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A feedback loop between hypoxia and matrix stress relaxation increases oxygen-axis migration and metastasis in sarcoma

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Abstract

Upregulation of collagen matrix cross-linking directly increases its ability to relieve stress under the constant strain imposed by solid tumor, a matrix property termed stress relaxation. However, it is unknown how rapid stress relaxation in response to increased strain impacts disease progression in a hypoxic environment. In this study, we demonstrated that hypoxia-induced expression of the cross-linker procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) in sarcomas resulted in increased lung metastasis. Short stress relaxation times led to increased cell migration along a hypoxic gradient in 3D collagen matrices, and rapid stress relaxation upregulated PLOD2 expression via TGF β -SMAD2- signaling, forming a feedback loop between hypoxia and the matrix. Inhibition of this pathway led to a decrease in migration along the hypoxic gradients. In vivo, sarcoma primed in a hypoxic matrix with short stress relaxation time enhanced collagen fiber size and tumor density and increased lung metastasis. High expression of PLOD2 correlated with decreased overall survival in sarcoma patients. Using a patient-derived sarcoma cell line, we developed a predictive platform for future personalized studies and therapeutics. Overall, these data show that the interplay between hypoxia and matrix stress relaxation amplifies PLOD2, which in turn accelerates sarcoma cell motility and metastasis.

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Keywords

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Introduction

Cell migration through the extracellular matrix (ECM) is required for cancer metastasis(1). With recent advances in analyzing and mimicking the tumor ECM, the role of ECM properties in cancer cell migration has been more thoroughly investigated, mainly focusing on the increased stiffness of the developing tumor (2). The natural ECM has a hierarchical structure that responds to different stress and strain according to the ECM's viscoelastic properties. Viscoelasticity describes how a material responds to deformation, and is composed of elastic and viscous characteristics. When elastic materials are deformed, a strain is generated simultaneously. Viscous materials, on the other hand, show a time dependency upon deformation. That is, the resulting strain is a function of time. Consequently, when a viscoelastic material is deformed, a time dependent strain will ensue. Similarly, when the mechanical load is removed the deformation will gradually return to its original form due to the entropy driven nature in the viscoelastic material, and the time that is needed to recover a portion of its original form is often called relaxation time(3,4). It is worth noting that stress relaxation is often related to the application of a constant strain. Relevant to the tumor microenvironment, stress relaxation has been shown to be correlative with solid stress (5,6), which is the mechanical stress that is contained and transmitted by the solid and elastic elements of the ECM and cells (i.e. tumor microenvironment)(7). The increase in solid stress causes short stress relaxation times in the tumor ECM. Additionally, the increase in solid stress occurs from an increase in tumor pressure (8), which was attributed to the collagen matrix in the tumor (6). The pressure increases causes a decrease in vascular permeability (5), exacerbating the hypoxic environment (9). Interestingly, different tumors have been shown to have unique stress relaxation profiles (10). Stress relaxation affects numerous cellular behaviors, including cell motility (11), mesenchymal stem cell differentiation (12), and the cell's ability to remodel the ECM (13). However, stress relaxation remains an understudied viscoelastic property of tumor ECM.

Polymer based hydrogels are currently utilized to recapitulate the three-dimensional (3D) cellular microenvironments (14). In cancer research, hydrogels are designed to mimic the chemical and physical properties of the tumor ECM. Several studies have investigated the effect of stress relaxation on cell behavior (4,15,16) and particularly in cancer (5,10). In sarcoma, collagen is one of the most abundant components of the ECM (17); and elevated expression of the collagen cross-linker, procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 (PLOD2), is associated with a more metastatic phenotype (18,19). In this study, we investigated the role of stress relaxation in sarcoma cell migration and metastasis using collagen matrix with varying stress relaxation.

Finally, intratumoral hypoxia occurs as the tumor outgrows its blood supply. Hypoxia is a key regulator of cancer development (20) and correlates with poor prognosis for sarcoma patients (21). Specifically, hypoxia has been shown to regulate PLOD2 expression in

sarcoma, leading to a metastatic phenotype (18,22,23). This hypoxic triggered PLOD2 has been shown to modify the ECM. Recently, we have shown that hypoxic gradients, which are present in sarcoma ECM, induce cell migration in the direction of higher oxygen, by modifying the ECM. (16,18,24). These modifications can impact mechanical properties of the matrix, one of which is stress relaxation.

To understand how stress relaxation modulates sarcoma progression, it is important to study it independently from other matrix properties (stiffness, porosity and fiber density) that have been shown to modify cell behavior. By isolating a single factor this platform can truly be used to explore a stress relaxation change in hypoxia. Here, we decouple stress relaxation from other matrix properties in collagen gels, in both hypoxic and non-hypoxic environments. Using murine and human undifferentiated pleomorphic sarcoma cells (UPS), we show that shorter stress relaxation time in hypoxic gradients leads to faster cell migration along an increasing gradient of oxygen tension causing an increase in PLOD2. The PLOD2 increase is a combinatorial effect of hypoxia and the quicker stress relaxation stimulating PLOD2 upregulation through the transforming growth factor beta (TGF- β)- SMAD2- signaling axis. This amplification feedback loop was not observed in non-hypoxic conditions. Upon implanting the quicker and slower stress relaxation hydrogels in a murine model, we show increased in collagen fiber size and density in the solid tumor, as well as in metastasis events.

Materials and Methods

Cell Culture and Primary Tumor Cell Extraction:

KIA was derived from murine model of sarcoma, LSL-Kras^{G12D/+}, Ink4a/Arf^{fl/fl} as established previously (18). KIA cells were authenticated as previously reported (18). KIA-GFP was expanded under standard culture conditions in high glucose DMEM with 10% FBS and 1% penicillin/streptomycin. hUPS cells were extracted from a primary human tumor sample treated at the Hospital of the University of Pennsylvania. A clinical pathologist, following obtaining written informed consent from the patients, authenticated the tumor. Studies were conducted in accordance with U.S. Common Rule, and were approved by IRB protocol #822382. hUPS cells were extracted by incubating the primary tumor with a 3 mg/mL collagenase solution in DMEM for 45 min at 37°C. The tumor was then spun down and the supernatant was aspirated and replaced with 0.05% trypsin EDTA solution and again incubated for 15 minutes at 37°C. The digestion is then stopped with complete cancer media. The tumors again were spun down, aspirated, and replaced with serum free media. The tumor is washed 2 more times and then triturated through a 40 μ m cell trainer into a 50 mL conical tube. The cells are then spun down and plated. Cells were selected by continuous culture for up to 8 passages prior to authenticate the line. All cell lines were used before passage 20 and were tested for mycoplasma every quarter.

Collagen Gel Preparation and Cell Encapsulation:

Collagen gel was prepared as previously reported (16). Briefly, to make 1 mL of 3 mg/mL collagen solution, 35.96 μ L of M199 10 $\times$ medium (Thermo Fisher Scientific) was mixed with 11.51 μ L of 1M NaOH until it turned dark pink. Next, we used acid soluble rat tail

collagen I at 9.61 mg/mL (Corning #354249). A total of 312 μ L of collagen was added to the dark pink solution and mixed thoroughly. A total of 640.35 μ L of M199 1 $\times$ medium (Thermo Fisher Scientific) was then mixed with the solution to create our final collagen gel solution. The solution was then incubated on ice for 2 hours. For cancer cell encapsulation, we first prepared cell pellets of hUPS-GFP and KIA-GFP cells (7.5×10^5 cells) in a 1.5 mL eppendorf tube. We then mixed the pellet with 0.5 mL of collagen solution by gentle pipetting to give a homogeneous cell suspension. After mixing, the solution was pipetted into a 96 well plate (BD Bioscience) and incubated for 30 min at 37 $^{\circ}$ C. Non-hypoxic gels were made with 45 μ L of collagen and hypoxic gels were made with 90 μ L of collagen.

