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Direct reprogramming of mouse fibroblasts to cardiomyocyte-like cells using Yamanaka factors on engineered poly(ethylene glycol) (PEG) hydrogels

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Abstract

Direct reprogramming strategies enable rapid conversion of somatic cells to cardiomyocytes or cardiomyocyte-like cells without going through the pluripotent state. A recently described protocol couples Yamanaka factor induction with pluripotency inhibition followed by BMP4 treatment to achieve rapid reprogramming of mouse fibroblasts to beating cardiomyocyte-like cells. The original study was performed using Matrigel-coated tissue culture polystyrene (TCPS), a stiff material that also non-specifically adsorbs serum proteins. Protein adsorption-resistant poly(ethylene glycol) (PEG) materials can be covalently modified to present precise concentrations of adhesion proteins or peptides without the unintended effects of non-specifically adsorbed proteins. Here, we describe an improved protocol that incorporates custom-engineered materials. We first reproduced the Efe *et al.* protocol on Matrigel-coated TCPS (the original material), reprogramming adult mouse tail tip mouse fibroblasts (TTF) and mouse embryonic fibroblasts (MEF) to cardiomyocyte-like cells that demonstrated striated sarcomeric α -actinin staining, spontaneous calcium transients, and visible beating. We then designed poly(ethylene glycol) culture substrates to promote MEF adhesion via laminin and RGD-binding integrins. PEG hydrogels improved proliferation and reprogramming efficiency (evidenced by beating patch number and area, gene expression, and flow cytometry), yielding almost twice the number of sarcomeric α -actinin positive cardiomyocyte-like cells as the originally described substrate. These results illustrate that cellular reprogramming may be enhanced using custom-engineered materials.

Keywords

direct reprogramming; stem cell niche; cardiomyocyte; poly(ethylene glycol); laminin; stem cell microenvironment

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1. Introduction

Heart failure is one of the most common health problems in the United States. It will eventually affect ~20% of all 40-year-old Americans [1]. Innovative solutions are necessary to reduce the costs of heart failure, which currently stand at ~\$39.2 billion per year [1]. Cell-based therapies, or introduction of healthy cells into cardiac scar tissue, have modestly improved various measures of cardiac function [2–4]. The best donor cell source is still under debate, with much room for optimization across cell types [5]. Current cardiomyocyte derivation strategies include differentiation from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), resident cardiac progenitor or stem cells, or direct reprogramming, which bypasses the pluripotent state. ESC and iPSC (collectively, PSC)-derived cardiomyocytes are expandable, and iPSC-derived cardiomyocytes could be autologously sourced. However, their populations must be carefully purified to avoid risks of teratoma formation, and chromosomal defects arising over long periods in culture add to tumorigenic risks [6]. Currently, iPSC derivation is relatively inefficient (0.01–1.4%) [7]. The full derivation, expansion, and purification of pluripotent stem cell derived cardiomyocytes can take several months [8, 9].

Several direct reprogramming strategies [10–13] have reported bypassing the pluripotent state, enabling autologous derivation of cardiomyocyte-like cells in a fraction of the time needed for iPSC cell production. This has been achieved by inducing transcription factors important for cardiac development (for example; Tbx5, Mef2c, Gata4, Myocd and/or Hand2) [11, 13, 14] or using microRNAs [12] to control signaling pathways. In addition to implanting cells reprogrammed *in vitro*, similar techniques have been used to reprogram scar tissue to cardiomyocytes *in vivo* [12, 14–16]. The direct reprogramming protocol described by Ding and colleagues (Efe *et al.* [10]) combines a carefully timed induction of Oct4, Sox2, Klf4, and c-Myc (OSKM), inhibition of the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pluripotency pathway, serum withdrawal, and BMP-4 mediated cardiogenic induction, resulting in beating patches of cells in as little as 11 days after OSKM induction [10]. The rapid derivation process is especially relevant in cases of myocardial infarction, as the inflammatory response begins within 24 hours [17] and scar formation progresses quickly from there [18].

Extracellular matrix (ECM) proteins in the cell microenvironment affect pluripotent differentiation via integrin signaling, and are well known to direct differentiation down varied pathways [19]. However, the optimal surface density and types of proteins are often determined by trial and error. Efe *et al.* found that Matrigel (~60% laminin-1, ~30% collagen IV, ~8% entactin, ~2% other proteins according to product literature) improved cardiac direct reprogramming over Geltrex, gelatin or fibronectin coatings, but no mechanism was proposed [10]. In contrast, Thomson and colleagues revolutionized PSC culture by measuring ESC growth factor receptor expression and tailoring the media formulation accordingly [20, 21]. Recently, Koche *et al.* analyzed global gene expression of partially reprogrammed mouse embryonic fibroblasts (MEFs, 3 days into OSKM induction), reporting upregulation of laminin-binding integrin subunits $\alpha 6$ (4.0-fold) and $\beta 4$ (13.3-fold) and downregulation of collagen IV binding subunits $\alpha 1$ (4.3-fold). RGD motif-binding integrin subunits (e.g. $\alpha 5$ and αV) did not show major changes [22]. Following Thomson's example, we engineered PEG materials to activate the upregulated $\alpha 6$ and $\beta 4$ integrins' pathways via laminin interactions. The use of laminin also avoids the batch-to-batch variation in protein content seen with Matrigel [23, 24]. Given that the integrin subunits $\alpha 5$ and αV did not show major changes in expression during OSKM expression [22], we expected that fibronectin or linear RGD peptides, respectively, would enhance cell adhesion to PEG and perhaps stimulate cell signaling through these integrins.

In the original direct reprogramming protocol, Matrigel was adsorbed to tissue culture polystyrene (TCPS) prior to cell seeding [10]. However, adsorption may cause unfolding of ECM proteins, resulting in altered cell signaling [25–28]. Serum proteins from the media also adsorb to TCPS, competing with the intended ECM proteins [29] and activating unintended signaling pathways that may vary by batch of serum. Poly(ethylene glycol) (PEG) is resistant to non-specific protein adsorption, but may be covalently modified to present bioactive molecules at precise quantities and in their native conformations [30, 31]. Because of the protein resistant properties, PEG is often used as a silent “background” material for probing specific effects of certain molecules without confounding adsorbed serum proteins [32, 33]. In addition, PEG can be tuned to adjust the matrix stiffness [34], incorporate bioactive moieties [35–37], and degrade at a controlled pace [38], all of which can affect stem cell differentiation outcomes [39, 40]. For this study, we engineered PEG cellular microenvironments that potentially provide better control over adhesion molecule concentration, conformation, and stability than is possible with the TCPS substrate initially described by Efe *et al.* [10].

