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PRECLINICAL STUDY

HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment

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Abstract Development of effective and durable breast cancer treatment strategies requires a mechanistic understanding of the influence of the microenvironment on response. Previous work has shown that cellular signaling pathways and cell morphology are dramatically influenced by three-dimensional (3D) cultures as opposed to traditional two-dimensional (2D) monolayers. Here, we compared 2D and 3D culture models to determine the impact of 3D architecture and extracellular matrix (ECM) on HER2 signaling and on the response of *HER2*-amplified breast cancer cell lines to the HER2-targeting agents Trastuzumab, Pertuzumab and Lapatinib. We show that the response of the *HER2*-amplified AU565, SKBR3 and HCC1569 cells to these anti-HER2 agents was highly dependent on whether the cells were cultured in 2D monolayer or 3D laminin-rich ECM gels. Inhibition of $\beta 1$ integrin, a major cell–ECM receptor subunit, significantly increased the sensitivity of the *HER2*-amplified breast cancer cell lines to the humanized monoclonal antibodies Trastuzumab and Pertuzumab when grown in a 3D environment. Finally, in the absence of inhibitors, 3D cultures had substantial impact on HER2 downstream signaling and induced a switch between PI3K-AKT- and RAS-MAPK-pathway activation in all cell lines studied, including cells lacking *HER2* amplification and overexpression. Our data provide direct evidence that breast cancer cells are able to rapidly adapt to different environments and signaling cues by activating alternative pathways that regulate proliferation and cell survival, events that may play a significant role in the acquisition of resistance to targeted therapies.

Keywords Breast cancer cell lines · Drug response · Targeted therapy · 3D cell culture · HER2 signaling

Abbreviations

lrECM	Laminin-rich extracellular matrix gel
2D	Two-dimensional
3D	Three-dimensional
HER2	Human epidermal growth factor receptor type 2
EGFR	Epidermal growth factor receptor

Introduction

Cancer cells do not exist and function autonomously but are immersed in a complex microenvironment composed of non-epithelial cells, such as fibroblasts, endothelial and immune cells, and are embedded in a matrix of extracellular proteins [1]. The extracellular matrix (ECM) has been shown to provide biochemical signals and architectural constraints that influence intracellular signaling cascades

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and play a pivotal role in determining the behavior of normal mammary epithelial cells in designer microenvironments [2–5]. Albeit less sensitive to environmental perturbations than their nonmalignant counterparts, cancer cells, nevertheless, show distinct regulation in signal transduction under different culture environments [6–10] with significant implications for the study of drugs targeting specific signaling pathways [11–17].

The human epidermal growth factor receptor type 2 (HER2) is amplified and overexpressed in about 15–20% of all breast cancers and associated with aggressive disease and poor prognosis [18]. With the advent of Trastuzumab (Herceptin, Genentech Inc.), a monoclonal antibody targeting HER2 that has been shown to significantly improve survival of patients with HER2 positive disease [19], several novel HER2-targeting agents have been identified and developed. These include Pertuzumab (Omnitarg, Genentech), a monoclonal antibody inhibiting the dimerization of HER2 with other HER receptors, and Lapatinib (Tykerb, GlaxoSmithKline), a dual small molecule tyrosine kinase inhibitor targeting HER1 (EGFR) and HER2. Despite the great promise of targeted therapy for the treatment of patients with breast cancer, *de novo* and acquired resistance remain major obstacles in the clinic [20]. Hence, further in vitro studies are required for the elucidation of molecular mechanisms that could explain—and help overcome—resistance to targeted drugs. For this purpose, the use of cell systems providing a context that more closely recapitulates the in vivo-like signaling in breast cancer cells would be desirable to increase the likelihood of translating results of culture models into patient care.

Here, we employed a 3D cell culture model in which breast cancer cells are grown on top of laminin-rich ECM (lrECM), allowing tumor-like colony formation to occur as well as cell–ECM interactions [3, 21]. The aim of the current study was twofold: (1) to determine whether ECM and 3D architecture modulate the Trastuzumab, Pertuzumab and Lapatinib's response of breast cancer cell lines that harbor *HER2* gene amplification and overexpression compared to monolayer culture; and (2) to investigate whether cell–ECM interactions have an impact on HER2 signaling under the conditions described.