To alter the stress relaxation, gels were incubated with PBS 24 mg/mL microbial transglutaminase (mTG) for 2 hours at room temperature. After 2 hours, the solutions were aspirated and the gels were cultured in normal culture media. 200 μ L of media was added to the hypoxic gels and 100 μ L was added to the non-hypoxic gels.

Control over Oxygen Levels:

The oxygen concentration is controlled as previously described(16). In short, to create a hypoxic hydrogel, the key parameters including diffusion coefficient of the collagen hydrogel material, boundary conditions, and cell oxygen consumption rate, were optimized. Collagen was chosen due to its relatively low oxygen diffusion coefficient (25). Additionally, polystyrene Petri-dish also has a very low diffusion coefficient (26). Thus, the polystyrene creates an impermeable barrier on the sides and bottom of the hydrogel placed within this common cell culture Petri-dish, limiting diffusion of oxygen and nutrients only in one direction- from the culture medium on the top of the hydrogel. Last, the oxygen consumption rate was modulated to balance the diffusion of oxygen, based on the height of the hydrogel and medium, with the cell consumption rate. This oxygen consumption rate is dependent on the concentration of the encapsulated cells in the hydrogel.

Invasive Oxygen Measurements:

Dissolved oxygen levels were monitored non-invasively and invasively as previously reported (16,24). A commercially available sensor patch (PreSens) and a Needle-Type Housing Fiber-Optic Oxygen Microsensor (PreSens) were used for the non-invasive and invasive experiments, respectively. For the non-invasive oxygen measurements, the sensor patches were placed at the bottom of a 96 well plate and the gel was formed on top of it. Invasive oxygen sensors were used to measure the oxygen concentration on day 3. The needle was placed into the gel until it hits the bottom and the oxygen was measured every 0.25 mm for the non-hypoxic gel and every 0.5 mm for the hypoxic gel.

Matrix Degradation:

Analyzing protease-mediated matrix degradation was performed as previously published(16) by incorporating 10 μ g/mL of DQ collagen (Invitrogen) into polymer solution prior to cell encapsulation. On day 3 DQ Collagen was analyzed by fluoresce microscopy (Axio Observer Z1 Zeiss) and measured using fluorescence spectrophotometry at a wavelength of 495 nm excitation and 515 nm emissions (Molecular Devices). High resolution fluorescence

and light microscopy images were taken and overlaid to determine the localized protease activity in the cells' surrounding.

Cell Tracking and Drug Treatment:

For cell tracking, a previously developed method (16,24,27) was used to assess sarcoma cell migration. KIA-GFP cells were encapsulated in hydrogels with different oxygen levels and mTG treatment. On day 3, cells were tracked with live-cell, 3D confocal microscopy (LSM 780; Carl Zeiss) equipped with an incubator (5% CO₂ and 37 °C). The time-lapse and z-stack images (>200 µm thickness) were collected every 30 minutes up to 24 hours at five randomly selected positions. Cells that did not start in frame were not included in the analysis in order to properly optimize the experiment. The images were analyzed with Imaris spot analysis software (Imaris 8.2; Bitplane). The 3D migration analysis was performed with a previously developed strategy (16,24,27). In brief, a minimum of 100 individual cells were tracked to generate x, y, and z coordinates at each time point. These were then sorted to only include cells present at time 0. The time that each of the sorted cells were in frame was calculated and the most common time was used to pick cells for tracking analysis, maximizing the sample size of cells in the analysis. Velocity, speed and Mean Squared Displacements were calculated using code adapted from previously established methods for triplicate tracking trials (n=3) (16,24,27). The statistical analysis was performed using MATLAB (Mathworks, Inc.) to calculate the mean, SD, and SE of the mean. Where appropriate, a t test was performed to determine significance (GraphPad Prism 4.02; GraphPad Software, Inc.). Graphed data are presented as average ± SEM. Significance levels were set at *P<0.05.

For minoxidil and TGF-β inhibitor treatment, cells were cultured in hydrogels under the conditions and methods described above for 3 days. On day 3, 0.5 mM minoxidil or 10 µM TGF-β inhibitor dissolved in KIA cell culture media was added to the wells containing the hydrogels and the cells were tracked for 24 hours. Untreated cultures served as controls. Cell tracking and data analysis were performed as described above.

Traction Force Microscopy

Collagen gel solution was prepared as stated above. One micron sized FluoSpheres that are Carboxylated modified (Thermo Fischer) were rinsed with phosphate buffered saline 3 times and then mixed with the cell pellet prior to hydrogel formation. On day 3 of culture z-stacks of cells and beads were imaged in 1 min interval for 20 minutes to capture bead movement on a confocal microscope (LSM 780, Zeiss). The tracking analysis performed above, IMARIS and MATLAB to track the bead movement through the gel. In order to calculate the force on the beads previously published formula for a particle moving through a stress relaxation hydrogel was used(28).

Rheology and Stress Relaxation Analysis:

For all rheological experiments an AR1500EX rheometer (TA Instruments) was used. A time sweep was performed on the mTG and control gels at 1% strain and 10 Hz to monitor the storage (G') and loss modulus (G'') at 37°C. A solvent trap with DI water, or an

immersion cup with PBS was used to prevent evaporation. Stress relaxation experiments were performed for strains from 0.5–1% for 2 minutes. During the stress relaxation test there was a strain ramp of 5 seconds prior to relaxation. Data recorded after the completion of the strain ramp was analyzed. This strain was chosen to stay within the linear viscoelastic region of the hydrogel, to not permanently deform the hydrogel. As previously reported a single element Maxwell model was used to fit the stress relaxation constants (16,28).

In brief the equation for a single spring in series with a single dashpot that describes viscoelastic fluids was fitted to stress relaxation curves:

$$\sigma = \sigma_0 e^{-t/\tau}$$

In the above equation σ_0 is the initial stress, τ is the time constant of stress relaxation, and t is the time. We have previously shown to fit the relaxation of collagen gels using this equation(16). Frequency sweep was performed at 1% strain from 0.5 to 10 Hz to study the change in moduli in response to increasing frequency. Creep and recovery test was also performed. All samples were initially stressed at 10 Pa and relaxed for 2 minutes followed by a strain recovery test. This stress was chosen to stay within the linear viscoelastic region of the hydrogel, to not permanently deform the hydrogel. In the strain recovery test the applied stress was removed and strain was monitored over time. A four element Maxwell-Voight model was used to fit the relaxation constant as previously performed (3). A stress strain curve was generated by getting the corresponding stress value at a given strain.

Reflective Microscopy and Second Harmonic Generation (SHG):

To analyze collagen fibers in the collagen gels reflective microscopy was used. Images were collected using an LSM 780 (Zeiss) microscope. A 40x oil immersion objective was used to capture the images and were configured to use the 405 nm light to illuminate and be collected to visualize the collagen fibers. To examine collagen in the murine tumors or metastatic lungs, histological slides from the center of the tumor were imaged using SHG microscopy as previously(18). SHG microscopy was carried out with a LSM 510 (Zeiss) microscope equipped with a Chameleon Ultravision II Laser Module (Coherent). A wavelength of 810 nm was used to image H&E slides of tumors and light was collected at 488 nm wavelength.

Collagen Fiber Morphology, Orientation, and Cell Aspect Ratio Analysis:

Collagen fiber orientation was adapted from a previously developed method using MATLAB (Mathworks) (16) of collagen on mTG and control hydrogels. Collagen fiber width and length were analyzed using a ct-Fire analysis, a previously established method (16,29) on SHG images of tumors. The software segments the image and calculates intensity gradients within subregions of images and uses them to track the overall directions of fibers. To study cell aspect ratio, code was developed to fit an ellipse around the cell and the aspect ratio is the ratio of the major to minor axis of cells on day 3 in the mTG and control hydrogels.