2. Materials and Methods

2.1 Mouse protocols and fibroblast culture

All animal protocols were approved by Washington University Institutional Animal Care Use Committee. A pair of mice homozygous for doxycycline-inducible OSKM factors (Jackson Labs, stock number: 011011) was bred to start a colony for tail tip (TTFs) and embryonic fibroblasts (MEFs) [41]. To harvest tail-tip fibroblasts (TTFs), adult tails were sterilized in 70% ethanol, skinned, and minced thoroughly. Minced pieces were incubated in 3 mL of 0.05% trypsin-EDTA (Invitrogen) at 37°C for 30 min then neutralized with 7 mL of fibroblast media (FB media: DMEM+10% fetal bovine serum+1% antibiotic-antimycotic). Tail pieces were collected by centrifugation, resuspended in 3 mL of FB media, and transferred by tweezers to gelatin coated wells (1 mL/well at 0.1 mg/mL, preincubated 1 h at room temperature) at a ratio of one tail per 2 wells of a 6 well plate. Care was taken to dry the well surfaces by aspiration prior to placing the pieces, and to evenly space the pieces in the well. After allowing 4 h for tails to adhere at 37°C, 0.2 mL of FB media was added per well. The next day, 3 mL/well of FB media were added. Once TTFs became confluent, they were trypsinized, strained through a 70 μ m cell strainer, and replated (2 wells/T75 flask). Once confluent, the flask was trypsinized and frozen at –80 (1 cryovial per initial mouse tail).

For preparing mouse embryonic fibroblasts (MEFs), day 13.5 embryos were harvested and the visceral organs removed to minimize the presence of cardiac precursor cells. Embryos were minced, incubated at 37°C for 30 min in 7 mL of 0.05% trypsin-EDTA and then triturated using a 10 mL pipet. Trypsin was neutralized with 23 mL of FB media, divided into T75 flasks (2–3 embryos/flask), and cultured in 15 mL of FB media. Once confluent, cells were passaged 1:3 and the passage 2 cells were trypsinized and frozen at –80°C (3 cryovials/confluent flask).

2.2 PEG hydrogel fabrication and functionalization of culture surfaces

All experiments were performed using 24-well plates. Thirty minutes prior to gel fabrication, wells were treated with a plasma cleaner (Harrick Plasma) set to medium (350 mTorr for 5 min) to reduce meniscus formation in the wells during gel formation. Plates were then UV-sterilized for 30 min and gel precursor solutions were added immediately. PEG derivatives were synthesized as previously described [42–44]. For gel fabrication, PEG₈-Vs and PEG₈-Am solutions (both 20% w/v in PBS, pH 7.4) were sterile-filtered (0.22 μ m filter) and diluted in 0.03 M NaOH in PBS (pH 8.75, NaOH enhanced reaction kinetics

and further reduced meniscus formation) for a final PEG concentration of 12.43% (w/v). The precursor mix was aliquoted at 200 μ L/well and allowed to crosslink in a humidified incubator (37°C) for 1–2 days.

Protein and peptide surface concentrations are reported as 1 $\times$ or 5 $\times$. When referring to Matrigel or laminin, 1 $\times$ represents 11.4 μ g/cm², the protein surface concentration of Matrigel used in Efe *et al.* [10]. When referring to the RGD peptide, 1 $\times$ (354 μ g/cm²) is the approximate surface density of RGD on the PEG microspheres previously tested with HL-1 cardiomyocytes [45]. See Supplementary Table S2 for more information. For functionalization with Matrigel (BD Biosciences, 356234) or laminin-1 (Sigma, L2020), protein stocks were first diluted in PBS. If RGD peptide (GCGYGRGDSPG, Genscript) was included, it was diluted in the same tube as the protein. Each well received 200 μ L of protein/peptide solution. For TCPS conditions, (non-plasma treated) 24-well plates were incubated at room temperature for 1 h to permit protein adsorption (according to Matrigel product literature). The pre-formed PEG hydrogels were incubated with the protein/peptide mixtures overnight at 37°C to permit the slower, covalent Michael-type reaction to occur. Fibroblast seeding occurred the next day.

2.3 Cell culture and reprogramming

All reagents were purchased from Invitrogen unless otherwise noted. Passage 3 fibroblasts were seeded at day –1 (minus one) onto the functionalized 24-well plates at 3,600 MEFs or 9,700 TTFs/cm² and incubated overnight in FB media to ensure complete adhesion. Reprogramming commenced the following day (day 0), and media was changed every 24–48 hours. Media formulations and reprogramming timelines were adapted from Efe *et al.* [10]. Reprogramming timeline #4 (Fig. 1a) was the most efficient at producing beating in initial testing with MEF, and was used in all experiments thereafter. From days 0–5, cells received knockout DMEM (KO DMEM, 10829018) + 2 μ g/mL doxycycline (made fresh for each experiment, Sigma, D9891) + 0.5 μ M JAK inhibitor I (JI1, EMD, 420099) + 15% embryonic stem cell qualified FBS (ES-FBS, 10439-024) + 5% knockout serum replacement (KSR, 10828028) + 0.1 mM β -mercaptoethanol (21985-023), + 1% Glutamax (35050-061) + 1% non-essential amino acids (11140-050) + 1% embryonic stem cell qualified nucleosides (Millipore, ES-008-D). For days 6–8, doxycycline was removed, ES-FBS concentration reduced to 1%, and KSR concentration increased to 14% (all other components remained the same). From days 9–14, cells were given chemically defined medium (CDM) with BMP4: RMPI-1640 (21870-084) + 20 ng/mL BMP4 (Stemgent, 03-0007) + 0.5 $\times$ N2 (17502-048) + 1 $\times$ B27 w/o vitamin A (12587-010) + 0.05% BSA fraction V (15260-037) + 0.5% Glutamax + 0.1 mM β -mercaptoethanol. From day 15 onward, cells were given CDM without BMP4. RW.4 mouse embryonic stem cells (ATCC), a gift from the Sakiyama-Elbert laboratory, were cultured according to previously described methods [46].

