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SHORT COMMUNICATION

Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab

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Three-dimensional (3D) cell culture techniques are frequently used to model alterations in tissue architecture critically important for tumor development. Here, we report on a detailed comparison of a spheroid model of human epidermal growth factor receptor (HER2) overexpressing cancer cells with the traditional monolayer culture. In 2D culture, HER2 and HER3 form heterodimers, whereas in multicellular spheroids HER2 homodimers are formed. These homodimers localize in membrane rafts, resulting in enhanced inhibition of the proliferation of cancer cells with trastuzumab (Herceptin), a monoclonal antibody specifically targeting HER2. Within the tumor spheroids, HER2 homodimerization leads to enhanced activation of HER2 and results in a switch in signaling pathways from phosphoinositide 3-kinase (PI3K) to mitogen-activated protein kinase (MAPK). Diminished PI3K signaling is accompanied by the activation of the integrin β4/Rac1/PAK 2 signaling cascade. We propose that the described 3D culture system may better reflect some *in vivo* aspects of HER signaling and can be used to further improve the understanding of the molecular mechanisms of trastuzumab action. Furthermore, the described human multicellular tumor spheroids may allow identification of new targets for the treatment of HER2-positive breast cancer patients who currently benefit suboptimally from trastuzumab treatment.
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The human epidermal growth factor receptor (HER) family consists of four receptor tyrosine kinases (epidermal growth factor receptor (EGFR), HER2, HER3 and HER4), which regulate cell survival, proliferation, differentiation and migration, and have been implicated in oncogenesis (Yarden, 2001). Several studies have shown that HER2-mediated signal transduction is induced by homodimerization or heterodimerization with other HER family members (Yarden, 2001).

HER2 gene amplification and protein overexpression have been identified in different tumor types (Wang and Hung, 2001), including 15–20% of breast tumors in which it is associated with poor prognosis (Owens *et al*., 2004; Yaziji *et al*., 2004; Wolff *et al*., 2007). Trastuzumab (Herceptin) is a humanized recombinant monoclonal antibody that offers a significant clinical benefit for patients with HER2-positive disease when administered either as monotherapy or in combination with chemotherapy (Hudis, 2007).

When investigating the role of HER2 in oncogenesis, the *in vitro* model system used is of fundamental importance, as studies have shown that the molecular behavior of cells can be altered in three-dimensional (3D) compared with two-dimensional (2D) culture (Shaw *et al*., 2004; Lee *et al*., 2007). 3D cell culture models of breast neoplasm were developed to investigate the aspects of cell–cell, cell–matrix interaction and loss of attachment to substratum in cancer development, and thus resemble a physiologically relevant *in vitro* model to investigate tumor development and behavior (Petersen *et al*., 1992; Yamada and Cukierman, 2007). These spheroids have since been used to investigate cellular signaling mechanisms and the effects of agent such as trastuzumab on neoplastic cell proliferation (Shaw *et al*., 2004; Lee *et al*., 2007; Ritter *et al*., 2007). Traditionally, HER2 signaling pathways and the effects of trastuzumab have been dissected using 2D culture systems. Earlier 3D culture systems used to investigate cell signaling in tumor cells have relied on an exogenously provided extracellular matrix (ECM) (Petersen *et al*., 1992; Lee *et al*., 2007). Our 3D model relied on anchorage-independent growth, in which tumor cells aggregate spontaneously, without supplying an exogenous ECM (Shin *et al*., 1975). Here, we use, for the first time, an anchorage-independent 3D model in comparison with traditional monolayer culture to specifically investigate endogenous HER2 signaling in cancer cells and elucidate the molecular action of trastuzumab on breast cancer cells *in vitro*.

In 2D culture of SKBR-3 cells, treatment with trastuzumab (10 μg/ml) for 7 days induced a slight reduction in proliferation, whereas in 3D, spheroid proliferation was inhibited by 48% (Figure 1a),

