Organ-specific ECM arrays for Investigating Cell-ECM Interactions During Stem Cell Differentiation

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ABSTRACT

Pluripotent stem cells are promising source of cells for tissue engineering, regenerative medicine and drug discovery applications. The process of stem cell differentiation is regulated by multi-parametric cues from the surrounding microenvironment, one of the critical one being cell interaction with extracellular matrix (ECM). The ECM is a complex tissue-specific structure which are important physiological regulators of stem cell function and fate. Recapitulating this native ECM microenvironment niche is best facilitated by decellularized tissue/ organ derived ECM, which can faithfully reproduce the physiological environment with high fidelity to *in vivo* condition and promote tissue-specific cellular development and maturation.

Recognizing the need for organ specific ECM in a 3D culture environment in driving phenotypic differentiation and maturation of hPSCs, we fabricated an ECM array platform using native-mimicry ECM from decellularized organs (namely pancreas, liver and heart), which allows cell-ECM interactions in both 2D and 3D configuration. The ECM array was integrated with rapid quantitative imaging for a systematic investigation of matrix protein profiles and sensitive measurement of cell-ECM interaction during hPSC differentiation. We tested our platform by elucidating the role of the three different organ-specific ECM in supporting induced pancreatic differentiation of hPSCs. While the focus of this report is on pancreatic differentiation, the developed platform is versatile to be applied to characterize any lineage specific differentiation.

INTRODUCTION

Pluripotent stem cells are promising source of cells for tissue engineering and regenerative medicine application. The process of stem cell differentiation is regulated by multi-parametric cues from the surrounding microenvironment including extracellular matrix (ECM), soluble factors, matrix stiffness, substrate topography and adjacent cell-cell interactions [1]. Stem cells interact with the ECM via several different cell surface receptors, of which the largest family is integrins [2]. This cell-ECM interaction allows cells to sense mechanical and biochemical cues and respond directly by inducing downstream cellular signaling, modulating the response of other integrins and influencing appropriate stem cell lineage commitment [3-6]. Hence it is of interest to quantify the contribution of ECM, alone and in synergy with the other microenvironmental factors, in modulating stem cell fate.

The ECM is a complex tissue-specific structure composed of collagens, proteoglycans, and glycoproteins. Multiple studies using variety of techniques (summarized in review by Rozario et al. [7]) have confirmed the effect of ECM on stem cell fate commitment. *In vitro* studies with isolated ECM proteins have shown the effects of laminin and fibronectin on definitive endoderm differentiation of mouse embryonic stem cells [8]. Furthermore, varying combinatorial ECM proteins such as fibronectin, laminin, and collagen type IV interspersed within collagen I hydrogel have demonstrated to influence the osteogenic and endothelial

differentiation of human embryonic stem cells [9]. Recognizing the need for a multi-matrix system in reconstructing the ECM microenvironment, there has been a focused effort in developing high throughput multicomponent ECM array platforms. Most of these platforms are ECM protein microarrays to facilitate the screening of stem cell fate after exposure to various ECM molecules in an adherent two-dimensional (2D) configuration [10-16]. Further recognizing the benefits of reproducing the three-dimensional (3D) culture microenvironment has initiated the development of 3D hydrogels on a chip by depositing cell-laden alginate solution using microarray spotting techniques [17, 18]. Multiplexed 3D cellular microarray was further developed for combinatorial screening of stem cell differentiation in response to multiple ECM and growth factors [14, 17-21]. Majority of these approaches use purified ECM proteins and its combinations in an attempt to reconstruct the cell environment. While promising, there is still inadequate information on engineering the complexity of the native, tissue-specific ECM in an *in-vitro* setting. To date, attempts to generate tissue-mimetic ECM that recapitulate the function of the ECM *in vivo* have had limited success using combinatorial single purified ECM [22, 23]. On the other hand, tissue/organ-specific ECM has been shown to expose the cells to a more physiologically relevant milieu with higher fidelity to *in vivo* condition and promote tissue-specific cellular development and maturation [24-26].

