The Role of Desmoplasia and Stromal Fibroblasts on Anti-cancer Drug Resistance in a Microengineered Tumor Model

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Abstract

Introduction—Cancer associated fibroblasts (CAFs) are known to participate in anti-cancer drug resistance by upregulating desmoplasia and pro-survival mechanisms within the tumor microenvironment. In this regard, anti-fibrotic drugs (i.e., tranilast) have been repurposed to diminish the elastic modulus of the stromal matrix and reduce tumor growth in presence of chemotherapeutics (i.e., doxorubicin). However, the quantitative assessment on

impact of these stromal targeting drugs on matrix stiffness and tumor progression is still missing in the sole presence of CAFs.

Methods—We developed a high-density 3D microengineered tumor model comprised of MDA-MB-231 (highly invasive breast cancer cells) embedded microwells, surrounded by CAFs encapsulated within collagen I hydrogel. To study the influence of tranilast and doxorubicin on fibrosis, we probed the matrix using atomic force microscopy (AFM) and assessed matrix protein deposition. We further studied the combinatorial influence of the drugs on cancer cell proliferation and invasion.

Results—Our results demonstrated that the combinatorial action of tranilast and doxorubicin significantly diminished the stiffness of the stromal matrix compared to the control. The two drugs in synergy disrupted fibronectin assembly and reduced collagen fiber density. Furthermore, the combination of these drugs, condensed tumor growth and invasion.

Conclusion—In this work, we utilized a 3D microengineered model to tease apart the role of tranilast and doxorubicin in the sole presence of CAFs on desmoplasia, tumor growth and invasion. Our study lay down a ground work on better understanding of the role of biomechanical properties of the

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matrix on anti-cancer drug efficacy in the presence of single class of stromal cells.

Keywords—Tumor microenvironment, Tranilast, Doxorubicin, Matrix stiffness, Cancer invasion.

ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AFM	Atomic force microscopy
APTES	2-Aminopropyl-3 triethoxy silane
CAFs	Cancer associated fibroblasts
DAPI	4',6-Diamidino-2-phenylindole, dihydrochloride
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDU	5-Ethynyl-2'-deoxyuridine
FAK	Focal adhesion kinase signaling
GA	Glutaraldehyde
PDL	Poly D-lysine
PDMS	Poly dimethoxy siloxane

INTRODUCTION

Breast cancer is known as the second most leading cause of death amongst women across the globe.⁵⁰ While the early stage of the disease can be treated with high success rate, the invasive and metastatic phase of the disease still suffer from poor therapeutic outcomes.⁵⁰ It is now widely accepted that the tumor microenvironment plays a crucial role in the disease progression as well as in inherent resistance to anti-cancer therapeutics during the metastatic cascade.^{40,63} Due to the complexity of the tumor microenvironment, chemotherapeutic drugs do not primarily perfuse through the parenchyma of the tissue in lethal amounts, primarily because of the high interstitial fluid stress, gradients of growth factors and hypoxia.^{28,63} Additionally, the signaling crosstalk between the stromal and cancer cells induce resistance by upregulating pro-survival mechanisms such as reduced cell death, enhanced proliferation and invasion, *etc.*^{10,13,49} In this regard, specific therapeutics are being developed to target the interactions between tumor cells and the surrounding stroma.⁶³ The stromal targeting drugs along with classical chemotherapeutics are now being considered as enhanced combinatorial treatment strategies compared to monotherapy regimes.⁶³

Amongst various stromal cells found within the breast tumor microenvironment, cancer associated fibroblasts (CAFs) are dominant in number.¹⁹ Previous

studies have demonstrated that CAFs play a significant role in inducing the microenvironment conducive for the progression of the disease.^{8,19,63,64} For instance, CAFs deposit abundant and aligned ECM proteins such as collagen, fibronectin, lysyl oxidase within the local tumor microenvironment as compared to their normal counterparts (i.e., mammary fibroblasts).^{8,64} High expression of ECM proteins leads to the formation of a desmoplastic stroma with elevated biophysical properties (i.e., stiffness), which subsequently promote tumor cell invasion, proliferation, and also reduces the functional efficacy of drugs due to upregulation of integrin and focal adhesion kinase (FAK).^{11,12,15,18,28,53}

