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Matrix identity and tractional forces influence indirect cardiac reprogramming

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SUBJECT AREAS:

REPROGRAMMING

BIOMEDICAL ENGINEERING

TRANSDIFFERENTIATION

Received
28 August 2013Accepted
25 November 2013Published
11 December 2013Correspondence and
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Heart regeneration through in vivo cardiac reprogramming has been demonstrated as a possible regenerative strategy. While it has been reported that cardiac reprogramming in vivo is more efficient than in vitro, the influence of the extracellular microenvironment on cardiac reprogramming remains incompletely understood. This understanding is necessary to improve the efficiency of cardiac reprogramming in order to implement this strategy successfully. Here we have identified matrix identity and cell-generated tractional forces as key determinants of the dedifferentiation and differentiation stages during reprogramming. Cell proliferation, matrix mechanics, and matrix microstructure are also important, but play lesser roles. Our results suggest that the extracellular microenvironment can be optimized to enhance cardiac reprogramming.

The Nobel prize winning discovery that four transcription factors [Oct4, Sox2, Klf4, and cMyc (OSKM)] could reprogram adult somatic cells to induced pluripotent stem cells (iPSCs) catalyzed efforts to search for other transcription factors that would reprogram somatic cells directly into different lineages^{1–3}. Transdifferentiation, or direct reprogramming, is a potentially compelling regenerative strategy because the reprogramming factors can be delivered directly into the damaged organ or tissue to induce regeneration in vivo. This approach is advantageous because no cell transplantation is required, the cells that reprogram into the functional cells are autologous, and complete integration of the reprogrammed cells is achievable. The first demonstration of this potential was carried out in the liver by Zhou, et al. in 2008⁴ followed by two more recent demonstrations of cardiac fibroblast transdifferentiation into cardiomyocytes in vivo^{5,6}. These latter studies underscore the potential and impact of this approach to heart regeneration. Additionally, the reprogramming efficiencies achieved in vivo were higher than those achieved in vitro^{7,8}, suggesting that some elements of the microenvironment actively influence the transdifferentiation process. In the context of stem cell differentiation, it is well established that some physical features of cells and their microenvironment can dictate cell fate, including cell shape, matrix rigidity, ligand density, traction forces, and micro- and nano-topography^{9–14}. However, the roles of the microenvironment during transdifferentiation or cell reprogramming are unknown.

Natural examples of transdifferentiation have been observed in urodele amphibians, and the process of lens regeneration in the newt is an often cited example. In this natural process of transdifferentiation, pigment epithelial cells first undergo dedifferentiation, and then go through a developmental program to differentiate into new lens cells¹⁵. This two-step process of dedifferentiation and/or differentiation is also observed in axolotl and newt limb regeneration. Efforts are ongoing to identify the activation signals, cellular sources, mitogens, and positional cues that drive these natural regenerative processes in order to understand and apply the same principles to humans¹⁶. Of particular interest, some studies have reported significant upregulation of pluripotency or reprogramming related genes like Sox2, Klf4, cMyc, and Sall4 in the blastema of regenerating limbs, which suggests that the cells in the blastema might be dedifferentiating towards a partially pluripotent cell phenotype^{17,18}. In 2010, Efe et al. described an indirect reprogramming method whereby the expression of the OSKM transcription factors were regulated temporally via inducible control to generate partially reprogrammed cells, which were then directed through a developmental program into cardiomyocytes¹⁹. Like the natural transdifferentiation process described above, this indirect reprogramming method also involved a two-step process of dedifferentiation and directed differentiation. While cardiomyocytes¹⁹, angioblast-like progenitors²⁰, and neural progenitors²¹ are so far the only cell types derived using indirect reprogramming strategies, the approach appears robust and poised to yield a broad spectrum of cell lineages in the near future.



One key element of the microenvironment that has yet not been investigated in the context of either direct or indirect reprogramming strategies is the extracellular matrix (ECM). Numerous studies over the past two decades have repeatedly demonstrated an instructive role for the ECM in the determination of cell fate^{9–12}, so we hypothesized that matrix factors would influence reprogramming as well. To examine a potential role for the ECM during cell transdifferentiation, we fabricated hydrogel substrates based on the natural protein biopolymers fibrin and type-I collagen, and assessed their ability to modulate the indirect cardiac reprogramming process originally developed by Efe et al.¹⁹. This reprogramming process can be divided into distinct dedifferentiation and differentiation phases (Fig. 1). During these phases, we quantified the number of

colonies and the number of contractile colonies to reflect the dedifferentiation and subsequent cardiogenic differentiation efficiencies, respectively. In addition, we also examined a role for ECM mechanical properties and cell-generated traction forces during the reprogramming process, altering the RhoA-ROCK-myosin light chain signaling axis by co-transducing constitutively active or dominant negative RhoA mutant transgenes with the OSKM vector. The findings in this study show that matrix identity and traction forces exert a significant influence on indirect cardiac reprogramming. On the other hand, for the range of variation studied in this system, our data suggest that matrix mechanics, microstructure and cell proliferation play lesser roles on the indirect reprogramming process.

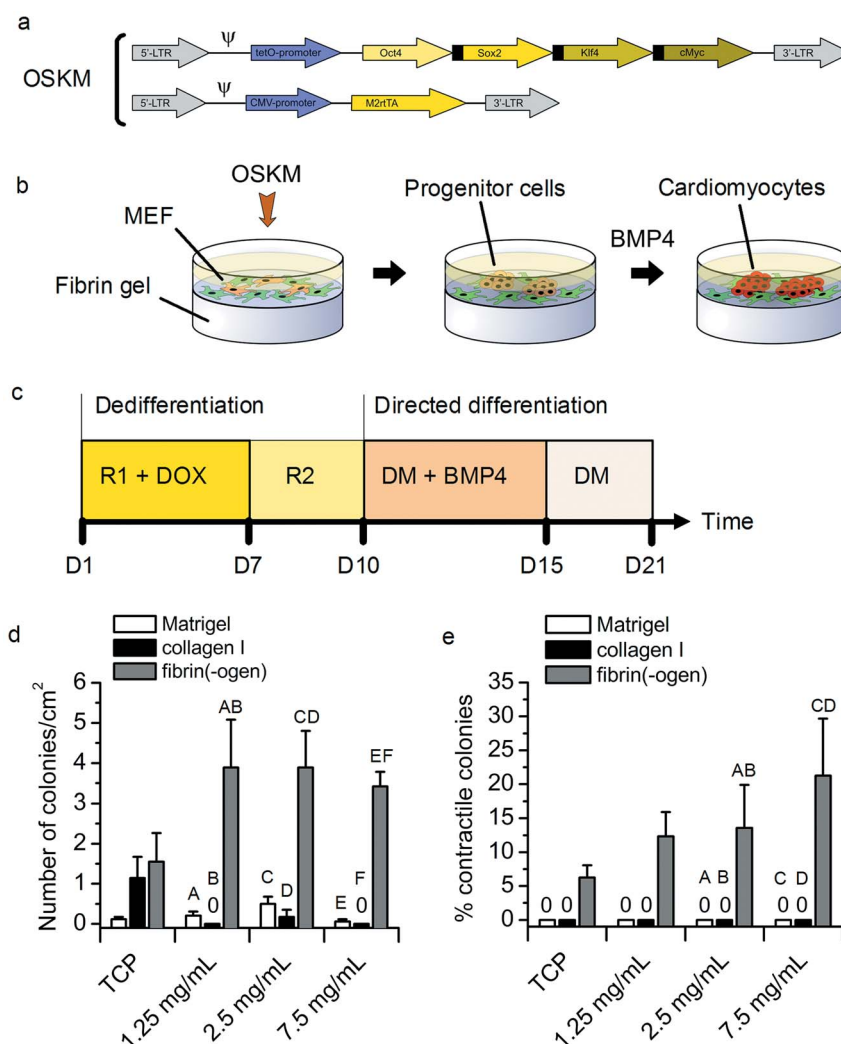


Figure 1 | Fibrin gels support indirect cardiac reprogramming better than Matrigel or collagen I gels. (a) A tetracycline inducible polycistronic Oct4-Sox2-Klf4-cMyc lentiviral vector and a tetracycline responsive transactivator lentiviral vector, collectively represented by the abbreviation OSKM, are transduced into cells in the indirect cardiac reprogramming protocol. (b) The indirect cardiac reprogramming protocol begins with plating of primary mouse embryonic fibroblasts (MEFs) on natural extracellular matrix (ECM) gels (depicted here is a fibrin gel) and transduced with the OSKM vectors. Dedifferentiating MEFs, in beige, begin to proliferate and form progenitor cell colonies. The progenitor cells are then differentiated into cardiomyocytes with BMP4. (c) Different cell culture media were used during the cardiac reprogramming process. Reprogramming medium 1 (R1) supplemented with doxycycline (2 μ g/mL) was used from day 1 to day 7 to initiate dedifferentiation. Reprogramming medium 2 (R2) was used to expand the progenitor cells from day 8 to day 10. Differentiation medium (DM) supplemented with BMP4 (10 ng/mL) was used to differentiate the progenitor cells into cardiomyocytes from day 11 to day 15. Total numbers of colonies per cm² (d) and percentage of contractile colonies (e) obtained from indirect cardiac reprogramming on Matrigel, collagen I and fibrin gels of various protein concentrations. Tissue culture plastic (TCP) coated with either Matrigel, collagen I, or fibrinogen was used as a control. The ANOVA tests on data in (d) and (e) indicate highly significant differences ($p < 0.0001$) across substrates of different identities. Matched symbols denote significant pair-wise differences ($p < 0.05$). Error bars represent \pm s.e.m., $n = 3$ independent experiments.



