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A Systems Mechanobiology Model to Predict Cardiac Reprogramming Outcomes on Different Biomaterials

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Abstract

During normal development, the extracellular matrix (ECM) regulates cell fate mechanically and biochemically. However, the ECM's influence on lineage reprogramming, a process by which a cell's developmental cycle is reversed to attain a progenitor-like cell state followed by subsequent differentiation into a desired cell phenotype, is unknown. Using a material mimetic of the ECM, here we show that ligand identity, ligand density, and substrate modulus modulate indirect cardiac reprogramming efficiency, but were not individually correlated with phenotypic outcomes in a predictive manner. Alternatively, we developed a data-driven model using partial least squares regression to relate short-term cell states, defined by quantitative mechanosensitive responses to different material environments, with long-term changes in phenotype. This model was validated by accurately predicting the reprogramming outcomes on a different material platform.

Collectively, these findings suggest a means to rapidly screen candidate biomaterials that support reprogramming with high efficiency, without subjecting cells to the entire reprogramming process.

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Author Contributions

Y.P.K. and A.J.P. designed the experiments. Y.P.K. conducted the majority of the experiments and analyzed the data. A.Y.R. pre-processed the cell traction images and analyzed Yap locus. X.X., Y.S., and J.F. provided the PDMS microposts, cell traction and Yap locus data on PDMS microposts. Y.P.K. and A.J.P. wrote the manuscript. All authors read and commented on the manuscript.

Competing Financial Interests

The authors have no competing financial interests to declare.

Data and materials availability

The data obtained in the current study are available on reasonable request from the corresponding author.

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Keywords

reprogramming; mechanotransduction; systems biology; heart regeneration

Introduction

Cell reprogramming was once thought to be impossible since adult somatic cells have reached the end of the developmental process. However, several lower vertebrates (axolotls, newts, and zebrafish) possess the ability to regenerate tissues and even entire limbs by reprogramming cells in injury sites into proliferating progenitor-like cells that subsequently differentiate into the cells necessary to restore tissue function [1, 2]. Inspired in part by such organisms and the landmark Yamanaka papers on iPS reprogramming [3, 4], transdifferentiation has now been demonstrated in several cell types, including reprogramming of pancreatic exocrine cells to β -cells [5], fibroblasts to muscle cells [6], lymphocytes to macrophages [7], and fibroblasts to neurons [8]. Candidate approaches to transdifferentiate fibroblasts into cardiomyocyte (CM)-like cells via exogenous expression of key cardiac transcription factors have also been reported [9–12]. Applying such approaches to transdifferentiate resident cardiac fibroblasts into CM-like cells *in situ* following a heart attack has even yielded improvements in cardiac function in animal models of myocardial infarction [11, 12]. A similar conversion was also recently achieved by inducing the expression of endogenous cardiac genes using only small molecules, rather than via transgene expression [13]. Therefore, reprogramming cells *in situ* to orchestrate tissue regeneration may obviate the need for exogenous cell delivery and thereby revolutionize current paradigms in regenerative medicine.

The efficiency of reprogramming in general, and cardiac reprogramming specifically, is low, which currently limits the translational applicability of this paradigm. Most efforts to improve efficiencies are focused on chemical genetic tools, but the observation that cardiac reprogramming *in vivo* is more efficient than *in vitro* suggests the microenvironment plays a critical role in transdifferentiation, an idea raised in Qian et al.[11] but not experimentally shown. Having previously demonstrated that biomaterials composed of different extracellular matrix (ECM) materials influence cardiac reprogramming efficiencies [14], here we attempted to decouple the physico-chemical features of the ECM to better understand their individual roles in modulating cardiac reprogramming. We used ECM-functionalized polyacrylamide as a nonfouling and tractable substrate suitable for systematic and independent variation of ligand identity, ligand density, and substrate elasticity [15]. Mouse embryonic fibroblasts (MEFs) cultured on these gels were subjected to an established indirect cardiac reprogramming process that transforms a committed cell into a progenitor-like cell state, through a brief tetracycline-controlled expression of Oct4, Sox2, Klf4, and cMyc (OSKM), followed by subsequent differentiation into CM-like cells [9]. Controlling orthogonal ECM cues altered reprogramming efficiencies, but no single cue could be directly correlated with reprogramming outcomes to predictably control efficiencies over this complex and relatively long duration morphogenetic transformation. Instead, we applied a systems biology approach [partial least squares regression (PLSR)] to relate quantitative short-term measurements of the mechanoresponsiveness of the cells [cell area, cell-

generated traction forces, and the nuclear localization of the Yes-associated protein (Yap)] on different ECM with the long-term changes in phenotype, and developed a data-driven model based on this relationship. When applied to a different ECM platform, our PLSR model based on a quantitative short-term 'cell state' signature was able to accurately predict reprogramming outcomes in the long-term. These results suggest an approach to screen highly efficient materials for current reprogramming strategies.

Materials and Methods

Cell culture

Mouse embryonic fibroblasts (MEFs) from Millipore were expanded to passage 5 with MEF media (DMEM + Glutamax, 5% FBS and 1% non-essential amino acids (NEAA) all from Gibco) on tissue culture plastic. Cells at passage 5 were then frozen down in 90% FBS (Gibco):10% DMSO (Sigma-Aldrich) freezing media and stored in liquid nitrogen until needed.