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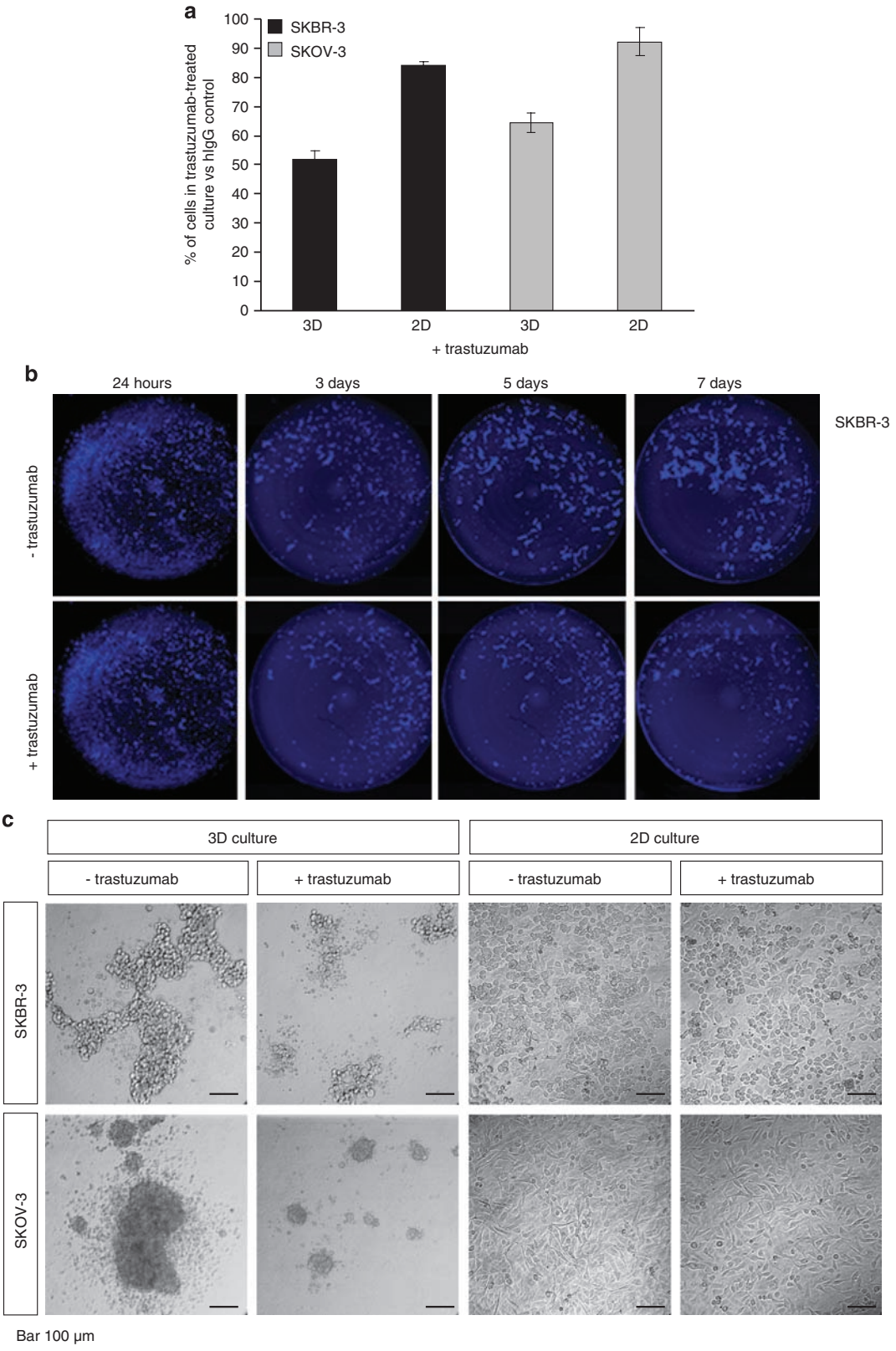
mirroring the clinical benefit of trastuzumab for HER2-positive breast cancer patients (Slamon *et al*., 1989). This inhibition was not because of stress conditions induced by the culture system, as data from an independent proliferation assay showed that SKBR-3 cells grew well in 3D culture (data not shown). Similar