Results

To address the potential roles of the ECM on indirect cardiac reprogramming, we selected natural ECM proteins that could be formed into 2-D hydrogels. Working with these tractable 2-D hydrogel systems allow several aspects of the extracellular matrix to be changed. For example, the mechanical properties of the matrix can be modulated by changing the protein concentration in the hydrogel, and the identity of the matrix can be varied by incorporating more than one ECM protein in different ratios. The natural ECM proteins selected for this study were Matrigel, collagen I, and fibrin. Prior studies on iPSC reprogramming had used Matrigel or collagen I to support the reprogramming process, so we reasoned that hydrogels fashioned from these two materials should support indirect cardiac reprogramming^{22,23}. Indeed, Matrigel had been demonstrated to support indirect cardiac reprogramming by Efe et al.¹⁹ Fibrin, on the other hand, has never been used to support reprogramming. However, fibrin was selected based on studies that showed it could mitigate the deterioration of cardiac function when injected into the infarcted myocardium^{24,25}. Potentially, the cardio-protective effects of fibrin might enhance cardiac regeneration if the reprogramming factors were introduced via a fibrin delivery system.

Cardiac reprogramming is more efficient on fibrin gels. We first performed a comparison between Matrigel, collagen I, and fibrin to determine which of these substrates could support the indirect cardiac reprogramming process. Fibrin gels readily supported both the dedifferentiation (indicated by number of colonies/cm², Fig. 1d) and cardiac differentiation (indicated by the % of contractile colonies, Fig. 1e) phases of the reprogramming process. Surprisingly, indirect reprogramming on Matrigel and collagen I gels in our hands was very poorly efficient, generating very small numbers of colonies (Fig. 1d) that were not contractile (Fig. 1e). When the fibrin gel concentrations were varied from 1.25 to 7.5 mg/mL, no significant differences in dedifferentiation or cardiac reprogramming efficiencies occurred (Fig. 1d, e). However, an increasing trend for cardiac reprogramming efficiency was observed with increasing fibrin gel concentration (Fig. 1e). Tissue culture plastic (TCP) coated with the various ECM proteins did not yield significant differences in terms of dedifferentiation efficiency (Fig. 1d), but only fibrinogen-coated TCP supported cardiac reprogramming (Fig. 1e).

Thrombin activated cell proliferation does not account for the enhanced cardiac reprogramming on fibrin gels. During the process of cardiac reprogramming, we observed that the MEFs were proliferating at different rates on the various gels. When the proliferation of MEFs cultured on 2.5 mg/mL gels of different identities was quantified, cells cultured on fibrin proliferated at a significantly higher rate than those cultured on either Matrigel or collagen I gels, and also higher than those cultured on fibrinogen-coated TCP (Fig. 2a). A prior study with induced pluripotent stem cells (iPSCs) suggested that dedifferentiation is a stochastic process that can be accelerated by cell proliferation²⁶, and so we investigated whether the more efficient cardiac reprogramming observed on fibrin gels could be explained by the enhanced rate of cell proliferation. Furthermore, it has been shown that thrombin in fibrin clots retains its activity and consequently enhances cell proliferation^{27,28}. Therefore, we focused on a role for thrombin in the cardiac reprogramming process.

First, we ascertained that the enhanced cell proliferation rate was indeed a result of the presence of thrombin by performing a proliferation assay of MEFs cultured on 2.5 mg/mL fibrin gels that had previously been soaked in PBS for a week, and of MEFs cultured on fibrinogen-coated TCP with 1 U/mL thrombin supplementation. When compared with the fibrin data, PBS-soaked fibrin gels supported significantly lower rates of MEF proliferation due to the removal of thrombin by the PBS soak (Fig. 2b). Supplementing thrombin to the culture medium of MEFs growing on fibrinogen-coated TCP stimulated significant increases in cell proliferation, but

not until day 7 (Fig. 2b). Thus, thrombin supplementation alone is not sufficient to explain the increased proliferation rates observed when culturing cells on fibrin gels. Furthermore, while thrombin supplementation also improved both the dedifferentiation and cardiogenic differentiation efficiencies of the reprogramming process when MEFs were cultured on fibrinogen-coated TCP, the differences observed +/- thrombin were not significant (Fig. 2c, d). Additionally, reprogramming on fibrin gels pre-soaked in PBS was not significantly different from reprogramming on the fibrin controls (Fig. 2e, f). Taken together, these data suggest that thrombin's effects on MEF proliferation are not the predominant determinant of the superior indirect cardiac reprogramming achieved on fibrin gels.

We also quantified the percentage of cells adherent to the gels and the spread cell areas on day 1 after initial seeding to determine if differences in these parameters correlate with the differential support of reprogramming observed in Fig. 1. Cells spread to the same degree on all of the different gels (Fig. 2g), thereby ruling out any potential effects from cell spreading. Quantification of initial cell adhesion revealed that a lower percentage of cells were adherent on day 1 on collagen I and fibrin soaked in PBS gels compared to the other gels (Fig. 2h). However, both Matrigel and collagen I gels support reprogramming to a much lower degree than fibrin gels (Fig. 1d, e), despite the fact that Matrigel and fibrin support cell adhesion equally (Fig. 2h). On the other hand, collagen I gels and fibrin gels soaked in PBS both show reduced adhesion relative to other conditions (Fig. 2h), but reprogramming on the fibrin + PBS soaked gels is equivalent to that on fibrin (Fig. 2e and 2f). Therefore, differences in initial adhesion did not correlate with the differences in reprogramming efficiencies.

Cardiac reprogramming is further enhanced by ascorbic acid. The results from the comparison of the various ECM gels validated fibrin as a supportive substrate for cardiac reprogramming. To further improve cardiac reprogramming on fibrin gels, we supplemented the media with ascorbic acid. Ascorbic acid had been used as a cardiogenic molecule in differentiating human embryonic stem cells²⁹, and it was also used to improve the efficiency of induced pluripotent stem cell derivation³⁰. We thus postulated that supplementing our reprogramming media with ascorbic acid might increase both the number of contractile and total colonies. Quantifying the number of colonies per cm² and the percentage that were contractile revealed that ascorbic acid increased the number of contractile colonies, but not the total number of colonies (Fig. 3a, b). This implies that ascorbic acid enhances the cardiac differentiation phase of the two-step reprogramming process, rather than the initial dedifferentiation phase. For an individual substrate, enhancement achieved by ascorbic acid supplementation was only significant (compared to unsupplemented reprogramming) on 2.5 mg/mL and 7.5 mg/mL fibrin gels. However, ascorbic acid supplementation significantly enhanced cardiac reprogramming on fibrin gels across the board relative to that achieved on fibrinogen-coated TCP. Ascorbic acid supplementation on fibrin gels also resulted in larger contractile colonies (Supplementary movies S1 and S2) and all contractile colonies (with and without ascorbic acid supplementation) immunocytochemically stained for sarcomeric alpha-actinin and cardiac troponin I (Fig. 3c and d). Non-contractile colonies did not stain for these markers.

We used qPCR to examine the temporal expression profiles of selected pluripotent genes, early cardiac genes, and late cardiac genes at discrete time points that included different phases of the reprogramming process. The expression levels for Oct4 were very high for transduced cells at early time points (day 4 and day 8) since we induced expression of Oct4 exogenously (Fig. 4a). However, high levels of Oct4 expression persisted for up to 8 days more (days 12 and 16), suggesting that expression of endogenous Oct4 had been stimulated. In contrast, the expression levels of endogenous Nanog