Materials and methods

Cell culture and drug treatment

AU565, SKBR3, HCC1569 and BT549 breast cancer cell lines (ATTC) were maintained following ATCC's instructions. For drug treatment in 2D culture, cells were seeded onto 8-well chamber slides in H14 medium with 1% FBS [22, 23]. For treatment in 3D cultures, single cells were seeded on top of Engelbreth-Holm-Swarm tumor matrix (Matrigel, BD Biosciences) in H14 medium with 1% FBS and 5% Matrigel [9, 22]. AU565, SKBR3 and HCC1569 were plated at a density of 2.1×10^4 cells/cm^2 and BT549 at 1.6×10^4 cells/cm^2 as described previously [9], and drugs or controls were added on day 4 after cell plating.

Cells were treated with the humanized monoclonal antibodies against HER2 Trastuzumab (21 μg/ml; Herceptin, kindly provided by Genentech, Inc.) or Pertuzumab (25 μg/ml; Omnitarg, kindly provided by Genentech, Inc.), with the dual small-molecule inhibitor targeting EGFR and HER2 Lapatinib (1.5 μM; Tykerb, kindly provided by GlaxoSmithKline), the β1 integrin inhibitory rat monoclonal IgG1 antibody AIIB2 (160 μg/ml; originally supplied by Carolyn Damsky, UCSF), or a non-specific rat IgG1 (25 μg/ml) (Pierce Biotechnology) as control for the inhibitory antibodies or DMSO as control for Lapatinib. Cells were analyzed after 48 h of drug treatment for proliferation and after 72 h for apoptosis.

Proliferation and apoptosis assays

Proliferation of cells grown in 2D or 3D cultures was measured by 5-Bromo-2-deoxyuracil (BrdU) incorporation using the 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I (Roche) following the manufacturers' instructions. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Apoptosis was assayed in cells grown on top of 3D lrECM by immunofluorescent staining of cleaved Caspase 3 (Asp175) (Cell Signaling Technology) as described previously [9]. Nuclei were counterstained with DAPI.

Confocal images were acquired at the colony midsection of cells grown on top of 3D lrECM using a Solamere Technology Group spinning disk confocal system as described previously [9]. For cells grown in 2D culture, fluorescent images were acquired on a Zeiss Axioplan 2 Imaging microscope and AxioCam camera. All images were analyzed using Image J (National Institutes of Health). Proliferation and apoptotic indices were determined by quantifying the proportion of cells positive for BrdU or cleaved Caspase 3 [6, 7]. A minimum of 200 cells was evaluated for each condition. For each drug or control, proliferation assays were repeated at least three times and apoptosis assays at least two times. Analyses were performed with the observer blinded to the identity of the cell line and culture conditions. Bar charts show the mean percentage of BrdU incorporation in the drug treated relative to the control-treated cells. A homoscedastic Student's t-test was computed for differences between groups; a two-tailed p value of <0.05 was considered significant.

Western blotting

Cells were extracted from 3D lrECM cultures using ice-cold PBS/EDTA [6]. Protein cell lysate preparation and western blotting were performed as described previously [9]. Membranes were blocked with bovine serum albumin and probed with antibodies against HER2 (#2242), phospho-HER2 (Tyr1221/1222) (#2243), HER3 (#4754), phospho-HER3 (Tyr1289) (#4791), EGFR (#2232), phospho-EGFR (Tyr992) (#2235), HER4 (#4795), phospho-HER4 (Tyr1284) (#4757), AKT (#4685), phospho-AKT (Ser473) (#4058), MEK1/2 (#9126), phospho-MEK1/2 (Ser217/221) (#9121) (Cell Signaling Technology) and β1 integrin (#610467) (all 1:1000). Blots were incubated with a horseradish peroxidase-linked secondary antibody and visualized with a FluorChem 8900 imager (Alpha Innotech) using chemiluminescence (Pierce).

Results

Response of breast cancer cell lines to HER2-targeting agents is dramatically altered depending on culture conditions

To assess whether ECM and tissue architecture have an impact on the sensitivity or resistance of human breast cancer cells to HER2-targeting agents of different mechanisms of action, we analyzed three *HER2*-amplified cell lines, AU565, SKBR3 and HCC1569, and as control BT549 cells, which lack *HER2* gene amplification and overexpression, to Trastuzumab, Pertuzumab or Lapatinib in 2D vs. 3D lrECM cultures. The cell line panel selected was reflective of each of the different molecular subtypes of breast cancer cell lines: AU565 and SKBR3 cells belong to the Luminal, HCC1569 cells to the Basal A and BT549 to the Basal B molecular subtypes [24] (Table 1).