2.4 Cell counts and quantification of beating patches

Phase contrast microscope images (10 $\times$ magnification) were taken with a CCD camera (Magnifire, Olympus) and used to perform cell counts at days 0 and 3 for analysis of initial adhesion and proliferation. Cell counts for three randomly selected images were averaged to determine the cell density of a given well. For beating patch quantification, 2 $\times$ images of the entire well were taken by phase microscopy. Wells were then carefully examined at 4 $\times$ for beating patches, and these were outlined on the 2 $\times$ images using Microsoft Paint. Outlined beating patches filled with white using Adobe Photoshop, and any bright regions of the image not associated with beating patches were filled with black. Images were then thresholded and quantified in Matlab (Mathworks) to determine the number and size of the beating patches and total beating area of the well. For late stage cell counts, cells were

enzymatically dissociated using trypsin at days 15 (n = 3–6) and 18 (n = 3–6) and counted using a hemocytometer.

2.5 Live-cell calcium imaging

Calcium-sensitive dye Fluo-2 (medium affinity, 10 mM in dimethyl sulfoxide, excitation/emission 488/515 nm, TefLabs) was mixed (1:1 volume ratio) with 20% (w/v) Pluronic F-127 (Invitrogen), diluted to 10 μ M in DMEM, and applied to cells for 30 min at 37°C/5% CO₂. Cells were washed three times in PBS and incubated for 15 min in DMEM to allow complete de-esterification of the Fluo-2. Cells were imaged in Tyrode's solution (1.33 mM CaCl₂, 1 mM MgCl₂, 5.4 mM KCl, 135 mM NaCl, 0.33 mM NaH₂PO₄, pH 7.4) containing 5 mM glucose and 5 mM HEPES by fluorescence microscopy (IX70, Olympus) using a Magnafire camera (Optronics, 10 frames/second). The resulting images were analyzed using a custom Matlab script that tracked fluorescence localization over different time intervals. Noise was removed through a Prewitt edge detection filter and a series of structuring element filters (2-pixel lines at 90, 0, then 45 degrees). A 10 × 10 pixel averaging filter was then used to remove biased pixels that were unrelated to the signal of interest. Finally, activation events were detected in each pixel by setting a fluorescence threshold. An activation map was formed by overlaying temporal data with a gradient color scheme.

2.6 Quantification of gene expression

Gene expression was evaluated by quantitative real time RT-PCR using an Applied Biosystems StepOnePlus or *ViiA* machine. On day 18 of reprogramming, cellular RNA was purified with TRIzol reagent (Invitrogen, 15596-026) by following the manufacturer's instructions. RNA concentration and quality (260/280 nm absorbance ratio) was verified using a NanoDrop spectrophotometer. Genomic DNA was removed and RNA was reverse transcribed to cDNA (10 ng RNA/ μ L) using the QuantiTect Reverse Transcription Kit (Qiagen, 205313). Pre-validated primers for mouse GAPDH, sarcomeric α -actinin (*Actn2*), cardiac troponin T (*Tnnt2*), and Nanog (*Nanog*) transcripts were obtained from Qiagen (Quantitect Primer Assay). Forward and reverse primer sequences designed using IDT software and experimentally validated were β -actin (*ActB*), sarcomeric α -actinin (*Actn2*), myosin light chain-atrial isoform (*Mlc2a*), potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channels 1 and 4 (*Hcn1*, *Hcn4*), T-box transcription factor Tbx3 (*Tbx3*), and contactin-2 (*Cntn2*). Primer sequences are in Supplementary Table S1. cDNA was amplified in triplicate reactions using Quantitect SYBR Green PCR Kit (Qiagen) following manufacturer's instructions. Expression levels were normalized to GAPDH or β -actin internal control genes, or to sarcomeric α -actinin to exclude effects from non-cardiomyocytes, using the $2^{-\Delta C_t}$ method [47].

2.7 Immunocytochemistry

For immunocytochemistry, cells were fixed in 4% paraformaldehyde (500 μ L/well) for 15 min. Triton-X 100 (Sigma) was diluted 1:1000 in blocking solution (10% normal goat serum + 1% BSA in PBS) for permeabilization (300 μ L/well, 30 min at room temperature). Sarcomeric α -actinin primary antibody (mouse IgG, Sigma A7811) was diluted 1:1600 in the blocking solution and applied to the cells (300 μ L/well, overnight at 4°C). Cells were washed 3× in PBS (5 min each) before incubation in secondary antibody diluted 1:1000 in blocking solution (300 μ L/well, 1 h at room temperature, Alexa Fluor 594 goat anti-mouse, Invitrogen, A-11005). Cells were washed 3× more in PBS before application of DAPI as a nuclear counterstain (100 ng/mL, 300 μ L/well, 30 min at room temperature). Wells were washed once in PBS then imaged in PBS.

2.8 Flow cytometry

At day 18, cells were enzymatically dissociated into a single-cell suspension using 0.25% trypsin-EDTA (Invitrogen), fixed in 1% paraformaldehyde in PBS at 4°C with agitation for 20 min, permeabilized with 1% saponin (Sigma, S4521) in PBS at 4°C with agitation for 20 min, and blocked with 5% normal goat serum (NGS) + 0.5% saponin in PBS at 4°C with agitation for 20 min. Sarcomeric α -actinin primary antibody (mouse IgG, Sigma A7811) was applied to the cells for 30 min at 4°C with agitation (1:1600 in 2% NGS + 0.5% saponin in PBS). Negative controls had no primary antibody at this step. AlexaFluor 647 goat anti-mouse secondary IgG (Invitrogen, A21235) was applied to the cells for 30 min at 4°C with agitation (1:200 in 2% NGS + 1% saponin in PBS). DAPI (1:125 in PBS) was applied as a nuclear counterstain. Data were acquired on a FACSCanto II (BD Biosciences), with at least 10,000 counts per condition. Data were analyzed using FlowJo software (Tree Star). Negative controls were used to determine gating parameters.

2.9 Statistics

Two-condition comparisons were made using Student's t-test, whereas multiple comparisons were made using ANOVA with post-hoc Tukey HSD. Normality of qPCR datasets was tested using the Shapiro-Wilks test. Non-normal datasets were analyzed using the non-parametric Wilcoxon rank sum test for two-condition comparisons or the Kruskal-Wallis test with post-hoc Tukey HSD for multiple comparisons. In figures, data are presented as mean $\pm$ standard error of the mean. Text references to data are mean $\pm$ 1 standard deviation.