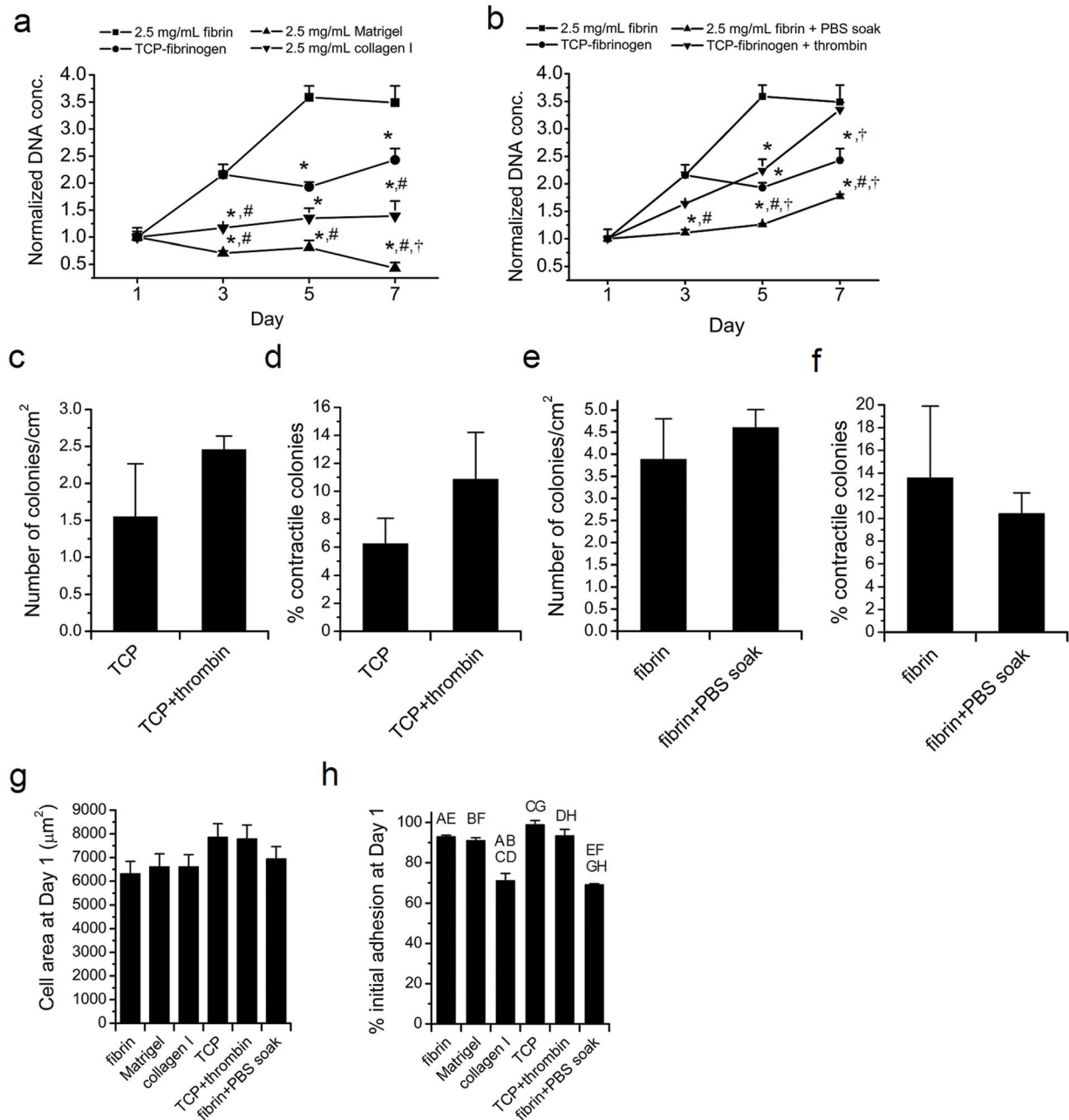


Figure 2 | Cell proliferation is enhanced by thrombin in fibrin gels, but does not fully explain fibrin's superior support of indirect cardiac reprogramming. (a) Cell proliferation of MEFs determined by DNA concentration for cultures on fibrinogen-coated TCP (●) and 2.5 mg/mL Matrigel (▲), fibrin (■), and collagen I (▼) gels. The ANOVA test indicates significant differences across the different substrates ($p < 0.0001$). * Significance between TCP-fibrinogen or Matrigel or collagen I gels, and fibrin gels ($p < 0.05$). # Significance between Matrigel or collagen I gels, and TCP-fibrinogen ($p < 0.05$). † Significance versus collagen I ($p < 0.05$). (b) Cell proliferation of MEFs cultured on TCP-fibrinogen supplemented with 1 U/mL thrombin (▼) and 2.5 mg/mL fibrin gels pre-soaked in PBS for 1 week (▲). The ANOVA test indicates significant differences across the different substrates ($p < 0.0001$). * Significance versus fibrin gels ($p < 0.05$). # Significance versus TCP-fibrinogen ($p < 0.05$). † Significance versus TCP-fibrinogen supplemented with thrombin ($p < 0.05$). No significant differences in the total number of colonies per cm² (c) and percentage of contractile colonies (d) were observed between fibrinogen-coated TCP and fibrinogen-coated TCP supplemented with thrombin conditions. No significant differences in the total number of colonies per cm² (e) and percentage of contractile colonies (f) were observed between fibrin and PBS pre-soaked fibrin conditions. No significant differences for cell area at day 1 were observed across the different matrices (g), $n = 100$. Significant differences were however observed for the percentage of cells that initially adhered at day 1 (h) ($p < 0.0001$ via ANOVA). Matched symbols denote significant ($p < 0.05$) differences. Error bars represent \pm s.e.m., $n = 3$ independent experiments unless indicated otherwise.

gradually increased to a peak at day 12, and then decreased to sub-baseline levels by day 16 (Fig. 4a). The expression profiles of Oct4 and Nanog indicated that 7 days of OSKM transgene expression on fibrin gels was sufficient to induce dedifferentiation of the cells but incomplete reprogramming to an iPSC phenotype since the expression of Nanog was not sustained.

The expression profiles of Mesp1, a cardiac mesoderm transcription factor, and Gata4, a transcription factor involved in the formation of the heart tube, were also examined with qPCR. There was a sharp increase in expression of Mesp1 observed at day 12 (Fig. 4b), presumably due to the directed differentiation initiated at day 10 (Fig. 1c). The expression levels of Mesp1 at day 12 across the condi-

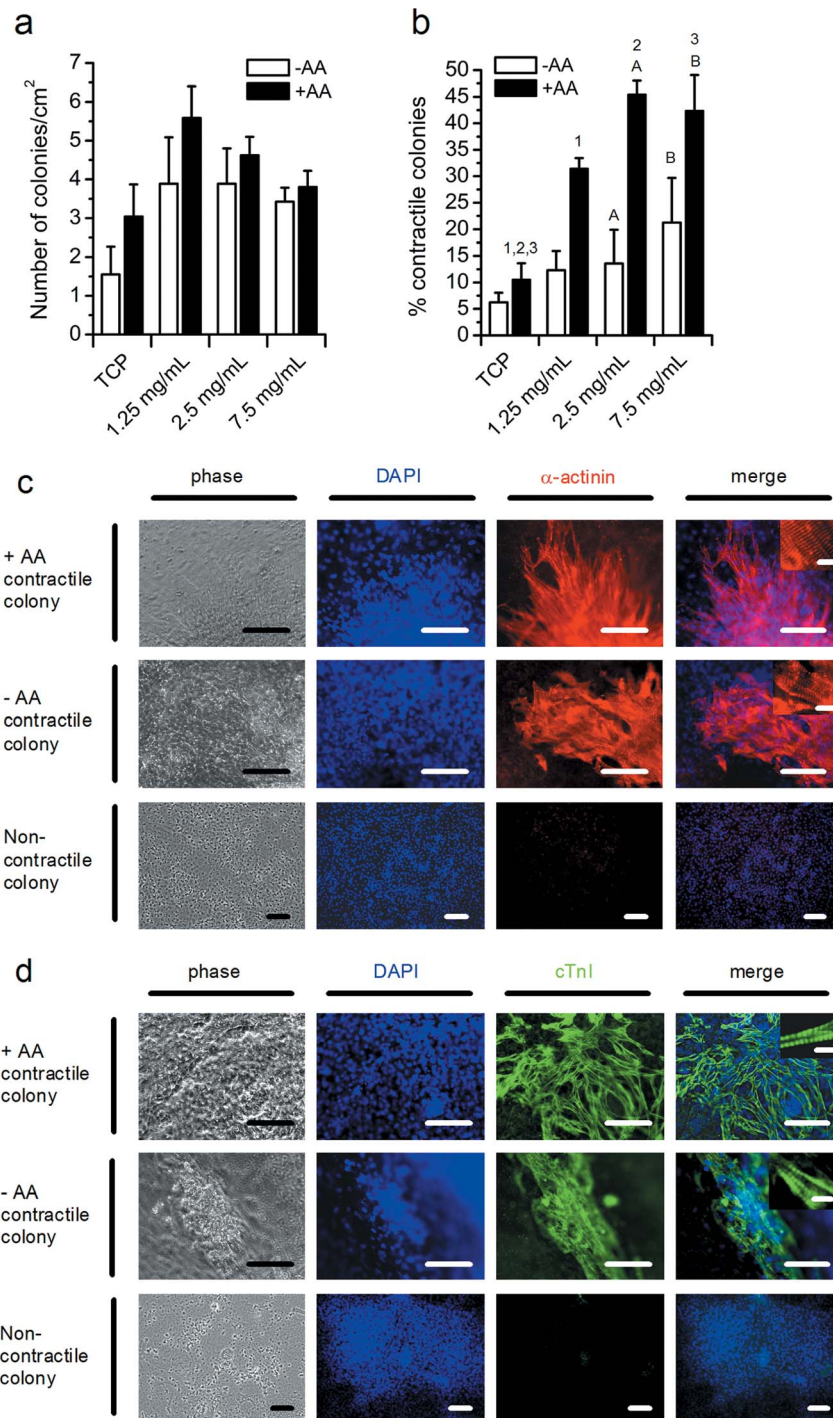


Figure 3 | Ascorbic acid further enhances indirect cardiac reprogramming on fibrin gels. (a) No significant differences in the total number of colonies per cm^2 were found between cultures supplemented with ascorbic acid (+AA) versus those without (−AA) (mean \pm s.e.m. for $n = 3$ independent experiments). (b) The number of contractile colonies increased with ascorbic acid supplementation when reprogramming was performed on fibrin gels. TCP substrates in both (a) and (b) were coated with fibrinogen. The ANOVA test indicates significant differences between treatment conditions (−AA vs. +AA, $p < 0.0001$) and between different protein concentrations ($p = 0.0015$). Matched symbols denote significant ($p < 0.05$) differences via pair-wise post-hoc analysis. Error bars represent \pm s.e.m., $n = 3$ independent experiments. The reprogrammed MEFs were examined by immunocytochemical staining of cell nuclei (DAPI), cardiac troponin I (cTnI) (c), and sarcomeric α -actinin (α -actinin) (d). Higher magnification insets show characteristic cardiac striations. Scale bars 100 μm , inset scale bars 5 μm .

tions were significantly different ($p = 0.03$); however the sample sizes were insufficient to determine significant pair-wise differences. At day 16, the levels of *Mesp1* for fibrin gels dropped to baseline, whereas those for *Gata4* at both day 12 and day 16 were above baseline (Fig. 4b). These expression profiles for *Mesp1* and *Gata4*

recapitulate the sequential activation of transcription factors that occur during embryonic cardiac development^{31,32} suggesting that reprogramming on fibrin gels in the presence of ascorbic acid enhanced cardiogenesis on a molecular level. Surprisingly, we found that the expression levels of late cardiac genes such as *Myl7*, *Myl2*,

