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Microenvironment rigidity modulates responses to the HER2 receptor tyrosine kinase inhibitor lapatinib via YAP and TAZ transcription factors

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ABSTRACT Stiffness is a biophysical property of the extracellular matrix that modulates cellular functions, including proliferation, invasion, and differentiation, and it also may affect therapeutic responses. Therapeutic durability in cancer treatments remains a problem for both chemotherapies and pathway-targeted drugs, but the reasons for this are not well understood. Tumor progression is accompanied by changes in the biophysical properties of the tissue, and we asked whether matrix rigidity modulated the sensitive versus resistant states in HER2-amplified breast cancer cell responses to the HER2-targeted kinase inhibitor lapatinib. The antiproliferative effect of lapatinib was inversely proportional to the elastic modulus of the adhesive substrata. Down-regulation of the mechanosensitive transcription coactivators YAP and TAZ, either by siRNA or with the small-molecule YAP/TEAD inhibitor verteporfin, eliminated modulus-dependent lapatinib resistance. Reduction of YAP *in vivo* in mice also slowed the growth of implanted HER2-amplified tumors, showing a trend of increasing sensitivity to lapatinib as YAP decreased. Thus we address the role of stiffness in resistance to and efficacy of a HER2 pathway-targeted therapeutic via the mechanotransduction arm of the Hippo pathway.

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C.H.L. participated in the study design, carried out all *in vitro* experiments, analyzed the results, and drafted the manuscript. F.A.P. participated in the design of the PA gel experiments and wrote the Matlab code for immunofluorescence staining analysis. J.L. and H.Z. carried out *in vivo* experiments. C.P. and V.M.W. participated in the design of the *in vivo* study. M.A.L. conceived of the study and participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

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Abbreviations used: AREG, amphiregulin; EGFR, epidermal growth factor receptor; HER2/3, human epidermal growth factor receptor 2/3; HGF, hepatocyte growth factor; IPTG, isopropyl β -D-1-thiogalactopyranoside; MET, hepatocyte growth factor receptor; PA, polyacrylamide; TCP, tissue culture plastic.

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INTRODUCTION

Human epidermal growth factor receptor 2 (HER2)-positive breast cancers account for ~15–20% of breast cancers, have poor prognosis, and are less responsive to hormone treatment than HER2(-) breast cancers (Kun *et al.*, 2003; Koboldt *et al.*, 2012). Although HER2-targeted therapies are one of the success stories in breast cancer treatment (Kim *et al.*, 2013), generating a durable drug response remains a challenge (Rexer and Arteaga, 2012). The tyrosine kinase inhibitor lapatinib is a potent inhibitor of catalytic activity of both epidermal growth factor receptor (EGFR) and HER2 (Medina and Goodin, 2008). Most tumor cells with elevated HER2 levels show high sensitivity to growth inhibition by lapatinib, but resistance often develops (Rusnak *et al.*, 2007).

Several mechanisms of lapatinib resistance have been reported, including compensatory activation of parts of the HER network. Compensatory up-regulation of HER3 activation, driven by Akt activity, confers resistance to lapatinib in HER2-amplified breast cancer

cell lines (Amin *et al.*, 2010). Incomplete inhibition of EGFR results in heregulin-driven feedback, sustaining EGFR activation, which contributes to lapatinib resistance in HER2(+) breast cancers (Xia *et al.*, 2013). Up-regulation of the membrane tyrosine kinase AXL sustains phosphoinositide 3-kinase (PI3K)/Akt signaling, conferring lapatinib resistance (Liu *et al.*, 2009). Hepatocyte growth factor (HGF) activation of its cognate receptor MET also is associated with resistance to lapatinib in HER2(+) gastric cancers (Chen *et al.*, 2012). Thus activation of redundant survival pathways can be induced, either intrinsically or extrinsically, by microenvironmental factors, such as growth factors.

The biophysical properties of tumors change during malignant progression, which also affects tumor cell functions. Thus, in addition to redundant signaling pathways, using integrin-blocking antibodies to alter how tumor cells perceived their microenvironments was shown to modulate the efficiency of cytotoxic agents (Weaver *et al.*, 2002). Use of an *in vitro* approach that compared responses of a number of HER2-targeted therapeutics (including lapatinib) in multiple HER2-amplified cell lines revealed that some cells were more sensitive to lapatinib in three-dimensional (3D) Matrigel than with two-dimensional (2D) cultures using tissue culture plastic (TCP; Weigelt *et al.*, 2010). Even in the relatively simplified 3D Matrigel cultures, there are multiple chemical and physical properties that contribute to those microenvironment-dependent drug responses. Accumulating evidence suggests that microenvironment rigidity can promote tumor progression and survival through activation of growth factor signaling pathways by enhancing integrin clustering and focal adhesion assembly (Paszek *et al.*, 2014; Rubashkin *et al.*, 2014) or through modulation of microRNA expression (Mouw *et al.*, 2014). Tissue rigidity also affects cytotoxic chemotherapeutic responses in hepatocarcinoma cells because increasing matrix stiffness promotes cellular proliferation and resistance to chemotherapeutic agents (Schrader *et al.*, 2011). Whereas this scenario is quite sensible in the context of cytotoxic chemotherapeutics, the effect of matrix rigidity on a HER2-targeted therapeutic such as lapatinib is less obvious.