3. Results

3.1 Replicating direct reprogramming to cardiomyocyte-like cells on Matrigel-coated TCPS

To verify that direct reprogramming using OSKM could be replicated in our hands, we first used adult mouse TTFs to test three possible interpretations of the published protocol [10] (Fig. 1a). Cells from all groups demonstrated noticeable morphological changes, notably a decrease in cytoplasm/nucleus ratio by day 6. By day 12, large cell clusters had formed, and were most prevalent in wells from timeline 4 (Fig. 1b). By day 14, beating patches were noted under timelines 3 and 4 only (Supplementary Movie 1). Timelines 3 & 4 gave similar numbers of beating patches by day 18, with timeline 4 having a slightly higher number. Given that timeline 4 was also shorter and less complex, it was used in all subsequent experiments.

The beating patches demonstrated spontaneously oscillating calcium transients, which were most prevalent at the outer edges of differentiated clusters or in patches of cells surrounding the clusters (Fig. 2a–c and Supplementary Movie 2). Beating patches derived from TTFs stained positively for striated sarcomeric α -actinin, a cardiac-specific marker (Fig. 2d–e). In agreement with the location of calcium transients, the most intense α -actinin staining was found in cells surrounding the differentiated clusters.

After demonstrating that the protocol is effective with adult somatic cells, MEFs harvested from the same mice containing the doxycycline-inducible OSKM cassette were utilized because they could be expanded to greater numbers in culture and reprogramming efficiency could be directly compared to the results of Efe et al. At day 21, the number of beating patches generated with MEFs on Matrigel-coated TCPS was similar to the number previously reported by Efe *et al.* (245 ± 83 in this study, versus 257 ± 17 beating patches per initial 100,000 MEFs seeded).

3.2 Adhesion and proliferation of MEFs undergoing reprogramming on PEG hydrogels

Unlike TCPS, PEG hydrogels resist non-specific protein adsorption, making them excellent background substrates for probing ECM interactions. To verify that direct reprogramming could be performed on PEG hydrogels, MEFs were cultured on surfaces presenting covalently immobilized Matrigel, laminin or RGD at varied concentrations (referred to as 1× or 5×: for detailed explanations of the surface concentrations see the methods section or Supplementary Table S2). As noted in previous studies [44], cells did not adhere to PEG surfaces without adhesion molecules. It is well known that the protein content of Matrigel varies by batch (~60% laminin, ~30% collagen IV, ~8% entactin and ~2% various growth factors and other proteins according to product literature) [48]. Consistent with this, we found that the ability of cells to adhere to Matrigel-functionalized PEG was Matrigel batch-dependent (data not shown). However, cells consistently adhered to laminin-functionalized PEG. RGD was incorporated in some conditions to promote better long-term adhesion (as discussed below). By 24 hours after seeding (day 0 of reprogramming), cell densities were similar on PEG-lam(5×) and TCPS coated with Matrigel or laminin. The densities were statistically higher than all other PEG groups and uncoated TCPS (Fig. 3a), indicating higher rates of initial adhesion and/or proliferation on these surfaces.

Early proliferation dictates the ultimate number of cells available for cardiac differentiation. To analyze the role of the adhesion molecules on proliferation of cells on PEG conditions during the first 3 days of OSKM induction, we quantified cell densities at day 3 and applied the following equation to determine the number of doublings per day:

$$d = \frac{\log_2 \left(\frac{N_t}{N_0} \right)}{t}$$

where d = doublings/day, N_0 = number of cells at day 0, N_t = number of cells after t days, and t = number of days (Fig. 3b). Day 3 was chosen as the endpoint because cells on PEG conditions reached confluency by days 4–5 and thus could not be readily counted. Rates could not be determined for TCPS conditions as they had reached confluency by day 3. For PEG conditions presenting RGD alone, the RGD concentration had an effect on cell proliferation (0.84 ± 0.07 doublings/day for 1× RGD versus 1.14 ± 0.15 for 5× RGD, $p = 0.01$ by t-test). PEG conditions presenting laminin alone at 1× or 5× concentration exhibited proliferation rates similar to the 5× RGD condition. This demonstrates the important role of integrin interactions during the direct reprogramming process. While beyond the scope of this particular study, a detailed temporal investigation of the cellular responses is an attractive future direction.

Over the course of reprogramming, cells on PEG-RGD(5×) continued to proliferate rapidly compared to other PEG groups, resulting in an extremely high cell density by day 9 (Fig. 3c). Interestingly, when laminin was added to PEG hydrogels that also contained 5× RGD, cell densities were noticeably lower. For example, cell density on the surface of the confluent cell monolayer alone was 2.75-fold higher for PEG-RGD(5×) than PEG-lam(1×)-RGD(5×) ($p < 0.01$ by t-test). By day 12, the dense cell layers had peeled off the PEG-RGD(5×) gels, forming compact aggregates at the edges of the wells (Fig. 4b). This occurred in 100% of the experimental replicates (supplementary Fig. S1). Thus, the PEG-RGD(5×) condition was not further analyzed. Other conditions enabled better long-term adhesion, but by day 18, cells of the 1× laminin, 1× RGD and, to a lesser degree, 5× laminin conditions had also begun peeling off the gel in isolated areas. Long-term adhesion appeared best on gels that presented both laminin and RGD (supplementary Fig. S1). No long-term adhesion issues were noted on any TCPS conditions.

Cells were counted by hemocytometer on days 15 and 18 for TCPS-Mat(1×), PEG-lam(5×), PEG-lam(5×)-RGD(1×), and PEG-lam(5×)-RGD(5×) to assess proliferation at a later stage of the reprogramming timeline. The cell density between the two days was not different for any condition, suggesting a decrease in proliferation (supplementary Figure S2). This finding is consistent with the decrease in progenitor marker (*Mesp1* and *Isl1*) expression reported by Efe *et al.* for that time frame, which suggests a transition from a proliferative progenitor to a less proliferative immature cardiomyocyte [10]. Counts also did not vary between PEG-lam(5×) with 1× versus 5× RGD (supplementary Figure S2). Counts were therefore pooled to increase the number of samples for a statistical analysis of late stage cell density (n=6–12). PEG-lam(5×)-RGD(1× or 5×) gels had a ~25% increase in cell density compared to the original condition used in Efe *et al.*, or PEG-lam(5×) without RGD (Fig. 4a, $p < 0.05$ by ANOVA with post-hoc Tukey HSD). Cell density on PEG-lam(5×) with RGD increased by a factor of 136.2 ± 23.7 compared to initial MEF seeding density. Densities on PEG-lam(5×) without RGD and TCPS-Mat(1×) increased by factors of 109.1 ± 20.5 and 108.3 ± 16.7 , respectively.

