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PLURIPOTENT STEM CELLS

Human Fibrinogen for Maintenance and Differentiation of Induced Pluripotent Stem Cells in Two Dimensions and Three Dimensions

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ABSTRACT

Human fibrin hydrogels are a popular choice for use as a biomaterial within tissue engineered constructs because they are biocompatible, nonxenogenic, autologous use compatible, and biodegradable. We have recently demonstrated the ability to culture induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium on fibrin hydrogels. However, iPSCs themselves have relatively few substrate options (e.g., laminin) for expansion in adherent cell culture for use in cell therapy. To address this, we investigated the potential of culturing iPSCs on fibrin hydrogels for three-dimensional applications and further examined the use of fibrinogen, the soluble precursor protein, as a coating substrate for traditional adherent cell culture. iPSCs successfully adhered to and proliferated on fibrin hydrogels. The two-dimensional culture with fibrinogen allows for immediate adaption of culture models to a nonxenogeneic model. Similarly, multiple commercially available iPSC lines adhered to and proliferated on fibrinogen coated surfaces. iPSCs cultured on fibrinogen expressed similar levels of the pluripotent stem cell markers SSea4 (98.7% ± 1.8%), Oct3/4 (97.3% ± 3.8%), TRA1-60 (92.2% ± 5.3%), and NANOG (96.0% ± 3.9%) compared with iPSCs on Geltrex. Using a trilineage differentiation assay, we found no difference in the ability of iPSCs grown on fibrinogen or Geltrex to differentiate to endoderm, mesoderm, or ectoderm. Finally, we demonstrated the ability to differentiate iPSCs to endothelial cells using only fibrinogen coated plates. On the basis of these data, we conclude that human fibrinogen provides a readily available and inexpensive alternative to laminin-based products for the growth, expansion, and differentiation of iPSCs for use in research and clinical cell therapy applications. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:512–521*

SIGNIFICANCE STATEMENT

As induced pluripotent stem cells approach clinical use for transplantation, the requirement for reagents that comply with regulatory practices needs to be addressed. To address this need, the use of human fibrinogen as a coating reagent for adherent culture of induced pluripotent stem cells was investigated. The data show that induced pluripotent stem cells cultured on fibrinogen maintain pluripotency, can be differentiated to the three germlayers, and can differentiate specifically to endothelial cells. As fibrinogen derived from human blood is readily available for clinical use, findings demonstrate an immediate solution to those developing regulatory-compliant cell therapy products for human application.

INTRODUCTION

Fibrinogen is a plasma protein that is the precursor to fibrin, an insoluble protein that forms a three-dimensional (3D) mesh that impedes the flow of blood, forming a clot. When isolated, fibrin forms a hydrogel, the properties of which can be altered based on its concentration [1]. When initially formed the hydrogel is adhesive, providing the basis for the clinical use of fibrin tissue sealants. The formation and degradation of fibrin in the body is regulated by well-known cascades of enzymes [2]. The ability of the body to degrade fibrin has resulted in a great deal of interest in the use of fibrin hydrogels as scaffolds for regenerative medicine therapies. This application

of fibrin has been accelerated in large part due to the availability of US Food & Drug Administration (FDA)-approved clinical grade fibrin sealants.

Fibrin gels are most synonymous with vascular engineering applications, where a variety of approaches have demonstrated the ability to form vessel-like structures in vitro and in vivo, angiogenesis in vivo and anastomosis after implantation [3]. Furthermore, fibrin hydrogels have shown potential use as an encapsulant for drug [4] and cell delivery [5, 6]. For example, bone marrow-derived mesenchymal stromal cells (MSCs) cultured in fibrin hydrogels were implanted in a cranial defect model of a rodent to develop bone mineralization and increased vascularization [5]. Autologous fibrin, in the form of autologous platelet-rich plasma, has already been used in multiple clinical trials as a means to deliver MSCs [7].

Previously, our group had reported using fibrin hydrogels as a scaffold for transplantation of induced pluripotent stem cell derived retinal pigment epithelium (iPSC-RPE) [8]. The results of this work showed that the partially differentiated iPSC-RPE can directly adhere to the fibrin gel, form a monolayer and express phenotype specific marker proteins [8]. Similarly, work from Willerths' group has demonstrated culture of iPSC-derived neural aggregates on fibrin hydrogels can be used to generate a mixed population of dorsal and ventral spinal neurons [9–11]. These examples highlight the ability to differentiate iPSC-derived progenitor-type populations to terminally differentiated cells using fibrin hydrogels. However, to the best of our knowledge, iPSCs themselves have not been cultured on fibrin hydrogels. Thus, we hypothesized that fibrin could serve as a valid scaffold for iPSC culture.

Although the emergence of 3D cell culture is an exciting and necessary advance for the future of regenerative medicine, the majority of cell culture today still occurs in two dimensions (2D). For therapeutic applications, and even at the basic research level, the need to generate cell banks, and in many cases for multistep differentiation procedures is still dependent on adherent cell culture in 2D.

For this reason, we also hypothesized that fibrinogen, the soluble precursor to fibrin, could serve as a novel surface coating for growth and differentiation of iPSCs. Our data demonstrate that both fibrin gels and fibrinogen coated surfaces are an excellent alternative to laminin coated surfaces providing the option of 3D cell culture, and a xeno-free, inexpensive, readily available material for the pretreatment of adherent surfaces for 2D culture of iPSCs and their differentiation in research and therapeutic applications.

Materials and Methods

Cells

The previously described iPSC line 006-BIOTR-0001, obtained from fibroblasts and reprogrammed with Sendai virus kit (Thermo Fisher, Waltham, MA; clone 1, Mayo Clinic) was used for all experiments [8, 12, 13]. Additional lines used to validate reproducibility of the fibrinogen coating included the previously described [13] IMR-90-4, obtained from a immortalized human fibroblast cell line and reprogrammed using lentiviral vectors (clone 4; WiCell; Madison, WI), and a new line, 018-BIOTR-0089 (clone 18), produced by reprogramming of human peripheral blood lymphocytes using episomal DNA transfection. mTESR (Stem Cell Technologies, Vancouver, BC, Canada) was used for iPSC growth, and ReLeSR (Stem Cell Technologies) was used to dissociate cells for passage. Passage was performed on iPSC colonies and replated at various dilutions (1:3–1:5) to maintain pluripotency and enable amplification.