Here we examined whether matrix rigidity affected lapatinib responses in HER2-amplified breast cancer cells, using polyacrylamide (PA) hydrogel-based culture substrates that enabled control over Young's elastic modulus. The Hippo pathway mechanotransducers, transcriptional coactivator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP) (Halder *et al.*, 2012), which are also oncogenes (Wang *et al.*, 2012), were required for modulus-dependent responses *in vitro*. Down-regulation of YAP *in vivo* slowed HER2-amplified tumor growth and improved sensitivity to lapatinib. YAP and TAZ did not mediate resistance by redundant activation of other HER-family receptors. Our results suggest that rigid microenvironments can modulate lapatinib resistance in HER2-amplified breast cancer cells via a YAP/TAZ-dependent mechanism.

RESULTS

Substrate elastic modulus is a modifier of lapatinib responses in HER2-amplified breast cancer cells

To facilitate further investigation of microenvironment-directed drug responses, we identified a breast cancer cell line and pathway-targeted drug combination that offered a potentially wide dynamic range of response. Previous work demonstrated that the use of 2D TCP versus 3D Matrigel culture microenvironments modulated the antiproliferative responses of four different HER2-targeted therapeutics that were used to treat four different HER2-amplified breast cancer cell lines. The combination of HCC1569 cells, a basal A subtype cell line (Neve *et al.*, 2006), and lapatinib demonstrated the

optimal differential response between TCP and 3D Matrigel (Weigelt *et al.*, 2010). We first validated our analysis methodology by showing that HER2-amplified HCC1569 breast cancer cells conformed to previous findings, that is, that they are more sensitive to the antiproliferative effect of lapatinib in 3D Matrigel than with cells on 2D TCP (Weigelt *et al.*, 2010). After plating on type 1 collagen-coated 2D TCP or in 5% "on-top" 3D Matrigel, cells were treated with dimethyl sulfoxide (DMSO) or 1.5 μM lapatinib, a dose that was comparable to the average concentration in patient blood serum (Burriss *et al.*, 2005). The magnitude of the antiproliferative effect of lapatinib was determined by measuring 5-ethynyl-2'-deoxyuridine (EdU) incorporation into nuclear DNA as a proxy for cell proliferation. Cells in 3D Matrigel were more sensitive to lapatinib than cells on TCP, with 21 ± 2.6 and $69.9 \pm 7.7\%$ EdU incorporation, respectively (Figure 1, A and B). Proliferation of the HER2-negative cell line BT549 was not affected by lapatinib treatment (Figure 1, A and B). The differential antiproliferative response between TCP and 3D Matrigel is partly explained by the distinct molecular compositions of the two culture microenvironments; indeed, it is known that the increased sensitivity in three dimensions is due partly to $\beta 1$ integrin-mediated extracellular matrix adhesion (Weigelt *et al.*, 2010). However, there are other potential microenvironment characteristics that bear scrutiny in this drug response context.

One of the major differences between TCP and 3D Matrigel is the rigidity of the culture substrate. Thus we examined whether rigidity is a modulator of responses to lapatinib in HER2-amplified breast cancer cells. Young's elastic modulus of Matrigel has been estimated at 400 Pa (Soofi *et al.*, 2009), on a par with that for normal breast tissue (Paszek *et al.*, 2005). In contrast, the elastic modulus of TCP is >2 GPa (Saruwatari *et al.*, 2005; Levental *et al.*, 2007), which is well outside the physiological range (Kolahi *et al.*, 2012). To examine the role played by matrix rigidity in lapatinib responses, cell culture substrates were fabricated from PA gels tuned to 400 ± 160 Pa and coated with a type 1 collagen to support cell adhesion. HCC1569 cells were more sensitive to lapatinib on 400-Pa PA gels than on TCP coated with type 1 collagen, with 50 ± 4.5 and $69 \pm 4.5\%$ EdU incorporation, respectively (Figure 1, C and D); BT549 cells were not affected by lapatinib or changes in rigidity (Figure 1, C and D). The half-maximal inhibitory concentration (IC_{50}) of lapatinib was threefold lower on 400-Pa PA gels than with TCP, 0.9 and 2.7 μM , respectively (Figure 1E). Thus HCC1569 cells responded to lapatinib in an elastic modulus-dependent manner, showing greater resistance to the antiproliferative effect of lapatinib on rigid matrices.

YAP and TAZ are required for the modulus-dependent lapatinib responses

YAP and TAZ are Hippo pathway transcriptional coactivators that interact with the Rho/Rock pathway (Halder *et al.*, 2012) and play an important role in transducing information about substrate rigidity from the plasma membrane into the nucleus, where a transcriptional response is generated (Dupont *et al.*, 2011). Consistent with their role in mechanotransduction, YAP and TAZ relocated from the cytoplasm into the nucleus as substrate stiffness increased (Figure 2, A and B). We assessed the effect of YAP and TAZ knockdown by small interfering RNA (siRNA) on modulus-dependent responses to lapatinib. Both YAP and TAZ knockdown (Supplemental Figure S1A) eliminated modulus-dependent lapatinib resistance on TCP (Figure 2C). Disruption of the TEAD-YAP interaction with 2 $\mu\text{g}/\text{ml}$ inhibitor verteporfin (Liu-Chittenden *et al.*, 2012) phenocopied the effect of YAP knockdown (Figure 2D). Indeed, increasing concentrations of verteporfin diminished the effect of modulus-dependent