The *HER2*-amplified AU565 cell line was significantly more sensitive to Trastuzumab when cultured on top of 3D lrECM compared to 2D culture conditions (55% 2D vs. 18% 3D proliferation inhibition, respectively as determined by BrdU incorporation; $p < 0.0004$). AU565 cells were significantly more resistant to Pertuzumab in 3D compared to 2D cultures (20% 3D vs. 46% 2D proliferation inhibition, respectively; $p < 0.002$) (Fig. 1a). Whereas the SKBR3 cells were resistant to Trastuzumab treatment when grown as 3D colonies, they were responsive in 2D monolayers (103% 3D vs. 62% 2D BrdU incorporation, respectively; $p < 0.0002$) (Fig. 1b). Lapatinib treatment resulted in a clear inhibition of proliferation in both cell lines, AU565 and SKBR3, and culturing conditions (Fig. 1a, b). In SKBR3 cells, however, this proliferation reduction was more pronounced when grown in the 3D vs. 2D culture model (68% 2D vs. 84% 3D proliferation inhibition, respectively; $p < 0.006$) (Fig. 1b).

Despite being established from pleural effusions of the same patient and having strikingly similar genetic and transcriptomic profiles (Spearman's rho for microarray-based comparative genomic hybridization and Pearson's r value for gene expression data between AU565 and SKBR3 [24] are 0.8388, $p < 0.0000001$ and 0.9689, $p < 0.0000001$, respectively), SKBR3 and AU565 cells had distinct responses to drug treatment with respect to culture conditions. For example, when grown on top of 3D lrECM, SKBR3 colonies were resistant to Trastuzumab treatment, whereas AU565 colonies were sensitive (103% SKBR3 vs. 45% AU565 BrdU incorporation, respectively; $p < 0.0001$). These findings suggest that although the SKBR3 and AU565 cell lines are nearly identical at the genetic and transcriptomic levels as well as at the level of HER family proteins (Table 1; Fig. 3) [9, 24], clearly they have diverged posttranscriptionally because they express distinct signal transduction pathways downstream of HER2 when grown in 3D lrECM gels (see below).

The *HER2*-amplified HCC1569 cell line harbors a *PTEN*-inactivating gene mutation, which has been shown to confer Trastuzumab resistance in patients and Lapatinib resistance in a xenograft model [25, 26]. As expected, HCC1569 cells were insensitive to Trastuzumab treatment in both 2D and 3D cultures (Fig. 1c). However, HCC1569 cells were significantly more sensitive to treatment with Pertuzumab or Lapatinib when propagated in 3D lrECM

Table 1 Breast cancer cell line characteristics

Cell line	Molecular subtype[a]	*HER2* amplification	EGFR[b]		HER2[b]		HER3[b]		HER4[b]	
			2D	3D	2D	3D	2D	3D	2D	3D
AU565	Luminal	+	+	+	+	+	+	+	−	−
SKBR3	Luminal	+	+	+	+	+	+	+	−	−
HCC1569	Basal A	+	+	+	+	+	±	±	−	−
BT549	Basal B	−	+	+	−	−	−	−	−	−

[a] Molecular subtypes according to Neve et al. [24]

[b] Protein expression level. 2D: two-dimensional; 3D: three-dimensional cell culture