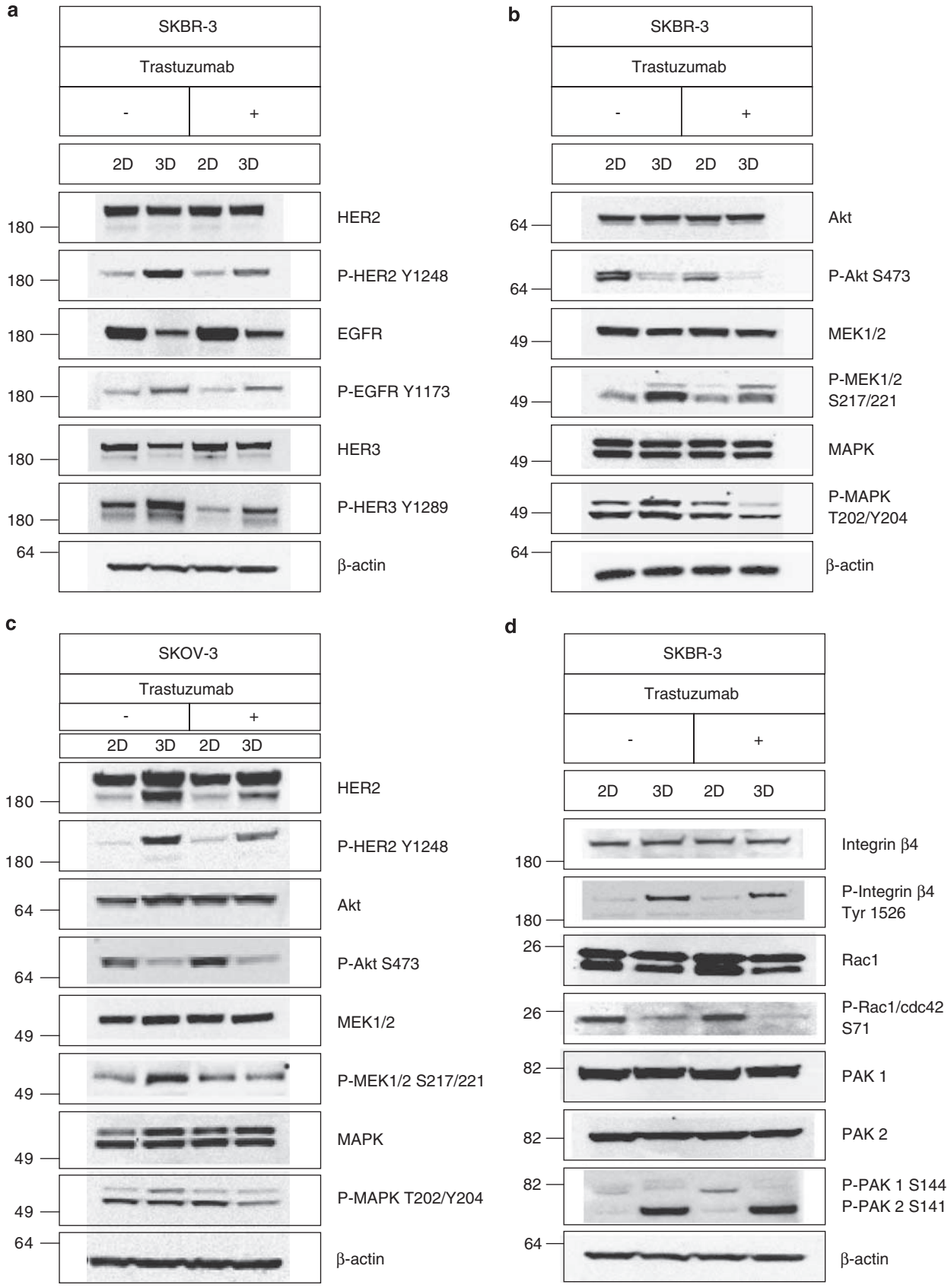


results were observed for the ovarian tumor line SKOV-3, indicating that these effects are not restricted to a specific cell line (Figure 1a). Inhibition of the proliferation in 2D culture depends on the confluency of the monolayer (data not shown). Furthermore, Hoechst 33342 staining of SKBR-3 cells in multicellular spheroids showed that the antiproliferative effects of trastuzumab were evident across the total cell population (Figure 1b). To further investigate the effects of trastuzumab on 3D culture of SKBR-3 cells, morphologic analysis of cell aggregation in the presence or absence of trastuzumab (10 μg/ml) was performed at different time points after treatment. Despite inhibiting the proliferation of SKBR-3 cells, trastuzumab did not interfere with spheroid formation (Figures 1b and c). Similar results were obtained using the SKOV-3 cell line, although a comparison of the cell morphology of both cell types grown in 2D and 3D undergoing trastuzumab treatment showed that in contrast to SKBR-3 cells, SKOV-3 cells formed very tight aggregates in 3D culture (Figure 1c).