Due to the significant role of ECM proteins and matrix properties (i.e., stiffness) in inherent drug resistance, many studies in the past have utilized anti-fibrotic drugs that directly target the desmoplastic stroma.^{6,35,48,58,60,68} Most of the previous *in vitro* studies in this regard have utilized two-dimensional (2D) monolayer of cancer cells, either alone or in co-culture with stromal cells, to study the influence of these drugs on tumor growth and invasion.^{4,6,17,24,33,44,46,56} These studies have provided valuable insight on cytotoxicity level of drugs and the biochemical pathways being influenced during the therapy.^{4,6,31,55} However, due to 2D nature of these platforms, the dynamic alterations in the biophysical properties of the matrix (i.e., stiffness) in the presence of anti-fibrotic drugs cannot be retrieved.³⁸ Additionally, the lack of a third dimension in 2D models does not enable recapitulation of the native characteristics of the tumor microenvironment, ultimately leading to notable differences in pharmacodynamic outcomes.³⁶ *In vivo* animal models, on the other hand, provide crucial insights on the role of the drugs in alleviation of stress, interstitial fluid pressure as well as deposition of stromal matrix proteins.^{35,42,45,60,68} However, due to the physiological differences between animal models and humans, clinical translation of the targeted drug has been limited.^{23,38} Additionally, the inherent complexities of *in vivo* models, does not enable quantitative assessment of the alterations of ECM matrix during tumor progression in the presence of a single class of stromal cells (i.e., CAFs).^{20,37,41} In this regard, microengineered 3D tumor models, integrated with novel biomaterials, provide enormous potential to mimic the complexities of tumor microenvironment with precise control on various factors including the spatial organization of cancer and stromal cells, matrix composition and so forth.^{25,38,65} Microengineered tumor models also enable better visualization of the dynamic changes within cell cytoskeleton and stromal matrix for enabling specific mechanistic studies.^{30,38}

In this study we developed a 3D microengineered platform, incorporating high density of tumor cell-embedded microwells, surrounded by stromal cells

such as CAFs. Due to the open top nature of the platform, we probed the matrix with atomic force microscopy (AFM) to assess the alterations of the ECM stiffness over the experimental period. Further, we studied the impact of combinatorial action of anti-fibrotic drug tranilast and doxorubicin on ECM remodeling, tumor growth and cancer cell invasion in the sole presence of CAFs. We focused our study on breast cancer in this work, however, due to highly versatile nature of the proposed platform, the model can be adapted to various other types of desmoplastic cancer.

MATERIALS AND METHODS

Materials

Poly dimethyl siloxane (PDMS Sylgard 184 Silicon Elastomer Kit, Dow Corning) was utilized to fabricate PDMS holders and stamps to engineer the 3D micropatterned breast tumor model. 2-aminopropyltriethoxy-silane (APTES), poly-D-lysine (PDL) and glutaraldehyde were utilized to surface treat the substrates (PDMS holders, glass bottom confocal dishes). PDMS stamps were treated with Pluronic F-127 to render them protein resistant. Tranilast was bought from TCI America and stock was prepared at the concentration of 100 mM in DMSO. Doxorubicin (alfa aesar) was diluted in DI water with stock solution of 1 mM.

Cell Culture

In our study we utilized three different breast cancer cell lines namely MDA-MB-231, MCF7 and MCF10A. MDA-MB-231 and MCF10A cells were transduced to express tdTomato fluorescence, provided by McCarty lab (Oregon Health & Science University). MCF7 cells on the other hand were obtained from Mouneimne lab at University of Arizona Cancer Center and expressed mCherry fluorescence. CAFs, which were isolated from human mammary gland tissue peripheral to invasive ductal carcinoma, were purchased as an immortalized cell line from ATCC (HTB-125). MDA-MB-231, MCF7 and CAFs were cultured in DMEM 1X media supplemented with 10% FBS, 1% PenStrep and 1% L-glutamine. MCF10A cells were maintained in a DMEM: F12 supplemented with 1% L-glutamine, epidermal growth factor, cholera toxin, hydrocortisone, insulin and 5% horse serum. For all experiments, the cell lines were cultured within T-75 flasks and maintained at 37 °C and 5% CO₂ with subsequent change of media every 2 days. The passage number used for various

experiments for different cell lines are as listed below: MDA-MB-231 (17-22), MCF7 (5-9), MCF10A (10-13), CAFs (54-62).

IC 50 Assay

Alamar blue assay (Thermo Fisher) was utilized to characterize the IC 50 values of different cells within 96 well plates after exposure to individual treatments for 48 h. To calculate IC 50 on 2D surface, the cells were trypsinized and plated within well plates at the cell density of 3×10^4 and 5×10^4 for MDA-MB-231 and CAFs respectively and were allowed to adhere to the well plate overnight. For studying IC 50 values of drug in 3D hydrogel, the cells at above mentioned cell densities were encapsulated within 4 mg/mL of collagen I and 30 μ L of the cell embedded gel was pipetted in 96 well plate. Tranilast and doxorubicin at various doses were prepared from stock in cell culture media and added to cells for 48 h. The media was removed, and the cells were washed with 1X PBS three times. Alamar blue was prepared at the dilution of 1:10 in cell culture media and added to cells for 3 h at 37 °C. The plates were read using a plate reader as per manufacturer's instruction.