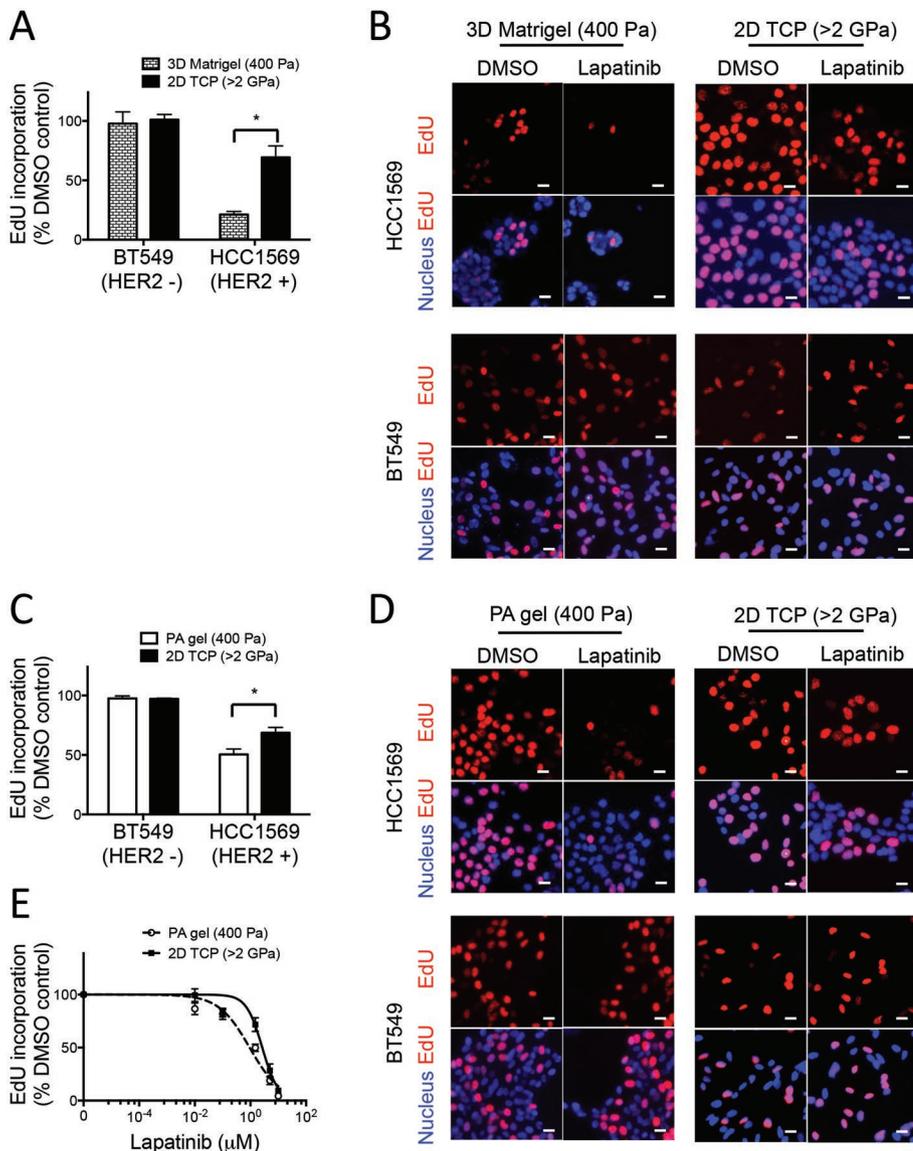


FIGURE 1: Substrate elastic modulus is a modifier of lapatinib responses in HER2-amplified cancer cells. Bar graphs show the relative incorporation of EdU expressed as a percentage of DMSO-treated cells in (A) HCC1569 and BT549 cells cultured on 2D TCP dishes or 3D in Matrigel for 48 h and then treated with lapatinib or DMSO for 48 h ($n = 3$; 500 cells/condition per experiment, $*p < 0.05$). (B) Representative images of HCC1569 and BT549 cells cultured on 2D TCP or in 3D Matrigel. EdU is pseudocolored red, and nuclear DNA is blue. (C) Bar graphs showing relative incorporation of EdU expressed as a percentage of DMSO-treated cells. HCC1569 and BT549 cells cultured on 2D TCP or 400-Pa PA gels for 48 h, followed by lapatinib (1.5 μM) or DMSO for 48 h ($n = 3$; 500 cells/condition per experiment, $*p < 0.05$). (D) Representative images of HCC1569 and BT549 cells cultured on 2D TCP or 400-Pa PA gels. EdU is pseudocolored red, and nuclear DNA is blue. Scale bars, 20 μm . (E) Dose-response curves used to calculate IC_{50} of lapatinib in HCC1569 cells cultured on 400-Pa PA gel (0.94 μM) vs. 2D TCP (2.66 μM). $n = 3$; 500 cells/condition per experiment, $*p < 0.05$.

lapatinib resistance in a synergistic manner with lapatinib (Supplemental Figure S2). YAP and TAZ were thus shown to be necessary for generating modulus-dependent lapatinib resistance.