Fibrin Gel Culture

Fibrin gels were made as previously described using a clinical grade kit (Evicel; Ethicon, Somerville, NJ) [8]. Briefly, a mixture of 30 mg/ml fibrinogen and 50 U/ml thrombin (final concentrations) was mixed in a well of a 12-well plate (total 100 μl), and a custom polycarbonate mold with parafilm lining was used to flatten the gel within the well and minimize the minicus effect. The 30 mg/ml fibrinogen concentration was chosen because it is comparable to fibrin glue concentrations and our previous work demonstrating iPSC-RPE culture [8]. The 50 U/ml thrombin concentration was chosen to provide some time for mixing and applying the mold prior to gelation. This resulted in a 4 cm^2 area gel, with a thickness ranging from 200 to 300 μm. The gel was allowed to fully polymerize for 2 hours at 37°C prior to washing with phosphate-buffered saline (PBS) and seeding the iPSCs. Culture media was supplemented with 50 U/ml aprotinin to investigate preservation of the gel.

Fibrinogen Extraction

Fibrinogen was extracted using two different previously published methods. These were ethanol precipitation [14] and cryo-precipitation [15]. Once precipitated, fibrinogen was reconstituted in 1 M Tris–HCL (pH 8.0) or Ca^{2+} and Mg^{2+} free PBS (CMF-PBS-PBS). We found the Tris–HCL solution resulted in more stable protein solubility and ease of coating. Samples were sterile filtered and aliquoted as a stock solution to prevent multiple freeze-thaws. Fibrinogen from Evicel was also used for comparison. Unfortunately, we found significant variability with commercially available research grade fibrinogen reagents (data not shown).

Clottable fibrinogen concentrations were confirmed using the Clauss method and were performed by Mayo Medical Laboratories (Rochester, MN). Total protein concentration was determined using a 660 nm Protein Assay (Thermo Fisher), in which Evicel was substituted as the standard. The purity of fibrinogen preparations was assessed using 10% SDS-PAGE gels stained with Coomassie G-250 solution (Bio-Rad, Hercules, CA). The gel was then photographed on a light box using a handheld camera.

Plate Coating

Fibrinogen stock solutions from the various sources were thawed at 37°C and diluted to working concentrations (ranging from 1 μg/ml to 1 mg/ml) in 1 M Tris–HCL. Evicel was diluted in PBS. A plating density of 0.3125 ml/cm^2 of surface area was used. Plates were incubated at 37°C for a minimum of 2 hours prior to use. After incubation, plates were washed three times with PBS prior to plating iPSCs.

Geltrex (Thermo Fisher) was used as a positive control. Frozen aliquots of Geltrex were thawed on ice, diluted 1:240 in Dulbecco's modified Eagle's medium/F12 media, and plated at a density of 0.3125 ml/cm^2 of surface area. Plates were incubated for at least 2 hours at 37°C, after which the solutions were aspirated and cells are immediately added in culture media.

Pluripotency Marker Expression

Cells on various coated surfaces were cultured for at least 48 hours prior to performing flow cytometry to analyze pluripotency

marker expression. Cells were dissociated using TrypLE (Life Technologies, Carlsbad, CA), incubated at 37°C up to 5 minutes, centrifuged at 800*g* for 4 minutes, resuspended in PBS, split evenly into two tubes (to accommodate an unstained control), and recentrifuged. Cells were fixed in PerFix-nc (Beckman Coulter, Brea, CA) per the manufacturer's protocol. Cells to be stained were mixed with a staining solution consisting of permeabilizing reagent, 1:10 Alexa 488 anti-human Nanog (BD, Franklin Lakes, NJ), 1:10 Alex 647 anti-OCT 3/4 (BD), 1:10 Phycoerythrin (PE) anti-SSEA4 (BD), and 1:10 PerCP-Cy5.5 anti-human TRA1-60 (BD). Samples were run on a Gallios Flow Cytometer (Beckman Coulter), using four channels: (Alexa 488) 488 nm excite, 550 nm band pass (PE), 561 nm excite, 582 nm band pass (PerCP), 561 nm excite, 695 nm band pass, and (Alexa 647) 633 nm excite, 660 nm band pass. A total of 1,000 cells were counted, with double positive cells required for confirmed expression.

Trilineage Differentiation

iPSCs were passaged from 60 mm plates using Accutase (Innovative Cell Tech, San Diego, CA) onto 6-well (for ectoderm) or 24-well plates (for endoderm and mesoderm) coated with the various substrates. Ectoderm differentiation was performed using STEMdiff Neural Induction Medium (Stem Cell Technologies) per the manufacturer's protocol. Y-27632 (Stem Cell Technologies) was added to the day 0 media only. After 9 days of culture, the cells were passaged from the 6-well plate using Accutase and replated onto appropriately coated 24-well plates. Differentiation was completed using the Neural Induction Medium until roughly 70% confluent. Endoderm differentiation was performed using the STEMdiff Definitive Endoderm Kit (Stem Cell Technologies) per the manufacturer's protocol. After day 5, cells were fixed in 4% paraformaldehyde (PFA). Mesoderm differentiation was performed using the StemDiff Mesoderm Induction Medium per the manufacturer's protocol. After day 5, cells were fixed in 4% PFA.

Immunofluorescent Staining

Fixed iPSCs were stained for pluripotency markers to assess clonal variation between culture substrates. Fixed cells were permeabilized in 0.2% Triton X-100 (Sigma–Aldrich, St Louis, MO) for 30 minutes at room temperature prior to incubation in blocking solution (DAKO, Santa Clara, CA). Respective wells were incubated with one of the following primary antibody combinations for 1 hour at room temperature: (a) 1:200 rabbit anti-Oct 3/4 (Abcam, Cambridge, MA; #Ab19857) and 1:100 mouse anti-SSea4 (Abcam; #Ab16287), or (b) 1:100 rabbit anti-Nanog (Cell Signaling, Danvers, MA; #4903) and 1:100 mouse anti-Tra1-60 (Abcam; #Ab16288). Wells were washed with washing solution (DAKO) thrice. Then, the secondary antibody cocktail was incubated for 30 minutes at room temperature: 1:200 anti-rabbit Alexa 594 and 1:300 anti-mouse Alexa488. Wells were again washed, stained with DAPI for 5 minutes and imaged using a Cytation 5 Imager (BioTek, Winooski, VT).