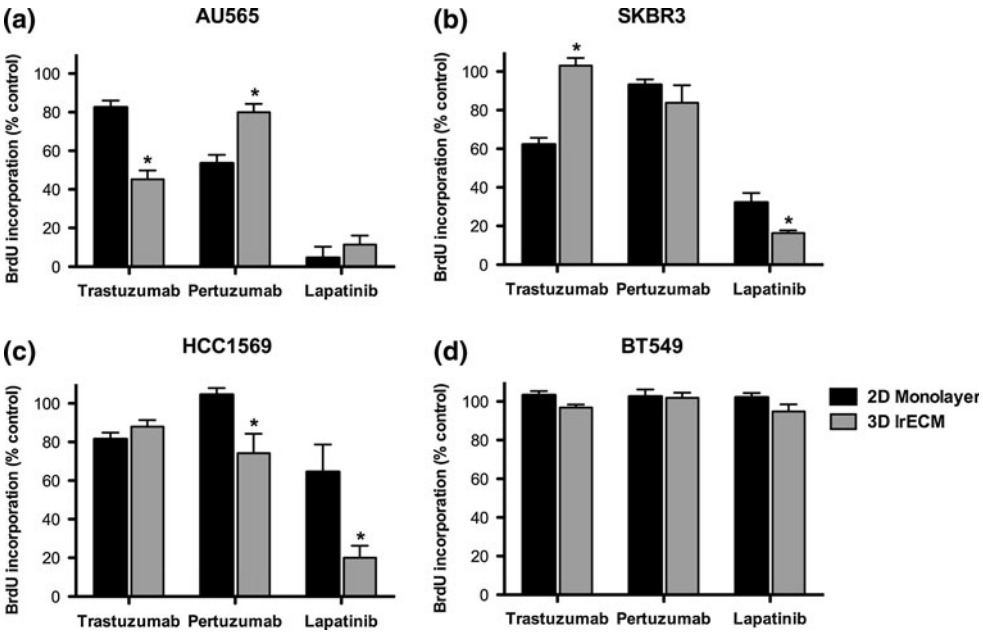

Fig. 1 Response of (**a**) AU565, (**b**) SKBR3, (**c**) HCC1569 and (**d**) BT549 cells to 48 h treatment with 21 μg/ml Trastuzumab, 25 μg/ml Pertuzumab or 1.5 μM Lapatinib in two- (*black bars*) and three-dimensional cell cultures (*gray bars*) as measured by BrdU incorporation. Bar charts show the mean percentage of BrdU incorporation in the drug treated relative to the control treated with non-specific IgG1 for the inhibitory antibodies and DMSO for Lapatinib. Error bars represent standard error of mean, experiments repeated 3 times; $*p < 0.05$

compared to monolayer cultures (BrdU incorporation; Pertuzumab: 104% 2D vs. 74% 3D, respectively; $p < 0.008$; Lapatinib: 65% 2D vs. 20% 3D, respectively; $p < 0.008$).

The Basal B BT549 cell line, which lacks *HER2* amplification and overexpression, was, as expected, resistant to the HER2-targeting drugs Trastuzumab, Pertuzumab and Lapatinib in both 2D and 3D culture environments (Fig. 1d). This result demonstrates that in cell lines lacking the molecular target of these drugs, culture conditions do not affect their response to the targeting agents.

These results demonstrate that 2D vs. 3D culture conditions significantly affect the response to humanized monoclonal antibodies against HER2, Trastuzumab and Pertuzumab, in *HER2*-amplified and overexpressing breast cancer cell lines.

Inhibition of cell–ECM interactions using β1 integrin inhibitory antibody increases sensitivity of breast cancer cells to HER2-targeting drugs in 3D cultures

Cell growth and survival are regulated by both growth factor receptors and integrin family of adhesion receptors [27]. β1 integrin subunit, a critical mediator of cell–ECM interactions, has been shown to play a role in progression and maintenance of mammary tumor growth in vivo [28], and its inhibition to arrest growth of breast cancer cells in 3D cultures and in vivo [6, 7, 29, 30]. Furthermore, previously we have reported that β1 integrin and EGFR signaling are coordinately regulated in 3D culture [6].

We sought to determine whether the response of the *HER2*-amplified cell lines, AU565, SKBR3 and HCC1569 to Trastuzumab and Pertuzumab could be increased in 2D and 3D cultures by concurrent inhibition of β1 integrin signaling using the β1 integrin inhibitory antibody AIIB2 [6, 7, 29]. For combination treatments, the same scheme was used as for single agent treatment. The addition of AIIB2 to Trastuzumab, Pertuzumab or Lapatinib had no significant additional effect on proliferation of the breast cancer cell lines AU565, SKBR3, HCC1569 and BT549 when maintained in 2D monolayers, with the exception of Lapatinib vs. Lapatinib plus AIIB2 treatment in SKBR3 cells (Supplementary Fig. 1). These results suggest a number of explanations: (1) that signaling pathways downstream of β1 integrin are not activated, (2) that cells cultured in 2D do not depend on β1 integrin for proliferation or survival or (3) that β1 integrin expression levels are downregulated in breast cancer cell lines maintained on tissue culture plastic. In contrast, in 3D lrECM, the combined treatment of AIIB2 with either Trastuzumab or Pertuzumab resulted in a significantly increased response compared to the single agents in all three *HER2*-amplified cell lines, as did the combination of Lapatinib and AIIB2 in SKBR3 cells ($p < 0.002$) (Fig. 2). In contrast, BT549 cancer cells were resistant to AIIB2 either as single agent or in combination with the HER2-targeting agents in 3D culture.