YAP knockdown in vivo increased sensitivity to lapatinib treatment

To test whether YAP similarly played a role in lapatinib responses in vivo, we used isopropyl β -D-1-thiogalactopyranoside (IPTG)-induced short hairpin RNA (shRNA) to knock down YAP in HCC1569

cells implanted in mice. Tumor volume was measured during the course of lapatinib treatment (Figure 3). Mice that received neither IPTG nor lapatinib (group A) had the maximum tumor volume (mean of volume, 1280 mm^3) by day 23. Mice treated with IPTG (group B) had significantly decreased ($p < 0.05$) tumor volume (mean of volume, 770 mm^3) compared with group A. Lapatinib treatment groups either with (group D) or without IPTG treatment (group C) had much smaller tumor volumes compared with groups A and B. Group D, which received lapatinib and had reduced YAP levels, had the smallest tumor volumes (mean of volume, 192 mm^3), even compared with group C (mean of volume, 269 mm^3); however, that difference was not statistically significant. These data demonstrate that YAP knockdown was sufficient to reduce growth of HER2-amplified cell lines in vivo, and they suggest that YAP knockdown and lapatinib together may have some synergistic benefit. More comprehensive animal studies are required, however, to clarify the independent versus synergistic effects.

Modulus-dependent lapatinib responses are driven by multiple factors

We sought to delineate other components of the molecular circuitry that enabled YAP to mediate the modulus-dependent response to lapatinib. Analysis of breast cancer data from the Cancer Genome Atlas (Koboldt *et al.*, 2012) showed that YAP mRNA expression correlated positively with expression of two known YAP targets, CTGF and amphiregulin (AREG; Zhang *et al.*, 2009), which is an EGFR ligand that has been attributed with multiple roles related to tumor invasion and drug resistance (Hurbini *et al.*, 2002; Higginbotham *et al.*, 2011; Supplemental Figure S3). HER2 (encoded by the ERBB2 gene) mRNA expression positively correlated with AREG and inversely correlated with EGFR and ERBB3 mRNA levels. Taken together, the results indicate that higher AREG expression correlated with YAP and HER2 expression in breast cancers.

We further examined AREG, which has been shown to mediate EGFR-HER3 heterodimer formation and activate the extracellular signal-regulated kinase (ERK)-Akt signaling pathway (Yotsumoto *et al.*, 2010). In addition, HER3-mediated PI3K/Akt activity was correlated with lapatinib resistance in HER2-amplified breast cancer cells (Garrett *et al.*, 2011). Paracrine AREG signaling in colorectal cancer cells also was shown to sustain ERK signaling and confer resistance to EGFR inhibitors (Hobor *et al.*, 2014).

AREG mRNA levels in HCC1569 cells showed a modestly increasing trend as the culture substrate rigidity was increased (Figure 4A),

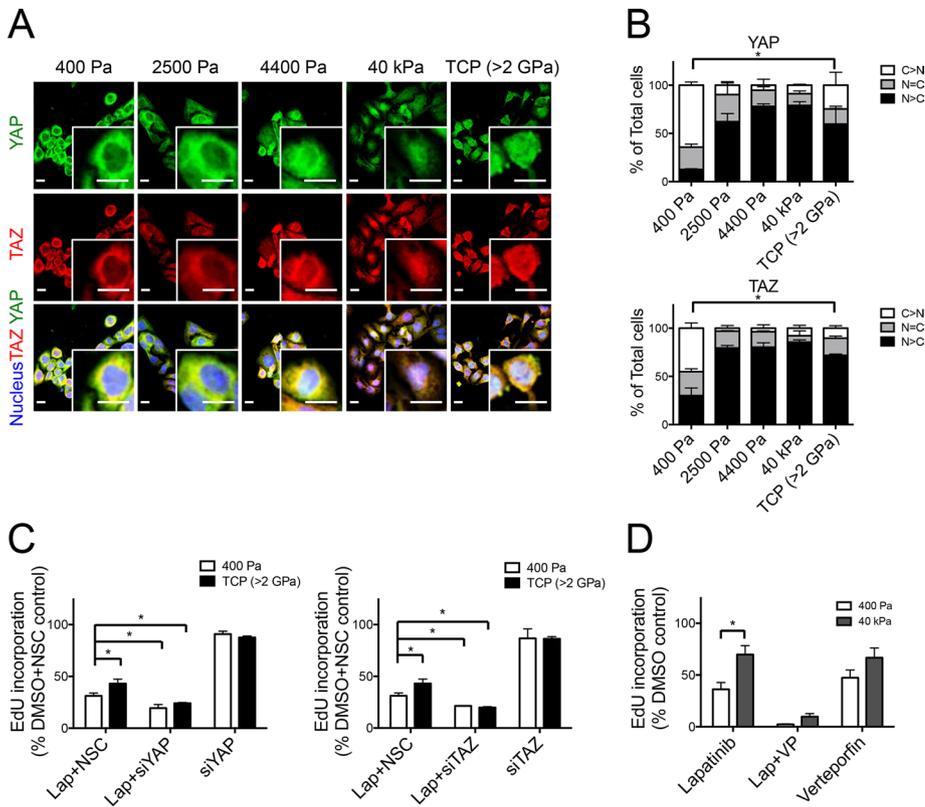


FIGURE 2: YAP and TAZ are required for modulus-dependent lapatinib responses. (A) Representative images of HCC1569 after 48 h culture on substrates of increasing stiffness: immunofluorescence stains represent YAP (green), TAZ (red), and nucleus (blue). Scale bars, 20 μm . (B) Bar graphs showing the proportions of single cells in which YAP and TAZ were located in the nucleus or cytoplasm or evenly distributed in both compartments as a function of stiffness ($n = 3$; 100 cells/condition per experiment, $*p < 0.05$). (C) Bar graphs showing the relative incorporation of EdU in HCC1569 cells cultured on 2D TCP and 400-Pa PA gel with YAP or TAZ knockdown by siRNA for 72 h and then treated with lapatinib (1.5 μM) or DMSO for 48 h. Results are expressed as a percentage of cells treated with DMSO and NSC siRNA-treated cells ($n = 3$; 500 cells/condition per experiment, $*p < 0.05$). (D) Bar graphs showing relative incorporation of EdU in HCC1569 cells cultured on 400-Pa and 40-kPa PA gels for 48 h and then treated with lapatinib (1.5 μM) together with verteporfin (2 $\mu\text{g}/\text{ml}$) or DMSO for 48 h. Results expressed as percentage of DMSO-treated controls ($n = 3$; 500/condition per experiment, $*p < 0.05$).