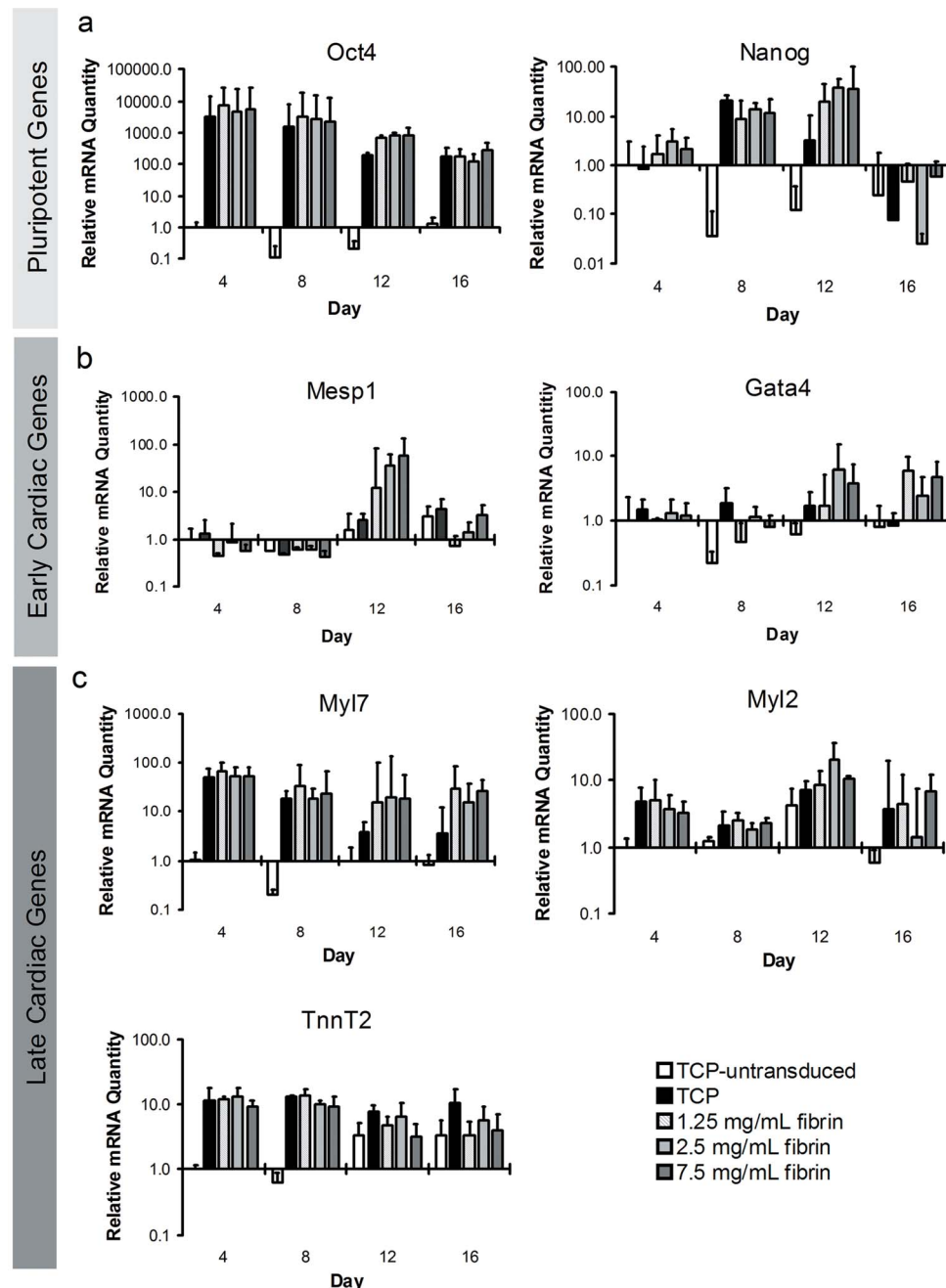


Figure 4 | MEFs undergoing indirect cardiac reprogramming on fibrin gels in the presence of ascorbic acid supplementation exhibit differential gene expression profiles. The expression profiles of MEFs cultured on various fibrin gels or fibrinogen-coated TCP were examined via qPCR at multiple time points, with the levels of (a) pluripotent genes (Oct4 and Nanog), (b) early cardiac genes (Mesp1 and Gata4), and (c) late cardiac genes (MyI7, MyI2 and TnnT2) assessed. * Significance versus TCP-fibrinogen. ANOVA indicates significant differences across groups in Mesp1 day 12 data ($p = 0.03$). Error bars represent \pm s.e.m., $n = 3$ independent experiments.

and TnnT2 were elevated throughout the cardiac reprogramming process (Fig. 4c). This suggests that reprogramming on fibrin gels with ascorbic acid supplementation primes the dedifferentiated cells towards the cardiac lineage.

Incorporating collagen I into fibrin replaces ascorbic acid. Several groups have suggested that induction of collagen synthesis by ascorbic acid is a key determinant of cardiogenesis^{33,34}. When the colonies that resulted from cardiac reprogramming on fibrin with ascorbic acid supplementation were stained for collagen I, the contractile colonies stained positive whereas non-contracting colonies were negative

(Fig. 5a). We postulated that ascorbic acid supplementation may be replaced by incorporating a small amount of collagen I into the fibrin gels. We began by making 75% fibrin-25% collagen I composite gels of various total protein concentrations and performed the cardiac reprogramming process with MEFs on the composite gels. Comparing the data between the composite gels with that of fibrin gels supplemented with ascorbic acid, we observed that the dedifferentiation efficiencies (number of colonies/cm²) were similar if not identical in both cases (Fig. 5b). The composite gels showed a gradual increase in cardiogenic efficiency (% contractile colonies) with increasing total protein concentration; at 7.5 mg/mL total protein



concentration, the composite gels achieved similar cardiogenic efficiencies similar to those achieved on pure fibrin gels supplemented with ascorbic acid (Fig. 5c).

We also made fibrin-collagen I composite gels with a constant 2.5 mg/mL total protein concentration, and varied the percentage of collagen I in those gels. Dedifferentiation efficiencies were similar and appeared not to depend on the amount of collagen I (Fig. 5d). However, for a fixed total protein concentration, the cardiogenic efficiency increased as the percentage of collagen I increased (Fig. 5e). In fact, the efficiency of cardiac reprogramming achieved on 2.5 mg/mL total protein concentration containing 75% collagen I and 25% fibrin was similar to that achieved with ascorbic acid supplementation.

Coupled with the results in Fig. 5c, these data suggested that the total amount of collagen I in the composite gels, rather than the amount relative to fibrin, determined the efficiency of cardiogenic reprogramming on the composite gels. To further test this idea, we also made fibrin-collagen I composite gels with a constant 7.5 mg/mL total protein concentration and again varied the percentage of collagen I. We then combined the data from this experiment with those from the other experimental conditions (1.25 mg/mL and 2.5 mg/mL total protein concentrations) on a single graph (Fig. 5f). These data illustrate that there is an optimal concentration of collagen I (~1.8 mg/mL) that makes the gels highly cardiogenic. Concentrations above and below this optimum decrease the cardiogenic efficiency of the gels.