Advancement in organ decellularization techniques have made it possible to extract organ/tissue specific ECM while closely preserving their native composition and microstructure [23, 26-28]. Specific to pancreas, our group [29] and Soker *et al.* [30] have demonstrated successful decellularization of pancreas scaffold from murine and porcine source respectively, along with more recent demonstration on human pancreas decellularization by Peloso *et al.* [31]. The resulting decellularized tissue/organ ECM have also been shown to be promising in regulating stem cell differentiation *in-vitro*, as a delivery platform to improve glycemic control and to study the comprehensive roles of ECM in mesenchymal stem cell phenotype commitment [23, 27, 32, 33]. More recently in the context of pluripotent stem cell (PSC), it was observed that exposure to organ-specific decellularized ECM scaffolds can enhance organ-specific maturation of PSC in lung [25, 34, 35] , heart [36-38], and kidney [39, 40]. These studies exposed the cells to a multitude of biochemical and biophysical properties contained within the decellularized tissue/organ scaffolds. In addition to the effect from ECM compositional differences, for instance when comparing hPSC seeded in decellularized lung versus kidney scaffolds [41], the cells also experience different tissue stiffness and topological structure, all of which influence stem cell fate [42-44]. There is currently lack of systematic quantitative studies to unravel the contribution of each factors related to organ-specific properties. Hence, it remains a challenge to study the effects of organ-specific ECM composition that leads to a desirable cell fate.

In this paper, we report the fabrication of an array platform to expose the organ-derived ECM to cells cultured in either adherent 2D configuration or aggregated 3D configuration. We demonstrate that these miniaturized arrays could function as a tool to investigate cell-ECM interaction during hPSC differentiation, while being compatible with rapid quantitative imaging using LI-COR scanner. We first derived organ-specific matrix by extracting ECM from decellularized pancreas, liver and heart using chaotropic agents. Characterization of resulting ECM extracts revealed the retention of complex and diverse ECM compositional profiles in all three organs. To elucidate the role of organ-specific ECM in influencing stem cell function, organ-specific ECM extracts were immobilized onto the array surface in 2D and 3D configurations. We demonstrated the utility of the ECM array in 2D configuration by examining

the cell-ECM adherence of human PSC derived pancreatic progenitor (PP) cells. Our result showed differential adherence profiles when hPSC-PP cells were plated onto the 2D configuration (2D ECM array), suggesting hPSC-PP cells were sensitive to organ-specific differences in the decellularized matrices. In the 3D configuration (3D ECM array) with aggregated PP culture, our platform showed excellent compatibility with enhanced maturation into insulin expressing cells in organ-derived ECMs compared to matrigel. Collectively, this platform demonstrated for the first time, the use of complex native-derived ECM in an array platform to rapidly interrogate stem cell fate and function. The developed platform will pave way for rational design of engineered tissue-specific stem cell niches and support the developing field of stem cell biology, tissue engineering and regenerative medicine.

METHODS

Organ decellularization and ECM extraction

We used a perfusion decellularization protocol reported previously [29], to obtain organ-specific decellularized pancreas, liver and heart scaffolds explanted from female ICR mice (Taconic, ages of 6-12 weeks) after systemic heparinization.. The decellularized organs were first lyophilized in Virtis Benchtop K freeze dryer, operating at 60 mTorr for 3 days. After lyophilization step, 30-50 mg dry weights of the lyophilized tissues from each organ were pulverized into ECM powder (Figure 1Aii)). This was followed by treatment with 50 µg/ml DNase (Roche) for 10 min and washed 3 times with PBS. For ECM extraction, each decellularized tissue were digested in high molar of chaotropic agents (4M guanidine HCl, 8M Urea in PBS) overnight with gentle shaking at 4ºC. Samples were be centrifuged at 13,000 g for 10 min at 4ºC to remove insoluble material and dialyzed on floating V-series membranes (Millipore) against PBS for one hour [45]. The final ECM solution was filter-sterilized through PVDF syringe filter units (0.22 mm, Millipore), and then stored at 4ºC until further use.

Surface modification and characterization

Patterning substrates were prepared by first coating glass slides or tissue culture plates with nitrocellulose [46]. Static water contact angle measurements were performed to assess the hydrophobicity of the coated surface. Matrigel was used as a control ECM to pattern the surface and test protein immobilization efficiency. Matrigel was applied to nitrocellulose-coated surface in droplets of 1µl-3µl volume containing 100 µg/ml concentration. After about 1 min, droplets were removed by aspiration and the substrate surface was blocked by washing twice with 1% bovine serum albumin (BSA)/PBS (Sigma Aldrich). To verify the efficiency of ECM protein patterning on nitrocellulose-coated surface, Lectin peanut agglutinin (PNA) –Alexa 647 (Invitrogen, Molecular Probes) was used to detect the immobilized ECM protein. PNA lectin stain was used at a concentration of 200 µg/ml in PBS and followed by three PBS washes. Microspots' diameter were measured using slide scanning images recorded with Metamorph 7.5.6.0 (Molecular Device) software on an Olympus IX81 inverted microscope. The fluorescence intensities (RFU) of the PNA lectin stain was quantified with LI-COR odyssey scanner using the 700nm wavelength channel.