Differentiated cells were stained using a similar protocol, but modified to include the following primary antibodies: (Ecto) 1:20 sheep anti-Pax6 (RND Systems, Minneapolis, MN; #AF8150; Endo), 1:200 rabbit anti-Fox A2 (Cell Signaling; #8186m), or (Meso) 1:200 rabbit anti-MixL1 (Millipore, Burlington, MA; #ABS232). Images were analyzed using Gen5 Imaging Prism (BioTek; Winooski, VT) software and differentiation efficiency was calculated as the total dual-stain positive cells divided by total DAPI positive cells.

iPSC-Endothelial Cells Differentiation

iPSC-endothelial cells (ECs) were differentiated as described previously [16], with the following modifications. iPSCs colonies (0.5–1 mm in diameter) were plated onto fibrinogen coated 6-well plates, 5–8 colonies per well, and cultured with mTeSR1 for growth (day 0). On day 4, the mTeSR1 media was replaced with Mesoderm Induction Media: B(P)EL [16] with 25 ng/ml Activin A (Miltenyi, Bergisch Gladbach, Germany), 30 ng/ml BMP4 (Miltenyi), 50 ng/ml VEGF (R&D Systems), and 1.5 μM CHIR (Tocris, Bristol, United Kingdom). The media was then replaced on day 7 with vascular specific media: B(P)EL with 50 ng/ml VEGF and 10 μM SB431542 (Tocris). The vascular specific media was replenished on day 11 and day 13 or 14.

On day 14 or 15, the ECs were purified using CD31-Dynabeads (Thermo Fisher) as described elsewhere [16]. Labeled cells were resuspended in Endothelial Growth Media (EGM2, Lonza, Basel, Switzerland) and plated on fibrinogen-coated T25 flasks for growth. Media changes were performed 1–2 days. iPSC-ECs were passaged every 4–7 days, based on confluence, using TrypLE and centrifugation at 300*g* for 3 minutes. Cells were used up to passage 5.

To verify EC differentiation, cells were cultured on 4-well chamber slides coated with fibrinogen. Cells were fixed in −20°C Methanol for 5 minutes and washed thrice with PBS (Ca^{2+}, Mg^{2+}). Cells were blocked using 6% goat serum in 0.3% Triton X in PBS. Cells were incubated with 1:50 anti-CD31 (BBA7, R&D Systems), 1:200 anti-VE-Cadherin (MAB9381, RND Systems) or 1:200 FITC-UEA-Lectin (Vector Labs, Burlingame, CA) overnight at 4°C. After washing, the CD31 stained cells were incubated with 1:200 FITC-anti-mouse secondary antibody for 1 hour at room temperature. After three washes in PBS (Ca^{2+}, Mg^{2+}), the slide was mounted using Fluoromount (Electron Microscopy Sciences, Hatfield, PA) and imaged using a Nikon E600 fluorescence microscope (Nikon, Tokyo, Japan).

RESULTS

iPSC Culture on Fibrin Gels

Based on our previous observation that iPSC-RPE attach to fibrin hydrogels, we sought to determine whether undifferentiated iPSCs could also be cultured on fibrin hydrogels. iPSCs colonies following digestion with TrypLE adhered to fibrin hydrogels made of 30 mg/ml fibrinogen concentration and 50 U/ml thrombin concentration (Fig. 1) within 24 hours of plating. The 30 mg/ml fibrinogen concentration was chosen based on our previous experience culturing iPSC-RPE [8]. iPSC colonies appeared rounded and cells appeared to proliferate over the next several days. As early as 24 hours, visible lines were detected within the hydrogel layer, suggestive of fibrin gel contraction. Previous reports have shown that cells cultured in fibrin can cause fractures within the gel due to contractile forces [17]. Thus, we tested whether the inclusion of aprotinin would better preserve the gel during culture. The inclusion of 50 U/ml of aprotinin into the culture media did not prevent the formation of lines within the gel (Fig. 1). With the inclusion of aprotinin, we were able to grow iPSCs up to 10 days (reaching confluence) and differentiate iPSCs out to 50 days without hydrogel collapse (data not shown).

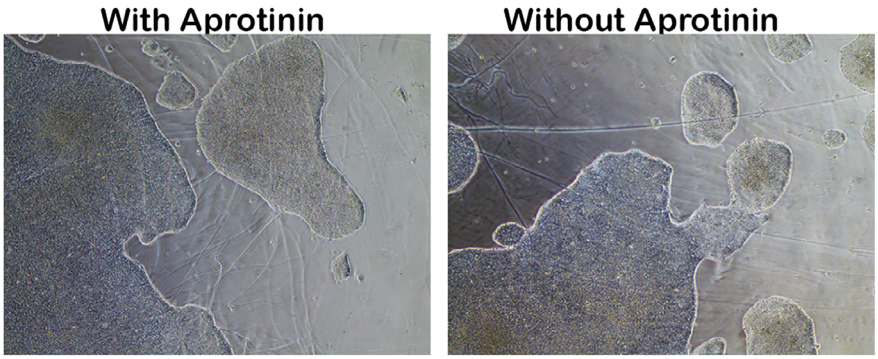

Figure 1. Induced pluripotent stem cells (iPSCs) attach to and grow on fibrin hydrogels. Representative image showing iPSC colony growing on fibrin hydrogel produced using (final) 30 mg/ml fibrinogen and 50 U/ml thrombin as described [8], with and without aprotinin. The inclusion of aprotinin did not reduce the appearance of lines within the gel. Magnification: ×4 objective, phase contrast.

Assessing Purity of Fibrinogen Extraction

Having demonstrated that iPSCs adhere to fibrin, we next sought to determine whether plates coated with fibrinogen, the precursor to fibrin, would support adhesion and growth of iPSCs. We have found that there is significant variability in commercial fibrinogen preparations with reference to purity and clottability. For this reason we compared fibrinogen obtained commercially with fibrinogen prepared in our laboratory from fresh frozen human plasma using either cryoprecipitation or ethanol precipitation. This further highlights the potential to use autologous fibrinogen with iPSC culture.

The clottable fibrinogen concentration of a commercial fibrinogen preparation (EVI) was 61 mg/ml. Cryoprecipitate (CPF) was 15.4 mg/ml, and ethanol precipitates (EPF1 and EPF2, respectively) were 6.7 mg/ml and 8.4 mg/ml (Fig. 2A). Total protein was determined using EVI as a standard based on the manufacturers claim for concentration (109 mg/ml) for CPF, EPF1, and EPF2, protein concentrations were 17.5 mg/ml, 9.7 mg/ml, and 9.3 mg/ml, respectively. Thus the percentage of clottable fibrinogen to total protein was 56% (EVI), 88% (CPF), 69% (EPF1), and 90% (EPF2; Fig. 2B).

To determine the purity of fibrinogen in each preparation we performed SDS-PAGE and stained the gels with Coomassie Blue. A shown in Figure 2B, all lanes had prominent bands representing α-(67 kDa), β-(54 kDa), and γ-chains (47 kDa) of fibrinogen (Fig. 2B). Compared with a positive control (lanes 9 and 10) depleted for plasminogen (92 kDa), von Willebrand factor (vWF; 20 kDa) and fibronectin (262 kDa), we observed that Evicel (lanes 4 and 5), CPF (lane 8) and EPF (lanes 2 and 3) had an additional band of ∼260 kDa, suggestive of fibronectin. That band was not observed in EPF2 (lanes 6 and 7). EPF also had additional bands migrating at ∼140 kDa.