Of note, no significant increase in apoptosis, measured by activated Caspase 3 staining, was observed in the breast cancer cell lines treated for 72 h in 3D lrECM with the HER2-targeting agents, AIIB2 or a combination of the two. Only in SKBR3 cells, Lapatinib treatment and in AU565 and SKBR3 cells, a combination treatment of Lapatinib and AIIB2 significantly induced apoptosis (SKBR3: Lapatinib vs. DMSO, $p < 0.009$; Lapatinib plus AIIB2 vs. DMSO, $p < 0.0003$; AU565: Lapatinib plus AIIB2 vs. DMSO, $p < 0.0003$, respectively) (Supplementary Fig. 2).

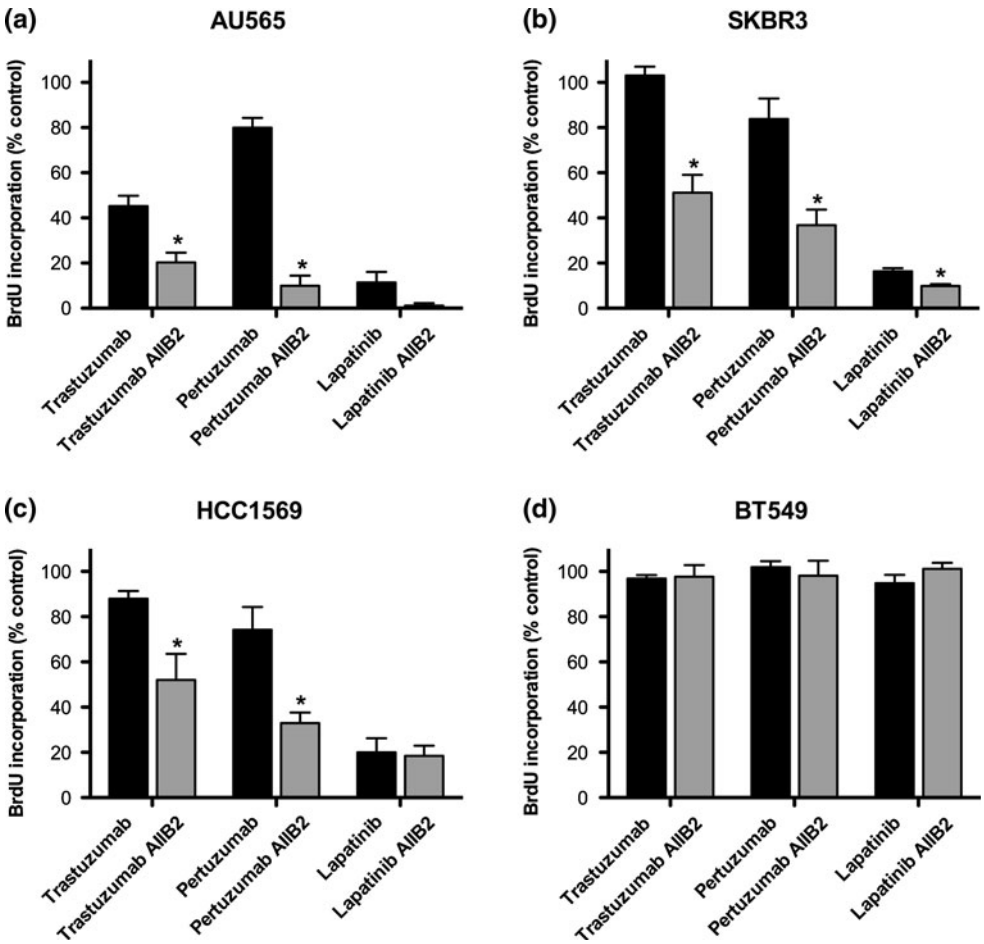

Fig. 2 Response of **a** AU565, **b** SKBR3, **c** HCC1569 and **d** BT549 cells grown on top of 3D lrECM cells to 48 h treatment with single agents: 21 μg/ml Trastuzumab, 25 μg/ml Pertuzumab, 1.5 μM Lapatinib (*black bars*) or in combination with the above plus 160 μg/ml AIIB2 (*gray bars*) as measured by BrdU incorporation. Bar charts show the mean percentage of BrdU incorporation in the drug treated relative to the control-treated cells (non-specific IgG1 for the inhibitory antibodies; DMSO for Lapatinib). Error bars represent standard error of mean, experiments repeated 3 times; $*p < 0.05$, *t*-test: single agent vs. single agent plus AIIB2

These findings suggest that the HER2-targeting monoclonal antibodies Trastuzumab and Pertuzumab primarily exert a cytostatic (i.e. reduction of proliferation) rather than a cytotoxic (i.e. induction of apoptosis) effect on the breast cancer cell lines cultured in our 3D model.