Scanning Electron Microscopy (SEM):

Collagen hydrogels, with and without cells, were fixed as previously described and processed for conventional SEM (30). Briefly, dime-size gels were fixed in 100mM cacodylate buffer, pH 7.4, containing 3% formaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose for 1 hr, washed, and osmicated at 4°C in Palade's fixative containing 1%OsO₄. Cells were then dehydrated through a graded series of ethanol; critical point dried using a Tousimis Model 795 critical point dryer (Rockville, MD); and coated with 4nm platinum using a Anatech Hummer 6.1 sputter coater (Hayward, CA). Samples were imaged in an FEI Quanta 200 ESEM (Hillsboro, OR) using the Everhart-Thornley secondary electron detector at 1.2keV under high vacuum.

Transmission Electron Microscopy (TEM):

Collagen hydrogels, with and without cells, were fixed as previously described (30). Briefly, for conventional electron microscopy (EM), gels were fixed in 100mM cacodylate buffer, pH 7.4, containing 3% formaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose for 1 hr, washed; and osmicated at 4°C in Palade's fixative containing 1%OsO₄. Cells were then washed; en bloc stained with Kellenberger's uranyl acetate over night; dehydrated through a graded series of ethanol; and subsequently embedded in epon. 80nm sections were cut on a LEICA UCT ultramicrotome; collected onto 300 mesh formvar/carbon coated grids; and post-stained in uranyl acetate and lead citrate. Images were recorded using a Soft Imaging System Magaview III camera mounted on a Tecnai 12 TEM operating at 100keV; and figures were assembled in Adobe Illustrator.

RT-PCR:

A quantitative real-time RT-PCR assay was used to assess gene expression. Total RNA was extracted from cells encapsulated in hydrogels with TRIzol (Invitrogen) in accordance with manufacturer instructions. Hydrogels were placed in 500 µL of TRIzol and homogenized with a microhomogenizer. The suspension was then centrifuged at 12,000 x g for 15 min and the supernatant was separated. 100 µL of chloroform was mixed with the solution by manual agitation for 20 s and the mixture was centrifuged at 12,000 x g for 10 min. The supernatant was isolated and mixed with 250 µL isopropyl alcohol and kept at -4 °C for 1 h. The precipitates were separated by centrifugation at 7,500 x g for 5 min and washed with 70% ethyl alcohol. Total RNA was quantified using an UV spectrometer and validated by lack of DNA contamination. One microgram of RNA was transcribed using reverse-transcriptase M-MLV and oligo(dT) primers (both from Promega), according to the manufacturer's instructions. TaqMan Universal PCR MasterMix and Gene Expression Assay (Applied Biosystems) was used according to the manufacturer's instructor for PLOD2, MMP2, MMP9 and HPRT1.

TCGA Analysis:

Gene Expression Profiling Interactive Analysis (GEPIA) (31) was used to generate survival curves as well as study trends in RNA-sequence data, based of querying the cancer genome atlas database. GEPIA also generated and provides all the statistics.

Generation of GpNLuc KIA stable cell lines:

KIA cells (70% confluent) were transfected with pRetroX-Tight-MCS_PGK-GpNLuc (Addgene plasmid #70185) using Lipofectamine 2000 (ThermoFisher scientific) in serum free medium as per the manufacturer's instructions. GpNLUC is a bioluminescent resonance energy transfer (BRET) reporter in which the luciferase bioluminescence excites the green fluorescent protein (32). Mixed populations were then FACS sorted for high GFP signal using a Sony Biotechnology SH-800 'chip' cell sorter at one cell per well in a 96 well plate. Stably expressing clonal populations were then utilized for downstream experiments.

In-vivo- primary tumor model:

The Johns Hopkins University's Institutional Animal Care and Use Committee approved all animal protocols. KIA cells, expressing GFP or GpNLuc that were encapsulated in quick and slow stress relaxation hypoxic hydrogels as reported above, were subcutaneously implanted in 8–10 week in nude mice. Caliper measurements were performed to track subcutaneous tumor growth. The volume of the tumor was calculated using the following equation:

$$V = \frac{1}{2} * L * W^2$$

where V is the volume of the tumor, L is the major axis, and W is the minor axis of the tumor (33). In addition to caliper measurements mice were injected intraperitoneally with 20 µg/mL of furimazine followed by luminescence imaging with IVIS Spectrum (PerkinElmer) within 5 minutes of injections. Average radiance (photons per second per centimeter squared per steradian) was observed at time point and tumor.

Additionally, primary tumors were generated as previously reported(18,24). In short, 1 million cells were injected into each flank of the mouse with either the KIA scrambled control line (KIA Scr) or PLOD2 knockdown line (PLOD2(-)). Tumors were allowed to grow for 20 days prior to extraction.

Tumor Rheology

Rheological measurements on the tumors using an AR1500EX rheometer (TA Instruments). A time sweep was performed at 1% strain and 5 Hz to monitor the storage (G') and loss modulus (G'') at 37°C. An immersion cup with media was used to prevent evaporation. Stress relaxation experiments were performed for 1% strain for 2 minutes with a strain ramp of 5 seconds prior to relaxation. In order to analyze the stress relaxation, the methodology listed above was used.

All samples were initially stressed at 20 Pa and relaxed for 2 minutes followed by a strain recovery test. Other studies have previously used a similar to measure mechanical properties in tumors (34,35). In the strain recovery test the applied stress was removed and strain was monitored over time. In order to analyze the stress relaxation, the methodology listed above was used.

In-vivo- metastatic lesions model:

The Johns Hopkins University's Institutional Animal Care and Use Committee approved all animal protocols. KIA cells, expressing GFP or GpNLuc that were encapsulated and cultured for 3 days in slow stress relaxation non-hypoxic gels and quick stress relaxation hypoxic hydrogels. Per side one quick stress relaxation hypoxic hydrogels or two slow stress relaxation non-hypoxic gels were subcutaneously implanted in 8–10 week in nude mice. Tumors were let to grow for 20 days, and then resected. Mice were left for additional 15 days after tumor resection, to allow lung metastasis to grow. On day 15, lungs were fixed and processed for histology.

Immunohistochemistry and Immunofluorescence Staining:

For murine tissue, following fixation the tissue was dehydrated with serial ethanol (70%–100%); embedded in paraffin; 5 μ m sections were cut; and subsequently treated with hematoxylin and eosin (H and E) stain, mason trichrome, or PLOD2. Immunofluorescent stains were performed as previously reported on gels(27). SMAD2/3 (Proteintech), vinculin (Sigma), phalloidin, and YAP (SantaCruz) were all used at a 1:200 dilution and counterstained with DAPI. An anti-rabbit Alexa Fluor 564 (Life Technologies) was used at 1:500 dilution and imaged with Zeiss LSM 780 or LSM 800 microscope. PLOD2 (Proteintech) was also used at 1:200 dilution for histology sections.

Quantification of immuno stains:

immunohistochemistry stains for PLOD2 were quantified as previously reported (36). In short, background was taken from an empty portion of the slide, the color deconvolution plug-in ImageJ (National Institutes of Health) was used to extract the DAB color of the image. Finally, the mean intensity was calculated from the DAB intensity over the entirety of the image.

For quantification of SMAD2/3 localization, IMARIS (Bitplane) was used to find the colocalization of SMAD2/3 and DAPI from a z stack images of the immunofluorescence stained cells. Surface area of the colocalization channel was then calculated using the surface package in IMARIS.

To quantify vinculin spots and actin filaments per cells the number of each was manually counted and normalized to the number of filaments or spots per cell.

Quantification of pore area using SEM:

SEM images of cells moving through the hydrogel were analyzed using ImageJ. The pore area was measured by drawing a region of interest over the pore.

Quantification of TEM:

ImageJ was used to measure the bands in TEM images of the collagen fibers. A line was drawn over the length of the light or dark segments to analyze the length of the banding.

