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Sorafenib resistance and JNK signaling in carcinoma during extracellular matrix stiffening

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1. Introduction

The tumor microenvironment plays an important role in providing a niche to nurture the growth of cancer cells [1]. Recently, stromal cells, growth factors, cytokines, and ECM proteins in the tumor microenvironment have been implicated in promoting resistance to chemotherapeutics as well [2, 3]. Specifically, certain stromal growth factors mediate cell proliferation in the presence of otherwise powerful chemotherapeutic drugs. For example, hepatocyte growth factor (HGF) imparts resistance to vemurafenib in melanoma [4, 5], and TGF-β induces the expansion of cancer stem-like cells, which are responsible for chemotherapy-resistance and relapses [6]. These growth factors are generally thought to be released by local stromal fibroblasts, which upon DNA-damage from treatments with a combination of mitoxantrone and docetaxel, or radiation stimulate prostate cancer cell proliferation and invasion through β-catenin signaling [7]. In addition to soluble growth factors, a change in adhesive ECM proteins in the tissue can confer resistance to chemotherapeutics via integrin-mediated signaling [8-11].

This evolution in the microenvironment during tumor progression is mediated by stromal fibroblasts, which differentiate into myofibroblasts [12] and cancer-associated fibroblasts (CAFs) [13], and remodel the ECM by breaking down the basement membrane and depositing fibril forming collagens [14-16]. The increase in crosslinked fibrous collagens results in tissue stiffening [17, 18], which stimulates cell proliferation [17, 19], invasion and intravasation [20, 21], disrupts cell-cell adhesion [22], and alters cell sensitivity to growth factors [23], while simultaneously limiting the diffusion of therapeutic agents into the tumor [24].

We hypothesized that these mechanochemical changes in the ECM during tumor progression may induce drug resistance in carcinoma. Testing this hypothesis required that we develop a new drug testing platform that included not only human cell lines and appropriate growth factors, but also tailored control over integrin-binding and ECM stiffness. Many groups are spurring the development of novel cell culture platforms for more rational and predictive drug discovery [25-27], however, we found that existing systems are either cumbersome to use, or have limited adaptability. In response, we adapted our previously published PEG-PC hydrogel system, an easy to use biomaterial, which is optically transparent, forms gels ranging from 1-10,000 kPa in Young's modulus, can be coupled with any protein or peptide of

interest, and rapidly polymerizes within 96-well plates [28]. This new method allowed us to quantify drug response in a high-throughput manner across a range of stiffness and integrin-binding conditions.

The role of stiffness in regulating drug response was explored by Schrader et al., who observed a reduction in apoptosis of cells on stiff substrates when treated with cisplatin [29]. Also, Zustiak et al. reported cell line-dependent stiffness sensitivity to paclitaxel [30]. Sorafenib was developed as a Raf kinase inhibitor [31], and unlike these previously tested drugs, there is no obvious link between this signaling pathway and ECM stiffness. Phosphorylation of ERK, a downstream effector of Raf kinase, has been implicated in controlling cell proliferation during ECM stiffening [19, 32], so we hypothesized that sorafenib efficacy could be hampered in stiff environments, contributing at least partially to sorafenib's modest clinical efficacy [33].

To capture the evolution of the tumor microenvironment during disease progression, we formed hydrogel environments with a range of stiffnesses, including either basement membrane-like ECM proteins [34], or a collagen-rich inflammatory ECM [21]. We examined whether stiff environments protected carcinoma cells from sorafenib treatment, and if this drug resistance was mediated by the canonical Rho-ROCK and β_1 integrin signaling pathways. Motivated by a targeted phospho-proteomic screen, we also quantified the role of ERK, Akt, JNK and p38 signaling during cell response to sorafenib on stiff substrates. Our results demonstrate the utility of our tunable, high-throughput PEG-PC biomaterial platform in drug screening, and identify an exciting new mechanism to increase the efficacy of sorafenib in stiff tumor environments.