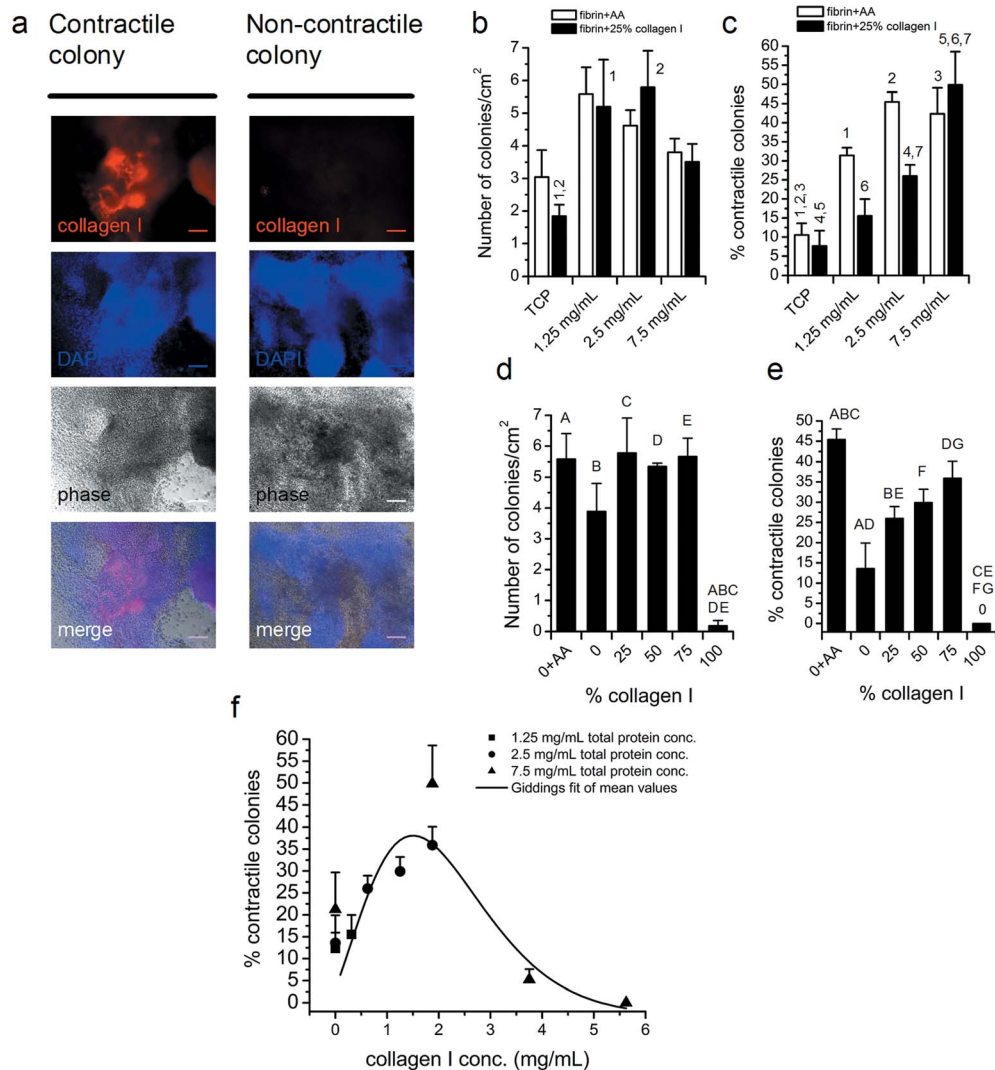


Figure 5 | Incorporation of collagen I into fibrin gels replaces ascorbic acid (AA) supplementation. (a) Colonies were examined by immunocytochemical staining for collagen I. Contractile colonies stained positive for collagen I, whereas non-contractile colonies did not. Total number of colonies per cm² (b) and percentage of contractile colonies (c) for reprogramming on fibrin-25% collagen I composite gels compared to fibrin gels with AA supplementation. The reprogramming efficiency on fibrin-25% collagen I composite gels approached that of fibrin gels + AA at 7.5 mg/mL total protein concentration. TCP substrates were coated with either fibrinogen or a 75:25 fibrinogen-collagen I solution. The total numbers of colonies (b) were not significantly different between the +AA and +25% collagen I conditions via ANOVA ($p = 0.77$). However, the differences in the % contractile colonies (c) were significant between the +AA and +25% collagen I conditions and across protein concentrations via ANOVA ($p < 0.0001$). Matched symbols denote significant ($p < 0.05$) differences via pair-wise post-hoc analysis. Increasing the percentage of collagen I in 2.5 mg/mL total protein concentration composite gels did not increase the total number of colonies per cm² (d), but did increase the percentage of contractile colonies (e). The reprogramming efficiency of MEFs cultured on 2.5 mg/mL composite gels containing 75% collagen I approached that achieved on pure fibrin gels + AA. ANOVA on (d) ($p = 0.0006$) and (e) ($p < 0.0001$) indicate significant differences across groups. Matched symbols denote significant ($p < 0.05$) differences via pair-wise post-hoc analysis. (f) Percentage of contractile colonies obtained from MEFs reprogrammed on various hydrogels composed of 1.25, 2.5, and 7.5 mg/mL total protein concentration. The data suggest an optimal collagen I concentration for the composite gels. Line plot is a Giddings fit of the mean values at a collagen I concentration. Error bars represent \pm s.e.m., $n = 3$ independent experiments.



Given these data showing the important role of collagen I in our improved reprogramming efficiencies, we returned to our earlier observation that MEFs on collagen I gels did not undergo reprogramming. Based on anecdotal observations, we postulated that large traction forces exerted by the MEFs on collagen I resulted in matrix remodeling and compaction, which in turn impeded reprogramming (or at least our ability to assess it). To address this hypothesis, a reprogramming experiment in which the collagen I gels were crosslinked with a 0.25% glutaraldehyde solution was performed. In this experiment, preventing the cells from compacting the collagen I matrix resulted in improved dedifferentiation and cardiogenesis. However, the dedifferentiation and cardiogenic efficiencies of MEFs cultured on these glutaraldehyde crosslinked collagen I gels were still significantly below that of ascorbic acid supplemented fibrin gels (Fig. 6). This may be due to the glutaraldehyde crosslinking destroying adhesion sites or signaling domains in collagen I, and consequently inactivating these active sites. The fibrin-collagen I composite gels may provide an alternative method of crosslinking collagen I that preserves the biological activity of the collagen molecule. This could potentially explain both the high dedifferentiation and cardiogenic efficiencies observed with the fibrin-collagen I composite gels.

Differences in matrix rigidity and microstructure are not sufficient to explain the enhanced cardiac reprogramming observed on fibrin gels. We investigated if the enhanced cardiac reprogramming observed on fibrin and fibrin-collagen I gels relative to pure collagen gels was simply due to differences in the mechanical properties of the gels. Quantification of the shear elastic moduli of the fibrin and collagen I gels showed how changing total protein concentration affected gel mechanical properties, and that there were in fact significant differences in the shear moduli values of the fibrin and collagen I gels for a particular protein concentration (Fig. 7a). However, despite very significant differences in the differential abilities of fibrin and collagen to support reprogramming (Fig. 1d, e), the ranges of shear moduli for the different concentrations of fibrin (9.5–212.4 Pa) and collagen I (3.6–283.9 Pa) gels used in this study were quite similar. Moreover, cardiogenic reprogramming efficiencies on fibrin gels in either the absence or presence of ascorbic acid did not vary significantly as a function of protein

concentration (Fig. 3b), even though these gels possessed a five fold difference in shear modulus (Fig. 7a). Finally, the cardiogenic reprogramming efficiency on fibrin-collagen I composite gels varied with composition (Fig. 5e) whereas the shear moduli of these gels are within the same order of magnitude (Fig. 7b).

Collagen I, fibrin, and fibrin-collagen I gels are all fibrillar in nature, and their microstructures and mechanical properties are intimately linked to the final protein concentrations of the gels. Therefore, changing the total protein concentrations and the different collagen I to fibrin ratios in the composite gel not only affects the mechanical properties of the gels, but also the microstructural features. Qualitative assessment of the microstructures of fibrin, collagen I, and various fibrin-collagen I composite gels (all of 2.5 mg/mL total protein) revealed them to be similar (Fig. 7c), incongruous with the significant differences and cardiogenic reprogramming efficiencies (Fig. 5e). Collectively, these data suggest that matrix rigidity and microstructure are insufficient to explain the observed differences in cardiac reprogramming as a function of material identity and composition, at least for the range of materials used here.

Traction forces modulate dedifferentiation during cardiac reprogramming. Previous studies have shown that cells cultured on different matrices exert different levels of traction forces³⁵. We hypothesized that the response to matrix factors observed during cardiac reprogramming is mediated by traction forces. To address this hypothesis, we co-transduced tetracycline-inducible RhoA mutant vectors (Fig. 8a) with the tetracycline-inducible OSKM vector to modulate the traction forces exerted by a cell during the reprogramming process. The constitutively-active RhoA mutant (RhoA-Q63L) increases traction forces in a cell when expressed, and conversely the dominant-negative RhoA mutant (RhoA-T19N) decreases traction forces when expressed³⁶. A tetracycline inducible eGFP control vector was used to account for any non-specific effects of additional lentiviral infection. Efforts to reprogram cells expressing the RhoA-Q63L mutant showed that increasing the traction forces exerted by a cell significantly decreased dedifferentiation efficiency. On the other hand, decreasing cell traction, with the RhoA-T19N mutant, slightly increased dedifferentiation efficiency (Fig. 8b). Cardiogenic efficiency however was not significantly affected by the modulation of traction forces during the dedifferentiation phase (Fig. 8c). Furthermore, ectopic expression of the RhoA mutants did not significantly influence proliferation rates (Fig. 8d), thus suggesting that the observed differences in dedifferentiation efficiency induced by the RhoA mutants (Fig. 8b) were not due to differences in proliferation.

Discussion

Reprogramming of somatic cells toward a specific lineage represents an attractive approach for regenerative medicine that could theoretically enable resident cells in situ to be induced to regenerate tissues and organs damaged by disease. Such an approach would obviate the need for transplanted cells, the numerous challenges associated with identifying an appropriate cell source, and potentially speed translation to the clinic. The powerful potential of this approach is arguably best exemplified in recent studies in which cardiac fibroblasts were reprogrammed into cells with functional cardiomyocyte-like properties that reduced scar formation and partially restored cardiac output^{5,6}. While most of the attention in the reprogramming literature has focused on the role of specific genetic factors and the epigenetic state of the cells, it has also been observed that reprogramming efficiencies are significantly higher in vivo than in vitro⁵, suggesting that elements of the cardiac microenvironment influence the reprogramming process. Our goal in this study was to focus on the role of the ECM in cardiac reprogramming in order to better understand the key features of the cellular microenvironment that influence this process.

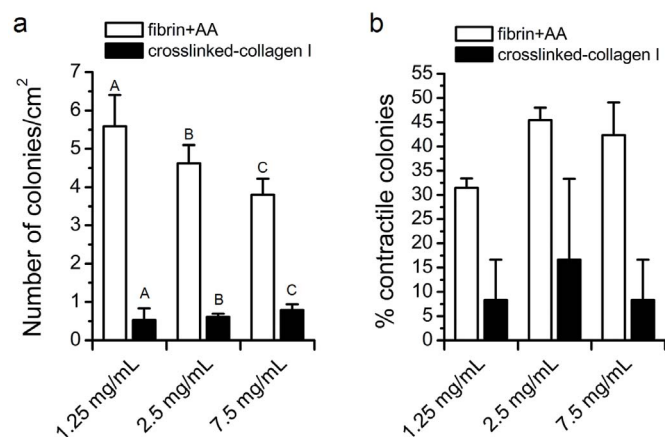


Figure 6 | Indirect cardiac reprogramming is attainable, albeit poor, on crosslinked-collagen I gels. The total number of colonies per cm² (a) and percentage of contractile colonies (b) show that reprogramming efficiencies on crosslinked-collagen I gels do not approach that fibrin with AA supplementation. ANOVA on (a) and (b) indicates significant differences between fibrin + AA and crosslinked collagen I groups ($p < 0.0001$ for a and $p = 0.0019$ for b). Matched symbols denote significant ($p < 0.05$) differences via pair-wise post-hoc analysis. Error bars represent \pm s.e.m., $n = 3$ independent experiments.