3.3 Functional assessment of direct reprogramming to cardiomyocyte-like cells on PEG surfaces

Many of the laminin receptors are upregulated during the first 3 days of OSKM reprogramming, whereas collagen receptors are downregulated [22]. Thus, we hypothesized that reprogramming would be equally as efficient on laminin versus Matrigel. Numbers of differentiated clusters were visibly increased on PEG-lam(5×)-RGD(5×) compared to PEG-RGD(1×), PEG-lam(1×)-RGD(1×) and PEG-lam(5×)-RGD(1×) (Fig. 4b).

Beating assays provide a simple yet well-accepted method for initially estimating cardiac reprogramming efficiency [10, 49–53]. On TCPS (Fig. 5a, red columns, Matrigel and laminin-1 coatings led to similar numbers of beating patches per cm^2 at day 18 (9.04 ± 3.05 versus 9.44 ± 2.42 respectively; $p = 0.71$ by t-test). When PEG was functionalized with 1× RGD, 1× laminin, or both (Fig. 5a, blue columns), beating patch numbers were similar to the TCPS conditions. A non-significant increase was noted when PEG-lam(1×) gels also presented 5× RGD. This trend was also seen on PEG-lam(5×) gels presenting 1× or 5× RGD (Fig. 5a, green columns). PEG-lam(5×)-RGD(5×) had more beating patches than all TCPS conditions and PEG with lower adhesion molecule concentrations. PEG-lam(5×) (no RGD) also promoted more beating than several of these groups, including the PEG-lam(1×) (no RGD) condition. Increasing laminin concentration from 1× to 5× on TCPS did not result in more beating, suggesting that adsorbed amounts of laminin were already near maximal at 1× solution concentration. On TCPS, laminin saturates around 850 ng/cm^2 [54], which is 13.4 and 67.1 times lower than our 1× and 5× concentrations. Therefore, the actual density of the laminin layer adsorbed on TCPS is likely similar for our 1× and 5× concentrations. Similar trends were noted when considering the total beating area instead of the number of beating patches (Fig. 5b). Reprogramming efficiency on Matrigel-functionalized PEG could not be assessed given the aforementioned adhesion inconsistencies, and Matrigelfunctionalized PEG gels were not studied further.

3.4 Gene and protein expression of directly reprogrammed cultures

Gene expression in day 18 reprogrammed cells was evaluated by reverse transcription quantitative real-time PCR (qRT-PCR) (Fig. 6a–b). Similar to what was seen in the beating patch counts, gene expression levels from PEG hydrogels with 5× laminin and 1×, 5×, or no RGD were statistically similar. Likewise, no differences were seen in expression levels on TCPS with 1× or 5× laminin (supplementary Fig. S3). Due to inherent high variability in qRT-PCR, data from similar conditions were pooled to increase sample numbers for statistical analysis. Results for each surface are presented individually as supplementary

materials (Fig. S3). The TCPS-Mat(1×) was left unpooled for direct comparison to the original Efe *et al.* condition, but had fewer samples for statistical tests.

Cardiac markers sarcomeric α -actinin (*Actn2*) and troponin T (*Tnnt2*) were upregulated compared to the MEF control on PEG-lam(5×)-RGD(0, 1, or 5×) and TCPS-lam(1 & 5×) (* $p < 0.05$ against MEFs by the Kruskal-Wallis non-parametric test for multiple comparisons, with post-hoc Tukey HSD) (Fig. 6a). While not statistically different from the other reprogrammed groups, the PEG-lam(5×)-RGD(0, 1, & 5×) presented the highest *Actn2* expression. A t-test comparison of *Actn2* levels between the PEG-lam(5×)-RGD(0, 1, & 5×) and the TCPS-lam(1 & 5×) conditions indicated a statistical difference ($p = 0.03$), providing rationale to further probe its expression by immunocytochemistry and flow cytometry.

Tnnt2 levels were similar on all conditions, but the trend towards higher expression on PEG-lam(5×)-RGD(0, 1, & 5×) than TCPS-lam(1× & 5×) was maintained. Pluripotency marker *Nanog* expression was statistically lower on PEG-lam(5×)-RGD(0, 1, & 5×) than TCPS-lam(1 & 5×). The pooled 5× laminin PEG groups were ~700 fold lower than *Nanog* levels of RW.4 mouse embryonic stem cells.

While Efe *et al.* reported higher gene expression of the atrial isoform of myosin light chain (*Mlc2a*) than the ventricular isoform [10], cardiomyocyte-like cells resulting from direct reprogramming strategies have not been well characterized with regard to the cardiomyocyte subtype generated. Conduction and nodal cells are rare but highly important to maintaining proper cardiac function. The ability to reprogram to this subtype may have future applications for diseases involving fibrosis around these areas. We compared expression of atrial (*Mlc2a*) [10] and conduction/nodal-enriched genes (*Hcn1*, *Hcn4*, *Tbx3*, and *Cntn2*) [55, 56] on PEG-lam(5×) (with and without 5× RGD) to the original condition described in Efe *et al.* (TCPS-Mat(1×)). Markers were normalized to α -actinin to take into the account the differences we see in reprogramming efficiency in these conditions (Fig. 6b). Interestingly, expression of conduction and nodal genes was generally higher on the TCPS-Mat(1×) condition, suggesting that there may be subtle but important roles that the microenvironment plays in the reprogramming processes to generate various cardiac subtypes.

Immunocytochemistry was used to quantitatively compare cardiac marker sarcomeric α -actinin expression on PEG conditions. Actinin/DAPI-positive pixel ratios for PEG hydrogels presenting 5× laminin with varied RGD concentration were double those presenting 1× laminin with varied RGD concentration (0.02 ± 0.01 versus 0.01 ± 0.01 , respectively. $p < 0.05$ by t-test) (Fig. 7a–c). Data for individual conditions can be found in supplementary Fig. S4.

Reprogramming efficiency was measured by flow cytometry for sarcomeric α -actinin (Fig. 8a). No significant differences were found between any of the PEG-lam(5×) groups, though averages for groups containing RGD were slightly higher. The percentage of sarcomeric α -actinin-positive cells was 1.72-fold higher on PEG-lam(5×)-RGD(1× or 5×) compared to TCPS-Mat(1×) (Fig. 8b, $6.35 \pm 0.69\%$ versus $3.70 \pm 1.03\%$, $p < 0.05$ against TCPS-Mat(1×) by ANOVA with post-hoc Tukey HSD test). Coupled with the late-stage cell density data (Fig. 4a), the PEG-lam(5×) conditions containing RGD yielded 7.73 ± 0.78 cardiomyocyte-like cells per originally seeded MEF, which is ~2-fold higher than TCPS-Mat(1×) and ~1.5-fold higher than PEG-lam(5×) *without* RGD (Fig. 8c, $p < 0.05$ against both conditions by ANOVA with post-hoc Tukey HSD).