Polyacrylamide substrate preparation

Square coverglasses (No. 1, 22 × 22 mm, VWR) were cleaned with piranha etch solution (1 part H₂O₂ (Fisher Scientific) to 3 parts sulfuric acid (Fisher Scientific)) for 30 mins and then rinsed with DI water for 5 minutes. The cleaned coverglasses were then functionalized with glutaraldehyde (Sigma-Aldrich) as in Aratyn-Schaus et al. [16]. Briefly, the cleaned coverglasses were immersed in 2% 3-aminopropyltrimethoxysilane (Sigma-Aldrich) in IPA (Fisher Scientific) for 10 minutes. The coverglasses were then removed and rinsed in ddH₂O. Subsequently, the coverglasses were immersed in 1% glutaraldehyde in ddH₂O for 30 mins. The coverglasses were then rinsed in ddH₂O and dried before storage in a dark and dry area. Functionalized coverglasses were stored for up to two months.

Polyacrylamide (PA) precursor solutions that result in gels of different elasticities were mixed and prepared as in Tse and Engler [17]. Briefly, 40% acrylamide (Bio Rad), 2% Bisacrylamide (Bio Rad), 10X PBS (Gibco) and ddH₂O were mixed in various ratios and degassed for 15 mins. TEMED (Bio Rad) and 10% APS (Bio Rad) were then added to the degassed precursor solution to initiate polymerization. This solution was filtered through a 0.22 µm PES syringe filter and 35 µL of the solution was placed on a perfluorodecyltrichlorosilane(FDTS, Gelest)-treated glass slide. The glutaraldehyde treated coverglass was then placed onto the drop and the solution allowed to polymerize for 30 mins. The coverglass with attached PA gels was then carefully removed from the FDTS-treated glass slide and immersed in DPBS (Gibco) before use.

Matrix proteins (collagen I (Advanced Biomatrix), fibrinogen (Sigma-Aldrich), fibronectin (BD Biosciences), or laminin-111 extracted from Engelbreth-Holm-Swarm sarcoma (Gibco)) were conjugated to the PA substrate with sulfo-SANPAH (Proteochem). The PA substrates were immersed in 50 mM of sulfo-SANPAH in 0.1 M MES (Fisher Scientific) 0.5 M NaCl (Fisher Scientific) buffer pH 6.0 and irradiated under a 365 nm lamp (2 mW/cm²) for 15 minutes. The UV irradiation was repeated with fresh sulfo-SANPAH solution and rinsed four times with the MES buffer. Matrix proteins diluted in D-PBS without calcium

and magnesium to the desired concentration were then applied to the PA substrates and incubated on a rocker for 2 hours at room temperature. The matrix protein solution was then removed and the PA substrates rinsed in DPBS three times. The matrix-conjugated PA substrates were used a day after conjugation or stored in 4 °C for maximum of one week.

Indirect reprogramming with OSKM

Lentiviral supernatant was prepared by co-transfecting HEK293FT (Invitrogen) cells with the tetO-FUW-OSKM viral vector (Addgene plasmid #20321) or FUW-M2rtTA (Addgene plasmid #20342) with the lentiviral packaging plasmids pLP1, pLP2, and pLP-VSVG. Viral supernatant was collected 48hrs after transfection and stored in -80 °C until required. MEFs were subjected to indirect reprogramming as previously described [9]. Briefly, passage 5 MEFs were plated on matrix-conjugated PA substrates the night before viral transduction. The next day (D0), MEFs were transduced with tetO-FUW-OSKM and FUW-M2rtTA viral supernatant for six hours and replaced with fresh MEF media. At D1, the transduced MEFs were then cultured with reprogramming media 1 (R1) consisting of 15% FBS (Gibco), 5% Knockout serum (KOSR, Gibco), 1% Glutamax (Gibco), 1% NEAA (Gibco), 0.1 mM 2-mercaptoethanol (BME, Gibco), KO-DMEM (Gibco) and 2 µg/mL doxycycline (Sigma-Aldrich) for 7 days. At D8, R1 was replaced with reprogramming media 2 (R2) consisting of 1% FBS, 14% KOSR, 1% Glutamax, 1% NEAA, 0.1 mM BME and KO-DMEM for 3 days. At D11, R2 was replaced with chemically defined media (CDM) consisting of 0.05% BSA fraction V (Sigma-Aldrich), 0.5% Glutamax (Gibco), 0.1 mM BME (Gibco), 0.5 × N2 supplement (Gibco), 1X B27 supplement (Gibco), RPMI-1640 (Gibco) and 10 ng/mL BMP-4 (Stemgent) for 5 days. At D16, CDM+BMP-4 media was later replaced with CDM media only, for 7 days. Quantification of lentiviral transduction efficiency on the different substrates was performed using viruses generated with the transfer vector tetO-FUW-eGFP (Addgene plasmid #73083) and FUW-M2rtTA (Addgene plasmid #20342).

Quantification of colonies and contractile colonies

Reprogrammed cells were examined with an Olympus CKX31 inverted microscope and the number of colonies and contractile colonies were quantified manually. The total number of colonies resulting from exogenous OSKM expression was counted at D10. This number divided by the substrate area is then normalized with the number of colonies per cm² observed on a reference material performed in the same experimental study (2 kPa polyacrylamide substrate conjugated with 50 µg/mL of collagen I) to obtain the normalized colonies per cm² (NCC) values. The range of values obtained for the reference material is 21.25 to 9.25 colonies per cm². This range arises from the variability introduced by the reagents, cells, and the experimental workflow. The number of contractile colonies was counted at D21. The percentage of contractile colonies (PCC) is the ratio of the number of contractile colonies to the total number of colonies multiplied by 100.