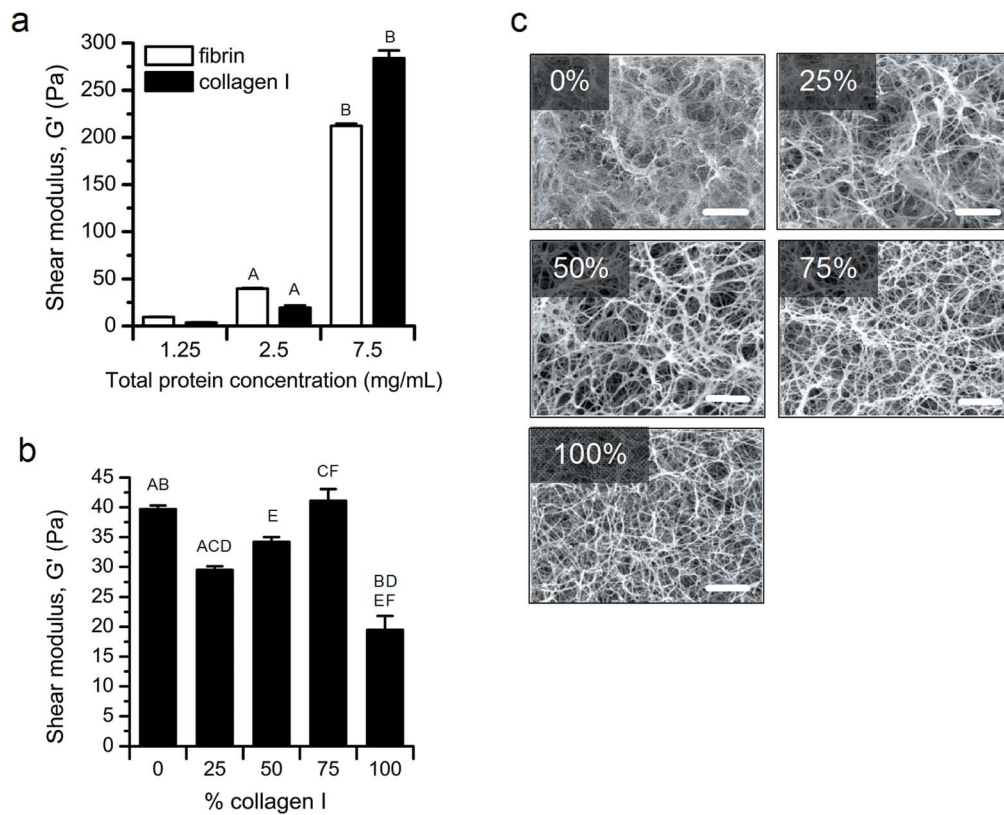


Figure 7 | Matrix modulus and microstructure do not account for differences in indirect cardiac reprogramming observed on different substrates. (a) Shear modulus of fibrin and collagen I gels of various total protein concentrations. ANOVA indicates significant differences between fibrin and collagen I gels ($p = 0.0002$). Matched symbols denote significant ($p < 0.05$) differences via pair-wise post-hoc analysis. (b) Shear modulus of 2.5 mg/mL fibrin-collagen I composite gels with various percentages of collagen incorporated. ANOVA indicates significant differences between groups ($p < 0.0001$). Matched symbols denote significant ($p < 0.05$) differences via pair-wise post-hoc analysis. Error bars represent \pm s.e.m., $n = 3$ independent experiments. (c) Matrix microstructure examined by scanning electron microscope of the various 2.5 mg/mL fibrin-collagen I composite gels.

Using simple hydrogel substrates made from naturally-derived proteins and an indirect cardiac reprogramming protocol that utilizes the same 4 factors originally used to create iPSCs, we found that ECM identity and cell-generated traction forces play major roles in reprogramming MEFs into cardiomyocyte-like cells. Fibrin and fibrin-collagen composite substrates supported cardiac reprogramming much more efficiently than either pure collagen I or Matrigel substrates. Systematically varying the concentration of the protein matrices and characterizing their physical properties revealed that the ECM's mechanical properties and microstructure play lesser roles in the reprogramming process, as does the degree of cell proliferation.

In dissecting why fibrin and fibrin-collagen substrates offer better support for cardiac reprogramming, we initially discovered that MEFs proliferate to a greater degree on fibrin gels than they do on collagen I, Matrigel, or fibrinogen-coated TCP. In the induced pluripotent stem cell (iPSC) field, cell proliferation has been demonstrated to accelerate the formation of iPSC colonies²⁶. Since iPSC reprogramming factors were used in our cardiac reprogramming protocols, elevated cell proliferation could also potentially accelerate the dedifferentiation process. Our findings suggested that thrombin was responsible for the enhanced proliferation observed when cells were cultured on fibrin gels. Thrombin, a known mitogenic agent, has been shown to increase cell proliferation through activation of the p38 MAP kinase pathway^{37,38}. Additionally, activation of the p38 MAP kinase pathway by NaCl supplementation has been shown to increase iPSC reprogramming efficiency³⁹. However, although both dedifferentiation and cardiogenic efficiency were increased when thrombin was supplemented during reprogramming on TCP, the

increase was not significant (Fig. 2c, d). Additionally, when thrombin was leached out of fibrin gels, both dedifferentiation and cardiogenic efficiency did not change significantly (Fig. 2e, f). When considered together, these data suggest that thrombin stimulated increases in cell proliferation are not the major reason for the observed enhancement of cardiac reprogramming on fibrin gels. Furthermore, since the generation of the intermediate progenitor-like colonies was not accelerated by an increase in thrombin-stimulated cell proliferation (Fig. 2c), our data suggest that the cells derived in this reprogramming protocol are likely not iPSCs.

In previous studies, ascorbic acid was found to reverse cellular senescence and thereby enhance iPSC reprogramming efficiency as observed by a significant increase in the number of colonies³⁰. However, in our studies, supplementation of ascorbic acid did not significantly increase the number of colonies, but rather significantly increased the efficiency with which those partially reprogrammed colonies were converted into beating contractile ones on fibrin gels (Fig. 3b). We postulated that the ascorbic acid induced synthesis of collagen I by the fibroblasts, and that the presence of this new collagen I accounts for the enhancement in cardiogenic efficiency induced by ascorbic acid treatment. In support of this hypothesis, a composite matrix containing both fibrin and collagen I was found to support enhanced cardiogenic reprogramming without ascorbic acid supplementation. More importantly, these data imply that matrix identity is critically important in determining cell fate during indirect reprogramming. Regardless of the overall total protein concentration or the ratio of fibrin to collagen I in our composite gels, we found that, an optimum concentration of collagen I (~ 1.8 mg/mL) was required for maximum cardiogenic efficiency (Fig. 5f).