4. Discussion

In this study, we demonstrated that the efficiency of direct reprogramming to cardiomyocyte-like cells can be nearly doubled by using custom-tailored PEG culture substrates. By using protein-resistant PEG, we were able to specifically probe the effects of covalently bound ligands on direct reprogramming without the confounding effects of adsorbed serum proteins. Ultimately, this led to the development of a culture surface that produced about twice as many cardiomyocyte-like cells as the originally described substrate. This type of precise control over microenvironment definition is a promising frontier in the field of undifferentiated PSC expansion [57]. Global gene expression analyses have also allowed researchers to specifically tailor media to meet the needs of pluripotent stem cells [20, 21], and this strategy should have a similar impact on the design of materials to control cell microenvironment. To date, many laboratories still use Matrigel to trigger integrin signaling pathways and define the cell microenvironment. Replacement of Matrigel with synthetic surfaces [58, 59], biologically inspired peptides [60–62] or purified whole proteins has allowed or enhanced PSC expansion in multiple studies. When ECM components are analyzed individually, laminin [19, 63], vitronectin [64], and E-cadherin [65] have shown to promote undifferentiated human PSC expansion on TCPS, while fibronectin and collagen IV have been reported to cause their differentiation [19]. Interestingly, while the laminin-111 isoform (used in this study) allows undifferentiated expansion of human PSCs [63], it has also caused differentiation of mouse PSCs [66], suggesting differences in integrin expression/signaling/ligand interactions between species.

While many groups are studying substrate designs for differentiating PSCs to cardiomyocytes, the cells of this protocol start as “pre-iPSCs”, which are phenotypically different from iPSCs [67]. The cardiac direct reprogramming literature has not yet examined the importance of ECM signaling, making comparisons between various protocols rather complicated. As mentioned earlier, we began with the insight that Koche *et al.* [22] provided into pre-iPSC integrin expression 3 days after OSKM induction. Taking that study into account, we designed the PEG substrates to have a high concentration of laminin (without the collagen IV that is present in Matrigel). However, laminin alone was not sufficient to support cell adhesion to PEG during the full 18 days of our protocol, possibly due to matrix metalloproteinase degradation. Because RGD-binding integrins were not strongly affected by OSKM induction [22], we incorporated RGD to promote long-term cell adhesion. While the presence of RGD positively affected cell proliferation on PEG hydrogels, its effects on cardiac gene or protein expression were not significant.

Recently, MacLellan and colleagues demonstrated that the timing of ECM protein presentation is important for cardiac maturation. First, they performed an immunohistochemical analysis of developing mouse and human hearts. This showed distinct ECM differences between stem cell niches (mostly collagen IV and laminin) and the surrounding myocardium (mostly collagen I and fibronectin) into which the maturing cells migrate [68]. Using this information, they differentiated mouse ESCs on TCPS and found upregulation of *Flk1* (a VEGF receptor but also a cardiac progenitor marker) and ultimately higher expression of the α -MHC (cardiomyocyte marker) on collagen IV and laminin coatings (as compared to collagen I or fibronectin). By isolating *Flk1*⁺ progenitor cells and re-plating them onto new ECM protein coatings, they showed that fibronectin was better at promoting cell cardiomyocyte *maturation* than collagen IV or laminin. Interestingly, Kamp and colleagues have demonstrated that cardiogenesis from iPSCs is robustly increased by providing “Matrigel overlays” at several timepoints during differentiation [69], further indicating the importance of timing in ECM presentation. While a temporal analysis of cellular phenotype during reprogramming is beyond the scope of this study, we did note variations in cell numbers that were caused by the material. For example, adhesion/

proliferation between days -1 and 0 was higher on TCPS (Fig. 3a), but final cell numbers were highest on PEG materials presenting RGD (Fig. 4a). Future studies should include a robust characterization of temporal gene expression over the course of the reprogramming process. That information could then be used to design substrate materials that further improve cardiac cell differentiation efficiency.

A goal of this study was to highlight the differences in the reprogramming process on two very different surfaces. An attractive future direction for this research is the development of a fully synthetic reprogramming microenvironment (i.e. replacing all ECM protein components with peptide sequences to activate similar signaling pathways). Ultimately this will eliminate cost barriers and possibilities of immunogenicity and/or disease transmission [70]. However, at this stage of our research, using whole laminin instead of laminin-derived peptides (such as YIGSR and IKVAV) allowed us to more directly assess differences relating to potential changes in protein conformation on the two materials.

Hydrogel substrates allow manipulation of multiple variables beyond what was examined in this study. In addition to ligand type and density, one could also study effects of hydrogel chemistry, nanotopography, degradation/remodeling, and elastic modulus. The substrates compared in this study (PEG and TCPS) differ drastically in stiffness, with PEG being much softer than TCPS. When substrate stiffness is adjusted to mimic that of various tissues (muscle, bone, or neural), mesenchymal stem cells are known to differentiate down the corresponding pathway [39]. The storage modulus of this type of gel is ~7 kPa [71], which is similar to heart tissue [72]. Substrates of similar stiffnesses (11 kPa) have been shown to support embryonic cardiomyocytes better than substrates of drastically different stiffnesses (34 kPa) [73]. While the adhesion protein concentration dependence (seen only on PEG) confirmed that integrin-dependent pathways are in some part responsible for the improvement, it is possible that the heart-like elastic modulus also played a role. Further studies should be undertaken to examine the interplay of these two factors in greater detail.

To compare our data to Efe *et al.*, we terminated our studies at day 18. However, many factors are known to affect the maturation of PSC-derived cardiomyocytes, including duration in culture [74] embryoid body size and culture condition [75, 76], and growth factor signaling [77]. Electrophysiological analysis and global gene expression analyses could be used to assess the maturity at this and later time points [78]. Based on the aforementioned studies, we expect that the mechanical and integrin-activation properties of the PEG gels could be adjusted to yield specific cardiac phenotypes.