2. Materials and methods

2.1. Cell culture

All supplies were purchased from Life Technologies (Carlsbad, CA) unless otherwise noted. Human breast cancer cell lines (MDA-MB-231, BT549, and SkBr3) were generous gifts from Dr. Shannon Hughes at the Massachusetts Institute of Technology, and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) at 37ºC and 5% CO_2. Human hepatocellular carcinoma (HCC) cells (HEP3Bs, American Type Culture Collection, Manassas, VA) were cultured in modified Eagle's medium (MEM) supplemented with 10% FBS and 1% P/S at 37ºC and 5% CO_2.

2.2. 96-well hydrogel platform

Glass-bottom 96-well plates (no. 1.5 coverslip glass; In Vitro Scientific, Sunnyvale, CA) were plasma treated (Harrick Plasma, Ithaca, NY) and subsequently methacrylate-silanized with 2 vol% 3-(trimethoxysilyl) propyl methacrylate (Sigma-Aldrich, St. Louis, MO) in 95% ethanol (adjusted to pH 5.0 with glacial acetic acid) for 5 min, washed 3 times with 100% ethanol, and dried at 40 °C for 30 min. PEGDMA (Mn 750, Sigma-Aldrich), from 0.6-9.1 wt%, was combined with 17 wt% 2-methacryloyloxyethyl phosphorylcholine (PC) (Sigma-Aldrich) in phosphate buffered saline (PBS). These PEGDMA crosslinker concentrations tune the Young's moduli of the resulting gels from 6 to 400 kPa [28]. Solutions were sterilized with a 0.2 µm syringe filter (Thermo Fisher Scientific, Waltham, MA) and degassed by nitrogen sparging for 30s. Free-radical polymerization was induced by addition of 0.05 wt% ammonium persulfate (APS) and 0.125 vol% tetramethylethylenediamine (TEMED, Bio-Rad Laboratories, Hercules, CA). Hydrogels of 40 µL per well in the 96-well plates were polymerized under nitrogen for 10 min.

Post-polymerization, hydrogels were allowed to swell for 24 hours in PBS, then treated with 100 µL of sulfo-SANPAH (ProteoChem, Denver, CO; 0.6 mg/mL in pH 8.5 HEPES buffer) under UV light for 20 min, rinsed twice with HEPES buffer, and followed immediately by incubation with protein mixtures overnight. ECM protein mixtures were defined as either "basement membrane" composed of 46% human collagen IV (Neuromics, Edina, MN), 46% human fibronectin (EMD Millipore, Billerica, MA), and 8%

mouse laminin at 5 μg/cm^2, buffered in pH 7.0 PBS, or "collagen rich" composed of 65% rat tail collagen I, 33% human collagen III (FibroGen, San Francisco, CA), and 2% fibronectin at 5 μg/cm^2, buffered in pH 3.8 PBS. Post-protein coupling, the gels were rinsed twice with PBS, UV-sterilized for 1 h, and rinsed with sterile medium before cell seeding.

2.3. Quantification of drug resistance

Cells were seeded onto gel surfaces at a density of 31,000 cells/cm^2 in serum-free medium supplemented with 20 ng/mL of platelet-derived growth factor (PDGF-BB, eBioscience, San Diego, CA) and 20 ng/mL of epidermal growth factor (EGF, R&D Systems, Minneapolis, MN). After 24 h, cells were treated with sorafenib (LC Laboratories, Woburn, MA) from 0 to 120 μM, diluted in growth factor-supplemented serum-free medium. After 24 h, we measured cell proliferation with CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) at 490 nm (BioTek ELx800 microplate reader, BioTek, Winooski, VT). The concentration of sorafenib that reduced cell proliferation by 50% (IC-50) was calculated with Prism v5.04 (GraphPad Software, La Jolla, CA). In some experiments, sorafenib was also co-administered with: an anti-β_1 integrin antibody (clone P5D2, R&D Systems, 0.5 μg/mL), p160ROCK inhibitor (Y-27632, R&D Systems, 10 μM), EGF receptor (EGFR) inhibitor (AG1478, AG Scientific, San Diego, CA, 1 μM), JNK inhibitor (SP600125, LC Laboratories, 20 μM), p38 inhibitor (BIRB796, LC Laboratories, 1 μM), or ERK inhibitor (FR180204, Sigma-Aldrich, 20 μM). Dimethyl sulfoxide (Sigma-Aldrich) was used as a vehicle control in all experiments. We also verified that cell proliferation measurements approximately linearly correlated to cell count (Suppl. Fig. 1).