As a significantly enhanced response to trastuzumab treatment in spheroids was observed, we investigated whether the HER2 signaling pathway differed in 2D culture. Interestingly, we detected a significantly enhanced phosphorylation of HER2, which was induced simply by culturing SKBR-3 cells in polyHEMA-coated plates. Levels of phosphorylated HER3 and EGFR were increased in multicellular spheroids, whereas total levels of HER2 and HER3 were comparable under both culture conditions. Total EGFR was slightly reduced in 3D culture (Figure 2a). mRNA expression of HER-family ligands did not differ significantly (data not shown). Treatment with trastuzumab substantially reduced HER2 and HER3 phosphorylation (Figure 2a). As HER2-mediated signaling has been associated with both the alpha serine/threonine protein kinase (Akt)/phosphoinositide 3-kinase (PI3K) (Zhou *et al*., 2004) and mitogen-activated protein kinase (MAPK) (Timms *et al*., 2002) signaling pathways, we evaluated the activation of these pathways after enhanced HER phosphorylation. Activated Akt was detected in 2D culture, but downregulated in 3D culture (Figure 2b). In contrast to the downregulation of Akt, levels of activated MEK1/2 and MAPK were enhanced in tumor spheroids (Figure 2b). Increased HER2 phosphorylation in 3D and a similar shift in signaling pathway activities between 2D and 3D cultures were observed in several other cell lines, including SKOV-3 (Figure 2c), BT474, Calu-3 and KPL-4 (Supplementary Figure). Treatment with trastuzumab reduced phosphorylation of MEK1/2 and MAPK in 3D culture (Figure 2b) compared with earlier studies, performed with 2D monolayer cultures, which focused on trastuzumab effects on the Akt/PI3K-mediated pathway (Yakes *et al*., 2002; Longva *et al*., 2005). The revelation that the Akt signaling pathway, is less activated in 3D spheroids, may have profound implications for future research and confirms the importance of this model system. Consistent with earlier data (Longva *et al*., 2005), Akt phosphorylation was not inhibited by trastuzumab in SKOV-3 cells, suggesting that a mechanism exists to reduce cellular response to trastuzumab (Nahta and Esteva, 2006).

Integrins regulate different cellular functions by mediating adhesion to an ECM, inducing cytoskeletal rearrangements and activating intracellular signaling pathways, including p21-activated kinase (PAK) (Schoenwaelder and Burridge, 1999). Integrin β4 phosphorylation was significantly increased in SKBR-3 cells grown in 3D culture for 48 h without the presence of an ECM (Figure 2d). Guo *et al*., (2005) reported earlier that integrin β4 signaling was necessary for tumor cell proliferation and suppression of apoptosis at the onset of HER2-initiated mammary tumorigenesis, but that these effects were not solely the result of ECM ligand binding to integrin β4 . Mammary tumors expressing wild-type integrin β4 lacked signs of tissue organization, which is similar to our *in vitro* 3D culture model. This is in contrast to the study by Weaver *et al*. (2002), in which the focus was on polarized 3D tumor models . Analysis of the integrin β4 downstream targets Rac1 and PAK showed that in 2D culture, Rac1 was highly phosphorylated at serine 71, a target phosphorylation site by which Akt kinase inactivates Rac1 (Kwon *et al*., 2000). No phosphorylation of Rac1 was detectable in the spheroids; instead, there was a significant phosphorylation of PAK2 at serine 141, which was not inhibited by

Figure 1 Trastuzumab inhibits cell proliferation but not aggregation in 3D culture. (**a**) SKBR-3 (American Type Culture Collection, Manassas, VA, USA) and SKOV-3 cells (Leopoldo Luistro, F Hoffmann-La Roche, Nutley, NJ, USA) were seeded in 96-well plates at densities of 1.2×10^4 cells/well and 1.5×10^4 cells/well, respectively, for 3D culture, and 24 h before treatment, at 6×10^3 cells/well and 4×10^3 cells/well for 2D culture. To promote 3D culture formation, cells were seeded in wells pre-coated with 100 μl 0.5% poly-HEMA (Polysciences, Eppelheim, Germany) in 90% ethanol and air dried at 37 °C for 4 days before use. Cultures were incubated for 7 days (SKBR-3) or 6 days (SKOV-3) with either 10 μg/ml trastuzumab (F Hoffmann–La Roche, Penzberg, Germany), 10 μg/ml human IgG (Dianova, ImmunoResearch, Hamburg, Germany), or buffer (5 mM histidine, 60 mM trehalose, 0.1 mg/ml polysorbate 20, pH 6.0). Cell viability was determined with CellTiter-Glo Reagent (Promega, Mannheim, Germany). Aggregates were resuspended by pipetting and luminescence was recorded using SpectraFluor Plus (Tecan Austria GmbH, Groedig, Austria). Proliferation assays were performed in triplicate, each with five replicates; data are shown as ± s.d. of three experiments. (**b**) Fluorescence microscopy images of Hoechst 33342-stained SKBR-3 cells in 3D culture with or without 10 μg/ml trastuzumab (bar = 100 μm). After 24 h, 3 days, 5 days and 7 days incubation, cells were stained with 10 μg/ml Hoechst 33342 (Molecular Probes, Invitrogen, Karlsruhe, Germany) for 10 min at 37 °C. Images were captured using ImageXpress (Molecular Devices, Sunnyvale, CA, USA) and a ×2 objective. Analysis was performed with MetaXpress 2.0 Software (Molecular Devices). (**c**) Phase contrast micrographs of SKBR-3 and SKOV-3 cells grown in 2D or 3D culture ± 10 μg/ml trastuzumab. SKBR-3 cells were seeded at a density of 1.2×10^4 cells/well in poly-HEMA-coated 96-well plates with or without 10 μg/ml trastuzumab. Cell aggregate morphology was evaluated using an Axiovert 135 (Zeiss, Jena, Germany), a CoolSNAP K4 camera (Visitron Systems, Munich, Germany), and MetaMorph Software Version 7.0.3 (Molecular Devices).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trastuzumab (Figure 2d). This residue is required for full PAK2 activation and is consistent with Rac1 activity (Jung and Traugh, 2005). These observations indicate that the Rac1/PAK2 signaling pathway could serve as a compensating survival pathway for SKBR-3 cells in 3D culture, in which only low levels of phosphorylated Akt are present (Figure 2b).