Comparatively, Geltrex consists mainly of extracellular matrix proteins, including laminin, collagen type IV, and heparin sulfate proteoglycans. Newer formulations of Geltrex come in reduced growth factor forms in order to reduce lot-to-lot variability.

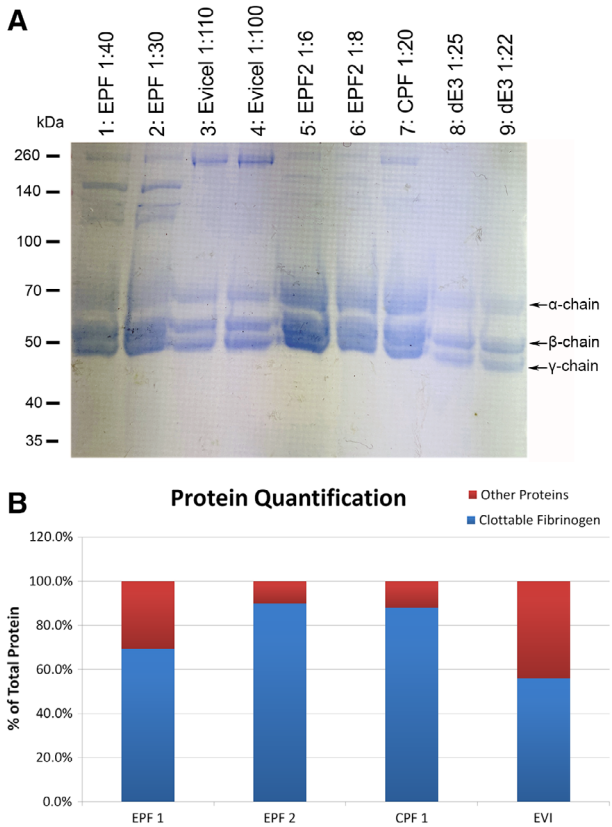

Figure 2. Purification of fibrinogen from fresh frozen plasma. **(A):** Coomassie Blue stained SDS-PAGE gel showing the various preparations of fibrinogen. Lane labels indicate dilution factor for each preparation. Lanes 2 and 3 are the first ethanol precipitated fibrinogen (EPF) and lanes 6 and 7, the second (EPF2). Lanes 4 and 5 are commercially available fibrinogen (Evicel or EVI). Lane 8 is cryoprecipitated fibrinogen. Lanes 9 and 10 are commercially available fibrinogen depleted of fibronectin, von Willebrand factor, and plasminogen. **(B):** A graph showing the values of clottable fibrinogen and total protein in fibrinogen preps produced in the lab.

Plating iPSCs on Fibrinogen Extract, Titration

Titration was performed to find the threshold concentration required for iPSC attachment using a range of 10 μg/ml to 2 mg/ml of clottable fibrinogen concentration. iPSCs were passaged as whole colonies on the various substrates. A minimum of 100 μg/ml of clottable fibrinogen concentration was necessary for consistent cell attachment (Fig. 3 shows the EPF2 group). However, after 2 days, only modest growth was observed for this coating concentration. As clottable fibrinogen concentration increased, more iPSC coverage was noted after 3 days of culture. No observable differences were detected at concentrations above 1.0 mg/ml. Similar consistency of iPSC attachment and growth was seen with EVI and CPF at 1.0 mg/ml clottable

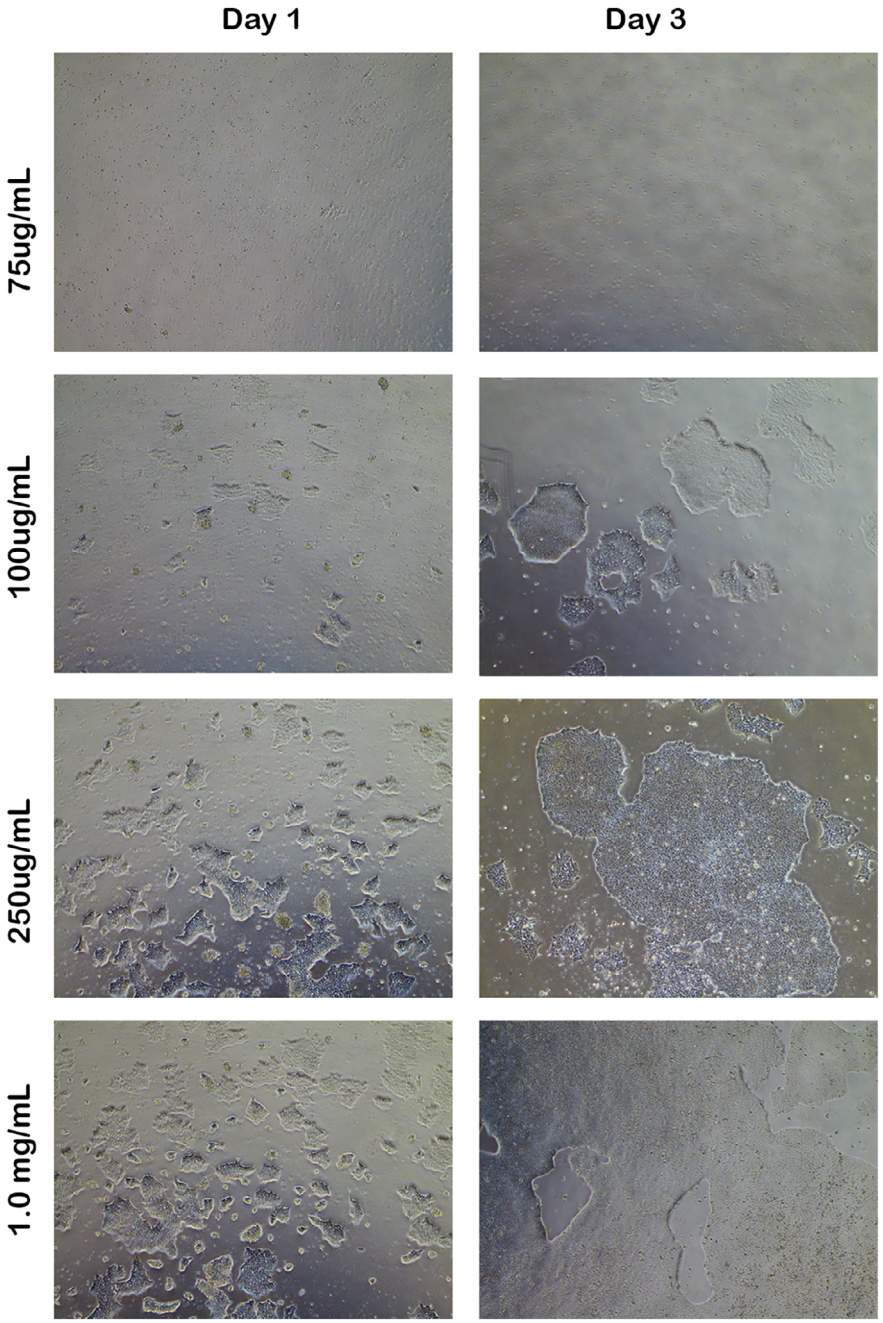

Figure 3. Titration of fibrinogen concentration for induced pluripotent stem cell (iPSC) culture. Representative images of iPSCs cultured on various coating concentrations of fibrinogen at day 1 and day 3. Panels show clone 1 iPSC colonies on plates coated with EPF2. Concentrations below 100 μg/ml did not exhibit iPSC attachment. Concentrations at and above 1 mg/ml exhibited consistent attachment and high proliferation over time.