Of the currently reported cardiac direct reprogramming strategies, we chose to work with the Efe *et al.* protocol for several reasons, many of which are also outlined in a recent review by Morris and Daley [79]. The ability to expand cells in culture is important for treating large myocardial injuries. In the Ieda *et al.* method, cells are converted directly to non-proliferating cardiomyocytes. In the Efe *et al.* protocol, the cells travel through a proliferative progenitor state, yielding greater numbers of cardiomyocytes [10]. Indeed, in this study we were able to double the number of cardiomyocytes produced, in part due to enhanced proliferation on the PEG-lam(5×) gels containing RGD. The progenitors formed by this protocol may also have vascularization potential [10, 80]. Indeed, brief OSKM induction combined with VEGF stimulation has separately been shown to directly reprogram human pre-iPSCs to endothelial cells [81]. Finally, the Efe *et al.* method reduces the time to beating (~2 weeks versus 4–5 weeks) compared to the Ieda *et al.* method, which may be important clinically as the cardiac remodeling cascade starts immediately following infarction [17, 18].

On the other hand, several groups have reported *in vivo* infarction scar reprogramming since their original *in vitro* paper [12, 14–16]. This feat has not yet been performed using the Efe *et al.* protocol, and it can be argued that teratoma risks are higher for the Efe protocol because the cells are more likely to revert to a pluripotent state. However, the OSKM induction in this protocol is transient, and further studies may allow development of methods that ameliorate the risk of teratoma formation [82]. In this study, we were able to reduce the already low *Nanog* expression 2.3-fold by using defined PEG substrates instead of TCPS presenting the same ECM molecules. This leads us to believe that it will ultimately be possible to prevent the formation of pluripotent cells. However, it will also be important to demonstrate the effects of cell microenvironment on the efficiency of the Ieda protocol.

Some groups have reported difficulties reproducing the Ieda *et al.* protocol [83], and to our knowledge no studies have yet been published using the Efe *et al.* protocol. Robust induction and proper stoichiometry of the reprogramming factors are highly important for achieving reprogramming using the Ieda *et al.* protocol [84] and presumably the Efe *et al.* protocol as well. This can be difficult to achieve using viral induction methods. The availability of mice carrying a doxycycline-inducible polycistronic OSKM cassette [41] eliminated variability between experiments, providing an excellent cell source for probing cell-material interactions in direct reprogramming. While demonstration of direct reprogramming in human cells is more clinically relevant, the genetic homogeneity of cells from transgenic mice is a powerful advantage, especially when detecting small but important statistical differences within the data.

The original publication also reported ~40% cTnT-positive cells, which was an additional reason we initially selected this protocol over the others. However, we found cells cultured on TCPS-Mat(1×) to be only ~4% α -actinin-positive. This discrepancy may be attributed to one or several of the following: 1) temporal variations in the expression of cTnT versus α -actinin during reprogramming, 2) differences in the robustness of the cTnT antibody versus the α -actinin antibody, 3) subtle differences in culture protocols between laboratories, or 4) variations in flow cytometry analysis between laboratories. Interestingly, a recent study reporting direct reprogramming of human fibroblasts to cardiomyocytes found that α -actinin expression was consistently lower by flow cytometry than cTnT across 9 different conditions [85]. While this new and growing field may still need to adopt more robust and consistent metrics, our data clearly demonstrates a solid point: PEG surfaces can be engineered to affect direct reprogramming outcomes, and in this case, nearly double the number of beating cardiomyocyte-like cells. Based on the gene expression data, we suspect that the expression of cardiac troponin-T may be similar between the PEG-lam(5×)-RGD and TCPS-Mat(1×) treatments. It is entirely possible that sarcomeric α -actinin in combination with cardiac troponin-T marks a more mature cardiac phenotype than cardiac troponin-T alone. This would explain how two reprogrammed conditions might yield similar expression of troponin-T but varied expression of α -actinin.

The field of cellular reprogramming has progressed at lightning speed since the discovery of induced pluripotency in 2006 [86]. Unlike traditional PSC differentiation strategies, direct reprogramming has the potential to eliminate the need for *in vitro* cell expansion, differentiation, and cell transplantation. Biomaterials that control the cellular microenvironment and eliminate confounding signals caused by adsorbed serum proteins have the potential to enhance our understanding of the requirements for both *in vitro* and *in vivo* direct reprogramming.

5. Conclusions

PEG culture substrates were designed to match integrin expression during the initial stages of OSKM-mediated direct reprogramming of fibroblasts to cardiomyocyte-like cells. Through a combination of increased reprogramming efficiency and increased proliferation, PEG hydrogels presenting high concentrations of laminin and RGD peptide yielded twice as many cardiomyocyte-like cells as the originally reported substrate (Matrigel-coated TCPS). RGD peptides enabled better cell adhesion, stimulated proliferation, and did not impede reprogramming. The general methodology, using gene expression to guide materials design, may be applicable to a wide variety of reprogramming strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effects of varied induction timelines on adult tail tip fibroblasts

A) Multiple reprogramming media timelines were tested to clarify the protocols described by Efe et al. Media with doxycycline (dox) promoted expression of the Yamanaka factors Oct4-Sox2-Klf4-c-Myc (OSKM) in tail tip fibroblasts (TTFs) derived from transgenic mice. Timeline 4 produced the most beating clusters. In timeline 4, six days of dox-induced OSKM were coupled with pluripotency inhibition by Jak-Stat Inhibitor (JI1) in high serum medium. This was followed by three days of recovery in low serum medium also containing JI1, and then six days of induction of cardiomyocyte differentiation in chemically defined (no serum) medium containing bone morphogenetic protein 4 (BMP4). Abbreviations: Fibroblast (FB) medium, Knockout (KO) medium, Chemically Defined (CD) medium, ES-qualified fetal bovine serum (FBS). B) TTFs became smaller and more rounded under the influence of OSKM, and cells in timeline 4 produced clusters of very small cells by day 12 that ultimately began beating.

Figure 2. Characterization of cardiomyocytes directly reprogrammed from adult tail-tip fibroblasts

Reprogrammed cells formed beating patches visible by phase contrast microscopy (A, day 19). (B) A time-based map of an activation wavefront from the beating patch in (A) was made using the calcium-sensitive dye Fluo-2. The wavefront starts at the right side of the cluster (dark red) and travels through the perimeter of the cluster (demonstrated by color changes to orange and then yellow). Calcium traces from individual regions of interest (ROIs) are shown in (C). (D and E) Immunocytochemistry of day 18 TTF-derived cardiomyocytes revealed expression of sarcomeric α -actinin (red) within the beating patches and in cells attached to the adjacent substrate.