Quantification of metastatic lesion:

The number of metastatic nodes was counted manually from hematoxylin and eosin histological sections of the lungs. Additionally, using ImageJ the area of metastasis was calculated and normalized to the area of the lung, to determine percent area covered by metastasis.

Statistical Analysis:

Statistical analysis was performed using MATLAB (Mathworks, Inc), or Prism 4 (GraphPad Software, Inc.) to calculate the mean, standard deviation, and standard error mean. T test and one way ANOVA were performed where appropriate to determine significance (GraphPad). Biological and technical replicates are indicated throughout the figure captions. All graphical data are reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Results

Design and Characterization of Stress Relaxation Collagen Gel Platform

To generate collagen gels with varying stress relaxation times, we fabricated a collagen hydrogel with defined fiber density by standardizing the ice incubation time prior to gelation as previously described (16). Once the hydrogel structure was established, microbial transglutaminase (mTG) was added on top of the completely formed hydrogel, to further crosslink the collagen fibers and modulate the stress relaxation time of the hydrogel. We hypothesize that mTG crosslinks the existing structure at primary amines in between the already formed collagen fibrils. The stress relaxation time is the time it takes for a material to return to normal after a strain is applied (Figure 1A). The stress relaxation time is determined by fitting the Maxwell equation to the stress relaxation curve. Mathematically, the Maxwell model is used to understand viscoelastic materials, such as the ECM. In brief, the equation is derived for a single spring, elastic component, in series with a single dashpot, viscous component. It is derived from adding the stresses from the elastic (Hookean) modulus with the Newtonian viscosity. This equation relates the stress change with time at a constant strain (see methods). It has been used previously to model biological tissues(4). The stress relaxation time is determined by fitting the Maxwell equation to the stress relaxation curve. Low concentration of microbial-transglutaminase (mTG; 24 mg/mL) was chosen to allow for crosslinking of the collagen fibers as a means to change stress relaxation without altering the stiffness of the collagen gel (Figure 1B–D). Consequently, the stress relaxation time decreased by half an order of magnitude (Figure 1B–D). In figure 1D we can see that the modulus, tensile elasticity G' , plateaus faster in the mTG treated gel, corresponding with a quicker stress relaxation. It should be noted that tests of the mTG crosslinked gels and non-treated controls using higher stains show a difference in the stress relaxation with no difference in the modulus between the two hydrogels (Supplementary Figure 1). The stress relaxation time was also verified using a creep and strain recovery test (Supplementary Figure 2A–B); and a similar decrease in stress relaxation was observed. Furthermore, hydrogels response to frequency was evaluated to explore the difference in slope when frequency is plotted versus G' . The greater the rate of change in G' , as frequency increases,

confirms a quicker stress relaxation material (12). (Supplementary Figure 2C–D). This range occurs with a strain below 30% and a stress under 30 Pa.

To further characterize the hydrogel scaffold, reflection microscopy and scanning electron microscopy (SEM) were performed on the mTG treated hydrogel and the non-treated control. Interestingly, we could not observe any differences between the two hydrogel structures at the length scale of cells (Figure 1E). Additionally, using transmission electron microscopy (TEM) we could not detect a significant difference in the length of the light and dark segments of the collagen fibers, thus revealing no difference in the fibril organization of the collagen fibers (Figure 1E). These light and dark segments are indicative of the how collagen alpha helices are arranged in the superstructure of collagen. Overall, using mTG to modify the stress relaxation of the collagen gels through additional crosslinks to the fibers, from the above reflective imaging, SEM, and TEM analyses, we could not detect change in the hydrogel structure (Supplementary Figure 3). We therefore conclude that there is no significant structural difference, at the cellular length scale, between the mTG hydrogel and the control.

Morphology and Migration of Sarcoma Cells in Hydrogels with Controlled Stress Relaxation profile

Having established the hydrogel platform, the influence of stress relaxation on sarcoma cell migration was explored in both hypoxic and nonhypoxic gradients. In order to investigate the effect of stress relaxation on cell migration, we used murine (KIA) autochthonous genetically engineered mouse model (GEMM) (16,24,37). The KIA cells were chosen due to their highly migratory and invasive behavior as well as the ability to metastasize from subcutaneous tumors(16,18,24,38). To establish oxygen gradients, we used our previously reported approach, in which we balanced the diffusion coefficient of the material, boundary conditions, and cell concentration (16). We measured oxygen tension throughout the hydrogels' depth in all conditions and confirmed the presence of hypoxic and nonhypoxic gradients in all conditions after 72 hours (Figure 2A). Additionally, we compared the morphology of the cells in the nonhypoxic region of the hypoxic hydrogels to the cells in nonhypoxic hydrogels and found no difference in cell aspect ratios in the two different gels with similar oxygen tension (Supplementary Figure 4). We further confirmed no change in the stress relaxation of the collagen hydrogels after 72 hours in both hypoxic and nonhypoxic conditions, showing that the stress relaxation time is consistent over the course of the experiment (Supplementary Figure 5). KIA cells were found to migrate more in both hypoxic and mTG treated gels compared with untreated gels (Figure 2B–C).

We have previously demonstrated that a large cell aspect ratio is consistent with a more migratory phenotype (16). Thus, we examined the motility of KIA cells in the three-dimensional space on the third day after encapsulation (16), and found there were minimal differences in the instantaneous cell velocities between conditions (Supplementary Figure 6). Next, we examined the migration persistence measured by the mean square displacement (MSD), and found no significant increase in the non-hypoxic conditions (Figure 2D). However, in the hypoxic gradient, we noticed a slight increase in the cell migration persistence in the x and y directions, but a stark increase in the z direction in the mTG

treated gels (Figure 2E). This demonstrates that the cells in quicker stress relaxation gel migrate faster.

Finally, to examine how the cells are interacting with the matrix, we performed traction force microscopy. For this, 1 μm carboxylated polystyrene beads were incorporated into the matrix with cells and were tracked in 3D space as cells deform the matrix. The beads were large enough to neglect Brownian displacements and are effectively tethered to the collagen matrix fibers due to the reactive carboxyl groups(39). Thus, any movement of the beads through the matrix is a result of cell-mediated matrix deformation. We plotted the trajectories of the beads in 3D space and found that their displacement decreased in the quicker stress relaxation hydrogel (Figure 2Fi–ii). The amount of force that the cells are applying on the matrix was then calculated from the displacement of the beads (28). We found that cells in the quicker stress relaxation hydrogels apply less force onto the matrix compared with control gels (Figure 2Fiii). Small forces have been previously reported for migrating cancer cells (40,41) as well as dividing cells (42,43), further supporting the biological relevance of these measurements.

PLOD2 Increase in mTG Treated Gels

We next analyzed why shorter stress relaxation time induced an increase in sarcoma cell migration. All analyses below were performed after cells were cultured for 3 days in the hydrogel. To study the whether cell-material interactions are enhanced in mTG treated gels, we looked for 1) vinculin spots, which are indicative of focal adhesions (44); 2) actin fiber rearrangement and localization (45), and 3), YAP nuclear localization (46). We could not detect significant differences among the KIA cells due to changes in stress relaxation or hypoxia (Supplementary Figure 7). As a result, we conclude that cellular responses to changes in the stress relaxation of the collagen gels examined here are not YAP mediated.