fibrinogen concentration, typically reaching confluence by day 4 of culture. This growth rate was similar to that seen with iPSCs plated on Geltrex coating at similar dilutions.

iPSCs on Fibrinogen Maintain Pluripotency Markers Similarly to iPSCs on Geltrex

iPSCs cultured on fibrinogen were confirmed to maintain pluripotency markers (Fig. 4A). Cells were passaged up to three times with the fibrinogen reagent prior to performing pluripotency marker analysis. To test heterogeneity of iPSC culture on fibrinogen, three uniquely different iPSC lines were cultured on fibrinogen coated plates and tested for pluripotency markers. These lines differed in the method of reprogramming (Sendai virus, Lentivirus, or episomal DNA) and origin of donor tissue (fibroblast versus lymphocyte). Immunofluorescent staining of iPSC colonies on EVI, EPF, and Geltrex (GT) was positive for Oct4, SSea4, Nanog, and Tra1-60 (Fig. 4A shows representative clone 1 images). FACs analysis of ≥1,000 cells was used to quantify cell expression of pluripotency markers (Fig. 4B). A marker was considered positive only if double positive in combination with another marker. Combining data from all three clones, SSea4 expression was positive in 98.7% ± 1.8% on EVI,

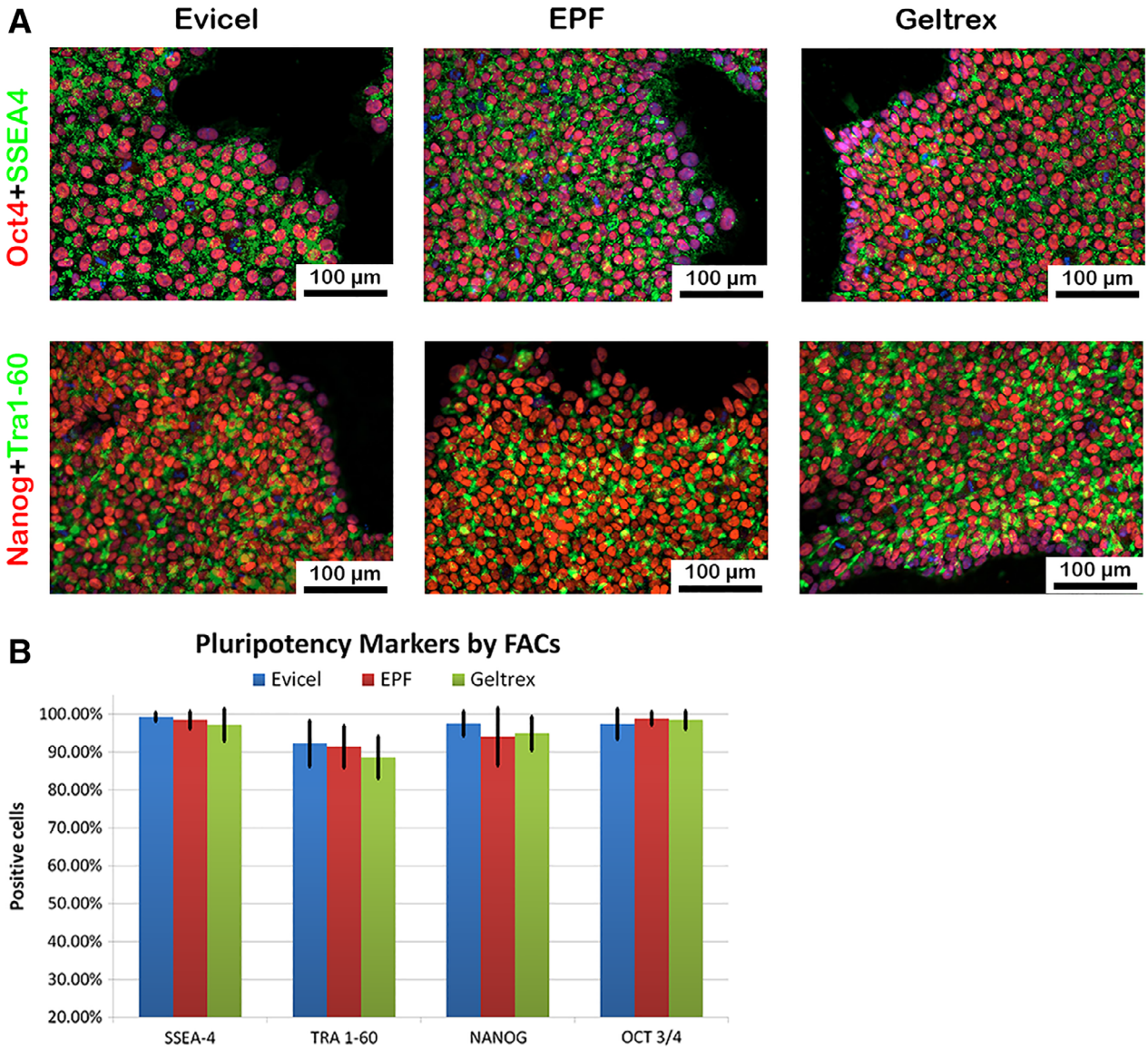

Figure 4. Pluripotency marker assessment of induced pluripotent stem cells (iPSCs) on various substrates. **(A):** Images of immunofluorescent staining of pluripotency markers (Oct4, SSea4, Nanog, Tra1-60) on iPSC colonies on Evicel, ethanol-precipitated fibrinogen (EPF), or Geltrex coated plates. The images are of clone 1 iPSC colonies. **(B):** Graph depicting flow assisted cytometry-based quantitative expression of pluripotency markers by iPSCs cultured on Evicel, EPF, or Geltrex. No statistically significant differences were detected ($n = 4$; $p > .05$).