Next, the nature of the HER2 signaling complex in 3D culture was investigated by analyzing proteins that co-immunoprecipitated with HER2. Significantly lower HER3 levels and concordantly lower levels of PI3K co-precipitated with HER2 in 3D when compared with 2D culture (Figure 3a). As HER3 is a major adaptor protein for recruiting PI3K to HER2 heterodimers, these results indicate a reduction in HER2/HER3 heterodimerization. In contrast, increased levels of Grb2 and Shc (MAPK pathway upstream signaling molecules) were co-precipitated with HER2 in 3D cultures (Figure 3a), providing further evidence of a switch in signaling pathways downstream of HER2. This switch is likely to be associated with the reduction or loss of HER2/HER3 heterodimerization. A time-dependent loss of Shc and Grb2 co-precipitates from 3D culture samples was observed after trastuzumab treatment (Figure 3b). These results suggest that HER2 forms homodimers in 3D culture. Trastuzumab binds to HER2 homodimers and inhibits the activation of these pathways, thus inhibiting cell proliferation (Hudziak *et al*., 1989). A transient increase in EGFR co-precipitation with HER2 was observed at earlier time points (4–8 h) in 3D culture, but after 24 h this difference was not discernable (Figures 3a and b). Transient HER2 co-precipitation

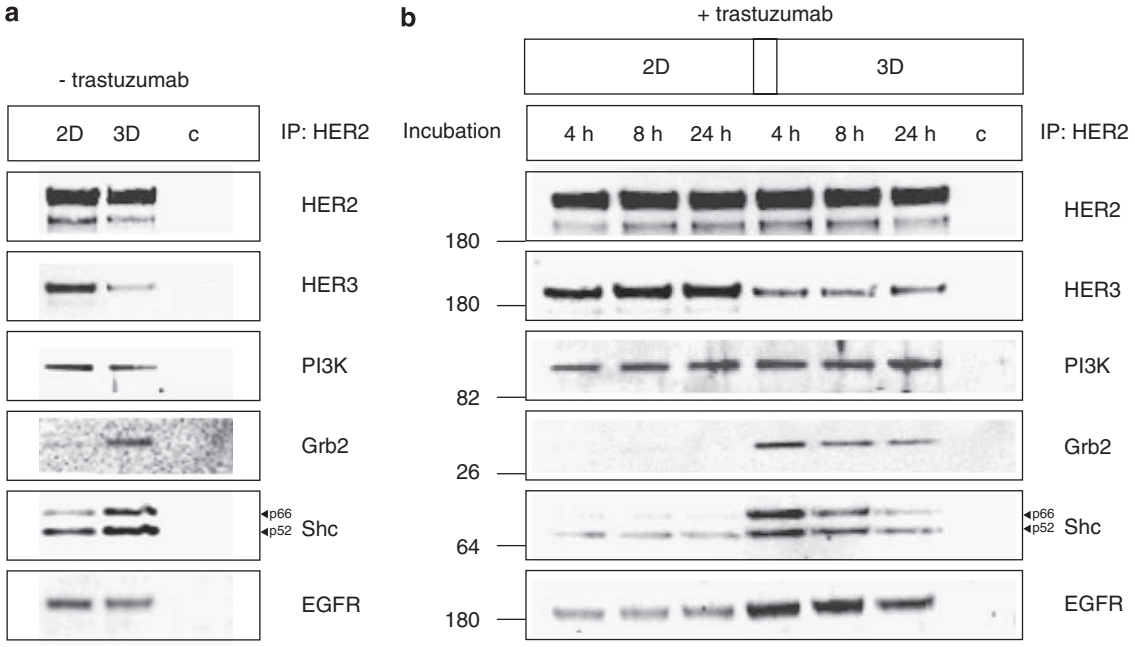

Figure 3 HER2/HER3 heterodimers are decreased in 3D culture. SKBR-3 cells were incubated in 2D and 3D (**a**) for 24 h without trastuzumab and (**b**) treated with 10 μg/ml trastuzumab as described earlier for 4, 8 and 24 h. Cells were then lysed in Tris buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1 μg/ml aprotinin and leupeptin, 10 μl/ml saturated PMSF in ethanol) containing 1% Triton X-100. Lysates (120 μg) from the post-nuclear fraction were incubated overnight at 4 °C with anti-ErbB2 Affibody Molecule (ab31892, Abcam). An equal amount of anti-ErbB2 Affibody Molecule was incubated in lysis buffer as a control. The precipitates were washed four times with ice-cold Tris buffer containing 0.5% Triton X-100 then resuspended in 100 μl 1 × loading buffer, and analysed by SDS–PAGE and immunoblotting for HER2, HER3, PI3K, Grb2, Shc and EGFR. Control represents incubation of anti-ErbB2 Affibody Molecule in lysis buffer.