Previous studies linked an increase in expression of collagen cross-linkers, such as PLOD2, to TGF- β driven SMAD2/3 nuclear localization (47,48). Specifically, in breast cancer, TGF- β and SMAD2/3 have been linked to cell mechano-sensing through PDZ-binding motif (TAZ) signaling, where TAZ specifically is required for nuclear accumulation of SMAD2/3 (49). Moreover, in stiffer matrices, cells have more nuclear SMAD2/3 (50). In pancreatic cancer, increased TGF- β expression leads to more migratory cells (51); while in prostate cancer an increase in TGF- β RNA expression, correlates to an increase in patient mortality (52). We thus hypothesized that a quicker stress relaxation time upregulates TGF- β expression enabling nuclear localization of SMAD2/3; which in turn further amplifies PLOD2 expression. To test this hypothesis, we first examined the expression pattern of SMAD2/3 in the different conditions. We found a significant increase in SMAD2/3 nuclear localization in the mTG hydrogels (Figure 3A–B). We then examined PLOD2 expression in the different conditions with and without inhibition of TGF- β . Without inhibition of TGF- β , we found higher PLOD2 expression in mTG treated gels, regardless of oxygen levels, compared to untreated controls. KIA cells embedded in mTG treated hypoxic gel exhibited the highest PLOD2 expression, suggesting an additive effect of these two cues. When TGF- β inhibitor was added, PLOD2 expression levels dropped (Figure 3C). Overall, we conclude that these results link TGF- β to PLOD2 expression.

To explore the consequences of increased PLOD2 expression, we performed SEM and analyzed how cells interact with the collagen matrix, and found that the pores around the cells are larger in mTG treated hydrogels on day 3 (Figure 3D). We sought to determine if the size of cell-associated-pores is due to cell-mediated matrix degradation. Using DQ collagen assays we did not detect differences in protease activity either overall or locally next to cells (Supplementary Figure 8A–B). Moreover, we did not observe significant differences in matrix metalloprotease (MMP) 2 and 9 expression (Supplementary Figure 8C); or in the G' of the different gels on day 1 and day 3 of culture (Supplementary Figure 8D). Taken together, these results suggest that the change in the size of cell-associated-pores was not protease mediated.

To explore changes in cellular migration due to increased PLOD2 expression, we performed tracking studies of KIA-GFP cells treated with a TGF- β inhibitor or with a PLOD2 inhibitor (18,24), and fluorescently labeled PLOD2-deficient cells. When KIA cells were treated with TGF- β inhibitor, we observed a decrease in migration of KIA cells in mTG hypoxic gradients to a level similar to the KIA cells in hypoxic gradients in the z direction (Figure 3Ei); further corroborating the impact of decreased PLOD2 expression with TGF- β inhibition. Additionally, when PLOD2 was inhibited with minoxidil, or knocked down (Figure 3E ii-iii); we found a decrease in MSD in the z direction, in both the mTG hypoxic gel and hypoxic gel, to a level below the untreated controls. The PLOD2 specific inhibitor and the knockdown cell line, are impeding the hypoxic and TGF- β contributions to PLOD2 expression. We thus conclude that the increase in migration, in the quicker stress relaxation gels, is due to an increase in PLOD2 expression induced by TGF- β stimulation through SMAD2/3 activation. We also confirm a combinatory effect of hypoxia in upregulating that migration via PLOD2 expression.

Quicker Stress Relaxation in Hydrogels Leads to Increased Collagen Fiber Size/Density and Metastasis

To examine the effect of matrix stress relaxation on sarcoma growth and metastasis *in vivo*, hypoxic control and mTG treated constructs were implanted subcutaneously into nude mice (Figure 4A). Sarcoma cell migration in response to an oxygen gradient was previously established *in-vivo* (16,18,24). In this model we examine the co-regulation of stress relaxation with hypoxia. For the *in vivo* assays we hypothesize that quicker stress relaxation is caused by PLOD2 upregulation in hypoxic conditions, resulting in more cell migration and metastasis. To test this hypothesis, we use hypoxic hydrogel encapsulated cells as a substitute for subcutaneous KIA tumors, which are generally hypoxic when grown beyond 300 mm²(24). Specifically, the hydrogel model allows us to directly manipulate stress relaxation of the material. KIA cells, transfected with a bioluminescent resonance energy transfer (BRET) reporter, were used for all *in vivo* experimentation (32). Tumors were allowed to grow for up to 20 days and monitored using IVIS imaging, before the animals were euthanized. mTG treated gels displayed enhanced fluorescent signal over time compared to control hypoxic gels (Figure 4B). This observation correlated with an apparent increase in tumor volume (Figure 4C). However, tumor volume, from the mTG hydrogel, was not significantly greater at any of the examined time points.

We next analyzed PLOD2 staining and found it to be significantly increased in the mTG tumors compared with controls. (Figure 4D). This confirmed the phenotype that we observed *in-vitro*. Finally, we analyzed the collagen fiber density and architecture in the day 20 tumors using mason trichrome and second harmonic generation (SHG) microscopy and image analysis with the ct-FIRE code suite (29). Masson's trichrome stain showed more collagen fibers in the tumors generated from the mTG treated hypoxic hydrogels (Figure 4E). By analyzing the SHG images we further found higher collagen fiber width, length and density in the mTG tumors (Figure 4F–G and Supplementary Figure 9). Together, these data demonstrate that quicker stress relaxation time increases collagen content and fiber architecture through upregulation in PLOD2 expression.

Additionally, to further confirm the effect of PLOD2 on stress relaxation, subcutaneous tumors were generated in nude mice using the KIA scramble control cells (KIA Scr) and KIA cells with a PLOD2 knockdown (PLOD2(–)). Cells were injected subcutaneously to form the tumors, and the tumors were then allowed to grow for 20 days (Figure 4H). The growth of these tumors was similar to what was seen with the hydrogel implantation, where we saw no difference in the tumor volume over time between the KIA Scr and PLOD2(–) tumors (Figure 4I). At day 20 the storage modulus (G'), and stress relaxation time was measured (Figures 4J–L). From these experiments, we observed that the tumors had the same G' but different stress relaxation times. The stress relaxation time was also confirmed using a creep and recovery test (Supplementary Figure 10). Overall, when PLOD2 crosslinking is knocked down the tumor has a slower stress relaxation time. This confirms the hypothesis about the importance of PLOD2 crosslinking in generating a quicker stress relaxation.

Quicker Stress Relaxation in Hydrogels Augments Metastasis

As previously loss of PLOD2 expression disrupts pulmonary metastasis and since lungs are the main metastatic site for sarcoma (18), we next examined the impact of stress relaxation on lung metastasis. We prepared nonhypoxic and mTG treated hypoxic constructs and implanted them after 3 days in culture. This time in culture was to allow transcriptional and translational changes responsible for the phenotype to take place prior to implantation. We speculated that while hypoxic conditions are rapidly generated in subcutaneous tumors, priming the cells to the quicker stress relaxation time would enhance their metastatic potential. After 20 days, tumors were resected and mice were monitored for an additional 15 days (Figure 5A). This model with extended time for tumor growth, allows for KIA cells to migrate out of the hypoxic hydrogel into the vasculature and disseminate to the lungs and form metastasis. After 15 days, metastatic lesions were found in the lungs in both conditions, but were more extensive in the mice that were implanted with the mTG treated hypoxic constructs (Figure 5B) including the number of metastatic lesions per lung as well as the area covered by metastasis (Figure 5C). Additionally, SHG imaging was performed on lung regions showing that there was more collagen deposited in the metastasis originated from the mTG treated hypoxic constructs (Figure 5D). This is indicative of more mature tumors. We observed no difference in the collagen fiber length and width in the lungs (Supplementary Figure 11).