97.7% ± 3.2% on EPF, and 95.8% ± 6.4% on GT ($p = .45$). TRA1 60 expression was positive in 92.2% ± 5.3% on EVI, 92.4% ± 5.2% on EPF, and 88.1% ± 5.0% on GT (0.35). NANOG expression was positive in 96.0% ± 3.9% on EVI, 94.5% ± 6.4% on EPF, and 93.3% ± 7.3% on GT ($p = .71$). Oct4 expression was positive in 97.3% ± 3.8% on EVI, 99.1% ± 1.4% on EPF, and 98.4% ± 1.8% on GT ($p = .40$). No significant differences were detected.

iPSCs on Fibrinogen Differentiate to Each of the Three Germline Layers

All three clones of iPSCs cultured on fibrinogen were confirmed to differentiate to each of the three germ layers when cultured in appropriate differentiation media (Fig. 5A shows representative clone 1 images). Cells were passaged up to three times with the fibrinogen reagent prior to performing differentiation. Immunofluorescent staining of iPSC colonies on EPF and GT showed positive staining for FoxA2 and Sox17 for cells undergoing endoderm differentiation throughout individual colonies. Mesoderm differentiation resulted in iPSCs cultured on EPF and GT expressing CD31 and NCAM. Ectoderm differentiation resulted in iPSCs cultured on EPF and GT expressing Nestin and Pax6.

To quantify efficiency of differentiation, differentiated cells were fixed and stained for the following nuclear markers: DAPI for all germ layers, FoxA2 for endoderm, Mixl2 for mesoderm, and Pax6 for ectoderm. Colonies were randomly selected and quantified for the percent of marker positive cells relative to DAPI positive cells. Data for each clone was kept separate due to the highly variable efficiency of differentiation amongst clones (Fig. 5B). Clone 1 cells on EPF demonstrated 31.1% ± 6.0% endoderm positive staining, 97.8% ± 2.0% mesoderm positive staining, and 46.9% ± 22.2% ectoderm positive staining while

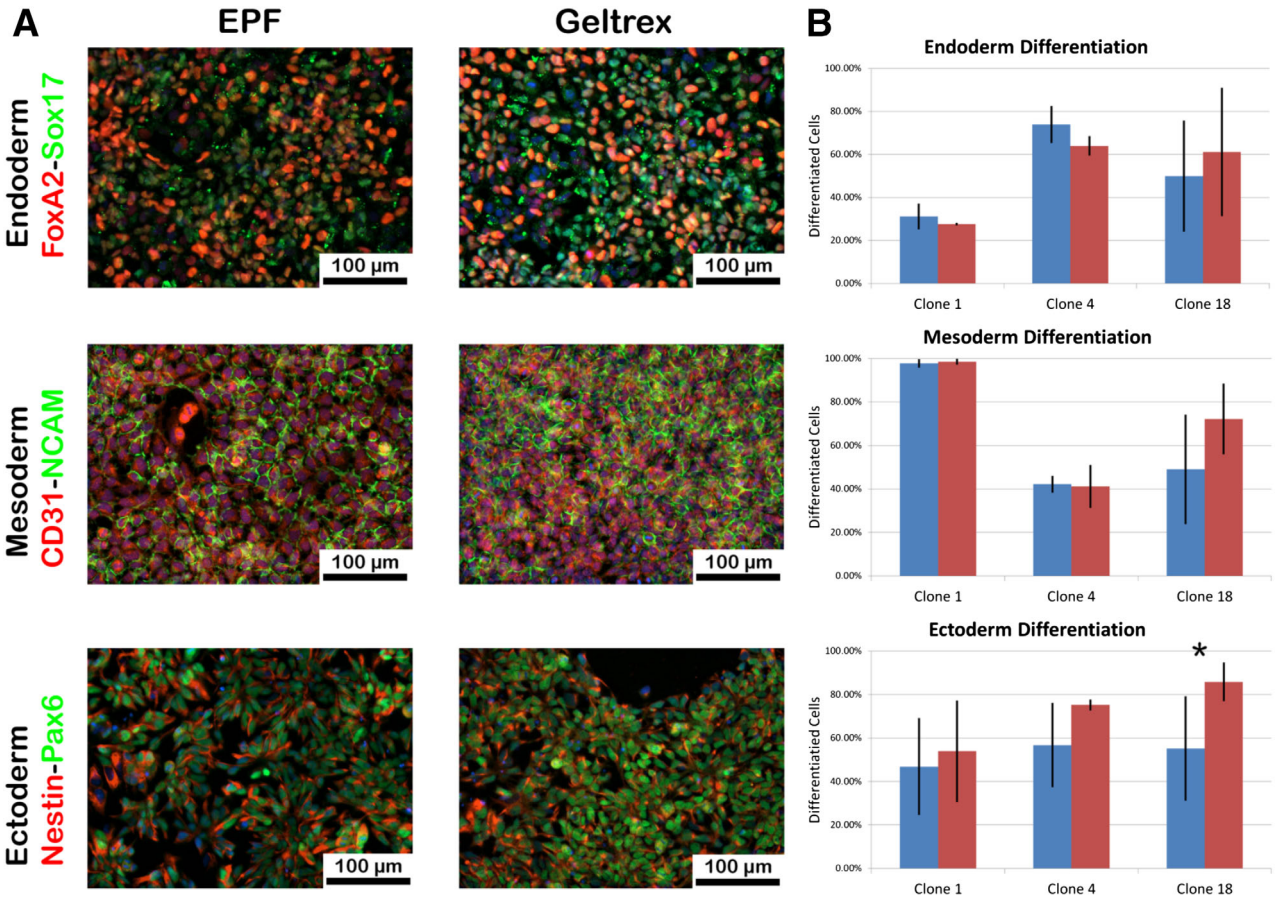

Figure 5. Trilineage differentiation of induced pluripotent stem cells (iPSCs) on various substrates. **(A):** Immunofluorescent staining of selective germlayer differentiation by iPSC colonies (clone 1) on ethanol precipitated fibrinogen (EPF) or Geltrex coated plates. Endoderm differentiation was detected by FoxA2 (RED) and Sox17 (GREEN). Mesoderm differentiation was detected by CD31 (RED) and NCAM (GREEN). Ectoderm differentiation was detected by Nestin (RED) and Pax6 (GREEN). **(B):** Graphs depict efficiency of germlayer differentiation by iPSCs cultured on EPF (blue bars) or Geltrex (red bars) using nuclear stains for anti-Pax6 (Ecto), anti-Fox A2 (Endo), or anti-MixL1 (Meso). The only statistically significant difference was detected in ectoderm differentiation of clone 18 cells (n = 3; p = .005).