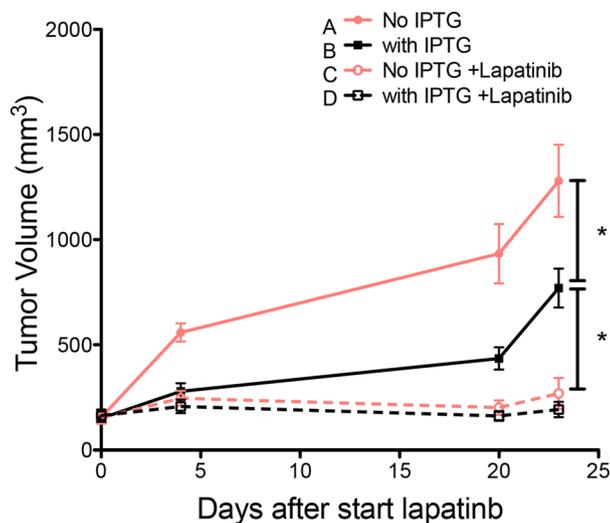
but cell membrane surface AREG protein level increased only $\sim 4\%$ (Figure 4B). To mimic the presence of a paracrine source of AREG, we added exogenous recombinant AREG (5 ng/ml) to cells, which caused increased nuclear YAP localization, even on compliant 400-Pa surfaces (Figure 4C), and on compliant surfaces conferred lapatinib resistance to the cells (Figure 4D). Simultaneous addition of exogenous AREG and the EGFR receptor inhibitor, erlotinib, reduced the resistance phenotype, demonstrating that exogenous AREG was exerting its effect partly through EGFR (Figure 4D). Knockdown of AREG by siRNA (siAREG) did not affect other ligands of EGFR, such as EGF or transforming growth factor- α , nor did it affect the receptors EGFR and HER2 (Supplemental Figure S1B). YAP knockdown with siRNA decreased AREG expression 25%, suggesting that YAP modulates AREG (Figure 4E). These data together suggest that AREG is putatively involved in the modulus-dependent lapatinib responses. However, direct targeting of AREG via siAREG showed no significant effect on modulus-dependent lapatinib responses (Figure 4F). Because AREG was reported to cause activation of HER3 (Yotsumoto *et al.*, 2010), we examined modulus-dependent changes in HER3 phosphorylation at 1 and 48 h after attachment, as well as from 49 other receptor tyrosine kinases. Without lapatinib treatment,

HER2 showed a higher phosphorylation on compliant substrates (400 Pa) within the first hour and increased phosphorylation after 48 h. However, consistent with the notion that AREG did not play a significant role in modulus-dependent responses, HER3 was unchanged within the first hour and was decreased by 48 h (Supplemental Figure S4). With lapatinib treatment, both HER2 and HER3 showed decreased phosphorylation within 1 h, and then HER3 showed a subtle increase in phosphorylation by 48 h on stiffer substrates (40 kPa). The results taken together show that modulus-dependent lapatinib resistance cannot be explained from a single YAP-AREG circuit.

DISCUSSION

Here we demonstrate that the mechanical property of microenvironments may influence resistance to and efficacy of the HER2 pathway-targeted therapeutic lapatinib in HER2-amplified breast cancer cells. Although engineered culture substrates necessarily oversimplify the tumor microenvironment compared with in vivo, they can reveal important mechanistic elements of cellular responses by winnowing down the possible candidate pathways involved in a given response. We specifically probed the property of elastic modulus on cells at low density on PA gels to minimize the confounding effects of cell-cell contact. YAP activation is regulated also by cell-cell contact (Zhao *et al.*, 2007); high cell density inhibits YAP activation by inducing YAP phosphorylation. In our engineered system, YAP and TAZ activation correlated with resistance to lapatinib, and when YAP was knocked out in orthotopically implanted tumors grown in mice, tumor growth slowed and they became more sensitive to lapatinib. The resistance phenotype is not exclusively modulus dependent, but by isolating and studying that one physical property of the matrix, we showed that the Hippo pathway is likely an important component of resistance in HER2-targeted kinase inhibitors.