TCGA Analysis and Patient Relevance of High PLOD2 Expression

To study the clinical significance of PLOD2 expression in sarcoma, the cancer genome atlas (TCGA) database was queried to analyze survival rates, as well as correlations from patient RNA-seq data. 261 patients were queried, and the results were pooled using GEPIA to generate RNA-seq correlations, disease free survival, and overall survival (31). We explored the correlation between upregulation of SMAD2, TGF- β , and hypoxia inducible factor 1- α (HIF1- α) and PLOD2 with patient disease free survival and overall survival. There is no significant difference in the overall survival, or disease free survival, of patients who are high expressing for SMAD2 and TGF- β 1 (Figure 6A–B). In addition, while we do not see an increase in overall survival in patients who are high expressing for HIF1- α , we detect an increase in their disease free survival (Figure 6C). Interestingly, we could detect a small but significant decrease in disease free survival for high PLOD2 expressing patients; and a significant increase in mortality rates, from 40% for low expressing PLOD2 patients to 20% for high expressing PLOD2 patients (Figure 6D). To confirm the TCGA data, we explored the ability of the mTG hydrogel platform to test the response of primary patient UPS cells, recently derived from human tumors (hUPS). The oxygen profiles in the gels on day 3 were similar to what we observed for encapsulated KIA cells (Figure 6E). We observed distinct phenotypes in the different hypoxic and non-hypoxic gels treated with or without mTG (Figure 6F); and an increase in aspect ratio in the more migratory cells (Figure 6G), which also similar to what was observed with KIA cells. Finally, there is no difference in cell migration between the non-hypoxic conditions (Supplementary Figure 12A); but there was a significant increase in migration in the mTG hypoxic gel condition compared to the control hypoxic gel in the z direction (Figure 6H and Supplementary Figure 12B). There was no difference in the velocity of hUPS cells in the hydrogels (Supplementary Figure 13). Overall, hUPS and KIA cells show similar trends.

Proposed mechanism of response to quicker stress relaxation

We propose a novel mechanism for cellular response to quicker collagen stress relaxation time in the hypoxic sarcoma microenvironment (Figure 7). As the tumor grows beyond its blood supply, hypoxic regions develop, which induces PLOD2 expression via HIF1- α upregulation, thereby leading to metastasis (18,24). This enhanced crosslinking quickens the stress relaxation time of the collagen matrix, which in turn stimulates TGF- β - shuttling of SMAD2 to the nucleus, further increasing PLOD2 expression. We propose a feedback loop between hypoxia and matrix properties, resulting in an amplification of cell migration and metastasis in sarcoma.

Discussion

The current study demonstrates the importance of stress relaxation time as a new mechanical property that is impacted by the hypoxic environment and in turn enhancing sarcoma motility and metastasis. Stress relaxation is a key factor that links the viscous and elastic components of the ECM. This is the first reported direct correlation between hypoxia and matrix properties that jointly regulate sarcoma fate.

The collagen hypoxic hydrogel platform was used to explore this mechanical property and biological impact. Using collagen gel and mTG we can control for faster stress relaxation without modulating the hydrogels' stiffness and micron level structure. With this system, by controlling cell concentration and hydrogel thickness, we are able to change oxygen tension to achieve hypoxic (1–12%) and non-hypoxic (15–20%) gradient hydrogels. Overall, this platform allows us to modulate oxygen and stress relaxation without altering the micro and nano-scale collagen fiber architecture; thereby allowing us to create a platform that more accurately mimics *in vivo* ECM to study finite properties of the tumor micro-environment. From our studies, we observed an increase in hypoxic sarcoma cell migration along the oxygen gradient from low to high tensions. This is expected as we have previously shown that sarcoma cells persistently migrate in the direction of higher oxygen concentrations(16,24). Importantly, the cells in the hypoxic hydrogels with the quickest stress relaxation time, exhibited the most robust migration within the oxygen gradient as well as an elongated phenotype quantified by aspect ratio. Using traction force microscopy we further found that less displacement and less force is applied in the quicker stress relaxation hydrogel. We speculate that cells need less force to move through the quicker stress relaxation gels, thus able to migrate faster and to longer distances compared with controls.

To explore the molecular mechanisms behind the increase in cell migration, we begun by analyzing YAP and could not detect significant differences among the KIA cells in the different conditions. Unlike epithelial cancer cells where YAP localization changes in stiffer gels during EMT(53), KIA cells, which derived from a genetic model of murine UPS, are already mesenchymal therefore they do not go under EMT. We speculate that KIA cells do not respond to changes in stress-relaxation via YAP. As a result, we conclude that cellular responses to changes in the stress relaxation of the collagen gels examined here are not YAP mediated. It is important to note, that our studies did not explore the response of KIA cells to changes matrix stiffness and that our gels are soft (100 Pa), and thus we cannot exclude that KIA maybe YAP-sensitive in stiffer matrix conditions. We were able to deduce that the quicker stress relaxation increased PLOD2 expression in cells via TGF- β and SMAD2. This increase in PLOD2 via TGF- β and SMAD2 had an additive effect when coupled with hypoxia. Using SEM we found that the pores around the cells are larger in quicker relaxing hydrogels on day 3. This ability to create larger pores is due to the lower polymer to crosslinking ratio, which requires less force to move the fibers. Previous studies have shown that an increase in crosslinking leads to an increase in cell migration(54) as well as it being a critical factor in tumor progression (18,55). As we further found that the change in the size of cell-associated-pores was not protease mediated, we hypothesize that pore size and cell movement in mTG treated hydrogels is most likely due to cells physically deforming the collagen fibers.

We next explore the impact of stress relaxation on tumor progression and metastasis. Tumors generated from hypoxic hydrogels with quicker stress relaxation times exhibited an increase in collagen fiber size and density as well as PLOD2 expression. Moreover, when generating tumors from PLOD2 knockdown KIA cells, we could not observe change in tumor volume and stiffness but found slower stress relaxation compared with control tumors. This confirms that loss of PLOD2 crosslinking affect stress relaxation in sarcoma, similarly to what we

observed in the *in-vitro* experiment, and with tumors generated from hydrogel implantation. Finally, to examine impact on metastasis, we performed a survival assay where fully grown primary tumors were removed, allowing mice to live for additional 15 days, examining pulmonary metastasis. We found more extensive metastasis with more collagen deposition in the mice that were implanted with the quicker stress relaxed hydrogels, further corroborating our migration observations *in vitro*.

Finally, the clinical relevance of these observations was determined by querying publically available TCGA global expression data. We found a dramatic decrease in patient survival in those who had high expression levels of PLOD2. Using this new hypoxic collagen gel platform, the response of primary patient hUPS cells was found to be similar to what was predicted using the KIA cell line, demonstrating the ability of this platform to screen future therapeutics to inhibit migration and subsequently minimize cancer progression.

This is one of the first studies to demonstrate the effect of stress relaxation on tumor growth and metastasis in a physiologically mimicking *in vitro* system. We therefore propose that our platform has substantial predictive potential and will enable a more thorough investigation of migratory phenotypes in primary human samples. This platform can further be adapted to explore stress relaxation in oxygen gradients as they affect other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature Reviews Cancer* 2003;3:362 [PubMed: 12724734]
2. Swaminathan V, Mythreye K, O'Brien ET, Berchuck A, Blobe GC, Superfine R. Mechanical stiffness grades metastatic potential in patient tumor cells and in cancer cell lines. *Cancer research* 2011;71:5075–80 [PubMed: 21642375]
3. Xia Z, Patchan M, Maranchi J, Trexler M. Structure and relaxation in cellulose hydrogels. *Journal of Applied Polymer Science* 2015;132
4. Chaudhuri O, Gu L, Klumpers D, Darnell M, Bencherif SA, Weaver JC, et al. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nature Materials* 2015
5. Stylianopoulos T, Martin JD, Chauhan VP, Jain SR, Diop-Frimpong B, Bardeesy N, et al. Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors. *Proceedings of the National Academy of Sciences* 2012;109:15101–8
6. Nia HT, Liu H, Seano G, Datta M, Jones D, Rahbari N, et al. Solid stress and elastic energy as measures of tumour mechanopathology. *Nature biomedical engineering* 2017;1:0004
7. Nia HT, Datta M, Seano G, Huang P, Munn LL, Jain RK. Quantifying solid stress and elastic energy from excised or in situ tumors. *Nat Protoc* 2018;13:1091 [PubMed: 29674756]