on GT demonstrated 27.6% ± 0.2% endoderm positive staining, 98.6% ± 1.3% mesoderm positive staining, and 53.9% ± 23.3% ectoderm positive staining. Clone 4 cells on EPF demonstrated 73.9% ± 8.6% endoderm differentiation, 42.2% ± 3.9% mesoderm differentiation, and 56.7% ± 19.4% ectoderm differentiation as on GT resulted in 63.9% ± 4.1% endoderm differentiation, 41.1% ± 9.8% mesoderm differentiation, and 75.2% ± 2.5% ectoderm differentiation. Clone 18 cells on EPF resulted in 50.0% ± 25.9% endoderm differentiation, 49.0% ± 25.2% mesoderm differentiation, and 55.2% ± 24.0% ectoderm differentiation whereas on GT resulted in 61.2% ± 29.4% endoderm differentiation, 72.2% ± 16.3% mesoderm differentiation, and 85.8% ± 9.0% ectoderm differentiation. Between the two coating reagents, only the ectoderm differentiation with clone 18 was statistically significant (p = .005).

iPSC-ECs Can Be Differentiated on Fibrinogen Coated Plates

To demonstrate a potential application of using fibrinogen coated surfaces for iPSC use, we differentiated iPSCs to endothelial cells based on a previously published protocol [16]. iPSC-EC were chosen because of the rapid differentiation protocol and the clinical need to obtain high volumes of ECs for potential application. All surfaces during the differentiation were coated with fibrinogen. According to a previously published protocol [16], iPSCs colonies were first induced to mesoderm and then to vascular specification (Fig. 6A). iPSC-ECs were purified using CD31-dynabeads, and replated on fibrinogen-coated flasks for expansion in EGM2 media. iPSC-ECs appeared in their characteristic phenotype as spindle shaped with rounded nuclei (Fig. 6B). iPSC-ECs obtained from this protocol stained positive for CD31 and UEA-lectin (Fig. 6C).

Discussion

The work reported herein was inspired by our recent studies on the use of fibrin hydrogels as a scaffold for RPE transplantation [8]. The ability to culture iPSCs on a fibrin hydrogel would be advantageous toward that work, and to the best of our knowledge, this had yet to be demonstrated in the literature. Although the use of fibrin hydrogels as a scaffold for growth of iPSCs certainly opens the door for a variety of 3D models of differentiation and tissue development, for our own studies using RPE and ECs, repeated passage was necessary to achieve the desired state of differentiation and 3D culture and repeated passage on fibrin gels was difficult. For this reason we set out to determine

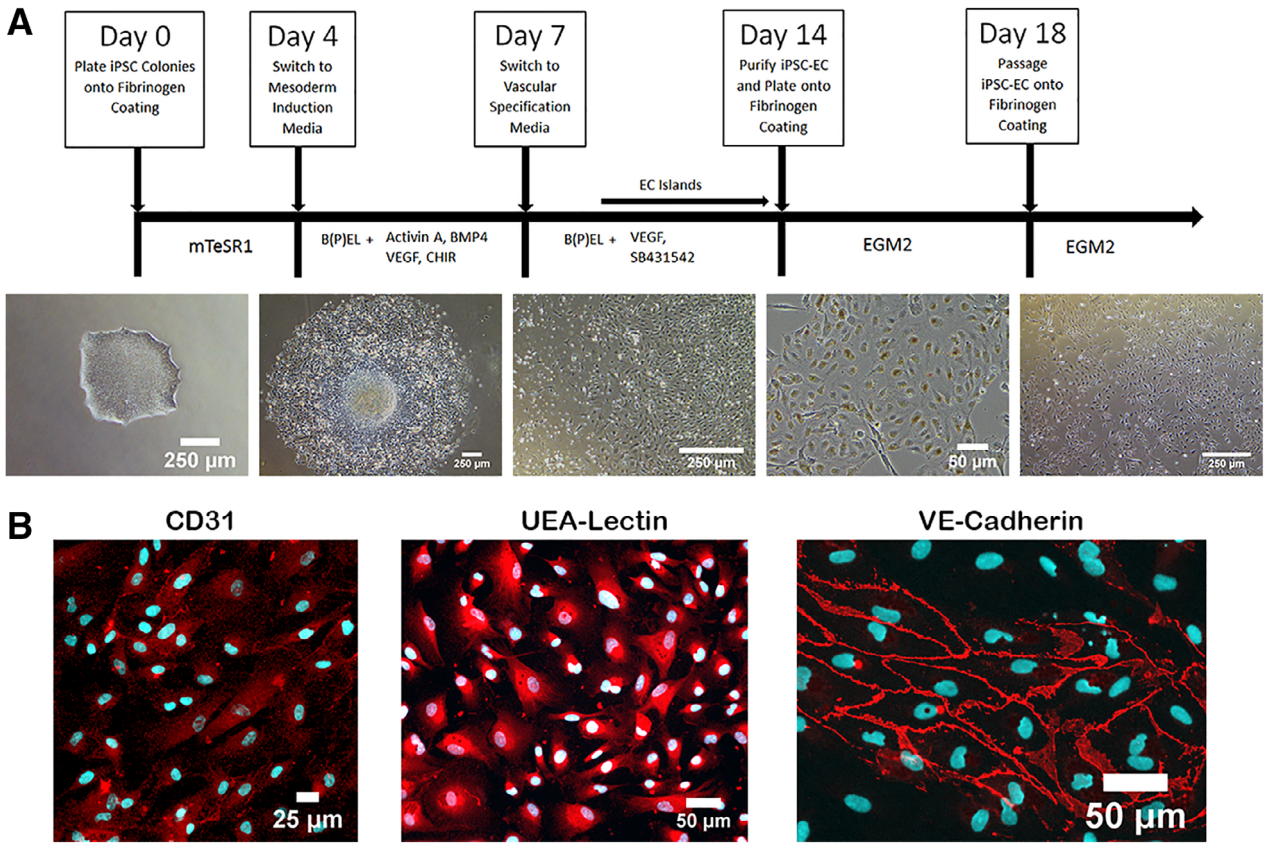

Figure 6. Induced pluripotent stem cell (iPSC)-endothelial cell (EC) differentiation on fibrinogen. **(A):** Phase contrast images of iPSC-ECs at various stages of differentiation and culture. **(B):** Immunofluorescent staining of iPSC-EC for the endothelial cell markers as indicated: CD31, UEA-Lectin, and VE-Cadherin (RED) or nuclear marker DAPI (CYAN).

whether iPSCs could be cultured on surfaces coated with the immediate precursor to fibrin: fibrinogen. Fibrinogen is easily prepared from plasma and so, at least for human iPSCs, fibrinogen represents a nonxenogeneic option for coating plates used to expand or differentiate iPSCs for research or commercial use. Our results showed that iPSCs cultured on fibrinogen coated surfaces are no different in maintaining pluripotency markers or differentiating to the three germ layers when compared with plates coated with murine Engelbreth–Holm–Swarm (EHS) tumor cell basement membrane extracts. This was verified using three distinct iPSC cell lines. Furthermore, to highlight one use of this technology, we successfully differentiated iPSC-ECs using fibrinogen coated plates.