Immunocytochemistry and microscope imaging

Cells were fixed with fresh 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.01% TX-100 (Sigma-Aldrich) for 10 minutes then rinsed with DPBS for 3 times. Fixed cells were blocked with 10% goat serum (Gibco) for 1 hour, and then exposed to primary antibodies against cardiac troponin I (Millipore), alpha-actinin (Sigma-Aldrich) or Yap

(Santa Cruz Biotech.) at a dilution of 1:500 in 10% goat serum. Secondary antibodies [Alexa Fluor 488 goat antimouse IgG (Invitrogen) and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen)] were applied at a dilution of 1:400 to label the bound primary antibodies. Cells were then finally stained with DAPI (Invitrogen). Stained cells were then imaged on an Olympus IX81 inverted microscope equipped with a Hamamatsu ORCA-R2 (model C10600) digital CCD camera.

Quantification of Yap translocation

The state of Yap translocation across the nuclear membrane was determined by multiple observers in a blinded manner using the following protocol. Briefly, images of cells immunostained with DAPI and antibodies to Yap were thresholded with ImageJ. The images were false colored and then overlaid, and the resulting overlay images used to identify cells with a nuclear Yap locus.

Intracellular Ca^{2+} characterization

Calcium transients in the reprogrammed cardiomyocytes were imaged by staining the cells with the Fluo-4 NW calcium indicator (Invitrogen) following the manufacturer's protocol. The calcium transients were imaged at room temperature on an Olympus IX81 inverted microscope equipped with a Hamamatsu ORCA-R2 (model C10600) digital CCD camera. Fresh isoproterenol hydrochloride (10 μM) (Sigma-Aldrich) or acetylcholine (10 μM) (Sigma-Aldrich) was used as an agonist and calcium transients were imaged and recorded after application to the cells.

Material characterization and rheology

Polyacrylamide precursor solutions prepared as in the cell experiments were allowed to set between FDTS treated glass plates and 8 mm discs were punched from the polymerized gels. The 8 mm discs were then tested between parallel plate platens of a rheometer (TA instruments AR-G2) with an oscillation of 1% strain at 1 rad/s.

Fabrication and surface functionalization of poly-dimethylsiloxane (PDMS) micropost arrays (PMAs)

The PDMS micropost arrays (PMAs) were fabricated using microfabrication techniques as described previously [18, 19]. Briefly, a silicon micropost array master was fabricated by standard high-resolution photolithography and deep reactive ion etching (DRIE). The height of the Si microposts was controlled by varying etching time during DRIE. The Si mold used in this work contained hexagonally spaced posts with a post diameter of 1.83 μm , post heights of 0.7 μm , 6 μm , 8.4 μm and 14.5 μm , respectively, and a post center-to-center distance of 4 μm . The final PMAs were generated by replica molding. The PMAs were fabricated onto 18-mm round coverslips before transferred to standard 12-well tissue culture plates. To promote cell attachment, the top surface of the PMA was functionalized with collagen I (Advanced Biomatrix) using microcontact printing as described previously [18].

Western blot

To characterize Yap expression at the protein level, cells were lysed with ice-cold RIPA buffer and cleared by centrifuging at maximum speed for 5 minutes at 4 °C. The total protein concentration in each sample was determined using the Pierce BCA assay kit. Equal protein amounts from each sample or treatment were then prepared and reduced in Laemmli sample buffer. The reduced samples were loaded and run in 10% Novex mini-cell SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes (Bio Rad) after separation. The blots were then blocked in 5% BSA in TBS-T (blocking buffer) overnight at 4 °C. Primary antibodies in blocking buffer were applied to the blots at room temperature for 2 hours. Blots were then rinsed with TBS-T, and secondary antibodies in TBS-T were applied for 2 hours. Chemiluminescent detection was used to visualize the blots, which were subsequently detected via autoradiography film (Hyblot CL). The film was processed and imaged using a Fotodyne white light transilluminator. Primary antibodies used were anti-YAP (H-9) (Santa Cruz Biotech.) and anti- β -actin (Santa Cruz Biotech.) at a dilution of 1:100 and 1:2000 respectively. The secondary antibody mouse-HRP (Santa Cruz Biotech.) was used at a 1:10,000 dilution. Densitometry analysis of the Western blots was carried out with ImageJ.

Traction force microscopy and quantification of cell area

The methods for traction force microscopy were based on Aratyn et al. [16] and Tseng et al. [20]. Briefly, PA substrates were prepared as above with the addition of 40 nm fluorescent beads (Invitrogen) as displacement markers. MEFs were plated on the ECM-conjugated PA substrates and the next day imaged on an Olympus IX-81 inverted microscope fitted with an EICOM microscope incubator. Fluorescent images with and without the cells were taken and processed with ImageJ plugins developed by Tseng et al. The displacement vectors were obtained using the PIV plugin and the traction forces were computed from the displacement vectors with the FTTC plugin. The phase contrast images taken during the traction force microscopy were used to determine the cell area. Using these images, the cell boundary was traced in ImageJ and the area within the outline of the cell was quantified by the measure tool in ImageJ.

Partial least squares regression and prediction with PLS derived model

Mean values of three mechanoresponsive signals (traction, cell area, and Yap) of cells measured 48 hours post-plating were compiled into a 12 (conditions) by 3 (signals) matrix X . The corresponding reprogramming outcomes NCC and PCC were compiled into a 12 by 2 (outcomes) matrix Y . These matrices were then used as inputs for the PLS regression performed in Matlab using a code provided by Professor Herve Abdi at the University of Texas at Dallas (<https://www.utdallas.edu/~herve>). A plot of the first two principal components (Fig. 5a) is obtained by plotting the first two columns of the scores matrix T defined as $T=XP$ and $T^T T=I$, where I is the identity matrix and P is the loading matrix. The predicted outcomes plotted in Fig. 5b-c and Fig. 6b were calculated with $\hat{Y}=TBC^T$, where B is the regression weight matrix and C is the loading matrix for the reprogramming outcomes.