ECM characterization with biochemical assays and SDS-PAGE

After ECM extraction, individual organ's ECM were assessed for protein and ECM content as follows. For protein quantification, bicinchoninic acid (BCA) assay (Thermofisher) was done (n=3 for each organ) according to manufacturer's instruction. For ECM content, organ derived ECM extracts (n=3 for each organ) were analyzed with the Sircol Collagen and Blyscan GAG assay kits (Biocolor Life Sciences Assays, Carrickfergus, UK) according to the manufacturer's instructions in order to quantify the amount of each component present. The final content was normalized to the starting dry weight of each lyophilized decellularized organ.

For SDS-PAGE analysis, ECM samples were loaded at 4ug per well. 20ul of sample was mixed with 20ul of 2X NuPAGE LDS sample buffer + 5% β-mercaptoethanol and heated at 98ºC for 5 minutes. This resulting mixture was loaded onto a NuPAGE 3-8% Tris-Acetate Gel in 1X Tris-Acetate Running Buffer for 5 hours at 60V constant. Gel was stained using Bio-Rad Bio-Safe Coomasie stain for 4 hours, and destained overnight in distilled water. The resulting gel was imaged using The Bio-Rad ChemiDoc XRS+ system.

2D organ-specific ECM array fabrication

Each organ-derived ECM extracts were diluted into 100 µg/ml and 1 µl of ECM solution per droplet were spotted with a repeater pipette (Eppendorf) onto the nitrocellulose-coated glass slides or tissue culture plates. After about 1 min, droplets of ECM solutions were aspirated and blocked by washing twice with 1% BSA/PBS. Five microspots were generated in square arrays for a well in a 6 well plate, and six by four droplets were generated in rectangular arrays for a glass slide. After fabrication steps, the arrays can be stored in blocking buffer at 4ºC until further use. All arrays used in this study were used within 3 months after fabrication.

Atomic Force Microscopy (AFM) surface roughness and stiffness measurement

For surface roughness measurement, AFM imaging of the ECM protein immobilized surface (MFP3D, Asylum Research) was performed in PBS at room temperature at a scan speed of 1 Hz with a resolution of 256 × 256 pixels. The scan size was 10 micron x 10 micron regions. Surfaces were scanned with a silicon nitride conical tip of k = 0.8 N m^{-1} (Veeco, Ltd), and root mean square (RMS) surface roughness analysis was performed on 4 regions per sample [47].

For stiffness measurement, 3D ECM array containing organ-derived alginate microspots were prepared and placed in room temperature PBS for AFM measurements. All AFM force-stiffness measurements were taken using silicon nitride cantilevers (k = 0.2 N m^{-1}) attached to silica microspheres (r = 3400 nm). Force measurements were taken at 16 locations over a 4 x 4 grid and two microspot samples were measured per condition. The resulting force curves were analyzed within the AFM-MFP3D software using Hertzian-fit for indentation depth of <10 microns [48].

Human embryonic stem cell culture and differentiation into pancreatic progenitor cells

The H1 hESC lines used in these experiments were obtained from the University of Pittsburgh Stem Cell Core, and are part of the NIH hESC registry eligible for NIH funding. Undifferentiated H1 hESCs were maintained in feeder-free culture in mTeSR medium (StemCell Technologies) on hESC-qualified Matrigel (BD Biosciences)–coated tissue culture plates. Cultures were fed every day with mTeSR medium and passaged with Accutase (StemCell Technologies) at 70% confluency. We have previously reported differentiation of hESC to pancreatic progenitor cells [49-51]. Briefly, first step of pancreatic differentiation is definitive endoderm induction by using

100 ng/mL ActivinA (R&D Systems) with 25 ng/mL Wnt3A (R&D Systems) for 4 days. This is followed by pancreatic progenitor induction with 0.2 μM KAAD-cyclopamine for 2 days (R&D Systems) and 0.2 μM KAAD-cyclopamine with 2 μM retinoic acid (Sigma-Aldrich) for 2 days. For generation of PP aggregates, an alginate encapsulation differentiation platform was utilized as previously described [49]. Encapsulated cells were grown into small aggregates and treated with the same induction protocol to differentiate into pancreatic progenitor stage. A basal media of DMEM/F12 plus 0.2% BSA and B27 supplement was used for the differentiation. Cultures were performed with p55–p70 hESCs in 37ºC incubator, 5% CO2, and 100% humidity.