8. Stylianopoulos T, Martin JD, Snuderl M, Mpekris F, Jain SR, Jain RK. Coevolution of solid stress and interstitial fluid pressure in tumors during progression: implications for vascular collapse. *Cancer research* 2013;73:3833–41 [PubMed: 23633490]
9. Jain RK. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer cell* 2014;26:605–22 [PubMed: 25517747]
10. Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK. Role of extracellular matrix assembly in interstitial transport in solid tumors. *Cancer research* 2000;60:2497–503 [PubMed: 10811131]
11. Lo C-M, Wang H-B, Dembo M, Wang Y-I. Cell Movement Is Guided by the Rigidity of the Substrate. *Biophysical Journal* 2000;79:144–52 [PubMed: 10866943]
12. Chaudhuri O, Gu L, Klumpers D, Darnell M, Bencherif SA, Weaver JC, et al. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nature materials* 2016;15:326–34 [PubMed: 26618884]
13. Carey SP, Goldblatt ZE, Martin KE, Romero B, Williams RM, Reinhart-King CA. Local extracellular matrix alignment directs cellular protrusion dynamics and migration through Rac1 and FAK. *Integrative Biology* 2016;8:821–35 [PubMed: 27384462]
14. Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnology and bioengineering* 2009;103:655–63 [PubMed: 19472329]
15. Lou J, Stowers R, Nam S, Xia Y, Chaudhuri O. Stress relaxing hyaluronic acid-collagen hydrogels promote cell spreading, fiber remodeling, and focal adhesion formation in 3D cell culture. *Biomaterials* 2017;154:213–22 [PubMed: 29132046]
16. Lewis D, Tang V, Jain N, Isser A, Xia Z, Gerecht S. Collagen Fiber Architecture Regulate Hypoxic Sarcoma Cell Migration. *ACS Biomaterials Science & Engineering* 2017
17. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *Journal of Cell Science* 2010;123:4195–200 [PubMed: 21123617]
18. Eisinger-Mathason TK, Zhang M, Qiu Q, Skuli N, Nakazawa MS, Karakasheva T, et al. Hypoxia-dependent modification of collagen networks promotes sarcoma metastasis. *Cancer discovery* 2013;3:1190–205 [PubMed: 23906982]
19. Noda T, Yamamoto H, Takemasa I, Yamada D, Uemura M, Wada H, et al. PLOD2 induced under hypoxia is a novel prognostic factor for hepatocellular carcinoma after curative resection. *Liver International* 2012;32:110–8 [PubMed: 22098155]
20. Semenza GL. Targeting HIF-1 for cancer therapy. *Nature reviews cancer* 2003;3:721 [PubMed: 13130303]
21. Francis P, Namløs HM, Müller C, Edén P, Fernebro J, Berner J-M, et al. Diagnostic and prognostic gene expression signatures in 177 soft tissue sarcomas: hypoxia-induced transcription profile signifies metastatic potential. *BMC genomics* 2007;8:73 [PubMed: 17359542]
22. Du H, Pang M, Hou X, Yuan S, Sun L. PLOD2 in cancer research. *Biomedicine and Pharmacotherapy* 2017;90:670–6 [PubMed: 28415047]
23. Gilkes DM, Bajpai S, Wong CC, Chaturvedi P, Hubbi ME, Wirtz D, et al. Procollagen lysyl hydroxylase 2 is essential for hypoxia-induced breast cancer metastasis. *Molecular cancer research* 2013;11:456–66 [PubMed: 23378577]
24. Lewis DM, Park KM, Tang V, Xu Y, Pak K, Eisinger-Mathason TK, et al. Intratumoral oxygen gradients mediate sarcoma cell invasion. *Proceedings of the National Academy of Sciences* 2016;201605317
25. Abaci HE, Truitt R, Tan S, Gerecht S. Unforeseen decreases in dissolved oxygen levels affect tube formation kinetics in collagen gels. *American journal of physiology Cell physiology*;301:C431–40 [PubMed: 21543738]
26. Gao Y, Baca AM, Wang B, Ogilby PR. Activation barriers for oxygen diffusion in polystyrene and polycarbonate glasses: Effects of low molecular weight additives. *Macromolecules* 1994;27:7041–8
27. Lewis DM, Blatchley MR, Park KM, Gerecht S. O₂-controllable hydrogels for studying cellular responses to hypoxic gradients in three dimensions in vitro and in vivo. *Nat Protoc* 2017;12:1620 [PubMed: 28726849]

28. McKinnon D, Domaille D, Brown T, Kyburz K, Kiyotake E, Cha J, et al. Measuring cellular forces using bis-aliphatic hydrazone crosslinked stress-relaxing hydrogels. *Soft Matter* 2014;10:9230–6 [PubMed: 25265090]
29. Bredfeldt JS, Liu Y, Pehlke CA, Conklin MW, Szulcowski JM, Inman DR, et al. Computational segmentation of collagen fibers from second-harmonic generation images of breast cancer. *Journal of biomedical optics* 2014;19:016007-
30. McCaffery JM, Farquhar MG. [29] Localization of GTPases by indirect immunofluorescence and immunoelectron microscopy *Methods in enzymology*: Elsevier; 1995 p 259–79.
31. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Research* 2017
32. Schaub FX, Reza MS, Flaveny CA, Li W, Musicant AM, Hoxha S, et al. Fluorophore-NanoLuc BRET reporters enable sensitive in vivo optical imaging and flow cytometry for monitoring tumorigenesis. *Cancer research* 2015;75:5023–33 [PubMed: 26424696]
33. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer chemotherapy and pharmacology* 1989;24:148–54 [PubMed: 2544306]
34. Insana MF, Pellot-Barakat C, Sridhar M, Lindfors KK. Viscoelastic imaging of breast tumor microenvironment with ultrasound. *Journal of mammary gland biology and neoplasia* 2004;9:393–404 [PubMed: 15838608]
35. Wisdom KM, Adebawale K, Chang J, Lee JY, Nam S, Desai R, et al. Matrix mechanical plasticity regulates cancer cell migration through confining microenvironments. *Nature communications* 2018;9:4144
36. Lessey BA, Savaris RF. Comparison of HSCORE assessment of endometrial $\beta 3$ integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ). *Analytical and quantitative cytopathology and histopathology* 2013;35:210 [PubMed: 24341124]
37. Kirsch DG, Dinulescu DM, Miller JB, Grimm J, Santiago PM, Young NP, et al. A spatially and temporally restricted mouse model of soft tissue sarcoma. *Nature medicine* 2007;13:992
38. Yoon SS, Stangenberg L, Lee Y-J, Rothrock C, Dreyfuss JM, Baek K-H, et al. Efficacy of sunitinib and radiotherapy in genetically engineered mouse model of soft-tissue sarcoma. *International Journal of Radiation Oncology• Biology• Physics* 2009;74:1207–16
39. Bloom RJ, George JP, Celedon A, Sun SX, Wirtz D. Mapping local matrix remodeling induced by a migrating tumor cell using three-dimensional multiple-particle tracking. *Biophysical journal* 2008;95:4077–88 [PubMed: 18641063]
40. Wolf K, Te Lindert M, Krause M, Alexander S, Te Riet J, Willis AL, et al. Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *J Cell Biol* 2013;201:1069–84 [PubMed: 23798731]
41. Franck C, Maskarinec SA, Tirrell DA, Ravichandran G. Three-dimensional traction force microscopy: a new tool for quantifying cell-matrix interactions. *PloS one* 2011;6:e17833 [PubMed: 21468318]
42. Au - He L, Au - Sneider A, Au - Chen W, Au - Karl M, Au - Prasath V, Au - Wu P-H, et al. Mammalian Cell Division in 3D Matrices via Quantitative Confocal Reflection Microscopy. *JoVE* 2017:e56364
43. Ng MR, Besser A, Brugge JS, Danuser G. Mapping the dynamics of force transduction at cell–cell junctions of epithelial clusters. *Elife* 2014;3:e03282 [PubMed: 25479385]
44. Humphries JD, Wang P, Streuli C, Geiger B, Humphries MJ, Ballestrem C. Vinculin controls focal adhesion formation by direct interactions with talin and actin. *The Journal of cell biology* 2007;179:1043–57 [PubMed: 18056416]
45. DuFort CC, Paszek MJ, Weaver VM. Balancing forces: architectural control of mechanotransduction. *Nature reviews Molecular cell biology* 2011;12:308 [PubMed: 21508987]
46. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. *Nature* 2011;474:179 [PubMed: 21654799]
47. Laklai H, Miroshnikova YA, Pickup MW, Collisson EA, Kim GE, Barrett AS, et al. Genotype tunes pancreatic ductal adenocarcinoma tissue tension to induce matricellular fibrosis and tumor progression. *Nature medicine* 2016;22:497–505