Cerivastatin and Y27632 supplementation and Yap Knockdown with shRNA

To block Yap's nuclear translocation, MEF cultures were treated with cerivastatin (Sigma-Aldrich) (1 μ M final concentration) in R1 and R2 media until 10 days after viral transduction. The number of colonies with and without cerivastatin supplementation was determined at day 10 post-transduction. To block Rho/ROCK-mediated traction forces, Y27632 (Millipore-Sigma) was supplemented at 10 μ M final concentration in R1 and R2 until 10 days after viral transduction. The number of colonies with and without (only DMSO) Y27632 supplementation was determined at day 10 post-transduction. Knockdown of Yap expression was implemented with pGIPZ lentiviral shRNA vectors obtained from the University of Michigan Vector Core. These lentiviral shRNA vectors are identical to the vectors obtained from GE Dharmacon. Transfer vectors pGIPZ-V2LMM_45834 (shRNA1), pGIPZ-V2LMM_53798 (shRNA2), and pGIPZ-nonsilencing (GE Dharmacon) were co-transfected with the lentiviral packaging plasmids psPAX2 [a gift from Didier Trono (Addgene plasmid # 12260)] and pLP-VSVG in 293T/C17 cells (ATCC) to generate viral supernatant. The viral supernatant was collected 48 hours post-transfection, frozen and stored at -80°C . MEFs were transduced with the non-silencing and shRNA lentiviruses (MOI = 3) a day before the cardiac reprogramming process was initiated.

Statistical analysis

All experiments were carried out in biological replicates. The number of biological replicates observed in experiments was at least three and stated in figure captions if different. Statistical analyses were carried out with Prism 7.0 (GraphPad) with a one-way ANOVA. Pairwise comparisons between multiple groups were performed using Bonferroni's post-hoc test and differences between the means were deemed statistically significant when $p < 0.05$. The F values and degrees of freedom for the ANOVAs are provided in Supplementary Table 1.

Results

Cardiac reprogramming on synthetic polyacrylamide substrates

To demonstrate that indirect cardiac reprogramming could be supported by synthetic polyacrylamide (PA) substrates, we first carried out reprogramming on 2 kPa PA-substrates with 50 $\mu\text{g/mL}$ protein concentration of collagen I conjugated via sulfo-SANPAH chemistry [21, 22]. MEFs were reprogrammed on these substrates (shown schematically in Fig. 1a and representative phase images of the colonies in Supplementary Fig. 1), and expressed α -actinin and cardiac troponin I (cTnI) after 21 days. Striations were observed in both cardiac proteins at higher magnifications (Fig. 1b). Cells positive for these cardiac markers formed spontaneously beating contractile colonies, which when stained with Fluo-4AM enabled visualization and quantification of calcium transients (Supplementary Video 1). We further investigated whether the reprogrammed cells were responsive to chronotropic drugs by supplementing with either isoproterenol (Iso) or acetylcholine (Ac). The reprogrammed cells did not significantly increase their beating frequency in response to Iso (Fig. 1c), but did exhibit significant reductions in response to Ac (Fig. 1d). These results showed the reprogrammed cells were cardiomyocytelike, or induced CMs (iCMs). Because the PA cell

culture platform robustly supported cardiac reprogramming, we used it to further probe the ECM's effects.

Effect of ligand identity, substrate modulus, and ligand density on cardiac reprogramming

The chief advantage of PA-substrates to investigate the ECM's effects on reprogramming is the ability to ostensibly tune ligand identity, ligand density, and elastic modulus independently by respectively changing the ECM type conjugated to the substrate [23], altering the concentration of the ECM solution during the conjugation process [24, 25], or varying the stoichiometry of the PA precursors [17]. On 2 kPa PA-substrates, we conjugated 50 $\mu\text{g/mL}$ of collagen I, fibrinogen, fibronectin, and laminin-111 (laminin) and reprogrammed MEFs with the process described above (Fig. 1a). The total number of colonies and the percentage that were contractile were quantified at day 10 and day 21 time points respectively. Collagen I-conjugated PA-gels promoted significantly higher dedifferentiation efficiency (η_d) than the other ligand types tested as demonstrated by more colonies per cm^2 (NCC) that appeared on these substrates (Fig. 2a). Additionally, as reflected by the percentage of contractile colonies (PCC), the cardiogenic efficiency (η_c) on collagen I substrates was higher than the other substrates (Fig. 2b).

Having established collagen I as a more permissive ligand for reprogramming, we conjugated 50 $\mu\text{g/mL}$ of collagen I to PA-substrates of the following elasticities; 0.2, 2, 20, and 200 kPa. Since these elasticities span across the spectrum of rigidities found in both the developing and pathologic myocardium, this experiment investigated how reprogramming might be impacted at an injury site undergoing fibrotic remodeling. When MEFs were reprogrammed on substrates with different modulus and the number of colonies was quantified, we found that NCC increased to a peak on 20 kPa substrates and decreased thereafter, indicating that η_d was modulated by substrate modulus (Fig. 2d). On the other hand, η_c was not significantly modulated by substrate rigidity (Fig. 2e).