YAP has been attributed dual roles in tumor genesis in breast cancer. Studies in vitro show that exogenous expression of YAP in cells can promote cell growth, suggesting that YAP has a role as a tumor promoter (Wang *et al.*, 2012). Others studies reported potential tumor-suppressive roles for YAP. Loss of heterozygosity at the YAP locus in a number of luminal breast cancers and shRNA knockdown of YAP in some breast cancer cell lines suppress anoikis and promotes tumor growth in vivo (Yuan *et al.*, 2008). Moreover, YAP expression was reduced in some invasive carcinoma samples compared with normal breast tissues (Tufail *et al.*, 2012). The multiple roles of YAP in tumorigenesis may depend on the stage of progression of a given cell; for example, active YAP enhances tumor growth when expressed in mammary carcinomas but not when expressed in a nonmalignant mammary epithelial cell line (Lamar *et al.*, 2012). YAP expression in breast cancers may be subtype dependent (Kim *et al.*, 2014), and YAP is notably present in stromal



Summary of AUC data

	No IPTG	with IPTG	No IPTG +Lapatinib	with IPTG +Lapatinib
Number of values	9	10	10	10
Minimum	11947	4480	1149	1751
25% Percentile	12757	6177	2626	2843
Median	15820	7720	5811	3801
75% Percentile	21299	10862	7158	5350
Maximum	24153	14308	7368	7334
Mean	16680	8372	4938	4171
Std. Deviation	4747	3069	2359	1859
Std. Error	1582	970.5	745.9	587.8
Lower 95% CI	13031	6177	3250	2841
Upper 95% CI	20328	10567	6625	5501

FIGURE 3: YAP knockdown has a synergistic trend of inhibition with lapatinib in vivo. Tumor volume curves as a function of time and the summary table of area-under-the-curve data for different treatment groups. The tumor volume was measured during the course of lapatinib treatment on mice that did not receive IPTG or lapatinib (group A), mice treated with IPTG only (group B), mice treated with lapatinib only (group C), and mice treated with lapatinib together with IPTG (group D).

cells (Calvo *et al.*, 2013) and not only epithelial cells, which further complicates the in vivo situation. In addition, YAP and TAZ exhibit distinct functions; for example, YAP-knockout mice are embryonic lethal (Morin-Kensicki *et al.*, 2006), but TAZ- knockout mice can be viable, although the animals have kidney disease (Hossain *et al.*, 2007). Here we showed that either YAP or TAZ knockdown in vitro can eliminate modulus-dependent lapatinib responses and confer more sensitivity to lapatinib, suggesting redundant roles in the context of modulus-dependent response to lapatinib.

The photosensitizer verteporfin (Visudyne; Novartis, Basel, Switzerland) is used as photodynamic therapy for neovascular lesions in the eye (Michels and Schmidt-Erfurth, 2001). In the absence of photoactivation, verteporfin can disrupt TEAD-YAP association and inhibit YAP-induced liver overgrowth (Liu-Chittenden *et al.*, 2012), suggesting a pharmacological strategy for regulating the transcriptional activities of YAP, which requires TEAD-family proteins to bind to DNA. Our data show that verteporfin has a synergistic effect with lapatinib in vitro, indicating that there is a potential benefit to testing verteporfin in the context of lapatinib resistance.

Many studies into resistance to HER2-targeted cancer therapeutics have focused on intrinsic cellular mechanisms, such as compensatory pathway activation, but the tumor microenvironment will likely play an important role as well. Indeed, greater matrix rigidity

leads to increased resistance to the antiproliferative effects of lapatinib in HER2-amplified breast cancer cells on culture substrates engineered to mimic different levels of matrix rigidity. Several studies have shown in various cancer contexts that increasing matrix stiffness can both promote chemotherapeutic resistance (Schrader *et al.*, 2011; Sharma *et al.*, 2014; Zustiak *et al.*, 2014) and decrease sensitivity to Raf kinase inhibitors (Nguyen *et al.*, 2014); however, the mechanisms underlying these elastic modulus-dependent effects are not well defined.

Lapatinib resistance also has been linked to compensatory activation of HER3 (Amin *et al.*, 2010), but we did not find that HER3 was strongly activated in the context of increased matrix rigidity. To mimic levels found in patient serum, we used 1.5 μM lapatinib, a concentration considered high (Amin *et al.*, 2010), and measured HER3 phosphorylation 48 h after lapatinib treatment, whereas compensatory activation of HER3 may occur after 72 h and at a lower lapatinib concentration. Taken together, and experimental differences notwithstanding, these reports suggest that resistance to HER2-targeted kinase inhibitors is likely a multifaceted challenge still to be overcome.

That most failures in drug development are due to a lack of efficacy suggests that our preclinical development toolbox does a poor job of predicting compound activity in vivo (Baker and Chen, 2012). Multiwell TCP plates are still the substrate of choice for much of modern drug screening, which ignores an obvious lack of context (Labarge *et al.*, 2014). Some high throughput (HT)-compatible 3D culture systems are being developed to overcome this problem, but retooling of HT systems and improvements in image analysis algorithms remain significant barriers to wide-scale adoption. Adaptation of 2D hydrogels that are controlled for tissue-like elastic moduli and are conjugated with tissue-like molecular milieus to HT systems might present an intermediate step that can both take advantage of existing HT systems and recapitulate some key elements of in vivo microenvironments that are crucial for determinants of drug responses.

Our data show that microenvironment rigidity influenced lapatinib responses in HER2-amplified breast cancer cell lines and that YAP and TAZ are important in this context. Our findings underscore the importance of microenvironmental effects in drug development and suggest potential therapeutic benefits of verteporfin in HER2-targeted treatment.

