

Supporting Information

Sirtuin 6 Mediated Stem Cell Cardiomyogenesis on Protein Coated Nanofibrous Scaffolds

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1. Materials and methods

1.1 Preparation of 3D scaffolds

Poly(ϵ -caprolactone) (PCL) (molecular weight \sim 80,000) and 2,2,2-trifluoroethanol (TFE) were procured from Sigma- Aldrich, USA and SDFCL, India respectively. PCL was dissolved in TFE by stirring for 6 h at 25° C. Aligned nanofibers of neat PCL were prepared by electrospinning (ESPIN NANO, India) using 15% (w/v) polymer solution(27). The solution was filled in 5 mL syringes (gauge size 22) and placed in the spinneret at 25° C and 50% humidity. Aligned nanofibers were collected on an aluminum sheet using a drum collector rotating at a speed of 2400 rpm with voltage set at 15 kV and distance between the syringe and collector fixed as 24 cm.

Post fabrication, the fiber mats were sterilized by incubating in 70% ethanol for 2 min followed by UV sterilization for 30 min. Scaffolds were cut into circular discs according to the size of the well plates and coated by incubating in different protein solutions at fixed concentration. Excess solution was removed after 2 h and the scaffolds were left to air dry. Table 1 lists the different proteins and the concentration used for coating the nanofibers in this study. Hereafter, the following designations are used to describe the different samples: PCL refers to the uncoated neat PCL aligned fibers that serve as the control whereas PCL-Col, PCL-Fib and PCL-Mat refer to the aligned fiber mats of PCL coated with human collagen, human fibronectin, and basement membrane Matrigel respectively. Human collagen (cat no: C7624; Sigma Aldrich, USA) and human fibronectin (cat no: F0556; Sigma Aldrich, USA) were purchased from Sigma Aldrich whereas the Matrigel (Cat no: 356234) was procured from BD Corning, USA.

1.2 Characterization of scaffolds

The morphology of the scaffolds was characterized using a scanning electron microscope (SEM, ZEISS ultra 55) after sputtering with gold (~15 nm thick) at 5.0 kV accelerating voltage. The presence of the adsorbed protein layer on the nanofiber mats was visualized in the SEM. The amount of protein adsorbed on nanofiber scaffolds were quantified spectrophotometrically by BCA assay following manufacturer's protocol. The absorbance for the assay were measured at 562nm. The change in the chemical composition of the scaffolds after protein coating was observed by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR, PerkinElmer Frontier IR/NIR systems) in the range of 1750–950 cm^{-1} with a resolution of 4 cm^{-1} . An average of 12 scans was performed for each sample.

Dynamic mechanical analysis (DMA, TA instruments Q800) was used to study the mechanical properties of the nanofiber mats (7 mm \times 12 mm sample size) that was performed in a controlled force mode with a constant force of 3 N/min. The thickness of the mat used as 0.3 mm. The elastic modulus was taken as the slope of the linear region of the stress-strain plot. Three replicates were used for each sample and results are reported as mean \pm S.D.

The surface water wettability of the as-fabricated scaffolds and after protein coating was assessed using the contact angle goniometer (OCA 15EC goniometer, Dataphysics). The contact angle was measured using 2 μL drop of ultrapure water (Sartorius) placed on the surface of the scaffolds. Three independent measurements were noted and the data are shown as mean \pm S.D.

The loss of the protein coating on the PCL nanofibers was characterized *in vitro* as follows. The scaffolds of 10 mm diameter were placed in 48 well plate in complete cell culture (pH 7.4) medium at 37° C to mimic the cell culture conditions. The nanofiber mats were removed at Day 1, 3 or 5. Mats were dried and imaged using the SEM.

1.3 Stem cell studies

All stem cell studies were conducted with due approval of the Institutional Committee on Stem Cell Research of IISc. Primary hMSCs of passage 2 were obtained commercially from Lonza, USA. The cells were isolated from human bone marrow mononuclear cells of a 25 year female donor and cultured in 5% CO₂ atmosphere at 37° C, as reported recently(28). The expression of known hMSC markers such as CD73 (98.5%), CD90 (98.7%), CD105 (98.8%), CD14 (0.9%), CD19 (0.4%), CD45 (0.4%) and HLADR (0.3%) was confirmed by the supplier. Knockout Dulbecco's modified Eagle medium (DMEM, Sigma) containing 15% (v/v) MSC-qualified serum, 1% Glutamax and 1% (v/v) antimycotic antibiotics was used as the growth medium. Medium was refreshed every 3 days. The cells at 75% confluency were detached using 0.25% trypsin EDTA. All studies reported herein were conducted with cells of passages 4 to 6.