2.4. Immunofluorescent imaging

18 mm glass coverslips (Thermo Fisher Scientific, Waltham, MA) were plasma treated, methacrylate-silanized, and dried at 120 °C for 15 min. 80 μL PEG-PC hydrogels were polymerized with APS and TEMED between a methacrylated-silanized coverslip and an untreated coverslip for 20 min on the bench. After polymerization, the hydrogels were allowed to swell in PBS, and the non-treated coverslips were removed easily with fine forceps. Swollen gels on coverslips were transferred to 12-well

tissue culture dishes, coupled with protein mixtures as described above, rinsed 3 times with PBS, and UV-sterilized for 1 h prior to cell seeding.

Cells were seeded at a density of 10,000 cells/cm^2 in growth factor-supplemented serum-free medium and allowed to adhere for 24 hours. Cells were rinsed three times with warm PBS, fixed with 4% formaldehyde, permeabilized with Tris-buffered saline (TBS) containing 0.5% Triton X-100 (Promega), and blocked with AbDil (2 wt.% BSA in TBS with 0.1% Triton X-100, TBS-T). F-actin was labeled with Alexa Fluor 555-conjugated phalloidin for 1 h. Vinculin was labeled with a monoclonal mouse anti-vinculin antibody (Sigma-Aldrich) for 1 h, followed by an anti-mouse FITC secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Cell nuclei were labeled with DAPI (MP Biomedicals, Santa Ana, CA) for 5 min. Each sample was treated with ProLong Gold antifade reagent for 5 min before imaging on a Zeiss Axio Observer Z1 microscope with a 63x oil immersion objective (Carl Zeiss AG, Oberkochen, Germany), and images were compiled in ImageJ (NIH, Bethesda, MD).

2.5. Multiplex phospho-protein quantification

MDA-MB-231 cells ('231s') were seeded at 50,000 cells/cm^2 on 6 and 400 kPa 18 mm diameter coverslip-mounted gels in 12-well plates. Immediately after seeding, at 0 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 24 h time points, coverslips were transferred to a new 12-well plate on ice, the gels were washed once with ice-cold Bioplex cell wash buffer (Bio-Rad, Hercules, CA), and lysed with ice-cold lysis buffer (Bioplex cell lysis buffer, Bio-Rad) containing protease (EDTA-free Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN) and phosphatase (2x phosphatase inhibitors cocktail-II, Boston Bioproducts, Boston, MA) inhibitors. Separately, cells were allowed to adhere for 24 hours, treated with sorafenib, and lysates were collected at 0 min, 1 h, 5 h, 15 h, and 24 h time points after sorafenib treatment. Total protein concentration was quantified with a BCA protein assay (Thermo Scientific, Rockford, IL). Lysate concentrations were adjusted to 100 µg/mL, and the phosphorylation levels of ERK1/2, Akt, JNK, and p38 were quantified with a MAGPIX (Luminex, Austin, TX) with Bio-Plex Pro™ phospho-ERK1/2, phospho-Akt, phospho-JNK, and phopho-p38 magnetic beads (Bio-Rad), according to the manufacturer instructions.

2.6. Statistical analysis

A one-way analysis of variance (ANOVA) with a Tukey post-test was performed with Prism v5.04 (GraphPad Software). Data are reported as mean ± standard error. $p \leq 0.05$ is denoted with *, ≤ 0.01 with **, and ≤ 0.001 with ***.