The coating of vessels to promote adhesion of cells in culture is a well-established technique. In general, solutions used either contain a polycationic material such as polylysine, or when more specific properties are desired, extracellular matrix proteins are used. Laminin-rich extracellular matrices (ECM) coatings (e.g., Geltrex and Matrigel) which are derived from mouse EHS tumor cells have been popular for this use for decades as have various isoforms of collagen. In contrast, with few exceptions [18, 19], fibrinogen has not traditionally been used for coating of tissue culture vessels,. We suggest that this is due to the idea that coatings used to promote cell growth should mimic the ECM found in the in vivo environment. This idea is further fueled by the ready availability of products like Matrigel and Geltrex. In the culture of iPSCs, Matrigel and Geltrex have become the gold standard. For iPSCs, those who have sought to expand beyond these products in the quest to identify nonxenogeneic culture materials or to promote differentiation to cell types that do not readily form on laminin rich matrices have focused on investigating components within the traditional range of ECM [20, 21]. For instance, others have evaluated other ECM and blood components for culture of PSCs, including vitronectin [22]. Vitronectin is an obvious choice as it is both a blood and ECM component that contains RGD sequences known to interact with integrins [23]. In contrast to embryonic stem cells (ESC) [20, 21], less effort has been devoted to novel coatings for growth and differentiation of iPSCs. For either ESCs or iPSCs, fibrinogen has not been tested or considered in any studies that we have identified, presumably because it is not viewed as a true ECM component.

Despite this view, the physiologic role of fibrinogen is to form the fibrin clots that prevent bleeding following a wound and to promote wound healing. A key aspect of wound healing is the recruitment of cells to the wound to rebuild damaged tissues. Toward this end, it is well known that fibrin monomers (and fibrinogen) have peptide domains that promote growth factor and cellular attachment [19, 24–26]. In the stem cell field, a great deal of research is targeted at the use of adult and mesenchymal stem cells to fix damaged tissues. The wound healing response has provided the basis, for example, for the use of platelet lysates in MSC cell culture [27, 28]. We too have observed that platelet lysate is an excellent replacement for serum in the growth of cells differentiated from iPSCs [13]. For these reasons, the use of other components of the wound healing response such as fibrinogen/fibrin seems logical.

As iPSCs begin to move into industrial cell manufacturing for clinical use, a need for inexpensive, nonxenogeneic, GMP-compatible surface coating reagents that are abundantly available arises. Cross-species culture reagents can elicit an immune response [29] and increase the amount of testing required for release of therapeutic products to identify adventitious viruses (Q5A Viral Safety Evaluation) [30]. In efforts to evolve from laminin rich mouse ECM (Matrigel and Geltrex), most groups have focused on human laminin and vitronectin. Multiple publications have shown the ability to use these for culture of ESCs [22, 31, 32], and for laminins, iPSC culture. For example, Synthemax (Corning), a synthetic peptide with vitronectin elements, is a GMP-compatible product marketed for ESC culture and which has seen limited use in the generation of iPSCs [22]. Synthetic hydrogel models for 3D culture of poly(N-isopropylacrylamide)-co-poly (ethylene glycol; PNIPAAm-PEG) and polyvinylalcohol-co-itaconic acid hydrogels with Synthemax have also been demonstrated [33, 34]. Another recently released product for cell therapy grade use is recombinant human laminin (Laminin-521, BioLamina). Specifically, Laminin 521 has been demonstrated to work as a plate coating for culture of both ESC and iPSC lines [32, 35], as well as for differentiation of stem cells to other cell types [31, 36].

Despite the availability of these products, a fibrinogen coating reagent would be competitive. Fibrinogen offers the prospect of autologous use, with systems already on the market that have received regulatory approval [37]. However, the most valid argument for use of fibrinogen is cost and scale. Blood derived products reached a market of $23.5 billion in 2012. Commercial scale approaches to obtaining fibrinogen products have evolved over multiple decades to provide methods of isolation that are simple, quick, inexpensive, and of very high yield compared with recombinant methods of synthesis. For example, our data suggest that current commercial formulations of fibrinogen used as tissue glues in the clinic, are already an order magnitude less expensive than research grade recombinant laminin products needed to cover an equivalent surface area. This difference is further evident when considering a 3D culture system that requires a greater volume and concentration of the product to generate a scaffold. Purchasing recombinant proteins on such a scale is currently unrealistic. Furthermore, 3D culture with laminins and vitronectin requires additional synthetic matrices that are yet to be tested in the clinic [33, 38] and synthetic matrices are often associated with foreign body responses. In contrast, fibrin tissue glues which inherently produce a 3D structure when used, have demonstrated an excellent safety profile over a period of decades [39].

Conclusion

Our data demonstrate that fibrinogen is an effective surface coating reagent for iPSC culture and differentiation comparable to various laminin containing products. Human fibrinogen offers a nonxenogeneic option, is readily available in forms produced using GMP to clinical standards, and is an inexpensive alternative to other surface coatings. The ease with which fibrinogen can be produced from plasma also lends itself to the use of autologous formulations. The current use of fibrin tissue glues in the clinic and their low cost suggest easy scalability for use in both 2D and 3D culture of iPSCs. These attributes make fibrinogen an attractive substrate for manufacturing of iPSCs and cells differentiated from iPSCs for human cell therapy and other applications.

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Author Contributions

J.K.G.: provided conception and design of studies, financial support, collection of data, data analysis and interpretation, manuscript writing, final approval of manuscript; T.K., M.H., B.R., L.B., C.P.-A., K.N.S., M.M.M.: provided collection and/or assembly of data, data analysis and interpretation; Z.R., M.J.A., J.S.P.: provided financial support, final approval of manuscript; A.D.M.: provided conception and design of studies, financial support, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

J.S.P. declared employment, intellectual property rights and ownership interest with LAgen Laboratories LLC. A.D.M. serves as CEO and declared ownership interests in LAgen Laboratories LLC. M.J.A. has an equity/royalty relationship with AliveCor and consultant/advisory role with Audentes Therapeutics, Boston Scientific, Gilead Science, Invitae, Medtronic, MyoKardia, StemoniX, St. Jude Medical. However, none of the disclosures pertain directly to this article and none of the companies provided financial support for this article. The other authors indicated no potential conflicts of interest.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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