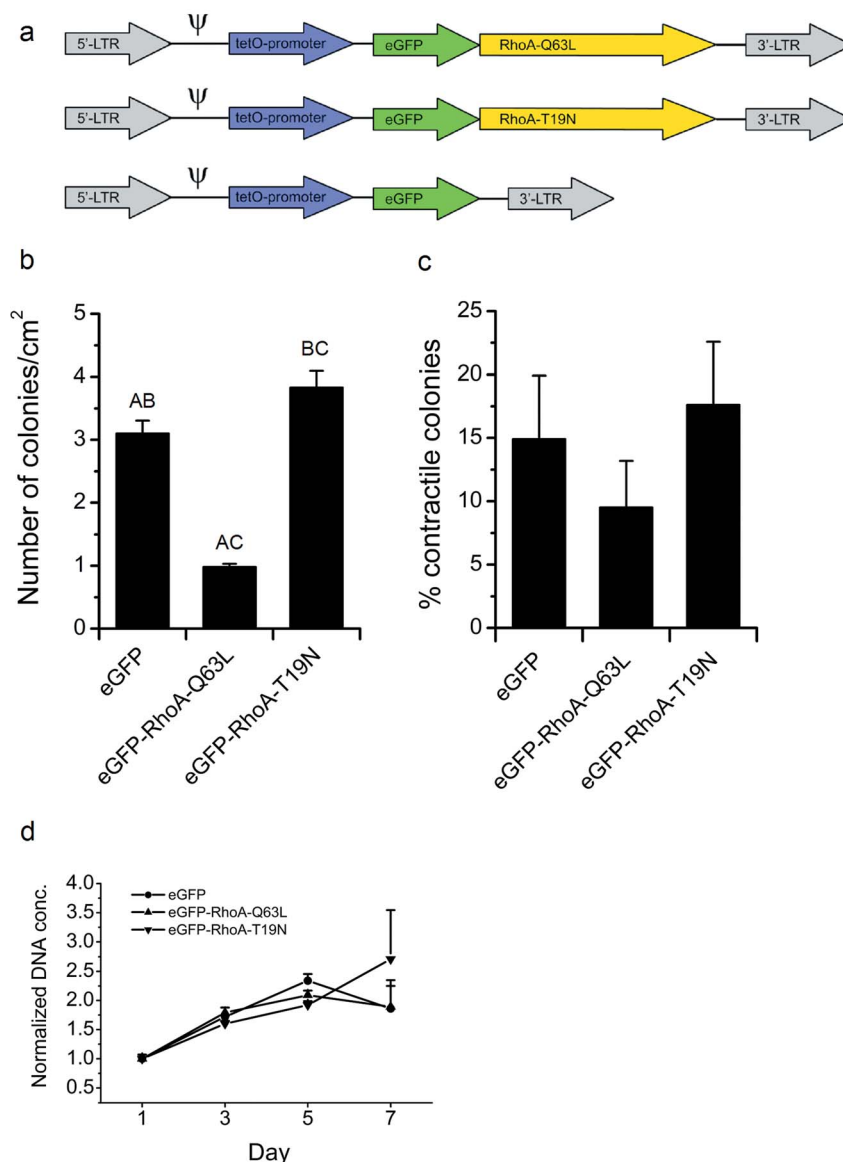


Figure 8 | Traction forces play a significant role in cell dedifferentiation during indirect cardiac reprogramming. (a) The RhoA mutant lentiviral vectors were co-transduced with the OSKM vectors to investigate the role of traction forces. RhoA-Q63L is a constitutively active RhoA mutant and RhoA-T19N is a dominant negative RhoA mutant. (b) Expression of RhoA-Q63L during the dedifferentiation phase of reprogramming significantly decreased the total number of colonies obtained. By contrast, expression of RhoA-T19N significantly increased the total number of colonies. ANOVA indicates significant differences across groups ($p < 0.0001$). Matched symbols denote significant ($p < 0.05$) differences via pair-wise post-hoc analysis. (c) Expression of the RhoA mutants during the dedifferentiation phase does not significantly shift the percentage of contractile colonies ($p = 0.46$ via ANOVA). (d) Proliferation rate of the different genotypes. No significant differences were detected via ANOVA ($p = 0.86$). Error bars represent \pm s.e.m., $n = 3$ independent experiments.

By incorporating collagen I into fibrin gels, our finding that the modulation of matrix identity affects the efficiency of cardiac reprogramming raises the question of whether collagen I or other collagens play a cardiogenic role during natural development. Collagens I, III, and IV have been found in murine and avian embryonic hearts^{40,41} but there is little evidence that these collagens play a role in the specification of cell fate in vivo. Collagen I, III, or IV knockout mice, although embryonic lethal, do not show an absence of cardiogenic differentiation^{42–44}. On the other hand, $\beta 1$ integrin-null mouse ES cells display an inability to differentiate into proper cardiac muscle phenotypes, indicating that interactions with the extracellular matrix through $\beta 1$ integrin are necessary for cardiogenic differentiation⁴⁵. This discrepancy in the roles of collagen and their receptors in vitro and in vivo implies that cardiogenic differentiation in vivo is substantially more robust than in vitro, likely due to the multiple

(and perhaps redundant) signaling cues intrinsic to the ECM. Nonetheless, these data suggest that collagen I (or other ECMs that bind to integrin dimers incorporating $\beta 1$) might play a major role in cardiogenesis. Since the density of ECM adhesive cues can alter the clustering of integrin receptors, which in turn can induce changes in downstream signals, ligand spatial presentation and mobility are important considerations as well, and are perhaps reflected in our observation of an optimal collagen I concentration to support reprogramming on fibrin-collagen composite substrates.

Besides the ECM's adhesive cues, its mechanical properties are another element that has repeatedly emerged as critical to engineer synthetic ECMs that can direct cell fate⁴⁶. The bulk mechanical modulus of the matrix has been correlated with changes in cell differentiation by several studies^{10,47,48}. Prior reports have also shown that substrates with elastic moduli values similar to that of the native



heart are optimal for supporting the contractile phenotype of embryonic cardiomyocytes from quail⁴⁹. However, recent evidence suggests that altering the spatial presentation of ECM adhesive ligands could recapitulate some effects previously attributed to changes in ECM modulus⁵⁰. In natural ECM gels, like the ones we have used here, matrix protein concentration and bulk mechanical properties are inextricably linked. Cells also locally remodel the matrix or exert tension to expose cryptic sites or cluster surrounding ligands^{13,51}. Thus, it is impossible to decouple all of these potentially instructive elements of the ECM using natural protein gels. Nevertheless, in our study, modulus per se did not appear to be a singular determinant of cardiac reprogramming efficiency since fibrin and collagen gels of comparable elastic moduli (Fig. 6) supported reprogramming to significantly differing degrees (Fig. 1). Instead, our data suggest that the ability of the cell to generate traction forces is crucial for cardiac reprogramming, and that the forces applied by wild-type MEFs were permissive for cardiac reprogramming. Inducing large traction forces through the expression of constitutively active RhoA decreased dedifferentiation and cardiogenic efficiency (Fig. 8b, c), and perhaps primed the dedifferentiated cells to a non-cardiac cell fate. By contrast, reducing traction forces through the forced expression of dominant negative RhoA increased dedifferentiation and led to more progenitor-like colonies (Fig. 8b). A similar traction force mediated bias was recently reported in the context of mesenchymal stem cell (MSC) differentiation, with high traction forces directing MSCs to an osteogenic lineage and low traction promoting their differentiation into an adipogenic lineage¹³.

Changes in ECM ligand identity, spatial distribution, and mechanical properties can all influence the state of traction within a cell, and thus further enhancements of cardiac reprogramming efficiencies may be realized by optimizing these elements. Our findings raise the question of whether or not optimal traction forces exist for the distinct phases of the reprogramming process (i.e., the initial dedifferentiation and subsequent differentiation). Reducing traction forces via the Rho kinase inhibitor Y27632 is already fairly standard practice to enhance the propagation of ES cells in culture⁵². Therefore, it is plausible that reprogramming into different lineages may potentially require a fairly uniform state of traction that can be readily optimized for the dedifferentiation process. However, the subsequent differentiation into different lineages will presumably require tuning traction forces according to the desired final cell and tissue type. Furthermore, the properties of the ECM that optimally support this second phase are likely to be different than those that best support the initial phase. The natural cardiac ECM present in adult higher vertebrates is likely optimized to support differentiated phenotypes. Likewise, the fibrotic ECM associated with the damaged myocardium following an MI may in fact represent a barrier to regenerative reprogramming. Therefore, in order for *in situ* reprogramming strategies to work optimally, it may be necessary to engineer new biomaterial substrates that recreate the natural developmental ECM. The ability to dynamically modulate the substrate to control cell fate determination^{53,54} may also be beneficial for reprogramming strategies as well. Such approaches have already shown some promise *in vitro* with respect to cardiomyocytes⁵⁵.

In summary, the present study has shown that fibrin and fibrin-collagen substrates are superior to Matrigel and collagen I in supporting indirect cardiac reprogramming. By deconstructing these natural ECMs into the specific elements that influence indirect reprogramming, we identified matrix identity and traction forces as the dominant determinants. It remains to be seen if transdifferentiation into other lineages can also be optimized by modulating these two key elements.

Methods

Mouse embryonic fibroblast (MEF) cell culture. MEFs were purchased from Millipore (Cat. #PMEF-NL) and expanded to passage 5 in MEF medium consisting of DMEM-Glutamax (Invitrogen Cat. #10569), 10% FBS (Invitrogen Cat. #10437), and

1× MEM NEAA (Invitrogen Cat. #11140). Prior to cell reprogramming, the MEFs were resuspended in freezing medium (90% FBS (Invitrogen Cat. #10437) and 10% DMSO (Sigma Cat. #. D2650)) and stored in liquid nitrogen.

Viral supernatant. A plasmid encoding a doxycycline inducible polycistronic Oct4-Sox2-Klf4-cMyc (OSKM) lentiviral vector (Addgene Cat. #20328) or encoding a doxycycline responsive transactivator (M2rTA) lentiviral vector (Addgene Cat. #20342) was transfected into 293FT cells (Invitrogen Cat. #R700) together with lentivirus packaging plasmids pLP1, pLP2, and pLP-VSVG (Invitrogen) using the Lipofectamine 2000 transfection reagent (Invitrogen Cat. #11668). The medium was changed 24 hours post-transfection and the supernatant collected 48 hours post-transfection. The virus containing supernatant was centrifuged at 3000 rpm at 4°C for 15 minutes. Aliquots of the supernatant were frozen and stored at -80°C.

Preparation of hydrogel substrates. Fibrinogen from bovine plasma (Sigma-Aldrich Cat. #F8630) was first dissolved in DMEM-Glutamax (Invitrogen Cat. #10569) at the desired protein concentration and then filtered with a 0.22 μm PES syringe filter. FBS (Invitrogen Cat. #10437) was then added to the filtered fibrinogen solution to a final concentration of 10%. 10 μL of thrombin from bovine plasma (Sigma-Aldrich Cat. #T6634) in ddH₂O of concentration (50 U/mL) was placed on the bottom of a well of a 12-well plate. 490 μL of the fibrinogen solution was then added to the bottom of the well, the contents mixed well and left to gel in the incubator at 37°C for 30 minutes.

Collagen I (high concentration, from rat tail; BD Cat. #354249) gels were prepared as recommended in the manual provided by the manufacturer. Briefly, the amounts of 10× PBS, 1 N NaOH, ddH₂O and collagen I to be mixed were first calculated based on the final concentration of collagen I and gel volume required using the equations in the manual. Next, ice cold 10× PBS, 1 N NaOH, and ddH₂O were mixed together in the amounts calculated. Collagen I was finally added to the mixture and 500 μL of the solution was placed into a well of a 12-well plate. The solution was left to gel in the incubator at 37°C for 30 minutes.

For fibrin-collagen I composite gels, the amounts of fibrinogen and collagen I were calculated based on the final gel volume, total protein concentration, and percentage of collagen I in the gel. Knowing the amount of collagen I permitted calculation of the amount of 10× PBS, 1 N NaOH, DMEM-Glutamax, and FBS (10%) using the formulas in the manufacturer's manual for collagen I. Fibrinogen was then dissolved in DMEM-Glutamax and filtered with a 0.22 μm PES syringe filter. The remaining components were then added in the following order: FBS, 10× PBS, 1 N NaOH, and collagen I. 10 μL of thrombin in ddH₂O (50 U/mL) was placed on the bottom of a well of a 12-well plate. 490 μL of the fibrinogen solution was then added to the bottom of the well, the contents mixed well and left to gel in the incubator at 37°C for 30 minutes.