3. Results

3.1. Carcinoma cell response to sorafenib on PEG-PC hydrogels

We created a high-throughput biomaterial platform to rapidly assay cell responses to chemotherapeutic drugs in different mechanochemical environments (Fig. 1). In particular, we focused on how these changes perturbed the efficacy of sorafenib, a Raf kinase inhibitor approved for thyroid, kidney and liver cancer, but which has had limited clinical success [33, 35, 36]. One potential cause of chemotherapy evasion by carcinoma cells might be the stiffening of the tumor environment itself, and so we quantified the responses of a liver cancer cell line and three breast cancer cell lines to sorafenib on PEG-PC of increasing stiffness. In all cell lines, we consistently observed a significant increase in sorafenib IC-50, the concentration at which the proliferation was dampened by half, with increasing substrate stiffness (Figs. 2A-B). We observed this phenomenon on both the basement membrane-like ECM (Fig. 2A), and the collagen-rich ECM (Fig. 2B), demonstrating that this stiffness-induced drug resistance is maintained even with alterations in integrin binding. Two of the breast cancer cell lines we tested, the SkBr3s and 231s, were the most drug resistant cell lines on the basement membrane ECM (Fig. 2A). When we normalized the IC-50s within each cell line to the softest condition, we observed that these two cell lines also showed the most stiffness-induced resistance to sorafenib, with 3.7 and 3 fold increases in IC-50 on the stiffest gels when compared to the softest condition, respectively (Suppl. Fig. 2A). On the collagen-rich mixture, again the 231 and SkBr3s were the most drug resistant cell lines (Fig. 2B), but, interestingly, the HEP3B cell line was the most stiffness sensitive (Suppl. Fig. 2B). Altogether, on the collagen-rich ECM, the SkBr3 and 231 cell lines are more sorafenib resistant across all gel conditions. Stiffness increases their sorafenib resistance, but they appear less stiffness sensitive than the HEP3Bs because the HEP3Bs are, overall, less resistant to sorafenib.

Neither initial cell adhesion to the gels, nor proliferation at 24 hours showed the same consistent trends as drug resistance, ruling them out as significant contributors to sorafenib resistance (Figs. 2C-D, Suppl. Fig. 3). We also cultured the 231 cells for five days prior to sorafenib treatment and found that the cells responded to sorafenib in the same manner as compared to dosing 24 hours post-seeding, but with larger IC-50s due to cell proliferation in the days prior to drug treatment (Fig. 2E). This result

demonstrates that the observed stiffness-mediated drug resistance is maintained at longer time points of culture.

3.2. Cytoskeletal tension and sorafenib resistance

Given the known role of substrate stiffness in influencing cell proliferation via the canonical Rho-ROCK pathway [19, 32], we hypothesized that intracellular tension was the most probable mechanism by which increasing stiffness protected cells from sorafenib. We quantified cell area and imaged F-actin organization in response to increasing substrate stiffness for the two most drug resistant cell lines (231 and SkBr3, Figs. 3A-D). Interestingly, on the collagen-rich ECM, cell spread area had a biphasic dependence on substrate stiffness, whereas cell spread area increased with stiffness on the basement membrane proteins. This result does not match the observed drug resistance results (compare Figs. 3B and D with Figs. 2A-B), and implies that intracellular tension does not exclusively explain the observed stiffness-mediated resistance on collagen-rich ECM. Figs. 3A-D also demonstrate that integrin binding (via ECM proteins) influences the sensitivity of cell area to substrate stiffness.

We further examined the potential role of intracellular tension in mediating stiffness-induced sorafenib resistance via ROCK activity. ROCK is a downstream effector of RhoA, a GTPase that regulates cell contractility and actin stress fiber formation [37]. We co-administered sorafenib with a ROCK inhibitor (Y27632) in both the 231 and SkBr3 cell lines on the collagen-rich ECM (Figs. 3E-F). When compared to the sorafenib alone condition (black lines), ROCK inhibition (red lines) dampened sorafenib resistance across all moduli, except for the stiffest condition; however, even in the presence of ROCK inhibitor, the IC-50s still increased with stiffness in both cell lines. Going further, we attempted to block cell adhesion to the collagen-rich ECM by co-administering sorafenib with a blocking antibody to the β_1 integrin subunit (blue lines). Blocking β_1 integrin was significantly effective in the 231 cells at all stiffnesses, but had no effect on sorafenib resistance in the SkBr3s, perhaps implying that SkBr3s can survive sorafenib treatment in low adhesive environments. Finally, we treated cells with an inhibitor to EGFR, given the known role EGFR activation in promoting resistance of several HCC cell lines to sorafenib [38], and given the fact that all these experiments are supplemented with EGF. EGFR inhibition (green lines) increased the efficacy of sorafenib modestly in both cell lines, but the trend of stiffness-