We next addressed the question of how ligand density, independent of substrate modulus, would influence reprogramming by conjugating collagen I to 2 kPa PA-substrates with a stepwise order-of-magnitude increase in protein concentration of 0.5, 5, 50, and 500 $\mu\text{g/mL}$. In contrast to substrate modulus, increasing ligand density exerted an increasing positive influence on η_d (Fig. 2g). However, as with modulus, ligand density did not significantly modulate η_c , although at the lowest ligand amount (0.5 $\mu\text{g/mL}$) no contractile colonies were observed (Fig. 2h).

Because we transduced the cells on the different substrates independently, we evaluated the lentiviral transduction efficiency on these substrates. We transduced the cells with an inducible eGFP reporter, constructed from the same backbone as the polycistronic OSKM vector, and the doxycycline responsive transactivator used with the OSKM vector. We found that the transduction efficiencies (% eGFP+ cells) across the substrates were relatively high and at similar levels (Supplementary Fig. 2). Additionally, the transduction efficiencies did not correlate with the reprogramming outcomes observed in the respective study groups, which indicated that the resulting outcomes was not due to the lentiviral transduction efficiency.

Together, our observations showed that the ECM's physico-chemical properties modulate the dedifferentiation and cardiogenesis phases of the reprogramming process. However, changes in the different micro-environmental elements mapped out a complex response landscape that a material property-based linear correlation was insufficient to predict. Contrary to several significant and highly cited reports that only a singular material property is a sufficient physical inducement of phenotypic outcomes [26, 27], we sought to adopt an alternative approach to predict reprogramming outcomes in response to diverse material cues by quantifying mechanoresponsive signals previously implicated in the ECM's control of cell fate (Fig. 2j). In particular, we singled out cell area [26], cell-generated traction [28, 29], and Yap nuclear locus [30] as potential underlying determinants of cell fate. We posited that these signals might also be involved in the regulation of reprogramming, and function to define a short-term cell state that predicts long-term phenotypic consequences, akin to the cue-signalresponse paradigm applied in other contexts [31].

Evaluating cell-generated traction forces and cell area to model cardiac reprogramming

Cell-generated traction forces and cell area were evaluated simultaneously 48 hours postplating MEFs on all the PA-substrates used in this study. Cell-generated tractions monotonically increased on substrates of increasing rigidity and increasing ligand density (Fig. 3a and Fig. 3b). The average total traction force on collagen I (2.30 μN) was significantly higher than the tractions on the other ECMs (0.09 μN on fibrinogen, 0.09 μN on fibronectin, and 0.11 μN on laminin) (Fig. 3c). The same trends were also observed for cell area on the various PAsubstrates (Fig. 3d-f). To extract meaningful correlations between these mechanoresponsive signals and the reprogramming outcomes, we then compared both traction and cell area with the values of NCC and PCC of corresponding PA-substrates. The relationship between both traction and cell area with NCC could be described fairly well with a power law equation (Fig. 3j and 3l). In contrast, no correlation was found between traction and cell area with PCC (Fig. 3k and 3m). Next, we modulated cell-generated traction forces of reprogramming MEFs on 2 kPa PA-substrates by inhibiting the Rho/ROCK pathway with Y27632 [32]. Neither NCC nor PCC were affected by Y27632 (Supplementary Fig. 3), suggesting that potentially other pathways are involved in the regulation of reprogramming.

Evaluating the Yap signaling axis to model cardiac reprogramming

The Yap signaling axis has been implicated in the response of adult stem cells to ECM elastic modulus, thus establishing a significant association between the physical properties of the ECM and the transcriptional machinery of cells [30]. We hypothesized that the ECM's ability to modulate lineage reprogramming was also dependent on the Yap pathway. We characterized the percentage of cells with nuclear Yap localization 48 hours post-plating by immunostaining and performing a blind characterization on the various substrates as carried out by others in the field [30, 33]. We observed that when the modulus of the substrate was increased from 0.2 to 2 kPa, there was a sharp increase of cells with nuclear Yap. As the modulus was increased further from 2 kPa to 200 kPa, the number of cells with nuclear Yap was reduced (Fig. 4a). The percentage of cells with nuclear Yap was low on substrates with low ligand density (0.5 $\mu\text{g/mL}$), but increased with progressively higher ligand densities (Fig. 4b). Significantly more cells had Yap in the nucleus on collagen I-conjugated

substrates as compared with substrates functionalized with other ECMs (fibrinogen, laminin, and fibronectin) (Fig. 4c). To more directly probe Yap's role in ECM-dependent control of reprogramming, we used a small molecular inhibitor and RNA interference. Treating cells with cerivastatin decreased the number of cells with nuclear Yap without decreasing total Yap protein expression levels (Fig. 4d), consistent with previous reports [34]. Subjecting cells to reprogramming in the presence of cerivastatin resulted in a significant decrease of NCC on collagen I-conjugated 2 kPa PA-gels (Fig. 4d). Knockdown of endogenous Yap with short hairpin RNAs also reduced the NCC of the reprogramming process on collagen I-conjugated 2 kPa PA-gels, the reduction corresponding with the level of protein knockdown (Fig. 4e). These results suggest that Yap is also central to the early reprogramming process. However, examination of the power law relationship between nuclear Yap localization and NCC revealed a less perfect fit as compared to that found for traction and cell area (Fig. 4f). No correlation was found between nuclear Yap and PCC (Fig. 4g).