Matrigel (BD Cat. #354230) gels were prepared as recommended in the manual provided by the manufacturer. Briefly, ice-cold stock Matrigel is diluted with ice-cold KnockOut DMEM/F-12 to the required protein concentration. The diluted Matrigel solution is then added to the bottom of the wells and left to gel in the incubator at 37°C for 30 minutes.

In all cases, hydrogels not used right away were kept hydrated by adding MEF medium into the wells and placing in the incubator.

Indirect cardiac reprogramming protocol. The reprogramming protocol used in this study was based on one previously developed by Efe et al.¹⁹. Frozen MEFs at passage 5 were thawed and seeded onto the various gels at a cell density of 3500 cells/cm² in MEF medium. After 6 hours (to allow cell attachment and spreading), cultures were incubated in MEF medium containing polybrene (Millipore Cat. #TR-1003-G) at a final concentration of 5 μg/mL. After another 30 minutes, the cells were transduced with viruses encoding OSKM and M2rTA at a MOI = 4. At 16 hours post-transduction, the medium was changed to reprogramming medium R1 comprised of KnockOut DMEM/F12 (Invitrogen Cat. #12660), 20% FBS (Invitrogen Cat. #10437), 4% KnockOut Serum Replacement (Invitrogen Cat. #10828), 1% Glutamax (Invitrogen Cat. #35050), 1% MEM NEAA (Invitrogen Cat. #11140), 0.1 mM of β-mercaptoethanol (Invitrogen Cat. #21985), and 2 μg/mL doxycycline (Sigma-Aldrich Cat. #D9891). R1 medium was changed everyday until day 7 and was replaced with reprogramming medium R2 comprised of KnockOut DMEM/F12, 1% FBS, 14% KnockOut Serum Replacement, 1% Glutamax, 1% MEM NEAA, and 0.1 mM of β-mercaptoethanol. R2 medium was also changed everyday and at day 10 replaced with differentiation medium (DM) comprised of RPMI-1640 (Invitrogen Cat. #21870), 0.05% BSA fraction V (Sigma-Aldrich Cat. #A8412), 0.5% Glutamax, 0.1 mM β-mercaptoethanol, 0.5× N2 supplement (Invitrogen Cat. #17502-048), 1× B27 minus vitamin A supplement (Invitrogen Cat. #12587-010). The DM was changed everyday and was supplemented with 10 ng/mL BMP-4 (Stemgen Cat. #03-0007) only from days 10 to 15. Figure 1c depicts the timeline of the reprogramming protocol and the corresponding medium used at specific timepoints. For those experiments involving ascorbic acid supplementation, a final concentration of 25 μg/mL of sodium L-ascorbate (Sigma-Aldrich Cat. #A4034) was maintained in all different media from day 1 until day 15 of the reprogramming protocol.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde in PBS after day 21 and permeabilized with 0.01% TX-100 and 10% goat serum in PBS. Primary antibodies to cardiac troponin I (Millipore Cat. #mab1691) and α-actinin (Sigma-Aldrich Cat. #7732) were used at a dilution of 1:500. Secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (Invitrogen Cat. #A-11001) and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen Cat. #A-11032) were used to label the primary antibodies



at a dilution of 1 : 400. The cells were then imaged on an Olympus IX81 inverted microscope equipped with a Hamamatsu ORCA-R2 (model C10600) digital CCD camera.

Quantitative PCR. At different time points, cells were detached from the different fibrin gels using a 50 FU/mL solution of nattokinase (Japan Bio Science Laboratory Co., Ltd., Osaka, Japan. NSK-SD). Nattokinase is a serine protease that degrades fibrin gels. RNA from the cells was then isolated using the SV Total RNA Isolation System (Promega Cat. #Z3100) and the concentrations of the isolated RNA from the different conditions were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The first strand cDNA was synthesized with equal amounts of RNA across samples using the ImProm-II Reverse Transcription System (Promega Cat. #A3800). The qPCR assays were performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using the KAPA SYBR Fast qPCR kit (Kapa Biosystems) and the primers for the studied genes (Supplementary Table S1). Assays for three biological samples were performed and for each sample, two technical replicates were carried out. The $\Delta\Delta C_t$ method was used to calculate the relative gene expression levels.

Quantification of colonies and contractile colonies. At day 10, before inducing cardiac differentiation, the total number of cell colonies (see Supplementary Figure S1 for example of a cell colony) in each well was counted under an inverted microscope (Olympus CKX31). At day 21, at the completion of the reprogramming protocol, the number of contractile cell colonies in each well was counted under an inverted microscope. The percentage of contractile colonies is then equal to the number of contractile cell colonies divided by the total number of cell colonies multiplied by 100. The contractility of a colony was assessed visually and robust contractions can be observed under the inverted microscope at 4 \times magnification (see Supplementary movies S1 and S2).

Quantification of cell proliferation. Cells were cultured on the various substrates or hydrogels with a density of 3500 cells/cm² in MEF medium. On days 1, 3, 5 and 7 the cells were retrieved using trypsin or nattokinase. The cells were spun down into a pellet and the pellet was then lysed. The total DNA from each sample was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The DNA concentration from each sample was normalized with the DNA concentration of day 1 from the same substrate type or cell genotype.

Quantification of cell area and cell adhesion at day 1. Cells were plated on the various substrates or hydrogels with a density of 3500 cells/cm² in MEF medium. On day 1 after plating, the cells were stained with a LIVE/DEAD cell imaging kit (Molecular Probes Cat. #R37601). The cells were then imaged for live cells (green fluorescence) with an Olympus IX81 inverted microscope equipped with a Hamamatsu ORCA-R2 (model C10600) digital CCD camera. The live cell images were processed in ImageJ to quantify the cell area on the various substrates or hydrogels (n = 100). The cells were next fixed with 4% paraformaldehyde, and stained with DAPI. The cell nuclei of the cells were then imaged with an Olympus IX81 microscope and the images were processed with ImageJ to quantify the percentage of initial cell adhesion at day 1.

Scanning electron microscopy of hydrogel microstructure. The fibrin and fibrin-collagen I composite precursor solutions were prepared as in the cell experiments and 10 μ L of the precursor solutions were cast on coverglass. The precursor solution was allowed to gel at 37°C for 30 minutes and immersed in PBS after gelation. The gels were then fixed with 2.5% glutaraldehyde in PBS for 20 min at room temperature. After rinsing the fixed gels in PBS, they were then immersed in 15% sucrose in ddH₂O for an hour. The gels were finally rinsed and immersed in ddH₂O. Next, these gels were flash frozen by immersing them in liquid nitrogen for 15 min. The gels were then subsequently lyophilized in a Micromodulyo freeze dryer (Thermo Scientific) overnight. Once dried, the gels were coated with a thin layer of gold (~10 nm) with a SPI sputter coater (Structure Probe, Inc.). The coated gels were then imaged with a Philips XL30 FEG scanning electron microscope at 5 kV.

Hydrogel rheology. The shear moduli of the hydrogels were measured using a 20 mm parallel plate setup on an AR-G2 rheometer (TA Instruments, New Castle, DE) with an oscillation of 1% strain at 1 rad/s. The hydrogel precursor solutions were prepared as in the cell experiments and introduced between the parallel plates before gelation. The solutions were allowed to gel at 37°C on a Peltier stage and gaps between the parallel plate and stage were sealed with mineral oil to prevent drying.

RhoA-mutant lentiviral constructs. The mutant RhoA and eGFP transgenes were subcloned from pcDNA3-EGFP-RhoA-Q63L (Addgene plasmid 12968), and pcDNA3-EGFP-RhoA-T19N (Addgene plasmid 12967) plasmids using a high fidelity DNA polymerase (Q5 high fidelity DNA polymerase, New England Biolabs). Primers were designed such that the transgenes were flanked with the restriction enzyme sites that match those on the multiple cloning site (MCS) of the receiving plasmid. The subcloned transgenes were inserted into the MCS of a tetracycline inducible backbone, tetO-FUW-MCS, derived from Addgene plasmid 20321 (tetO-FUW-OSKM) to construct the plasmids tetO-FUW-eGFP-RhoA-Q63L, tetO-FUW-eGFP-RhoA-T19N, and tetO-FUW-eGFP. Clones of the different constructs were then DNA sequenced to select the final desired construct.

Statistical analyses. All experiments were carried out in three independent samples unless indicated otherwise. One-way or two-way analysis of variance (ANOVA) was the primary statistical analysis method carried out followed by post-hoc pair-wise analysis utilizing the Bonferroni correction. For the post-hoc analysis, the means were considered significantly different when $p < 0.05$.

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Acknowledgments

We gratefully acknowledge funding from the University of Michigan for this study. Y.P.K. was supported by the American Heart Association through the Midwest Affiliate Postdoctoral Fellowship. R.K.S. was partially supported by a pre-doctoral fellowship from the NIH (NIDCR)-funded Tissue Engineering and Regeneration training program (T32-DE007057) at the University of Michigan.

Author contributions

Y.P.K. and A.J.P. conceived of the study and designed the experiments. Y.P.K., B.C. and R.K.S. performed experiments and analyzed data. Y.P.K. and A.J.P. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kong, Y.P., Carrion, B., Singh, R.K. & Putnam, A.J. Matrix identity and tractional forces influence indirect cardiac reprogramming. *Sci. Rep.* **3**, 3474; DOI:10.1038/srep03474 (2013).



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