induced drug resistance remained. Taken together, neither ROCK nor EGFR appears to regulate stiffness-mediated sorafenib resistance; however, β_1 integrin antibody may be a candidate for co-treatment with sorafenib in triple negative breast cancer (the subtype of the 231 cell line), but not HER2 overexpressing breast cancer (SkBr3 subtype).

3.3. Signaling pathways activated during ECM stiffening

Given that β_1 integrin antibody was the only effective co-treatment with sorafenib in the 231 cells, we investigated a subset of candidate signaling pathways, downstream of β_1, which might be interfering with sorafenib efficacy. We used a multi-plex MAGPix system to quantify the phosphorylation of three members of the MAPK family (ERK1/2, JNK, p38), and Akt of the PI3K pathway at multiple time points post-adhesion to the softest and stiffest substrates tested in the 231 cell line (Figs. 4A-D). On both the collagen-rich and basement membrane ECMs, we observed an early peak in phosphorylation of ERK1/2 and Akt post-adhesion, but there was no difference when comparing between the soft and stiff gel conditions. JNK phosphorylation was delayed on the basement membrane ECM when compared to the collagen rich ECM, and the activity of JNK and p38 was higher on the stiffer gel at all time points on the collagen-rich ECM. Therefore, changes in JNK signaling could partially explain differences in cell behavior on the two protein mixtures, and both JNK and p38 are promising candidates to explain sorafenib resistance on stiff substrates.

3.4. Combinatorial treatment of a JNK inhibitor and sorafenib on stiff substrates

To determine whether sorafenib treatment perturbed the activity of these signaling proteins, we allowed 231 cells to adhere to the two stiffness gels coupled with collagen-rich ECM for 24 hours, and performed the MAGPix assay at various time points directly following a 15 μM sorafenib treatment. Upon sorafenib treatment, ERK1/2 phosphorylation on the stiff substrate remained significantly higher than that on the softer gel at early time points post-dosing (Fig. 5A). Akt phosphorylation also peaked in the first hour after sorafenib treatment; however, there was no difference in Akt phosphorylation between the soft and stiff substrates, which further confirmed that Akt signaling was not involved in stiffness-mediated drug resistance (Fig. 5B). The observed peak in Akt might be due to the ability of Ras to mediate signaling

through PI3K/Akt pathway [39], while sorafenib inhibits the Ras/Raf/MEK/ERK pathway. JNK phosphorylation was highest on the stiff substrate, and, unlike ERK and Akt, did not change over time (Fig. 5C). Sorafenib treatment also reduced the stiffness sensitivity of p38 phosphorylation (Fig. 5D).

When considering the results in Figures 4 and 5 together, ERK, p38, and JNK were all potential candidates for involvement in stiffness-mediated sorafenib resistance. We co-administered sorafenib with inhibitors to each of these molecules (Fig. 5E), and found that JNK inhibition (blue line) both significantly increased sorafenib efficacy, and eliminated the impact of substrate stiffness. In hindsight, this result could have been anticipated, as JNK was the only signaling molecule both enhanced by substrate stiffness during cell adhesion (Fig. 4C), and unaffected by sorafenib treatment (Fig. 5C). With an expected synergistic effect in mind, we then co-administered sorafenib with JNK inhibitor, and either ERK or p38 inhibitors (Fig. 5F). Strikingly, we found that combining either p38 or ERK inhibitors alongside the JNK inhibitor and sorafenib treatment reversed the effect of co-administering the JNK inhibitor alone.

