURY LNA microRNA PCR System (Exiqon, Copenhagen, Denmark). Gene expression levels were quantified using primers for hsa-miR-141 (204504) and hsa-miR-200c (2044852) (Exiqon). Normalization was carried out with U6 RNA(#203907)(Exiqon).

NSG xenografts
The in vivo tumorigenicity assays were performed using NOD/SCID interleukin-2 receptor gamma chain null (NSG Il2rg−/−) mice, under unblinded conditions. The animals (n = 23) were randomized into four groups according to size, and 5 × 10⁵ cells were injected bilaterally in 100 μl phosphate-buffered saline/matrice. Group sizes were as follows: D492(WT), n = 5; D492EGFR, D492HER2 and D492HER2/EGFR, n = 6 for each group. Distilled tap water was given ad libitum, supplemented with 17β-estradiol at a concentration of 4 mg/l. For drug experiments, 23 mice were injected bilaterally with 5 × 10⁶ D492HER2 cells. Mice were treated intraperitoneally with 4 mg/kg trastuzumab, 20 mg/kg cetuximab or a combination of the two (n = 6 for each group) from day 0 (13 days post injection), once a week on days 0, 7, 14 and 21. All mice used in the experiment were locally bred at the Department of Comparative Medicine (Oslo University Hospital, Oslo, Norway). All procedures and experiments involving animals were approved by the National Animal Research Authority (http://www.fdu.no/).

The experiments were conducted according to the regulations of the European Federation of Laboratory Animal Science Association (FELASA), and performed according to the 3R principle (Replacement, Reduction, and Refinement). Mice were killed before the end of the experiment if tumor size exceeded 20 mm diameter, or if mice appeared unhealthy or in pain. Tumors were measured two times weekly, and after the experimental period, tumors were harvested, formalin fixed and embedded in paraffin.

Statistical analysis
All experiments were performed at minimum in triplicate. Graphs were generated in Microsoft Excel. Error bars represent the standard deviation of the mean.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES

1 Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA et al. Molecular portraits of human breast tumours. Nature 2000; 406: 747–752.

2 Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001; 98: 10869–10874.

3 Visvader JE, Stirling J. Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev 2014; 28: 1143–1158.

4 Oakes SR, Gallego-Ortega D, Ormandy CJ. The mammary cellular hierarchy and breast cancer. Cell Mol Life Sci 2014; 71: 4301–4324.

5 Gudjonsson T, Ronnov-Jessen L, Villadsen R, Rank F, Bissell MJ, Petersen OW. Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal basal epithelial cells for polarity and basement membrane deposition. J Cell Sci 2002; 115(Pt 1): 39–50.

6 Sigurdsson V, Hilmarsdottir B, Briem E, Sigurdsson H, Jarvinen S, Nordling S et al. a-ERBB2-induced epithelial-mesenchymal transition reduces mammary gland branching and promotes mammary tumor growth and metastasis. Breast Cancer Res 2014; 16: 238.

7 Stenmark HO, Oliner JD, Gumbiner B. Cell biology of ERBB receptor signaling. J Cell Sci 2005; 118: 3499–3512.

8 Bjornsti MA, Dalerba P. Stem cells in cancer. Nat Rev Cancer 2012; 12: 205–217.

9 Tam WL, Weinberg RA. Targeting cancer stem cells. Nat Rev Cancer 2015; 15: 748–760.

10 Tichadou S, Bisselet N, Chabot M, Landsman D, Schoenfield S, Salcedo X et al. Myoepithelial cells control breast tumor cell motility and invasion. J Cell Sci 2015; 128: 3154–3169.
47 Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M et al. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. Cancer Cell 2004; 6: 459–469.
48 Ingthorsson S, Sigurdsson V, Fridriksdottir A Jr, Jonasson JG, Kjartansson J, Magnusson MK et al. Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture. BMC Res Notes 2010; 3: 184.
49 Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods 2007; 4: 359–365.

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