Derivation of a predictive model using partial least squares regression

Our findings thus far demonstrate the reprogramming outcomes do not correlate well with individual material properties or single mechanoresponsive signals. Furthermore, while cell area and traction correlated well with NCC (Fig. 3), disrupting these with Y27632 had no significant effect on reprogramming (Supplementary Fig. 3). By contrast, Yap nuclear localization did not correlate well with NCC, but was paradoxically still required (Fig. 4). We therefore analyzed our data with a systems biology approach in order to generate a data-driven model [31, 35]. Quantitative measurements of traction, cell area, and nuclear Yap were compiled into a matrix defining the mechanoresponsive state of the cells at 48 hours postplating (the inputs, or X variables). Separately, the measured outputs NCC and PCC were compiled into a matrix of reprogramming outcomes (the outputs, or Y variables). These data were subjected to a PLSR analysis, where the co-variance between the X and Y matrices of data is maximized to find a linear regression and in the process projects the input and output variables to a new space. In this case, PLSR was able to cluster our data into 3 groups: one that correlated with high η_d and high η_c (Hi-Hi), one that correlated with high η_d and low η_c (Hi-Lo), and one that correlated with low η_d (Lo) (Fig. 5a-i).

From the principal components plot, we observed the effects of modulating ligand identity, ligand density, and substrate modulus. The initial increase in stiffness (from 0.2 to 2.0 kPa) resulted in increased η_d but a further stiffness increase (from 2.0 to 200 kPa) did not further increase η_d . On the other hand, increasing stiffness from 0.2 to 2 kPa increased η_c , which then decreased upon a further stiffness increase to 200 kPa (Fig. 5a-ii). Changing ligand density resulted in changes to both η_c and η_d simultaneously, with higher ligand densities resulting in very low η_c (Fig. 5a-iii). Furthermore, this PLSR-derived cell state-based model was able to predict the NCC and PCC of the training data set relatively well as shown by the Pearson correlation coefficients comparing the predictions to the measured outcomes (Fig. 5b-c). This multi-dimensional data-driven model resulted in significantly better predictability when compared to simpler correlations based on single material properties or cell signals, which at best could only predict one outcome well.

To test the predictive power of our PLSR-derived model, we performed indirect cardiac reprogramming on MEFs cultured on PDMS microposts and again quantified NCC and PCC at their respective time points day 10 and day 21 (Fig. 6a). We selected PDMS microposts as a mechanically tunable cell culture platform distinct from the PA-substrates used to derive the predictive model in part because they are made of a completely different base material (silicone vs. acrylamide) and rendered cell adhesive in a completely different manner (micro-contact coated collagen I vs. covalent conjugated collagen I). However, utilization of the PDMS microposts allowed quantitative evaluation of traction, cell area, and % nuclear Yap of cells at 48 hours post-seeding (Fig. 6b-d) to establish the cell state matrix of data required as an input to predict the reprogramming outcomes. The PLSR model developed for reprogramming on PA substrates was able to predict the measured reprogramming outcomes on this alternative PDMS platform remarkably well (Fig. 6e) as compared to a Log-Quadratic material model derived with the data in Figs. 2c-d.

Discussion

Lineage reprogramming has the potential to transform regenerative medicine, either as a means to generate an unlimited supply of potentially autologous cells for therapy or by obviating the need for exogenous cell delivery altogether via transdifferentiation *in situ*. We have shown here that biomaterial mimics of the ECM can potentiate this phenomenon, providing evidence that ECM stiffness, ligand identity, and ligand density all modulate cardiac reprogramming. The efficiencies of both dedifferentiation (η_d) and cardiomyocyte-like differentiation (η_c) were modulated in a non-intuitive fashion such that the reprogramming outcomes, NCC and PCC, were not linearly correlated with any singular material property (Supplementary Fig. 4). Using a systems biology approach to collectively analyze three early mechanoresponsive signals of an initial population of cells - Yap, cell area, and cell traction - with PLSR resulted in a data-driven model that predicts both phases of indirect cardiac reprogramming well.

The degree of cell spreading, the magnitude of cell-generated traction forces, and Yap's nuclear localization have all been previously implicated in studies linking ECM mechanochemical properties with cell fates [26, 28–30], and we hypothesized they could serve as early cell-based indicators of reprogramming outcomes. When examined individually, their values correlated reasonably well with the generation of progenitor cell colonies (NCC) during the dedifferentiation phase of indirect cardiac reprogramming (Figs. 3g, 3i and 4f). In particular, our data show that Yap is necessary for generating the progenitor cell colonies during the dedifferentiation process of indirect cardiac reprogramming, consistent with reports in the literature showing Yap's necessity for OSKM-based iPS reprogramming [36]. By contrast, these cell responses were not individually correlated with the subsequent differentiation into contractile colonies (Figs. 3h, 3j, and 4g). When integrated collectively into a PLSR-derived model, not only were Yap localization, traction force magnitude, and cell area at 48 hours cocorrelated with NCC (Supplementary Fig. 5), but they were also able to predict the subsequent differentiation phase (PCC) as well (Fig. 5c). The effectiveness of a PLSR model comprising just three short-term cell responses to define cell states that predict reprogramming outcomes was initially surprising, as we anticipated the need for greater complexity. However, it has been demonstrated that PLSR-

derived models require only 3–7 input parameters to predict with high efficiency [31]. More importantly, after training the model with data from cells and reprogramming outcomes on one material platform, we demonstrated its ability to predict reprogramming outcomes on an entirely different culture platform. The PLSR model's ability to predict day 21 phenotypic outcomes using observations of cells plated on candidate biomaterials obtained 48 hours post-adhesion may potentially translate to a significant time and cost advantage when coupled with high-throughput screens of different materials that can support directed differentiation programs [37, 38].