2D vs. 3D culture conditions induce distinct HER2 downstream signaling in breast cancer cell lines

Given that the baseline protein expression levels of the HER receptors in the breast cancer cell lines studied were similar in 2D and 3D cultures (Table 1; Fig. 3), we hypothesized that the differential drug response to the HER2-targeting agents was a result of distinct HER2 downstream signaling in cells cultured either in 2D monolayers or as tumor-like colonies in 3D lrECM. Upon phosphorylation of its tyrosine kinase domain, HER2 promotes proliferation by activation of the RAS-MAPK-pathway and survival through PI3K-AKT-pathway [31].

In all three *HER2*-amplified breast cancer cell lines, a significant difference in the signaling pathway activation downstream of HER2 could be observed depending on the culture microenvironment. In fact, the differences in HER2 downstream signaling of AU565 and SKBR3, cell lines remarkably similar in terms of their genomic and transcriptomic characteristics, may explain their distinct response to Trastuzumab and Pertuzumab in 2D and 3D. When grown on cell culture plastic, AU565 cells showed phosphorylation of AKT but not of MEK1/2, which suggests that under these culture conditions HER2 in AU565 cells signals primarily via the AKT-pathway. In 3D lrECM, however, a switch in the HER2 downstream signaling from the AKT- to the MAPK-pathway was observed as MEK1/2 but not AKT was phosphorylated (Fig. 3a). In SKBR3 cells propagated and treated in 2D cultures we found phosphorylation of AKT and MEK1/2 suggesting activation of both MAPK- and AKT-pathway downstream of HER2. In contrast, in 3D lrECM in the SKBR3 cell line, in a way akin to AU565 cells, HER2 signaling was mediated via the RAS-MAPK-pathway only because AKT was not phosphorylated under these conditions (Fig. 3b). Interestingly, phosphorylation of HER2 was significantly reduced in SKBR3 cells in 3D compared to 2D cultures, which may explain the Trastuzumab resistance observed in these cells grown on top of 3D lrECM [32]. Furthermore, our results provide evidence that despite *HER2* amplification and