Figure 2 HER2 and MAPK signaling as well as the integrin β4 and the Rac1/PAK 2 signaling pathways are activated only in 3D cell culture. Cells were seeded either at 2×10^5 cells/well in 6-well plates (3D) or 5×10^5 cells/well in 10 cm dishes (2D) and maintained for 48 h ± 10 μg/ml trastuzumab before incubation with 2 mM sodium orthovanadate (Na_3VO_4) for 5 min at 37 °C, washing with chilled PBS/2 mM Na_3VO_4, and then lysis in NP40 lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium desoxycholate, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1 μg/ml aprotinin and leupeptin, 10 μl/ml saturated phenylmethylsulfonyl fluoride (PMSF) in ethanol). In all, 25 μg of protein was resolved on gradient SDS–PAGE gels (Invitrogen), transferred to nitrocellulose membranes (Invitrogen) and immunoblotted with the following antibodies: HER2/*neu* (Dako Deutschland GmbH, Hamburg, Germany), phospho-HER2/*neu* Y1248 (Biosource, Invitrogen), EGFR, Grb2, PI3K, and Rac1 (Millipore GmbH, Schwalbach, Germany), phospho-EGFR Y1173 (Epitomics, Burlingame, CA, USA), HER3, Akt, phospho-Akt S473, MEK1/2, phospho-MEK1/2 S217/221, MAPK, phospho-MAPK T202/Y204, PAK 1, PAK 2, and phospho-PAK 1/PAK 2 (Cell Signaling Technology, Danvers, MA, USA), Shc (BD Biosciences, Heidelberg, Germany), caveolin 1 and integrin β4 (Santa Cruz Biotechnology, Heidelberg, Germany), phospho-integrin β4, phospho-Rac1, and β-actin (Abcam, Cambridge, UK). Equal loading was confirmed by reprobing membranes for β-actin. Precipitated proteins in SKBR-3 cells (**a**, **b** and **d**) and SKOV-3 cells (**c**) were visualized using the LumiLight^{PLUS} Western Blotting Kit (F Hoffmann-La Roche Diagnostics) and autoradiography.

with EGFR coincided with cell aggregation, suggesting that HER2/EGFR heterodimerization resulted from reseeding and reattachment during aggregation.

The mechanism by which HER2 overexpression contributes to oncogenesis has been the subject of several studies. The physical proximity of HER2 molecules on the cell surface is a significant factor and HER2-rich domains have been shown to colocalize with membrane rafts in the SKBR-3 breast cancer cell line (Nagy *et al*., 2002; Mocanu *et al*., 2005; Sottocornola *et al*., 2006). Membrane rafts are small, dynamic and lipid-rich regions that compartmentalize cellular processes (Pike, 2006). These are stabilized by gangliosides (glycophospholipids), which play a key role in modulating