Development of the 3D Tumor Model

The 3D tumor model was fabricated using micro-molding techniques.²⁷ Specifically to develop the model, we primarily fabricated PDMS stamps and holders using soft lithography techniques. While the holders were utilized as a substrate to immobilize collagen I hydrogel, stamps were engineered to micro-mold the gel and create a high-density array of microwells. Both PDMS platforms were prepared by mixing SYLGARD Silicone Elastomer Base and the SYLGARD Silicone Elastomer curing agent in the ratio of 10:1. The mixture was then vacuumed to remove air bubbles. The mixture was poured on a silanized silicon wafer and degassed for 30 min after which it was incubated overnight at 80 °C. PDMS holders were first treated with air-based plasma for 4 min and then immediately immersed in 2% of 2-(aminopropyl) triethoxysilane (APTES) prepared in 95% ethanol. The holders were incubated in APTES for 1 h at 60 °C. To remove unbound APTES, the holders were transferred to 100% ethanol solution, and sonicated using a water-based ultrasonic bath for 20 min. The holders were washed with 100% ethanol in five 10 min intervals followed by 1 h incubation at 80 °C. To allow covalent immobilization of collagen I, the PDMS holders were then incubated in 2% glutaraldehyde (GA) at room temperature for 1 h. GA was removed

FIGURE 1. (a) Schematic of the fabrication steps of the 3D microengineered high-density tumor model. (b) 3D view of the micropatterned breast tumor model. (c) Representative illustration of the proposed hypothesis of the study demonstrating the role of desmoplasia on tumor survival, growth and cancer cell invasion. Addition of antifibrotic drug tranilast along with doxorubicin downregulate ECM remodeling thereby reducing overall tumor progression.

by five consecutive 5-min washes with DI water and then incubated overnight at 80 °C. PDMS stamps were made protein-repellant by incubating within 1% Pluronic F-127 solution.

To fabricate the tumor model, rat tail collagen I (corning) was used at the concentration of 4 mg/mL. CAFs were mixed with collagen I at the optimized cell density of 2×10^6 cells/mL (Fig. 1a). PDMS stamps were removed from pluronic solution and washed three times with DI water. The collagen solution was then added to each stamp immediately and further inverted on top of the surface treated PDMS holders (Fig. 1a). The whole assembly was then kept for polymerization for 30 min at 37 °C (Fig. 1a). After polymerization of the gel, the stamp was lifted off gently and the microwells were seeded with cancer cells at a density of 7×10^6 cells/mL for 2–3 min (Fig. 1a). The cells from

unpatterned surface were removed by washing with media. The prepared samples were kept inside the incubator for 15 min to allow attachment of cells to collagen wells. After 15 min, the samples were immersed within 500 μ L of media in 24 well plate overnight. On day 1, freshly prepared media with drugs were added to the samples for total period of 48 h. The experimental groups included control (i.e., MDA + CAF without drug), DMSO (0.62%), tranilast (620 μ M), doxorubicin (280 nM) and tranilast + doxorubicin.

Cell Proliferation Assay

Tumor cell proliferation was quantified by using Click iT-Alexa Fluor-488-Edu Imaging Kit (Thermo Fisher) as per manufacturer's instruction. To quantify cancer cell proliferation, the DsRed positive cells

(MDA-MB-231) with EdU positive and DAPI stained nuclei were counted in Image J using cell counter plugin. To further calculate the percentage of proliferative cancer cells, the EdU positive cancer cells was divided by total number of DsRed positive tumor cells. Additionally, we also performed the assay on non-invasive MCF7 and normal mammary epithelial MCF10A cells for control and tranilast + doxorubicin experimental group across two experiments.

Cancer Cell Invasion Assay

To visualize invasion of tumor cells across all groups, prepared samples were imaged using fluorescence and phase contrast microscopy (ZEISS) on day 1, before addition of drug as well as on day 3 of the culture. Tile images were acquired from two random locations of the sample. Using ImageJ, we thresholded each image and utilized particle analyzer plugin to extract co-ordinates of each cell within an image. Next, we used a custom-written MATLAB code to perform delaunay triangulation modelling similar to previous reports.²⁶ The area of each triangle within delaunay plot was calculated using MATLAB. We calculated average area of all triangles and quantified area disorder for each delaunay plot using the following equation:

$$\text{Area disorder} = 1 - \left[1 + \left(\frac{\text{Standard deviation}}{\text{Average area}} \right) \right]^{-1}.$$

The invasion index was calculated based on the following equation:

$$\begin{aligned} \text{Invasion Index} \\ = \frac{(\text{Area disorder on Day 1} - \text{Area disorder on Day 3})}{\text{Area disorder on Day 1}}. \end{aligned}$$

Immunostaining

To visualize the deposition of collagen I and fibronectin within the matrix of the micropatterned model, primary monoclonal mouse antibodies against collagen I and fibronectin were used (Santa Cruz Biotechnology) at dilution of 1:200 and 1:100 respectively. To fluorescently stain the fibers, Alexa Fluor 488 secondary antibodies (Life Technologies) were used at the dilution of 1:400 and 1:200 respectively. The fluorescently labelled fibers were visualized using confocal reflectance microscopy (Leica Microsystems, SP8) available at KECK bioimaging center at ASU.