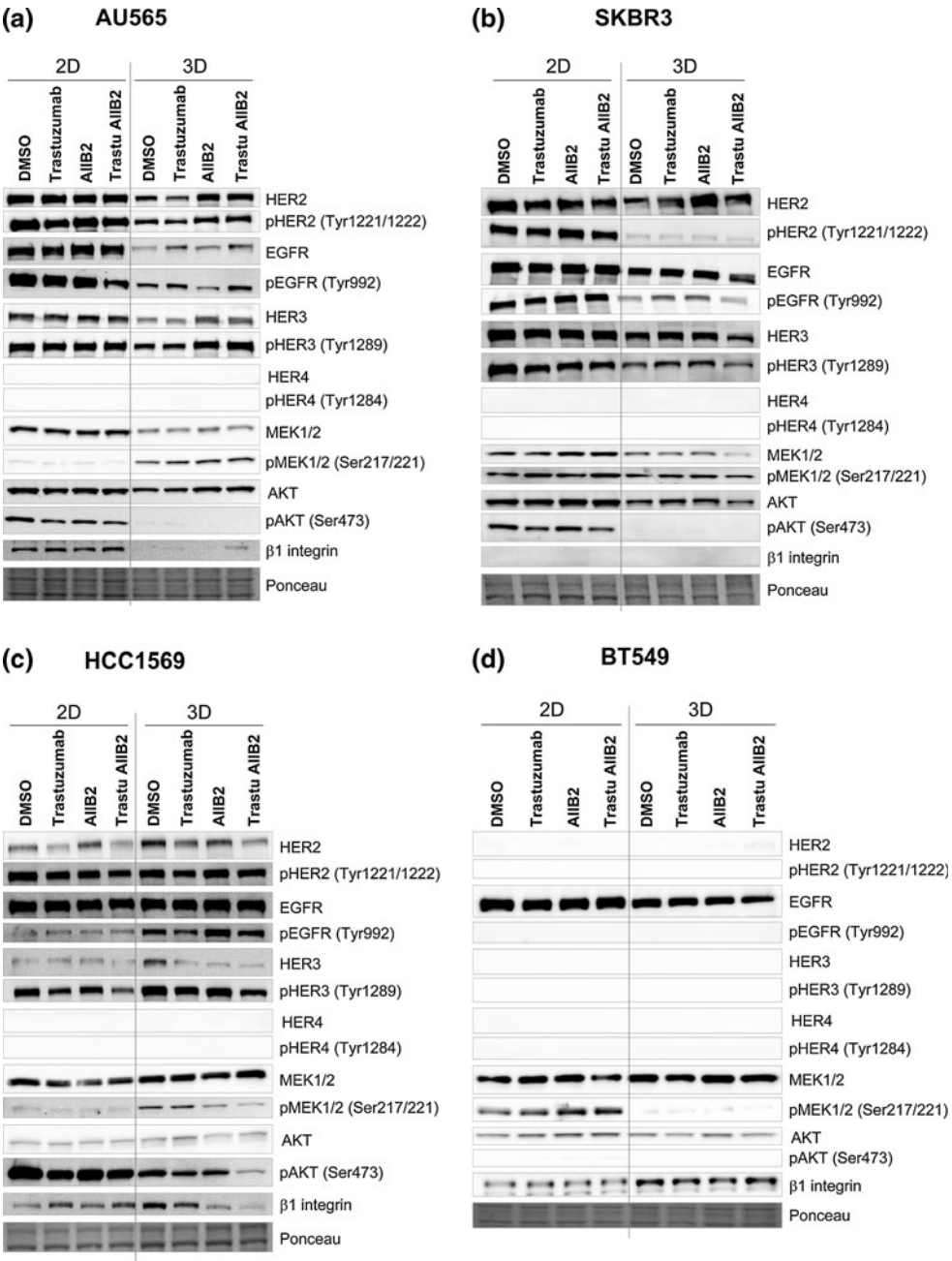

Fig. 3 Total and activated levels of HER2, EGFR, HER3, HER4, MEK1/2 and AKT, and β1 integrin detected by western blotting in (**a**) AU565, (**b**) SKBR3, (**c**) HCC1569 and (**d**) BT549 cells grown in two- (2D) or three-dimensional (3D) cultures treated for 48 h with DMSO control, 21 μg/ml Trastuzumab, 160 μg/ml AIIB2 or 21 μg/ml Trastuzumab plus 160 μg/ml AIIB2. Trastu = Trastuzumab

overexpression, this receptor does not constitute the main growth factor receptor mediating proliferation and cell survival in SKBR3 cells in 3D culture conditions.

The *HER2*-amplified HCC1569 cell line clearly showed enhanced phosphorylation of EGFR in 3D compared to 2D cultures. HER2 inhibition by Trastuzumab also induced a reduction of activated EGFR but not of HER3 or HER4 (Fig. 3c), indicating that EGFR may serve as the preferred dimerization partner for HER2 in these cells. Of note, none of the cell lines studied here expressed HER4 or activated HER4 as reported previously [24]. As mentioned earlier, the HCC1569 cell line harbors a *PTEN*-inactivating mutation, suggesting a constitutive activation of PI3K-AKT signaling [33]. Indeed, under both culture conditions, HCC1569 showed phosphorylation of AKT. In contrast, activation of the RAS-MAPK-pathway was found only in 3D and not 2D cultures (Fig. 3c).

Of note, although AU565 and SKBR3 cells cultured on top of 3D lrECM respond to the β1 integrin inhibitory antibody AIIB2 (Figs. 2, 3), we did not detect any β1 integrin protein expression under these conditions. In contrast, β1 integrin was expressed in HCC1569 cells and downregulated after treatment with AIIB2 or Trastuzumab plus AIIB2 in 3D lrECM (Figs. 2, 3). These findings

support our earlier work showing that response to AIIB2 does not necessarily correlate with total levels of β1 integrin expression in cancer cells [29].

Of the four members of the HER family, the non-*HER2*-amplified BT549 cells were found to express only EGFR in 2D as well as 3D culture environments; however, no EGFR phosphorylation and also no phosphorylated AKT were detected (Fig. 3d). Nevertheless, and despite the fact that BT549 cells were resistant to Trastuzumab, Pertuzumab or Lapatinib treatment in both culture conditions as measured by BrdU incorporation, western blot analysis revealed that a switch in cell culture environment also induced a switch in pathway activation in this Basal B cell line (Fig. 3d). BT549 cells showed phosphorylation of MEK1/2 when grown in cell culture plastic but not in 3D lrECM. Our results demonstrate that the microenvironment does affect PI3K-AKT- and RAS-MAPK-signaling cascades even in the absence of HER2 expression.