4. Discussion

Several groups have demonstrated a link between substrate stiffness and cell proliferation across a variety of cell types [30, 40-43], and many of these studies have linked stiffness sensing, the actin and microtubule cytoskeleton, and the classic Rho-ROCK pathway. This foundation of work has propelled us and others to determine if this pathway, which is known to control cell growth and survival, might also be responsible for drug resistance in stiff tumor environments. For instance, Zustiak et al found that paclitaxel, a cytotoxic microtubule stabilizing agent, eliminated stiffness-induced drug resistance in most tested cell lines [30]. However, chemotherapies that induce apoptosis via non-cytoskeletal pathways, are also affected by substrate stiffness. Schrader et al. showed that stiff substrates reduced HCC cell apoptosis when treated with cisplatin, which causes apoptosis by crosslinking cellular DNA [29]. These studies motivated us to look at how stiffness might perturb the efficacy of a common chemotherapeutic within another class of drugs, specifically, sorafenib, a Raf kinase inhibitor that targets the Raf/MEK/ERK pathway. We also observed a clear stiffness-induced resistance to sorafenib across multiple cell lines. Consistent with Schrader et al., the HCC cell line (HEP3B) was the most stiffness-sensitive cell line tested, and showed a positive correlation between cell proliferation (at 24 hours) and drug resistance

(Suppl. Fig. 3A). However, cell proliferation did not correlate with drug resistance in any of the other cell lines tested, which were all from breast carcinoma; thus the simplest explanation for our results in Figure 2, that cell proliferation on high stiffnesses was responsible for sorafenib resistance, does not hold. The IC-50s for most cell lines are higher on collagen-rich proteins than on basement membrane proteins, with the exception of the SkBr3 cell line, which has similar IC-50s on both protein mixtures (Figs. 2A-B). Yang et al. also found that binding of SkBr3 cells to laminin, which is a component in our basement membrane mixture, causes substantial resistance to anti-ErbB2 agents [11], possibly agreeing with our results in the SkBr3 cells. These results point to maximum possible sorafenib resistance in stiff, collagen-rich microenvironments, which represent highly progressed tumors. These results implicate tumor stiffening as a cause for the lack of success for sorafenib, which boasts a paltry 3-month survival increase in comparison to placebo in HCC [33].

Integrin binding can mediate cellular responses to substrate stiffness via RhoA activity, leading to stress fiber formation, focal adhesion assembly, actomyosin contractility, and cell spreading [37, 44, 45]. Although we did observe cell spread area changes in response to both stiffness and ECM protein (Figs. 3A-D), we quantified no change in stiffness-induced resistance trend when co-administered sorafenib with ROCK inhibitor. Instead, we examined whether integrin-binding mediated this stiffness-induced drug response via some other pathway. β_1 integrin has a high affinity for collagen [46], and increased signaling through β_1 integrin binding protects cancer cells (MDA-MB-231 and MDA-MB-435) against paclitaxel [8] and small cell lung cancer cells against doxorubicin, cyclophosphamide, and etoposide [47]. Reducing β_1 integrin binding with an antibody sensitized the 231 cells to sorafenib, but did not affect the SkBr3s (Figs. 3E-F). Park et al. also observed that SkBr3 cells were not responsive to β_1 integrin inhibition as compared to MDA-MB-231, likely because of their inherently low β_1 integrin expression [9].

Ezzoukhry et al. observed that inhibiting EGFR sensitized several HCC cell lines to sorafenib treatment [38]; however, we found that co-treatment of a pharmacological EGFR inhibitor with sorafenib in both MDA-MB-231 and SkBr3 cells only affected sorafenib efficacy on soft gels (Figs. 3E-F). Given that integrin binding to the ECM can enhance EGFR phosphorylation in the absence of ligand binding [48], it

is possible that, at lower stiffnesses, β_1 integrin predominantly mediates signaling through EGFR phosphorylation in the absence of ligand binding [49], but not at higher stiffness.