Atomic Force Microscope (AFM) Based Mechanical Characterization

The measurement for elastic modulus (i.e., stiffness) of the matrix were made on day 1 before addition of drug followed by day 3 of the culture after 48 h of the treatment. The matrix stiffness was measured by indenting MDA-MB-231 free areas within the platform and convoluting elastic moduli of ECM and CAFs since fibroblasts have been shown to match the stiffness of the substrate.⁵² Force-indentation curves were recorded with a commercial atomic force microscope (MFP-3D-BIO AFM, Asylum Research, Santa Barbara CA, USA) using sphere-conical probes ($k_{\text{nominal}} = 0.2 \text{ N/m}$, LRCH, Team Nanotec, Germany) with a half cone angle of 18.8° and sphere radius of 850 nm. Force-indentation curves were collected in 4×4 grids as force maps in an area of $90 \mu\text{m} \times 90 \mu\text{m}$ located in the center between four wells at 37°C with indentation speed of $2 \mu\text{m s}^{-1}$. The trigger force was selected to be 60–80 nN resulting in indentation depths of at least $10 \mu\text{m}$. The spring constant of each cantilever was determined before the experiment by thermal noise method.^{3,16} Three force maps per sample per day were collected. The first $10 \mu\text{m}$ indentation of each force distance curve was fitted to a non-adhesive elastic contact model for a conical indenter with a spherical tip.⁵⁴ Data analysis was done using MATLAB. The poison ratio of collagen was assumed to be $\nu_{\text{collagen}} = 0.5$.

Quantification of Expression of Proteases within 3D Microengineered Model

The quantification for expression of various proteases and their inhibitors was done by utilizing a commercially available RayBiotech human MMP1 antibody array (QAH-MMP-1-1) for measuring the values of MMP1, 2, 3, 8, 9, 10, 13 and TIMP-1, 2 and 4. To perform the assay, the samples were prepared and cultured in 10% serum containing media for 24 h and then washed three times with 1X DPBS to remove serum. The samples were then incubated in various drug conditions prepared in serum free media. The conditioned media was collected after 48 h and centrifuged at 14,000 rpm for 10 min to remove cell debris. The supernatant was collected and stored at -80°C until further use. To perform the antibody array all the samples were run as per manufacturer instructions. To get a comprehensive analysis, all conditions were repeated in triplicate technical and biological replicate.

Statistical Analysis

Unless otherwise stated, all the assays were repeated three times with three technical replicates per condition. The IC 50 results were analyzed using sigmoidal curves in GraphPad. The elastic moduli data were analyzed using two-way ANOVA with Sidak's multiple comparison test. All other data were analyzed using repeated one-way ANOVA with Tukey multiple comparison test. Due to unavailability of samples for some groups in few experiments, the statistical analysis was reported for EdU and Tunnel assay using ordinary one-way ANOVA. *p* value less than 0.05 was considered significant for all the results. The statistical analyses and data representation was performed using GraphPad Prism v 7.0. All the data was presented as mean $\pm$ standard deviation.

RESULTS

Microengineering of the 3D Breast Tumor Model

The 3D breast tumor model was fabricated by utilizing micromolding technique to create a high-density array of microwells within collagen I hydrogel as shown in Fig. 1a. The stromal region was fabricated by encapsulating CAFs within collagen I hydrogel while the tumor region was engineered by seeding tumor cells (MDA-MB-231) within the microwells (Fig. 1a). As shown in Fig. 1b, the tumor and stromal regions were accurately organized to mimic the native spatial organization of tumor microenvironment and thus study the invasive behavior and proliferation of tumor cells as well as change in biophysical properties of the ECM. The experimental groups were designed to study the influence of tranilast and doxorubicin on alterations of matrix properties and tumor progression either individually or in combination. The control condition included the co-culture of MDA-MB-231-cells and CAFs without the addition of any drug. The time period for the current study was kept constant for 3 days since CAFs exerted high traction force on collagen I which caused folding of hydrogel and disruption of the model for extended period of culture.

We hypothesized that within the control condition (i.e., no drug), the presence of tumor and stromal cells lead to remodeling of the matrix and elevated stiffness by deposition of proteins such as collagen I and fibronectin (Fig. 1c). The increase in the elastic modulus of the matrix eventually lead to enhanced tumor growth, invasive activity of MDA-MB-231 cells as well as resistance to cell death (Fig. 1c). On the other hand, addition of tranilast will reduce the fibrosis of the matrix due to its known inhibition of collagen synthesis and ECM turnover, leading to impaired

desmoplasia (Fig. 1c).³⁵ Reduced fibrosis of the matrix will downregulate the biomechanical signaling of the ECM, thereby enhancing the efficacy of doxorubicin and condensing tumor growth and invasion (Fig. 1c).