Discussion

Here, we demonstrate that signal transduction pathway activation downstream of HER2, and response to the HER2-targeting drugs Trastuzumab, Pertuzumab and Lapatinib of *HER2*-amplified breast cancer cell lines differ significantly between 2D and 3D lrECM culture environments. These observations have important implications for in vitro studies focusing on unraveling the mechanisms underlying *de novo* and acquired resistance seen in the majority of patients treated with anti-HER2 tailored therapy.

Our findings further imply that both cell–ECM interactions and 3D morphology of breast cancer cells have significant impact on the response to drugs targeting HER2. Of note, 3D tissue architecture itself has been found by our laboratory and others to modulate drug response of cancer cells [11, 34]. SKBR3 cells were shown to be more sensitive to Trastuzumab when maintained as floating aggregates using poly-HEMA-coated tissue culture dishes than in conventional 2D culture [13]. In contrast to our 3D lrECM model, in which breast cancer cells receive biochemical signals via cell–ECM interactions, poly-HEMA cultures induce anchorage-independent growth without the supply of an exogenous ECM. We have shown that cells on poly-HEMA are unable to induce acinar polarity in non-malignant mammary cells whereas cells on lrECM do [35]. These results could explain the difference in Trastuzumab response of SKBR3 between the poly-HEMA and our 3D model, providing additional evidence of the impact of ECM on the behavior of cancer cells.

One of the crucial mediators of cell–ECM interactions is β1 integrin, which in a way akin to growth factor receptors, regulates cell growth and survival in normal cells and has been implicated in breast cancer initiation and progression [6, 29, 30, 36, 37]. Addition of the β1 integrin inhibitory antibody AIIB2 to Trastuzumab or Pertuzumab resulted in a significantly increased response of AU565, SKBR3 and HCC1569 cells grown in 3D compared to single anti-HER2 agents (Fig. 2). This was not observed in 2D. Given that β1 integrin is expressed in cells cultured on 2D plastic (Fig. 3), our results further support the conclusion [7, for review see 5, 38] that suggest that β1 integrin and its downstream signaling cascade are not required for proliferation in cells propagated on tissue culture plastic and that regulation of signaling are fundamentally different in 2D and 3D conditions.

Our data provide evidence to suggest that the cell culture-dependent differential response to HER2-targeting drugs is a result of distinct HER2 downstream signaling cascade activation. Significantly and strikingly, all breast cancer cell lines studied here showed a switch in the activation between PI3K-AKT- and RAS-MAPK-pathways in 2D vs. 3D environments.

Extensive studies of normal mammary cells have revealed that the 3D lrECM model used here more accurately recapitulates the in vivo architecture, signaling and behavior of the mammary gland than conventional 2D models [2, 3, 5, 17]. There is burgeoning evidence to suggest that even for cancer cells, 3D cell culture models may also better mimic the tissue specificity, behavior and function of breast cancers than 2D models [15–17, 39]. This is supported further by our work demonstrating that the 3D morphology of breast cancer cell lines correlated well with the underlying gene expression patterns and functions [9], and that the response pattern observed of breast cancer colonies in the 3D lrECM model to the β1 integrin inhibitory antibody AIIB2 could be confirmed in vivo using nude mice [29, 30]. Here, we provide direct evidence that 3D culturing influences response to targeted therapies as well as the downstream signaling of the molecular target.

Most importantly, however, our data demonstrate that breast cancer cells are able to rapidly adapt to novel environments and signaling cues, and to shift signaling pathways regulating survival and proliferation, which clearly play a role in modulating the sensitivity to specific targeted therapeutic agents. Based on our results, one could hypothesize that distinct responses to targeted therapies of metastatic deposits in distinct anatomical sites may stem from different patterns of tumor–microenvironment interactions. We hypothesize that in order to maximize the likelihood of a targeted therapy to work in the clinic, one needs to understand not only the pathways directly inhibited by the therapeutic agent in cancer cells, but also off-target pathways that concurrently activate and may modulate the response to a given agent.

For the promise of targeted therapy to be fully realized, culture systems that better recapitulate in vivo conditions

are required. Our data demonstrate that 2D cultures, which until very recently almost exclusively used in functional genomics, epigenomics and drug sensitivity screens to determine dependence on the signaling of a given molecule or sensitivity to targeted agents, may not be representative of all stages of disease progression. Tumor–microenvironment interactions clearly change in different stages of breast cancer progression (i.e. in situ, invasive, metastatic), and, therefore, apart from the use of isogenic systems or large collections of cell lines, the impact of the microenvironment, ECM and 3D morphologies also need to be taken into account in testing therapeutic agents.

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