MATERIALS AND METHODS

Cell culture and drug treatment

HCC1569 (American Type Culture Collection, Manassas, VA) and BT549 breast cancer cell lines (a gift from Joe W. Gray, Oregon Health and Science University, Portland, OR) were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), and 1% penicillin/streptomycin/glutamine (Invitrogen). Genotypes of the cell lines were authenticated by STR profiling (DDC Medical, Fairfield, OH). For drug treatment in 2D cultures, cells were cultured in 24-well plates with RPMI 1640 with 1% FBS and 1% penicillin/streptomycin/glutamine for 48 h after initial adhesion and then treated with lapatinib (1.5 μM ; LC Laboratories, Woburn, MA) for an additional 48 h. For drug treatment in 3D cultures, cells were cultured in 24-well plates coated with Matrigel (BD Biosciences, San Jose, CA) following the so-called “on-top” protocol adapted from Lee *et al.* (2007), using a 5% Matrigel drip, and then drugs or control were added on day 4 after cell plating for an additional 48 h. Other pharmaceutical and recombinant protein modulators were added concurrently with lapatinib: verteporfin (Sigma-Aldrich, St. Louis, MO) was added at

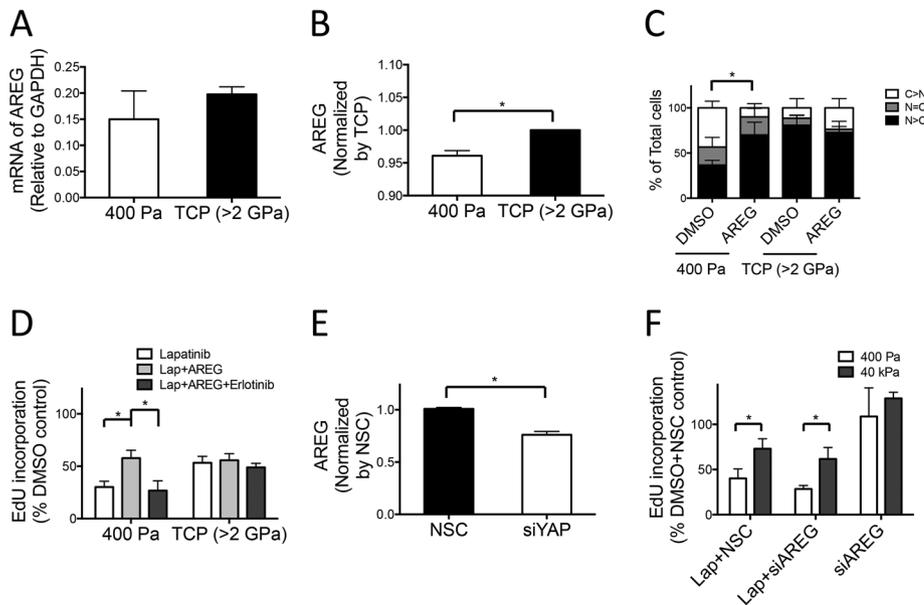


FIGURE 4: YAP-dependent amphiregulin protein regulation is involved in modulus-dependent lapatinib responses. Bar graphs showing (A) AREG mRNA expression level measured by qRT-PCR and (B) cell surface AREG measured by FACS in HCC1569 cultured on TCP or 400-Pa PA gels for 96 h ($n = 3$; 10,000 cells/condition per experiment, $*p < 0.05$). (C) Bar graphs showing the proportions of single cells in which YAP and TAZ were located in the nucleus or cytoplasm or evenly distributed in both compartments as a function of stiffness in HCC1569 cells cultured on TCP or 400-Pa PA gel for 48 h and then treated with AREG (5 ng/ml) for 48 h ($n = 3$; 100 cells/condition per experiment, $*p < 0.05$). (D) Bar graphs showing the relative incorporation of EdU, expressed as a percentage of DMSO-treated cells in HCC1569 cultured on TCP or 400-Pa PA gels for 48 h and then treated with lapatinib (1.5 mM), AREG (5 ng/ml), and erlotinib (1.5 mM) for 48 h ($n = 3$; 500 cells/condition per experiment, $*p < 0.05$). (E) Bar graphs showing intracellular AREG protein levels measured by enzyme-linked immunosorbent assay in HCC1569 with NSC siRNA or YAP knockdown by siRNA for 72 h ($n = 3$, $*p < 0.05$). (F) Bar graphs show the relative incorporation of EdU, expressed as a percentage of DMSO- and NSC siRNA-treated cells in HCC1569 cultured on 400-Pa and 40-kPa PA gels with AREG knockdown by siRNA for 72 h and then treated with lapatinib (1.5 μM) or DMSO for 48 h ($n = 3$; 500 cells/condition per experiment, $*p < 0.05$).

0.2, 2, and 10 $\mu\text{g/ml}$, recombinant human amphiregulin (Sigma-Aldrich) at 5 ng/ml, and erlotinib at 1.5 μM .

Tunable elastic modulus cell culture substrate fabrication

Polyacrylamide gels were polymerized on 12-mm-diameter coverslips etched with 0.1 M NaOH, adapted from Tse and Engler (2010). Acrylamide 3% and bis-acrylamide 0.06% were used to generate 400-Pa PA gels. Sulfo-SANPAH (0.5 mM; ProteoChem, Loves Park, IL) was added on PA gels and activated by ultraviolet light exposure for 10 min. PA gels were washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and then incubated with type 1 collagen at room temperature for 2 h (0.1 mg/ml in 50 mM HEPES from calf skin; Sigma-Aldrich). Gels were rinsed with copious amounts of phosphate-buffered saline (PBS) before placing them in 24-well plates treated with polyHEMA (0.133 ml at 12 mg/ml in 95% EtOH; Sigma-Aldrich) for cell culture.