Characterization of IC 50 Concentrations

Prior to proceeding with the experiments using microengineered model, we characterized the IC 50 values for each of the drugs (tranilast, doxorubicin) on both cell lines (MDA-MB-231, CAFs) using 2D cell culture and alamar blue assay. Based on the metabolic activity of cells across various concentration of drugs, relative IC-50 values were calculated using standard mathematical models. Our results demonstrated lower IC 50 value of tranilast for CAFs (620 μ M) as compared to MDA-MB-231 cells (987 μ M, Fig. 2a). Similar results have been also observed by previous studies, on pancreatic tumor and stellate cells seeded on 2D surfaces in presence of anti-fibrotic drug, pirfenidone, depicting enhanced influence of the drug on fibroblasts as compared to the tumor cells.²¹ On the other hand, doxorubicin had lower IC 50 value for MDA-MB-231 cells (280 nM) as compared to CAFs (370 nM) as shown in Fig. 2b. Previous studies in the literature have reported the IC 50 of doxorubicin for MDA-MB-231 cells in the range of 0.5 nM to 5 μ M.^{1,22,39,43,51,61,67} Consistently, our IC 50 value was within this previously reported range. Such a wide range of IC 50 for MDA-MB-231 cells can be due to multiple factors including passage number, culture conditions as well heterogeneity of the cell population. We also performed similar IC 50 assay within 3D collagen I hydrogel. As shown in Supplementary Figs. S1A and S1B, both MDA-MB-231 and CAFs demonstrated IC 50 values for tranilast and doxorubicin higher than those observed in 2D assay. In order to experimentally achieve 50% inhibition in cell metabolic activity, we therefore expanded the range of concentration of doxorubicin to as high as 10 μ M. Our results demonstrated IC 50 values for MDA-MB-231 and CAFs for Doxorubicin to be 2073 nM and 2108 nM respectively within 3D collagen I hydrogel (Supplementary Fig. S1C). Higher IC 50 values for the drug within 3D hydrogel assay can be possibly attributed to reduced diffusion of the drug, difference in cell phenotype and genetic make-up within 3D systems as compared to 2D systems.^{28,36,38} Similar analysis for tranilast could not be achieved as the drug remained insoluble at higher concentrations in cell culture media. Such insolubility of the tranilast can be explained due to its super hydrophobic nature; as also demonstrated by previous studies.³⁴ Since our main goal was to study the effect of combination of two drugs on desmoplasia, tumor growth and invasion, we proceeded with IC 50 values

FIGURE 2. (a) Metabolic activity of MDA-MB-231 and CAFs in response to different doses of Tranilast in a 96 well plate. (b) Doxorubicin induced metabolic activity of MDA-MB-231 cells and CAFs with different concentrations.

FIGURE 3. (a) Representative immunofluorescent images of collagen I within 3D matrix across all the groups. Inset represent a magnified view of fiber density across all the conditions. (b) Elastic moduli of matrices across all the groups on day 1 before addition of drug and day 3 after addition of drugs. (c) Table of average elastic modulus values across various drug conditions on day 1 and day 3 of culture. All values are written as mean $\pm$ standard deviation. *Represent the microwells molded in collagen. Scale bars represent 20 μm . (*Represents p value < 0.05).

obtained within our 2D assay. Therefore, based on our preliminary studies, we fixed the concentration of tranilast to be 620 μM and doxorubicin to be 280 nM for our future experiments. We also added blank DMSO vehicle (0.62%) as another control group.

Characterization of Desmoplasia

A major advantage of our 3D platform is the ability to measure the changes in biomechanical properties of the matrix during active invasion of cancer cells in the presence of the drugs as compared to traditional 2D

in vitro as well as *in vivo* models. To visualize the changes in matrix protein deposition, we immunostained the samples for collagen I and fibronectin on day 3 of the culture. Additionally, we measured the changes in stiffness of the matrix using AFM on day 1 before addition of drugs and on day 3 after 48 h of drug treatment. As shown in Fig. 3a, the stromal matrix of the control group demonstrated a high density of collagen I fibers as compared to the samples exposed to combinatorial drugs. Similar observation was made with respect to fibronectin assembly within stromal matrix (Fig. S1, Arrows). Additionally, both collagen

FIGURE 4. (a) Representative histograms showing the distribution of elastic moduli of the stromal matrix across various groups. (b) Representative color maps curves of stiffness across all the experimental conditions.

and fibronectin fibers were more punctuated in the presence of both drugs (Figs. 3a and S1). Such differences were also reflected in matrix stiffness across all culture groups (Figs. 3b and 3c, S2). Specifically, our results showed that, while both control and DMSO treated groups depicted significant increase in elastic modulus on day 3 ($E_{\text{control, day 3}} = 4.25 \pm 1.26$ kPa and $E_{\text{DMSO, day 3}} = 3.53 \pm 1.07$ kPa) as compared to day 1 ($E_{\text{control, day 1}} = 1.67 \pm 0.49$ kPa, $E_{\text{DMSO, day 1}} = 1.76 \pm 0.46$ kPa), tranilast, doxorubicin and tranilast + doxorubicin treated groups did not exhibit any significant change in the matrix stiffness (Figs. 3b and 3c, S2). Additionally, the elastic modulus for the tranilast + doxorubicin group on day 3 of culture was significantly lower than the control and DMSO group. ($E_{\text{control, day 3}} = 4.25 \pm 1.26$ kPa, $E_{\text{DMSO, day 3}} = 3.53 \pm$