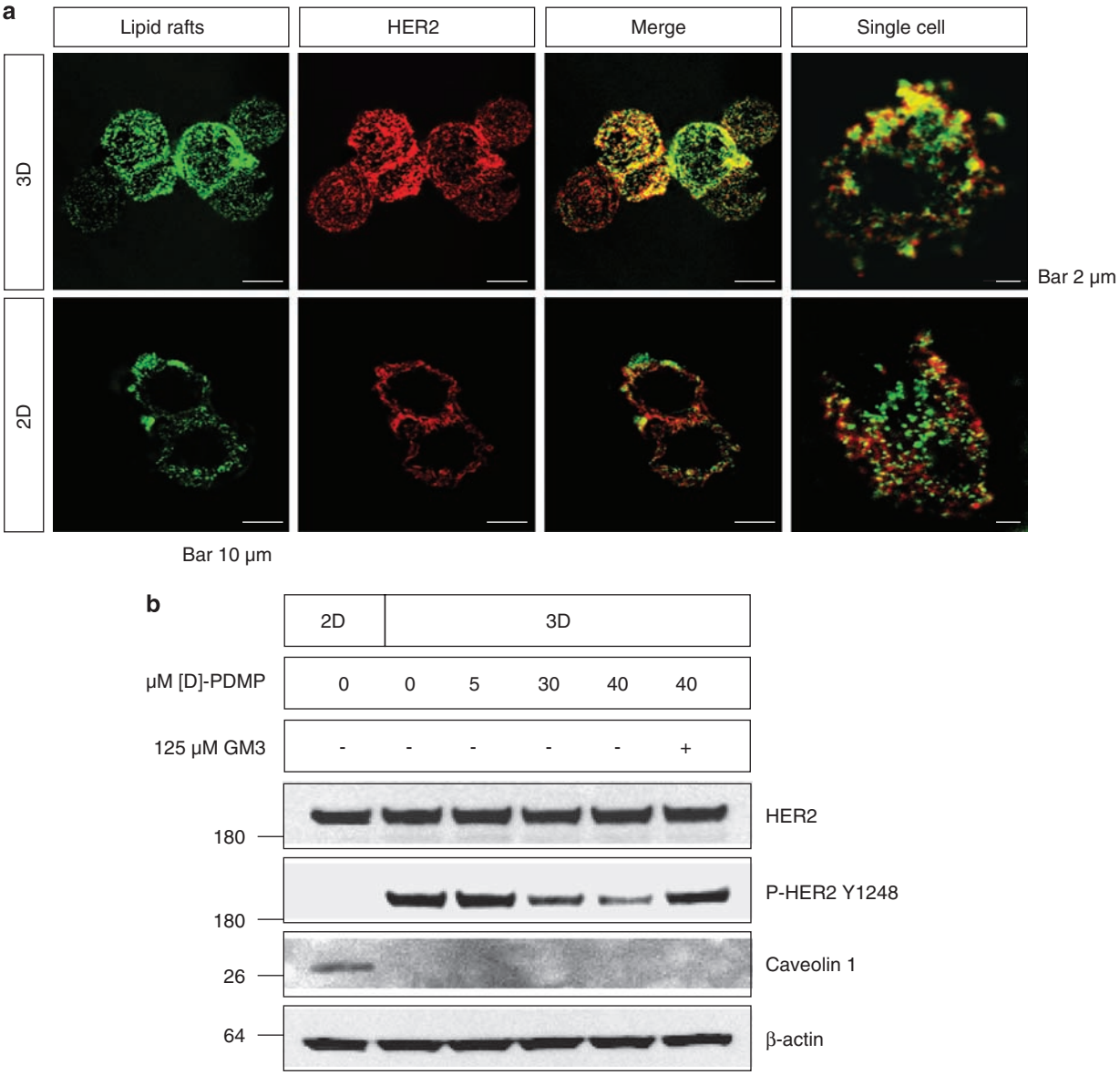

Figure 4 Persistent association and activation of HER2 in membrane rafts in 3D cell culture. (**a**) SKBR-3 cells were incubated for 24 h in 2D or 3D culture. Cell aggregates were pooled by centrifugation and then washed with ice-cold PBS before incubation with CT-B Alexa 488 conjugate at 1 μg/ml (Molecular Probes) in a complete growth medium for 20 min on ice to detect GM1 (visualized in green). After washing, cells were patched with anti-CT-B rabbit serum (Molecular Probes) for 20 min on ice and, after a final wash, were fixed in 3% paraformaldehyde for 15 min on ice. Cells were then incubated with trastuzumab (20 μg/ml in complete medium) for 30 min on ice, washed and then incubated with Alexa Fluor 594 goat anti-human IgG (Molecular Probes) to detect HER2 (visualized in red) before mounting using Gel/Mount (Abcam). Using a Leica TCS SP2 microscope equipped with argon and HeNe ion lasers and Leica Confocal Software 6.0, images were captured at 1024 × 1024 pixels and processed with MetaMorph Software Version 7.0.3 (Molecular Devices). Areas of yellow, which occur predominantly in 3D culture, indicate GM1/HER2 colocalization (bar = 10 μm for aggregate images and bar = 2 μm for single cell images). (**b**) SKBR-3 cells incubated with 5, 30 and 40 μM threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride ((D) PDMP) (Sigma) for 4 days in 2D culture followed by incubation in 3D for 24 h underwent lipid raft restoration by incubation with 125 μM ganglioside GM3 (Alexis, Lörrach, Germany) in 3D for 24 h. Whole-cell lysates were prepared and proteins (25 μg/lane) were immunoblotted with the indicated antibodies.