48. Remst DF, Davidson ENB, Vitters EL, Bank RA, van den Berg WB, van der Kraan PM. TGF-ss induces Lysyl hydroxylase 2b in human synovial osteoarthritic fibroblasts through ALK5 signaling. *Cell and tissue research* 2014;355:163–71 [PubMed: 24192939]
49. Varelas X, Sakuma R, Samavarchi-Tehrani P, Peerani R, Rao BM, Dembowy J, et al. TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nature cell biology* 2008;10:837 [PubMed: 18568018]
50. Taylor MA, Amin JD, Kirschmann DA, Schiemann WP. Lysyl oxidase contributes to mechanotransduction-mediated regulation of transforming growth factor- β signaling in breast cancer cells. *Neoplasia* 2011;13:406-IN2 [PubMed: 21532881]
51. Chen Y, Yu G, Yu D, Zhu M. PKC α -induced drug resistance in pancreatic cancer cells is associated with transforming growth factor- β 1. *Journal of Experimental & Clinical Cancer Research* 2010;29:104
52. Reis STd, Pontes-Júnior J, Antunes AA, Sousa-Canavez JMd, Abe DK, Cruz JASd, et al. Tgf- β 1 expression as a biomarker of poor prognosis in prostate cancer. *Clinics* 2011;66:1143–7 [PubMed: 21876965]
53. Zanconato F, Forcato M, Battilana G, Azzolin L, Quaranta E, Bodega B, et al. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. *Nature cell biology* 2015;17:1218 [PubMed: 26258633]
54. Anguiano M, Castilla C, Maška M, Ederra C, Peláez R, Morales X, et al. Characterization of three-dimensional cancer cell migration in mixed collagen-Matrigel scaffolds using microfluidics and image analysis. *PloS one* 2017;12:e0171417 [PubMed: 28166248]
55. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* 2009;139:891–906 [PubMed: 19931152]

Statement of significance

Findings demonstrate that mechanical (stress relaxation) and chemical (hypoxia) properties of the tumor microenvironment jointly accelerate sarcoma motility and metastasis via increased expression of collagen matrix cross-linker PLOD2.

Figure 1. Collagen stress relaxation gels. (A) Schematic of hydrogel response to stress relaxation (drawing not to scale). mTG crosslinked gels and non-treated controls were analyzed for (B) stiffness, (C) stress relaxation time using at 1% strain (n=5). (D) Representative stress relaxation curve from shear rheology. (Ei) Representative micrographs taken using reflection microscopy (upper panel, scale bars 20 μ m) and SEM (lower pane, scale bars 5 μ m l) of the two gels and representative micrographs taken using TEM. (Eii) Corresponding quantification of the length of the light and dark chains of the collagen fibers in the two gels from TEM images (n=3 with 10 images analyzed in each replicate). Scale bars are 100 nm.

Figure 2: Sarcoma cell migration increases with quicker stress relaxation time in hypoxia. (A) Oxygen gradients on day three of culture for the hypoxic and nonhypoxic hydrogels with KIA cells (n=3). (B) Representative light microscope images of KIA cells in hypoxic and nonhypoxic hydrogels with two different stress relaxation times. Inserts are high magnification of the boxed areas. (C) The aspect ratio of the KIA cells (n=3 with 10 images analyzed in each replicate). Migration of KIA cells in the (D) nonhypoxic and (E) hydrogels on day 3. (F) Trajectory of the beads moving through the (i) hypoxic and (ii) nonhypoxic fast relaxing gels, and (iii) quantification of the forces that cells applied to the matrix based on the bead trajectories. (n=3). Scale bars are 100 μm.

Figure 3: Increased expression of PLOD2 through TGF- β /SMAD2/3 in mTG treated gels. SMAD2/3 nuclear localization in KIA cells cultured in the different conditions for 3 days: (A) representative immunofluorescent images (red is SMAD2/3) and (B) Quantification of SMAD2 localization to nucleus (n=3 with 10 images analyzed in each replicate). (C). PLOD2 expression in KIA cells treated with and without TGF- β inhibitor (n=3). (D). Representative SEM images (*left*) and quantification (right; n=3 with 10 images analyzed in each replicate) of the KIA cells- associated-pores in mTG and control hypoxic gels (E) Mean squared displacement in the z direction of KIA cells in hypoxic mTG and control gels in (i) with TGF- β inhibitor; (ii) treatment with minoxidil; and (iii) knockout PLOD2(-) KIA cells. n=3 in each condition. Scale bars are 5 μ m.

Figure 4: Effect of quicker stress relaxation on tumors.

(A). Schematic of in-vivo experiments, using subcutaneous implantations of hypoxic hydrogels treated with or without mTG. (B) IVIS imaging was used to follow tumor growth and cell density via a BRET reporter and fluorescent intensity (unit is in radiant efficacy). (C) Tumor volume was calculated to track the growth of mTG and control tumors (n=5). (D). (i) Representative images and (ii) quantification of PLOD2 stain of day 20 tumors (n=3 with 10 images analyzed in each replicate). (E). Mason trichrome stain of day 20 tumors. Arrows indicate collagen fibers. (F) Representative SGH microscopy images of day 20 tumors and (G) quantification of collagen fiber width, length, and density (n=3 with 10 images analyzed in each replicate). (H) Schematic of subcutaneous tumor growth experiments KIA Scr and PLOD2(-) lines. (I) Tumor volume of KIA Scr and PLOD2(-) tumors (n=5). (J) G' of the tumors was measured showing no difference (n=5 tumors per condition). (K) A quicker stress relaxation time was seen when PLOD2 was knocked down (n=5 tumors per condition). (L) Representative curve of stress relaxation of KIA Scr and PLOD2(-) tumors.

Figure 5: Effect of quicker stress relaxation on metastasis:

(A) Schematic of survival surgery experiments. (B) Images of lungs extracted from the mice 15 days after tumor resection. (C) (i) Representative images of H&E histological sections of lung metastasis and their quantification including (ii) number of metastatic nodes and (iii) area covered by lesions. (D) (i) Representative SGH microscopy images of metastatic lesions in the lung and quantification of (ii) area of collagen (n=6). Scale bars are 20 μ m.

Figure 6: Patients data.

TCGA database analysis of disease free survival and overall survival of patients in relation to the expression of (A) SMAD2, (B) TGF-β1, or (C) HIF1-α and (D) PLOD2. hUPS cell migration increases with the quicker stress relaxation time in hypoxia. (E) Oxygen gradients on day three of culture of the hypoxic and nonhypoxic hydrogels with hUPS cells. (F) Representative light microscope images of the hUPS cells in hypoxic and nonhypoxic hydrogels with two different stress relaxation times. Inserts are high magnification of the boxed areas. (G) The aspect ratio of the hUPS cells (n=3 with 10 images analyzed in each replicate). (H) Migration of the hUPS cells in hypoxic hydrogel on day three showing MSD in the z direction.

Figure 7: Proposed biological response to quicker stress relaxation:

HIF1- α upregulation due to hypoxia increases PLOD2 expression instigating a quicker stress relaxation collagen matrix, which in turn additionally upregulates PLOD2 via TGF- β and SMAD2.