1.07 kPa, $E_{\text{Tranilast+Doxorubicin, day 3}} = 2.16 \pm 1.03$ kPa; Fig. 3c). These findings suggest enhanced anti-fibrotic activity when the two drugs were added together to the model. Figures 4a and 4b show histograms and 2D color-maps of the indented matrix on days 1 and 3. Stiffness histograms on day 1 demonstrated similar unimodal distributions, consistent to the data shown by Plodinec *et al.* for normal tissues (healthy human and MMTV-PyMT mice biopsies).⁴¹ For the untreated control group of our 3D tumor model, we found multi-peaks in the stiffness histogram on day 3. Bimodal stiffness with broad distribution as seen for control group has been associated with cancer biopsies indicating a biomechanical heterogeneity in diseased tissue.⁴¹ Narrower matrix stiffness distributions on day 3 for treated groups within our model suggested lower

level of interplay among CAFs and tumor cells. Therefore, the control group on day 3 demonstrated significant desmoplasia. Notably, the combinatorial addition of tranilast and doxorubicin drugs impaired these biophysical alterations.

Tumor Growth

Since the stiffness of the matrix was significantly modulated by the combinatorial action of two drugs, we next hypothesized that the proliferative behavior of tumor cells will also be altered upon exposure to varied drug treatments. To assess tumor growth, we utilized Alexa Fluor 488 EdU assay to fluorescently label replicating MDA-MB-231, MCF7 and MCF10A cells and further visualized them using fluorescent microscopy (co-expressing DsRed and GFP). As shown in Fig. 5a, MDA-MB-231 cells proliferated the most within the control group. While monotherapy with tranilast and doxorubicin reduced the tumor growth however, the reduction was not statistically significant (Figs. 5a and 5b). Further, quantification for EdU positive cells demonstrated a significant decrease in proliferative behavior of tumor cells (MDA-MB-231) due to the combinatorial action of tranilast and doxorubicin (Fig. 5b). Similar trend was observed when non-invasive MCF7 cells and normal mammary epithelial cells (MCF10A) were cultured with CAFs in presence and absence of drugs. Specifically, in the control group both MCF7 and MCF10A cells demonstrated high replicative ability (~ 90%, Supple-

mentary Figs. S4A and S4b). However, when tranilast and doxorubicin were added together, the proliferation rate for both cell types reduced significantly (MCF7 = $35 \pm 5.4\%$, MCF10A = $30 \pm 18\%$, Supplementary Figs. S4A and S4b). Thus, overall our results demonstrate reduced proliferative ability of invasive (MDA-MB-231) and non-invasive (MCF7) tumor cells as well as normal mammary epithelial cells (MCF10A) when the two drugs act in synergy in presence of CAFs as compared to untreated condition.

Cancer Cell Invasion

Based on our hypothesis, we further speculated a limited ability of cancer cells to invade the surrounding stroma in combinatorial treatment of drugs. To visualize the dispersion of tumor cells within the 3D matrix, we utilized phase contrast and fluorescent imaging along with delaunay triangulation. As shown in Figs. 6a and S3A, tumor cells demonstrated enhanced invasive capacity across all the groups on day 3 as compared to day 1 of the culture. Similar analysis was drawn from triangulation graphs (Figs. 6b and S3B), where tumor cells appeared more scattered within the stroma on day 3 as compared to day 1 of the culture. Quantification of area disorder demonstrated invasion of tumor cells into the matrix for all groups (Fig. S3B). However, quantification of invasion index across all the conditions, indicated a significant decrease in invasion of tumor cells in combinatorial treatment of tranilast and doxorubicin as compared to control. These results are in

FIGURE 5. (a) Representative immunofluorescent images of EdU assay depicting tumor cell proliferation across all experimental groups. (b) Quantification of proliferation of MDA-MB-231 cells within all conditions. Scale bars represents 50 μm . (*Represents p value < 0.05).