transmembrane signaling (Miljan and Bremer, 2002). When HER2 was activated by the HER3 ligand EGF and heregulin in SKBR-3 cells, an increase in the number of HER2 proteins recruited to membrane rafts was observed (Nagy *et al*., 2002). There is also an evidence that HER2 activity can be modulated by the ganglioside GM3 (Sottocornola *et al*., 2003) and by caveolin 1, a protein found in specialized plasma membrane-associated membrane rafts called caveolae (Engelman *et al*., 1998).

An earlier study showed that HER2 is weakly associated with membrane rafts in 2D culture of SKBR-3 cells, as crosslinking with cholera toxin B-chain (CT-B) diminished the membrane raft association of HER2 (Nagy *et al*., 2002; Mocanu *et al*., 2005). CT-B binds selectively to the GM1 ganglioside, a structural component of lipid rafts and caveolae. Both GM1 and GM3 are known to modulate HER2 homodimerization and heterodimerization activity (Nagy *et al*., 2002; Sottocornola *et al*., 2003). It is currently unknown how gangliosides modulate the dimerization of HER2, but earlier studies suggest that they may retain HER2 within the lipid raft, thereby modulating the local density of HER proteins and increasing the likelihood of HER heterodimerization and homodimerization and activation (Nagy *et al*., 2002; Sottocornola *et al*., 2003). To determine whether HER2 colocalized with membrane rafts in 3D culture, SKBR-3 cells were labeled in parallel with CT-B conjugated with the Alexa 488 fluorochrome and trastuzumab. Confocal microscopy of stained cells showed that HER2 colocalized with GM1 ganglioside in 3D cell culture but there was no significant colocalization in 2D cell culture after crosslinking with CT-B (Figure 4a). These experiments indicate that HER2 is more tightly associated with membrane rafts in 3D than in 2D culture.

To interfere with the assembly of lipid rafts, we used threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride, an inhibitor of ganglioside biosynthesis. Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride treatment depleted cell surface GM1 by 50% in 3D culture (data not shown) and reduced the levels of phosphorylated HER2 in a dose-dependent manner (Figure 4b). HER2 phosphorylation was restored with addition of exogenous ganglioside GM3, corroborating the relationship between HER2 activation and membrane raft association (Figure 4b). It is unclear how trastuzumab affects HER2 localization in these lipid rafts. Caveolin 1 was detected in cell extracts from 2D but not from 3D SKBR-3 cultures (Figure 4b) and was unaffected by trastuzumab (data not shown). Furthermore, caveolin 1 mRNA levels were downregulated in spheroids compared with 2D culture of SKBR-3 cells and caveolin-1 protein levels were reduced to differing degrees in 3D culture of additional cancer cell lines (data not shown). Caveolin 1, a main structural component of caveolae, has also been implicated in tumorigenesis, and various human tumors and cancer cell lines exhibit a decrease or loss of caveolin 1 expression (Razani *et al*., 2001). Caveolin 1 is potentially a key modulator of HER2 activity, possibly by interacting directly with HER2 or by localizing with HER2 in the caveolae. However, in tumor cells, caveolin 1 downregulation coupled with the localization of HER2 within membrane rafts may diminish the regulatory ability of caveolin 1 in relation to HER2.

Our results show a clear difference in HER2 molecular signaling in cells cultured in 2D versus 3D, which correlates with other studies using 3D cell culture models. These have highlighted differences in the behavior of cells cultured in this manner (Shaw *et al*., 2004; Lee *et al*., 2007), possibly as a result of the different architectural phenotype of the culture system (Shaw *et al*., 2004). We suggest that the reorganization of the cell surface that occurs in 3D culture induces changes in protein association and localization, offering alternative signaling platforms that may not be available in 2D culture. These analyses of 2D versus 3D culture underline the flexibility of oncogene switching between HER receptor family members, which may play an important role in the development of drug resistance.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)