We found that JNK was the key mediator of sorafenib resistance on stiff substrates (Figs. 4-5). Activation of JNK has been reported to mediate either pro-apoptotic or anti-apoptotic signaling pathways depending on stimuli [50, 51], with parallel contradictory roles *in vivo*, either supporting tumor growth [52-55] or suppressing tumorigenesis [56-59]. Our results indicate a role for JNK in enhancing cell survival during sorafenib treatment, and for the first time we show that JNK activation is regulated by substrate stiffness. The high activity of JNK on stiff substrates implicates high Rac1 activity and low RhoA activity [49, 60], and low RhoA activity is consistent with the overall lack of stress fiber formation observed in 231 cells [61], regardless of stiffness (Fig. 3A). Further, ROCK inhibition did not affect the stiffness-induced drug resistance. Finally, RhoA/ROCK can activate ERK [32], and indirectly activates PI3K-Akt pathway [62], supporting our observations of a lack of stiffness-dependent ERK or Akt phosphorylation (Figs. 4A-B).

Conversely, when we quantified phospho-protein activity in the presence of sorafenib and PDGF and EGF, we observed that ERK phosphorylation was higher on the stiff substrate. This is consistent with other observations that cells on stiff substrates are more sensitive to EGF stimulation in comparison to those on soft substrates (Figs. 5A-B) [19, 23]. However, this transient ERK activation on stiff substrates did not prolong cell survival in the presence of sorafenib treatment (Fig. 5E). Combining both p38 and JNK inhibitors alongside sorafenib reversed the effect of inhibiting JNK alone, suggesting negative crosstalk between JNK and p38. Other studies have reported this antagonism between p38 and JNK before [63, 64]. We observed this same rescue of stiffness-mediated sorafenib resistance when we co-administered JNK and ERK inhibitors, which is also supported by reports of JNK and ERK antagonism [65, 66].

Overall, these results elucidate a role for JNK in mediating resistance to sorafenib through β_1 integrin binding to collagen-rich environments (Fig. 6). β_1 integrin activation leads to Src-mediated phosphorylation of EGFR [49, 67], which activates Ras/Raf/MEK/ERK and PI3K/Vav2/Rac1/JNK. Inhibiting EGFR improved the efficacy of sorafenib on soft gels (Fig. 3E), which we attribute to low β_1

integrin affinity [49]. At high stiffness, however, EGFR inhibition had no effect, as integrin clustering increases, recruiting the FAK-Cas complex and activating Ras/Rac1/JNK [68-70].

In sum, the ability to capture the cell-matrix interactions present in the *in vivo* tumor microenvironment could profoundly influence our ability to understand true drug efficacy *in vitro.* Others have created similar high-throughput biomaterial platforms including ECM microarrays [71], contact-printed microarrays [72], PEG microwells [73], and 2D biomaterials in 96-well plates [30, 41]. The most promising of these approaches have each used polyacryamide (PAA) gels, but require either a complicated plate insert [41], or manually placing gels into individual wells [30]. Our method of casting PEG-PC gels allowed us to quickly make multiple uniform gels of varying stiffnesses in multiple 96-well plates at the same time, and does not require fabrication of any special devices (Fig. 1). Our high-throughput PEG-PC platform allowed us to identify β_1 integrin, and its downstream effector, JNK, as mediators of tissue stiffening-induced drug resistance. Co-administering sorafenib with inhibitors to either of these targets equally eliminated stiffness-induced resistance in the 231 cells (Suppl. Fig. 4).

5. Conclusion

We propose that systems like our high-throughput PEG-PC hydrogel platform are critical for future screening of potential chemotherapeutics, as well as for discovery of possible mechanisms for failed efficacy of previously promising targets. With our platform, we discovered that the efficacy of sorafenib in carcinoma could potentially be increased by co-administering inhibitors to β_1 integrin or JNK, which could not have been appreciated on traditional tissue culture plastic plates. Our results highlight the importance of incorporating relevant tissue stiffness and integrin binding ligands into the high-throughput drug screening process to increase the success of drugs in the development pipeline.

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