FIGURE 6. (a) Representative phase contrast and fluorescent images of tumor cell dispersion in control and tranilast + doxorubicin group on day 1 (before addition of drug) and day 3 (after addition of drug). (b) Representative triangulation graphs depicting tumor cell invasion into the stroma. (c) Quantification of invasion index of MDA-MB-231 cells. Scale bars represent 100 μm . (*Represents p value < 0.05).

fact similar to previous *in vivo* studies where metastasis was observed to be minimalistic only when tumors were subjected to combinatorial therapy of anti-fibrotic drug (i.e., pirfenidone) and doxorubicin.⁶⁰

Assessment of Proteases and Their Tissue Inhibitors Expression

To complement our matrix remodeling and stiffness results, we performed a comprehensive analysis of expression of various matrix remodeling factors including matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). Our results demonstrated that MMP1, a known interstitial collagenase, had highest concentration within the control group (1197 ± 508.789 pg/mL) which was not

significantly different from DMSO (668.93 ± 282.191 pg/mL) and doxorubicin group (673.98 ± 177.03 pg/mL), thereby suggesting enhanced ECM remodeling within these groups. However, upon addition of tranilast, concentration of MMP1 significantly decreased both in tranilast (291.3 ± 284.142 pg/mL) and tranilast + doxorubicin groups (316.926 ± 276.208 pg/mL; Fig. 7a). Besides MMP1, we also observed significant difference in the expression of TIMP2 across different culture conditions. As shown in Fig. 7b, the expression of TIMP2 within the control group (5449.974 ± 504.055 pg/mL) was significantly higher than tranilast + doxorubicin group (1389.667 ± 455.242 pg/mL). Furthermore, the doxorubicin group (7625.03 ± 4234.258 pg/mL) had significantly higher expression of TIMP2 as compared to

FIGURE 7. (a) Quantification of various proteases of MMP array for all culture conditions. (b) Quantification of concentration of different TIMPs across all culture conditions. (*Represents p value < 0.05).

DMSO (2222.955 ± 526.215 pg/mL), tranilast (2173.451 ± 1127.598 pg/mL) and tranilast + doxorubicin group. Besides MMP1 and TIMP2, we did not observe any significant difference for other proteases across all culture conditions.

DISCUSSION

In this study we utilized a 3D microengineered breast tumor model to study the influence of anti-fibrotic drug tranilast in combination with doxorubicin on desmoplasia, tumor growth and cancer cell invasion. Many previous studies have suggested that cancer cells activate stromal cells such as fibroblasts surrounding them to alter the microenvironment and make it more conducive for tumor progression.^{19,64} Fibroblasts in particular are known to get activated in presence of tumor cells and adopt a myofibroblast like phenotype (CAFs) similar to a wound healing process.^{19,64} Such a change in phenotype of fibroblasts leads to remodeling of the matrix and hence induction of fibrosis within the surrounding microenvironment.^{19,64} Therefore, to initially construct an *in vivo* like tumor microenvironment, the proposed model was compartmentalized into tumor and stromal regions by seeding MDA-MB-231 cells within microwells that were surrounded by collagen-based stroma encapsulating CAFs. Such a spatial organization of tumor-stromal region was purposefully chosen to mimic the native breast tumor microenvironment. The choice of collagen I hydrogel, to establish the model was motivated by the abundance of this ECM protein within native tumor microenvironment.⁹ Our proposed platform enabled dissection of the role of CAFs as the most dominant cell within the mammary carcinoma, on desmoplasia and tumor progression.

To disrupt the desmoplastic response of CAFs within breast tumor microenvironment, we studied the action of tranilast; a clinically approved drug in Japan and South Korea for treatment of fibrotic diseases

such as keloids.⁷ While in previous 2D *in vitro* studies, this drug has been shown to be paramount in influencing tumor cell viability as well as growth,^{4,7,17,46,56} not much has been studied about its efficacy on the dynamic change in biophysical properties of the ECM (i.e., stiffness) during active invasion. Additionally, a quantitative study on the impact of tranilast on matrix stiffness and tumor progression has been missing in the sole presence of CAFs. Therefore, our study was motivated by these critical knowledge gaps in the literature.

A major finding of our study was the limited increase in elastic modulus of the matrix in combinatorial treatment of tranilast and doxorubicin as compared to control after 48 h of exposure (Fig. 3b). Immunostaining of collagen I and fibronectin, further confirmed reduced density and assembly of these matrix protein fibers within combinatorial drug treatment, which are known to be regulators of ECM stiffness.^{8,19,64} Such an observation can be attributed to various factors including reduced proliferation of CAFs due to the action of tranilast,⁵⁹ downregulation of biochemical pathways and limited viability of tumor and stromal cells in the presence of doxorubicin.³⁹ While the mode of action of tranilast is still not clear, many previous studies have shown that this specific anti-fibrotic drug downregulates the proliferation of fibroblasts of various lineage including dermal, corneal and CAFs³² consistent with our alamar blue assay results (Figs. 2a and 2b). Additionally, it has been shown by previous *in vivo* studies that tranilast can downregulate the expression of growth factors such as CTGF, TGF β which play a crucial role in ECM protein synthesis and deposition, therefore limiting the increase in the stiffness of the matrix.^{2,35,56} Furthermore, due to the known cytotoxic effect of doxorubicin on cancer cells, the impaired crosstalk between MDA-MB-231 and CAFs can influence the autocrine/paracrine signaling and hence lead to reduced fibrosis.^{19,40,65,66} Such specific action of these drugs also explains the insignificant change of stiffness

within monotherapy groups, while the synergistic influence of these drugs can significantly reduce desmoplasia (Fig. 3b).