Proliferation assay

EdU incorporation and staining were performed according to the manufacturer's protocol (Invitrogen). Nuclei were stained with Hoechst 33342. Images were acquired by a Zeiss 710 LSM (Carl Zeiss, Jena, Germany) confocal microscope, and images were analyzed with ImageJ (National Institutes of Health, Bethesda, MD).

Drug response values are expressed as a percentage of DMSO-treated cells.

Transfection

Cells were transfected with YAP, WWTR1 (TAZ), AREG, or nonsilencing control siRNA (NSC; SMARTpool: ON-TARGET plus, GE Dharmacon, Lafayette, CO) with a fluorescein isothiocyanate label (siGLO Green Transfection Indicator; GE Dharmacon), using DharmaFECT 2 Transfection Reagent (GE Dharmacon) according to the manufacturer's protocol 72 h before assay performance.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde at room temperature for 10 min, blocked with PBS, 5% normal goat serum, and 0.1% Triton X-100 at room temperature for 30 min, and then incubated with primary antibodies anti-YAP (1:100; Santa Cruz Biotechnology, Dallas, TX) and anti-TAZ (1:200; Cell Signaling Technology, Beverly, MA) overnight at 4°C. Primary antibodies were visualized with fluorescent secondary antibodies raised in goats (1:500; Invitrogen) together with Hoechst 33342 (1:200; Sigma-Aldrich) incubated at room temperature for 2 h. Images were acquired with a Zeiss 710 LSM confocal microscope. Cell segmentation and single-cell fluorescence intensities were analyzed with Matlab script adapted from Pelissier *et al.*, 2014. For quantification of YAP/TAZ localization, the ratios of mean fluorescence intensity in the cytoplasmic (C) and nuclear (N) compartments of segmented cells were used. The cutoffs of \log_2

ratios were used to establish three classes: C > N ($X < -0.074$), N = C ($-0.074 < X < 0.074$), and N > C ($X > 0.074$).

Real-Time PCR

Total RNA was extracted with TRIzol (Invitrogen) and purified by RNeasy prep (Qiagen, Valencia, CA). cDNA was synthesized with SuperScript III RT (Invitrogen). Transcripts levels were measured by quantitative real-time PCR (qRT-PCR) with iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and Light Cycler480 (Roche, Indianapolis, IN). Primer sequences were as follows: YAP, 5'-AGCCAGTTGCAGTTTTTCAGG-3' and 5'-AGCAGCAATGGACAA-GGAAG-3'; TAZ (WWTR1), 5'-GGAGAAAACGCAGGACAAAC-3' and 5'-TCATTGAAGAGGGGGATCAG-3'; AREG, 5'-GTGGTGCT-GTCGCTCTTGATA-3' and 5'-ACTCACAGGGGAAATCTCACT-3'; and glyceraldehyde-3-phosphate dehydrogenase, 5'-AAGGTGAA-GGTCGGAGTCAAC-3' and 5'-GGGGTCATTGATGGCAACAATA-3'.

Flow cytometry

Cells were collected via EDTA-PBS (0.4% EDTA) treatment without trypsin on ice. After washing with PBS, cells were blocked with PBS containing 2% bovine serum albumin, 5% normal goat serum, and 5 mM EDTA on ice for 30 min. Cells were incubated with the primary antibody anti-AREG (1:100; R&D Systems, Minneapolis, MN) on ice for 30 min, washed with PBS, and then treated with the secondary

antibody on ice for 15 min. After two PBS washes, the level of AREG bound on cell membrane was measured with a FACSCalibur (Becton-Dickinson, San Jose, CA).

AREG enzyme-linked immunosorbent assay

The intracellular AREG protein level was measured according to the manufacturer's protocol (Abcam, Cambridge, MA), after 72 h in HCC1569 cells cultured on 2D TCP and 400-Pa PA gel with YAP knockdown by siRNA or nonsilencing control.

Animal experiments

Six-week-old female *nu^{-/-}* mice were obtained from Taconic (Germantown, NY) and housed five per cage with chow and water ad libitum in a controlled animal barrier. After 1 wk, the animals were injected subcutaneously into the upper flank with $(3.5\text{--}5) \times 10^6$ shRNA YAP HCC1569 cells. On day 13 after tumor injection, when the average tumor volume was 150–200 mm³, IPTG (Sigma-Aldrich) and lapatinib were administered for 2 wk. IPTG was mixed into the drinking water at 10 mM/1% glucose in light-protected bottles and changed every 2–3 d. Lapatinib was administered at 75 mg/kg/day body weight divided into twice-daily dosing by oral gavage. Tumor dimensions (width, height, and depth) were measured biweekly. At the time of killing, tumors were harvested and either immediately snap frozen or fixed in Formalin. Animals were monitored for toxicity by measuring weight, assessing overall activity, and performing necropsy. All experimental procedures were followed according to the University of California, San Francisco, Animal Welfare Committee's approved policies and guidelines.

Human phospho-receptor tyrosine kinase array

HCC1569 cells were cultured on 400-Pa and 40-kPa PA gels for 48 h, treated with lapatinib (1.5 μM) or DMSO, and then harvested at 1 or 48 h after lapatinib treatment. The phosphorylations of 49 different receptor tyrosine kinases were measured according to the manufacturer's protocol (ARY001B, lot 1323072; R&D Systems).

Statistics

Significance was considered $p < 0.05$ or better using *t* tests and Pearson correlations. Those tests and area-under-the-curve calculations were performed with Prism (GraphPad, La Jolla, CA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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