Cardiomyogenic differentiation was induced in the stem cells on the protein coated 3D nanofibrous mats that were cut to fit individually in wells of a 48 well plate. The fiber mats were sterilized by exposing to UV light for 30 min before conducting the cell culture studies. 1×10^4 cells per well were seeded in 0.2 mL serum-free knockout DMEM containing 3 μ M 5-azacytidine (Aza), a known cardiomyogenic factor(29). Aza-containing medium was completely removed after 24 h of incubation and the cells were washed with phosphate-buffered saline (PBS) to ensure complete removal of Aza. The medium was then changed to complete medium without Aza, as described in a recent study(28). Knockout DMEM medium supplemented with 10% FBS and 1% antibiotic was mixed with spent medium (SM) from H9c2 cardiomyocyte at 1:1 ratio to maintain the cells for up to 7 days. Medium was replenished every 2 days.

1.4 Assessment of stem cell response

The proliferation of the cells on the uncoated and protein coated fibrous scaffolds was evaluated at day 1, 3 and 7 by measuring the cellular DNA content using the Picogreen dsDNA

quantification kit (Invitrogen) as described previously(30). Briefly, cells were lysed with a lysis solution and the lysate was mixed with the Picogreen dye to determine DNA content by measuring the fluorescence intensity in a plate reader. Three independent biological repeats were conducted for cell proliferation assay.

Cardiomyogenic lineage commitment was assessed by immunostaining for cardiac specific marker proteins such as connexin 43 (Cx43) (Thermo Scientific, USA), cardiac myosin heavy chain (β -MHC) (Thermo Scientific, USA) and desmin (Abcam, USA). All the primary antibodies were raised in mice. Cells were fixed at day 7 with 4% (mass/vol) paraformaldehyde in PBS for 15 min. The samples were washed further with PBS and the cell membrane was permeabilized using 0.2% Triton-X solution. Blocking was done using 5% BSA at room temperature for 1 hr. The cells were incubated with the primary antibodies at a dilution of 1:200 overnight at 4° C. The primary antibodies were removed and washed thoroughly with PBS. This was followed by incubation with goat anti-mouse Alexa Fluor 488 conjugated secondary antibodies for Cx43 and desmin, and donkey anti-mouse Alexa Fluor 546 conjugated secondary antibody for β -MHC for 2 h at 25° C at a dilution of 1:400. The nuclei were stained with 0.5 μ g/ml of DAPI for 45 s at 25° C. The samples were mounted over a glass coverslip using the mounting agent DPX and imaged using a confocal microscope (LSM 710, Zeiss, USA).

For western blotting, cells were scraped from the scaffolds with 1 \times cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF and 1X protease inhibitor cocktail from Sigma) and completely lysed using a vortex mixer. The lysate was centrifuged at 12000 rpm for 10 min at 4° C to remove cell debris and the supernatant was transferred to a fresh tube. Lysates were diluted to normalize for the protein concentration determined using the Bradford's protein estimation method and were resolved by SDS-PAGE in a 10% gel at

constant voltage. The proteins were then blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Hybond 0.45 μm PVDF membranes) by sandwiching them between filter pads and performing overnight wet transfer. Membrane was blocked for nonspecific binding with 5% skimmed milk before further incubation with primary antibodies (1:1000) prepared in 5% BSA overnight at 4° C. HRP conjugated secondary antibodies were prepared at 1:5000 dilutions in 1% skimmed milk and were incubated for 1 h at room temperature. The blots were developed using a chemiluminescence imager (BioRad ChemiDoc Touch). Western blot was performed with antibodies for cardiac specific markers Nkx 2.5 (raised in rabbit), and desmin (raised in mouse) with Actin (HRP conjugated) or GAPDH (raised in rabbit) as the loading control. All the antibodies were purchased from Abcam, USA.

1.5 Live cell calcium imaging

hMSCs were cultured on protein coated aligned fibers for promoting cardiomyogenic differentiation for 7 days, as above. The differentiated cells were transfected with red fluorescent protein (pmCherry-N1, Clontech) received as gift from Dr. Annapoorni's Lab, IISc. Transfection was done using Lipofectamine 3000 (Life Technologies, USA) following manufacturer's protocol one day prior to co-culturing.

The differentiated cells were co-cultured with primary neonatal cardiomyocytes. All experimental protocols involving the use of animals were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of IISc constituted as per the article number 13 of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) rules laid down by the Government of India. Neonatal Sprague Dawley rats (0 – 1 day old) were procured from Central Animal Facility, IISc. Pups were sacrificed by decapitation and their hearts were harvested. The primary cardiomyocytes were isolated using cocktail of trypsin and collagenase type II(31). Pre-plating on uncoated dishes was done for 1.5 h to minimize the attachment of non-myocardial cells. The non-adherent primary

cardiomyocytes were then co-cultured on gelatin coated glass bottom dishes along with the transfected stem cell-derived cardiomyocytes. Primary cardiomyocytes and mCherry-transfected hMSC-derived cardiomyocytes were seeded at 2.5×10^6 /ml cells and 0.05×10^6 /ml, respectively, in 35 mm live cell imaging dishes. The cells were cultured in DMEM medium supplemented with 10% FBS. The cells were incubated in a humid 5% CO₂ incubator at 37° C for 36 h. The medium was replenished 4-6 hours before imaging.

mCherry transfected hMSC derived cardiomyocytes on different protein coatings were co-cultured with primary rat cardiomyocytes attached to the surface within 16-18 h of seeding. The cells in the co-culture looked healthy and imaged to visualize cytosolic Ca²⁺ spikes. The cells were loaded with Fluo-4 for 45 min at 37°C, washed and imaged in a confocal microscope (Zeiss LSM 880). The fluorescence emission from the transfected cells was detected with 594 nm excitation for expression of RFP and 488 nm excitation for Fluo-4 in a special chamber maintained at 37° C and 5% CO₂ incubator for live cell imaging. Fluorescence (F) during cell contractions was normalized to the basal cell fluorescence (F₀) and plotted as a function of time using Microsoft Excel.