To further develop a comprehensive understanding on ECM remodeling abilities of cells under various drug conditions, we also performed an antibody array analysis on various MMP and TIMP expression. Our results demonstrated reduced expression of proteases such as MMP1 within tranilast and tranilast + doxorubicin group suggesting limited remodeling ability of tumor and stromal cells due to the action of this drug. Many previous studies have demonstrated similar reduced expression of various other proteases including MMP2 and MMP9 under the influence of tranilast.^{5,14,57} For instance, Darakhshan *et al.* showed that when MCF7 and MDA-MB-231 cells were treated with tranilast, MMP9 mRNA expression was significantly reduced as compared to untreated cells.⁵ While most of these studies have been primarily 2D monoculture of tumor cells,^{5,14,57} similar assessment of MMP analysis for 3D co-culture of tumor and stromal cells has not been attempted in the past. Such discrepancy in cell culture conditions can be a possible explanation for differences in our findings from those of previous studies. Our results also demonstrated reduced expression of TIMP2 within tranilast + doxorubicin group as compared to control and doxorubicin. Since TIMP2 is known to play a significant role in inhibiting the action of various proteases,⁶² crosstalk between tumor cells and CAFs in control condition can possibly lower the expression of TIMPs to favor ECM remodeling; an observation made in Fig. 3 and Supplementary Fig. S2. However, in doxorubicin condition such a crosstalk between tumor cells and CAFs is altered due to limited number of tumor cells (i.e., less proliferative), thereby maintaining the expression and activity of TIMP2. On the other hand, in combinatorial group limited viability of tumor and stromal cells can minimize the overall expression of different proteins thus minimizing the concentration of TIMP2.

Another finding of our study was the reduced tumor growth and invasive behavior of MDA-MB-231 cells within the combinatorial treatment group as compared to untreated condition. Our results also demonstrated similar trend for non-invasive MCF7 and normal mammary MCF10A cells such that combinatorial action of tranilast and doxorubicin reduced their replicative ability significantly. We envision that these findings can be primarily correlated to our elastic modulus results where the combinatorial action of tranilast and doxorubicin treatment significantly reduced the stiffness as compared to control. Various studies have established that stiffness of the ECM can

provide biomechanical cues by controlling the activity of integrins as well as FAKs, which in turn can influence proliferation and migratory behavior of the cells.^{8,15,19,53} For instance, Schrader *et al.* utilized polyacrylamide gels (PAA) of variable stiffness and demonstrated tight regulation of proliferation of hepatocellular carcinoma cells by substrate stiffness due to upregulation of integrin $\beta 1$ and FAK signaling in stiff matrices.⁴⁷ Moreover, they demonstrated that the inhibition of integrin $\beta 1$ and FAK signaling, significantly reduced the proliferative ability of the cells.⁴⁷ Besides decrease in elastic modulus, the presence of doxorubicin in the combinatorial treatment can further target proliferating cells, leading to overall reduction of growth of cancer and normal mammary cells within our 3D microengineered platform. In another study by Rosa *et al.* MCF10A cells demonstrated enhanced migratory and wound closure activity upon seeding on PAA gels of high stiffness.²⁹ These findings were consistent even when tumor cell proliferation was inhibited with mitomycin-C.²⁹ Further inhibition of myosin contractility reduced the cell speed on stiff matrices, suggesting the role of integrin mediated myosin contractility on tumor cell invasion.²⁹

Several previous *in vivo* studies have demonstrated a significant decrease in tumor growth with monotherapy of doxorubicin as compared to combinatorial action with tranilast.^{35,42,60} We did not observe a significant reduction in tumor growth and stiffness in sole presence of doxorubicin or tranilast. Such differences in results can be attributed to various factors such as interstitial stress, leaky vasculature, and the presence of other cell types (i.e., immune cells) within *in vivo* models.^{35,42,60} In this regard, a previous study by Stylianopoulos *et al.* demonstrated that the administration of antifibrotic drugs such as tranilast and pirfenidone significantly reduced the interstitial stress that further enabled penetration of doxorubicin into tumor parenchyma, thereby influencing the tumor growth.^{35,42} Since our platform lacked tumor vasculature, doxorubicin did not encounter any limitation in terms of penetration within the matrix. It is also important to note that the aforementioned *in vivo* studies were performed for longer duration (i.e., 20–30 days) with continuous addition of tranilast as compared to our platform.³⁵ Nevertheless, our platform provides a unique ability to dissect the role of a single class of stromal cells on drug resistance. Additionally, we were able to visualize matrix remodeling as well as quantitatively assess the biomechanical changes during the course of experiment within our microengineered tumor model. In future, we aim to conduct further mechanistic and gene expression studies on cancer cells in the presence of different drug combination.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (<https://doi.org/10.1007/s12195-018-0544-9>) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

HS, KRE, CS, MA, GM, RR, MN declare no conflict of interest.

ETHICAL APPROVAL

No human and animal studies were carried out by the authors for this article.

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