Figure 3. Substrate effects on early stage cell adhesion and proliferation

The culture substrate had dramatic effects on cell adhesion and proliferation during the first 9 days of the protocol. A) Although surfaces were seeded equally, the efficiency of cell adhesion and/or proliferation was not the same. One day after MEF seeding (day 0 of reprogramming), similar cell densities were seen on PEG-RGD(5x) and TCPS surfaces (red bars) coated with Matrigel or laminin. These were statistically higher than other PEG gels and TCPS without protein coatings (* $p < 0.05$ by ANOVA with post-hoc Tukey's HSD test). B) Cell counts from PEG hydrogels containing only RGD or only laminin were used to calculate proliferation rates over days 0–3. The proliferation rate was positively influenced by RGD concentration (* $p < 0.05$ by t-test), but not laminin-1 concentration. C) When RGD was used alone at 5x (lower left), an exceptionally high cell density was noticeable by phase microscopy by day 9. Densities were not as high on PEG-RGD(1x) or any PEG group where laminin was included.

Figure 4. Cell density and morphology at late stages of reprogramming protocol

Addition of RGD to PEG hydrogels boosted cell proliferation. A) Cells were trypsinized and counted by hemacytometer at days 15 and 18. Within the same condition, the cell density did not vary by day. Counts also did not vary between PEG-lam(5×) with 1× or 5× RGD. Counts from these conditions were therefore pooled to increase the number of samples for statistical analysis ($n = 6-12$). PEG-lam(5×) gels with RGD yielded ~25% more cells per square centimeter than TCPS–Mat(1×) or PEG-lam(5×) without RGD (* $p < 0.05$ by ANOVA with post-hoc Tukey HSD test). B) Phase contrast images were stitched together to give a macroscopic view of the individual wells. Without laminin, cells on the RGD(5×) gel rolled off the plate forming a large clump at the edge of the well (white arrow). On gels with laminin, more clusters were seen on gels that also presented RGD at 5×. Clusters on gels with 5×laminin but no RGD had a more spread morphology than those on gels presenting RGD (bottom image).

Figure 5. Effects of culture substrate on cell beating at day 18 of reprogramming

The extent of cell beating, quantified manually by phase contrast microscopy, was influenced by the culture substrate. A) Beating patches are defined as synchronously contracting. PEG hydrogels with high surface densities of adhesion molecules (green bars) had greater numbers of beating patches than gels with low surface densities (blue bars) or any TCPS surface (red bars) (* $p < 0.05$ versus PEG-lam(5 $\times$)-RGD(5 $\times$); # $p < 0.05$ against PEG-lam(5 $\times$) by Tukey's HSD test). The numbers of beating patches on TCPS surfaces (red bars) were similar regardless of adhesion molecule type or concentration. Unlike the PEG gels, it was not possible to increase beating on TCPS by increasing the laminin concentration. B) PEG conditions presenting laminin at 5 $\times$ (with or without 5 $\times$ RGD) also had greater coverage of beating cells than all TCPS conditions or PEG-lam(1 $\times$) (* $p < 0.05$ by Tukey's HSD test).

Figure 6. Effects of culture substrate on gene expression at day 18 of reprogramming

A) Levels of cardiac (*Actn2*, *Tnnt2*) and pluripotency (*Nanog*) markers were analyzed by quantitative real time RT-PCR. Markers were normalized to the *GAPDH* internal control gene. TCPS-Mat(1x) levels are scaled to 1 to present fold induction against the original Efe *et al.* condition. Some conditions were pooled to increase the number of samples, with measurements for individual substrates presented as supplementary materials. No statistical differences or trends were found between any of the pooled conditions. In order to present the original Efe *et al.* condition, TCPS-Mat(1x) is presented separately and thus has fewer samples for statistical testing. *Actn2* and *Tnnt2* levels were statistically higher than MEFs for the TCPS-lam(1x&5x) and PEG-lam(5x)-RGD-(0,1&5x) (* $p < 0.05$ against MEFs by Kruskal-Wallis with post-hoc Tukey HSD). *Nanog* levels in PEG-lam(5x)-RGD(0,1,&5x) were statistically lower than TCPS-lam(1x&5x) (# $p < 0.05$ by Kruskal-Wallis with post-hoc Tukey HSD). B) Expression levels of cardiac sub-type genes were normalized to sarcomeric α -actinin (*Actn2*) to exclude effects from non-cardiomyocytes. Expression level of the atrial marker *Mlc2a* stays constant, while markers for cardiac nodal and conduction cell phenotypes (*HCN1*, *HCN4*, *Tbx3*, *Cntn2*) are generally downregulated on PEG-lam(5x) conditions when compared to TCPS-Mat(1x) (the condition in Efe *et al.*). TCPS+Mat(1x) (n=3) is normalized to 1 to present fold change of the PEG-lam(5x) conditions. No statistical differences or trends were seen in PEG-lam(5x) gels presenting 5x RGD (n=3) or no RGD (n=3), so the conditions were pooled for the purpose of statistical testing (n=6). (* $p < 0.05$ by Student's t-test.)

Figure 7. Immunocytochemical comparison of cardiac marker expression on PEG conditions with 1 $\times$ versus 5 $\times$ laminin

Wells were stained for the cardiomyocyte marker sarcomeric α -actinin, with DAPI as a nuclear counterstain. A) The number of α -actinin positive pixels was normalized to the number of DAPI positive pixels for each well. PEG conditions presenting laminin at 5 $\times$ had a higher α -actinin/DAPI ratio than conditions presenting laminin at 1 $\times$ (* p <0.05 by t-test). (B and C) sample images from the quantitative analysis performed in (A). Samples were pooled to increase the number of samples, with measurements for individual substrates presented as supplementary materials.

Figure 8. Quantification of α -actinin expression by flow cytometry

PEG-lam(5 $\times$) gels with RGD promoted higher efficiency reprogramming. A) Histograms for fluorescence intensity of sarcomeric α -actinin IgG negative controls (black) versus stained cells (grey) demonstrated well-separated peaks between stained cells and the negative control. At least 10,000 events are presented per condition. B) The average percentage of α -actinin positive cardiomyocytes in PEG-lam(5 $\times$) gels containing RGD was ~1.72-fold higher than the condition originally described in Efe et al. (* p <0.05 against TCPS-Mat(1 $\times$) condition by ANOVA with post-hoc Tukey-HSD). C) Combined with the proliferation data, the increased efficiency gave greater numbers of cardiomyocyte on PEG-lam(5 $\times$) gels containing RGD than PEG-lam(5 $\times$) gels without RGD or TCPS-Mat(1 $\times$) controls (* p <0.05 by ANOVA with post-hoc Tukey HSD).