The molecular mechanism behind ECM-mediated nuclear localization of Yap remains unknown. One proposed mechanism implicates the activation of ROCK via Rho-GTPases and GPCR signaling [30, 39]. Surprisingly, in our experiments, treatment of cells with a ROCK inhibitor, Y27632, did not result in significant reductions in Yap nuclear localization (Supplementary Fig. 3), suggesting that ECM cues do not modulate Yap exclusively through the Rho-ROCK signaling axis [30, 33]. A recent study demonstrated that activation of Rac1 promoted Yap nuclear localization and that modulation of Rac1 signaling, achieved by presenting combinations of different adhesive ligands, controlled the amount of nuclear Yap [40]. Our observed modulation of the Yap locus with different ligand identities and ligand densities may similarly be mediated by the Rac1 signaling axis. Cell area and traction force were also co-correlated with NCC, the first such evidence linking these metrics to cell reprogramming. Based on an observed co-correlation between cell area with Yap nuclear loci [30], a mechanistic link between the two could be argued. However, others have observed that cell area can be decoupled from Yap [40]. This suggests that cell area and traction might be representing signals independent of Yap. This may explain the observation that cell area and traction were anti-correlated with η_c , in contrast to Yap (Supplementary Fig. 5).

Our observation that microenvironmental cues modulate indirect cardiac reprogramming is in contrast to a recent report that mechanical stretching, and substrate stiffness had no effect on the efficiency of direct cardiac reprogramming, a transdifferentiation process achieved by exogenous expression of Gata4, Mef2c, and Tbx5 (GMT) [41]. However, it is possible that the range of modulus (1 to 62 kPa) and choice of ECM (Matrigel) examined in this recent study may not have modulated the cell state sufficiently to observe an effect. On the other hand, as reported by the same study, micrometer grooved substrates increased the efficiency of GMT reprogramming. These observations suggest that not all cardiac transdifferentiation processes are modulated by the same extracellular cues, and that translating observations based on material parameters from other studies might not lead to expected effects. However, it would be interesting to investigate whether a larger modulus range, ligand density, and ligand identity will modulate GMT reprogramming.

Although induced pluripotent stem cells (iPSCs) share the same reprogramming factors as the indirect cardiac reprogramming process used in our study, we do not necessarily expect the derivation of cardiomyocytes from established iPSC lines to be modulated in the same fashion by the extracellular cues observed in this study. In fact, efforts to improve cardiogenic differentiation from iPSCs have mainly focused on soluble cues, and perhaps where extracellular cues would exert their influence most is during the maturation process of

these iPSC derived cardiomyocytes (iPSC-CMs) as indicated by the choice of certain natural matrices for the engineering of heart tissues from these cells [42].

Finally, our findings caution that correlating single material properties with complex outcomes is potentially problematic, and might lead to observations that are perceived as inconsistencies. Specifically, we observed large modulation of NCC as a function of substrate stiffness for PA substrates (Fig. 2d), but not with PDMS microposts across a similar modulus range (Fig. 6e). These observations mirror those that sparked a debate of whether mesenchymal stem cell fate is indeed controlled by the modulus of biomaterials, a phenomenon observed on PA [15] but not on PDMS [43]. The differences in outcomes that we observed between PDMS and PA substrates of the same effective modulus could be due to the coupling strength of the ECM onto the substrate. Recent work showed that differences in this coupling strength will modulate the transmission of cell generated traction forces and lead to a modified perception of modulus [44]. Therefore, it is important to consider, and characterize, *all* physicochemical cues when trying to translate results and conclusions obtained from one material system to another. However, by measuring cell responses to different environments in an unbiased, material-agnostic manner, we circumvent the need to characterize *all* physicochemical parameters. Our results also demonstrate that a short-term phenotypic signature (a cell state) predicts a long-term phenotypic consequence without the need for prolonged culture experiments. Adopting models that focus on cell responses to the materials, rather than material properties per se, may yield consistent and translatable cell state-response relationships that are applicable across different materials, culture platforms, and potentially even *in vivo* microenvironments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

The polyacrylamide cell culture platform enables the influence of ligand identity, ligand density, and substrate modulus on the cardiac reprogramming process to be investigated. a) Schematic of the reprogramming process on 50 μ g/mL collagen I coated 2 kPa polyacrylamide substrates. Nanoscale fluorescent beads are only incorporated into the polyacrylamide gels as fiducial markers when performing traction force microscopy. MEFs are plated and transduced with OSKM viruses at Day 0. On day 1, doxycycline supplementation in reprogramming media-1 (R1) induces the exogenous expression of OSKM. This expression is switched off at day 7 when reprogramming media-2 (R2) is administered to replace R1. The progenitor cell colonies resulting from the dedifferentiation process are then allowed to expand to day 10. These colonies are then differentiated into cardiomyocyte-like cells by supplementation of BMP4 in a chemically defined differentiation media (DM). At day 15, BMP4 supplementation is withdrawn and the cells are cultured to day 21 with DM only. b) Contractile colonies immunofluorescent stain positive for the cardiac proteins α -actinin and cTnI (top panel, scale bar 50 μ m). These cardiac proteins display striations reminiscent of mature cardiomyocytes at a higher optical magnification (bottom panel, scale bar 10 μ m). c) Calcium transients of contractile colonies with and without 10 μ M isoproterenol supplementation. Colonies exposed to isoproterenol, on average, show a small but statistically insignificant increase in beating frequency. d)

Calcium transients of contractile colonies with and without 10 μ M acetylcholine supplementation. Colonies exposed to acetylcholine, on the other hand, show a significant decrease in beating frequency. Matched symbols denote statistical significance between the two groups, $p < 0.05$.