1.6 Role of SIRT6 in cardiomyogenesis

hMSCs were seeded on PCL-Col and induced to differentiate to cardiomyogenic lineage by treatment with 3μM Aza for 24 h in serum free media. Aza containing media was removed after 24 h and SIRT6 was transiently depleted using a specific siRNA. The siRNA sequence (SIRT6 siRNA - AAGAATGTGCCAAGTGTAAGA) was adapted from a previous report and was synthesized and supplied by Eurogentec(32).

SIRT6 siRNA was transfected using Lipofectamine RNAiMax (Thermofisher, USA) following manufacturer's protocol. Non-targeting siRNA was used for the transfection in control group. The hMSCs were then evaluated 96 h post transfection using immunostaining and western blotting

Inhibition of the Wnt signaling pathway was done using β -catenin inhibitor (Calbiochem, Germany) at a concentration of 10 μ M for 48 h. DMSO was used as the vehicle control . The β -catenin inhibitor was added 24 h post SIRT6 siRNA transfection . The effect of the Wnt signaling inhibition on differentiation of hMSCs during cardiomyogenesis was assessed at day 5 using immunostaining and western blots, as described above.

Figure S1

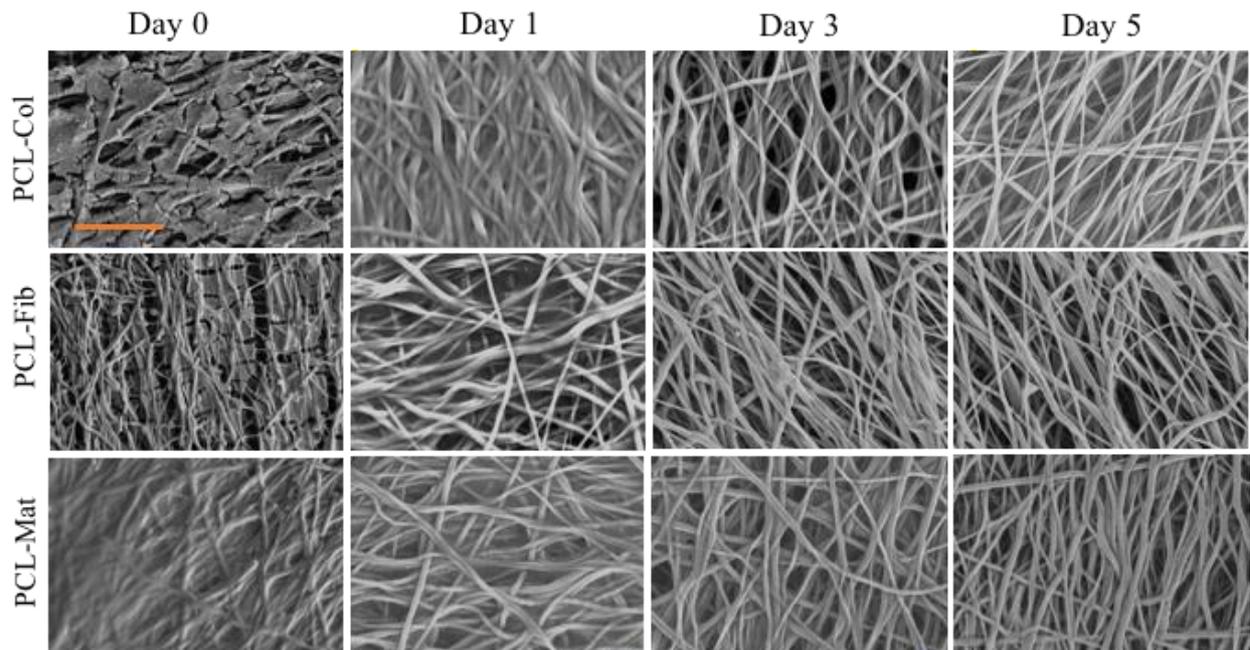


Figure S1: *In vitro* degradation of the protein 3D nanofibrous mats. The loss of ECM from the scaffolds were studied at different time point and confirmed using scanning electron microscopy. Ultra-structural micrographs representing the loss of collagen coating (a-d), fibronectin coating (e-h), and matrigel coating (i-l) progressively over a period of 5 days (0-5) when incubated in media at 37 °C.

Supporting videos V1-V4 are available online