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Figure 2:

Modulation of cardiac reprogramming by ligand identity, substrate modulus and ligand density. a-b) Dedifferentiation [normalized colonies/ cm^2 (NCC)] and cardiogenic [% contractile colonies (PCC)] efficiency as a function of ligand identity at 50 $\mu\text{g}/\text{mL}$ on 2 kPa polyacrylamide (PA) substrates. c) Representative images of colonies on different ligand identities. d-e) NCC and PCC as a function hydrogel modulus with collagen I at a ligand density of 50 $\mu\text{g}/\text{mL}$. f) Representative images of colonies on different substrate modulus. g-h) NCC and PCC as a function of collagen I ligand density on a 2 kPa PA hydrogel. i)

Representative images of colonies on different ligand densities. j) Establishment that the microenvironment influences the cardiac reprogramming process which suggests that a predictive model based on material parameters ligand identity, modulus, and ligand density can be derived (yellow arrow). However, the difficulty of characterizing these parameters for some materials lead us to posit that a material-agnostic predictive model based on the cell state defined by mechano-signals cell traction, cell area, and/or nuclear Yap locus will allow us to predict reprogramming outcomes more dependably on a variety of materials. $n = 6$ for all experiments. Matched symbols denote statistical significance between the two groups, $p < 0.05$. Scale bar is $200\ \mu\text{m}$.

Figure 3:

Cell generated traction forces and cell areas, measured 48 hours after plating, are dependent on extracellular cues. a-c) Quantification of cell traction forces on substrates of different modulus, ligand density and ligand identities. d-f) Quantification of cell area on substrates of different modulus, ligand density and ligand identities. g-i) Representative images of cell traction and relative cell area on all of the different substrates. Normalized colonies/cm² (NCC) (j) and % contractile colonies (PCC) (k) against cell traction plots to determine whether cell traction can predict reprogramming outcomes. Cell traction correlates only with dedifferentiation efficiency (j) and the relationship can be described well by a power law. NCC (l) and PCC (m) against cell area plots to determine whether cell area can predict reprogramming outcomes. Similarly, cell area correlates only with dedifferentiation efficiency (l) and the relationship can be described well by a power law. n = 15 cells were quantified. Matched symbols denote statistical significance between the two groups, $p < 0.05$.

Figure 4:

The Yap signaling axis is central to the modulation by the extracellular cues. a) Quantification of YAP nuclear locus show modulation as a function of modulus. Panel of immunostained images show typical YAP nuclear locus for selected substrates of different modulus. b) Quantification of YAP nuclear locus show modulation as a function of ligand density. Panel of immunostained images show typical YAP nuclear locus for selected substrates of different ligand density. c) Quantification of YAP nuclear locus show modulation as a function of ligand identity. Panel of immunostained images show typical

YAP nuclear locus for selected substrates of different ligand identities. d) YAP nuclear locus is inhibited by a small molecule inhibitor, cerivastatin. Western blot shows that the amount of total YAP protein is not changed with cerivastatin supplementation. Cerivastatin supplementation significantly abrogates dedifferentiation. e) Knockdown of Yap expression by shRNA reduces dedifferentiation efficiency. Western blot shows a reduction of Yap expression by shRNA1 and shRNA2 compared to a non-silencing (non) construct. shRNA1 reduces normalized colonies/cm² (NCC) more than shRNA2 due to lower Yap protein levels as shown by the densitometry analysis. NCC (f) and % contractile colonies (PCC) (g) against % nuclear Yap plots to determine whether nuclear Yap locus can predict reprogramming outcomes. Nuclear Yap correlates only with dedifferentiation efficiency (f) and the relationship can be described reasonably by a power law. A minimum of 150 cells per sample were used to quantify YAP cellular locus in a blinded manner. n = 3 was used in quantifying YAP cellular locus. n = 3 experiments were conducted for cerivastatin studies. n = 7 experiments were conducted for the Yap knockdown studies. Matched symbols denote statistical significance between the two groups, p < 0.05. Scale bar is 50 μ m.

Figure 5:

Partial least squares (PLS) regression of the mechano-signals cell traction, cell area, and Yap nuclear locus to the reprogramming outcomes derives a complete cell state-based predictive model for cardiac reprogramming. a) Plot of the first two principal components of the analyzed data reveals clusters of extracellular conditions that result in low dedifferentiation efficiency (Lo), high dedifferentiation efficiency but low cardiogenic efficiency (Hi-Lo), and the desired high dedifferentiation and cardiogenic efficiencies (Hi-Hi). Isolating the experimental conditions provides further insight into the effects of changing substrate

modulus (ii), ligand density (iii) and ligand identity (iv). The PLS derived cell-state model predicts both NCC (b) and PCC (c) well as evidenced by the Pearson correlation coefficients $r = 0.89$ and $r = 0.78$ respectively.

Figure 6:

The systems mechanobiology model predicts reprogramming outcomes on a different culture platform. a) Schematic of the experiment performed to predict the cardiac reprogramming outcomes on PDMS microposts. b) Inputs to the model, traction, cell area, and % nuclear Yap are quantified on PDMS microposts. c-d) Panels represents typical images or data obtained for cell area, traction and nuclear Yap. e) Measured normalized colonies/cm² (NCC) and % contractile colonies (PCC) on PDMS microposts compare well to the predicted reprogramming outcomes using the PLS cell-state model as compared to a Log-Quadratic material model. n = 19 cells were quantified for cell area and total traction force. n = 4 samples were used to quantify nuclear Yap locus. n = 3 samples were used to quantify NCC and PCC. Matched symbols denote statistical significance between the two groups, $p < 0.05$.