On-chip immunofluorescence assay and LI-COR Odyssey scanner analysis

Similar to regular immunofluorescence assay, attached 2D cells or encapsulated cells on 3D ECM arrays were first fixed for 15 min with 4% formaldehyde (Thermo Scientific). For 3D ECM array staining, we used dH_2O supplemented with 10mM $BaCl_2$ to replace PBS for all the washing steps because cation in alginate could be dissolved by displacement of other stronger ions in PBS. For 2D ECM array staining with ECM proteins, ImmEdge Hydrophobic Barrier Pen (Vector Laboratories) was used to confine the antibody solutions to staining specific microspot of interest. The blocking buffer was also supplemented with 10mM $BaCl_2$ when staining on 3D ECM arrays. Following fixing, samples were permeabilized with 0.1% TritonX100 for 15 min and blocked with Odyssey Blocking buffer (LI-COR Biosciences) for 1 hour. For primary antibodies, we used the following antibodies: goat anti-PDX1 (1:400, R&D Systems), goat anti-NKX6.1, rabbit anti-Cpeptide (1:200, Cell signaling). Antibodies were diluted to half of the suggested immunofluorescence concentration as the combination of robust Near-IR dyes and sensitive LICOR scanner can provide sufficient to reliably detect signals. Incubation time for primary antibodies was overnight in 4°C. Slides were washed three times (5–10 min) with PBS before secondary antibody staining. Infrared anti-goat and anti-rabbit IRDye800CW secondary antibody (1: 800, LI-COR Biosciences) and DRAQ5 (1: 10,000, Invitrogen) in blocking were then added to the arrays and incubated for 1 hour at room temperature. This was followed by PBS washing for three times and incubated in PBS. The plates were imaged on an Odyssey infrared scanner using slide or plate settings with sensitivity of 5 in both the 700 and 800 nm wavelength channels. Data were acquired by using Odyssey image studio software, exported and analyzed in Excel (Microsoft). Primary antibody stained values were background subtracted from microspots treated only with secondary antibody, and then normalized to cell numbers by dividing by the total DNA (DraQ5) fluorescence signal.

Cell-adherence evaluation on 2D ECM array

Cell adhesion assays were carried out on 2D ECM array on glass slide or tissue culture plastic. If glass slides were used, we used chamber glass slides (Nunc™ Lab-Tek™) for the purpose of cultivation here. hESC-derived PP cells were treated with 10 μM of the rock inhibitor (RI) Y-27632 for 4 hours before dissociation into single cells using accutase. Single-cell suspension of hESC derived-PP cells in serum free media (DMEM/F12 with B27) were plated into the patterned ECM microspots. For short term attachment, cell suspension were incubated for 2 hours in serum free medium to allow for cell attachment (shaking the plates every 15 minutes to redistribute the cells). The arrays were then gently aspirated to remove unattached cells and fixed for cell staining and counting. For long term attachment, after first 2 hours of cell attachment and removal of unattached cells, fresh culture medium with serum was added and the cells were allowed to attach overnight.

3D ECM array fabrication

Similar to the protocol described by Fernandes et al. [17], poly-*L*-lysine (PLL) (0.01% w/v) (Sigma Aldrich) and $BaCl_2$ (0.1M) were mixed in 1:2 volume ratio and spotted on the nitrocellulose coated surface using repeater pipette (Eppendorf). A mixture of 1.1% (w/v) low-viscosity alginate (Sigma-Aldrich), ECM extracts and hESC-PP cell suspension ($2.5x10^6$ cells/ml) in DMEM/F12 media was prepared and micro-spotted on top of the $BaCl_2$/PLL bottom layer using the repeater pipette.

Differentiation of PP aggregates in 3D ECM array

For hESC-derived PP aggregates differentiation in 3D ECM array, the PP aggregates were first generated using the alginate differentiation platform described above. Alginate differentiated PP aggregates were treated with 10μm of the rock inhibitor (RI) Y-27632 for 4 hours before decapsulation with 100mM EDTA. This was followed by passing the PP aggregates through 100 μm mesh-sized cell strainer to avoid larger aggregates being included in the microspots. Approximately 8000-12000 PP aggregates were mixed in 1ml of alginate and ECM extract solutions to micro-spot on top of the $BaCl_2$/PLL bottom layer using the repeater pipette. Subsequently, the cell-laden 3D ECM array was cultured in base maturation medium consists of DMEM/F12 plus 0.2% BSA and B27 supplement with nicotinamide (10 μM, Sigma Aldrich) and 10μm of the rock inhibitor (RI) Y-27632 for 2 days. After that, fresh maturation media with notch inhibition (30 uM DAPT, Santa Cruz) was added and cultured for another 7 days. The medium was changed daily for a total for 9 day culture period.

Cell viability assay: live/dead staining and quantification

Cell viability on the 2D and 3D ECM array was assessed via a Live/Dead assay (Invitrogen). Briefly, calcein AM (1 μM) and EthD-1 (2 μM) were added together with culture medium to the cell seeded arrays and incubated for 15 minutes under light protection in room temperature. Samples were washed twice with PBS and continued with epifluorescence microscopy to distinguish between live cells (green) and dead cells (red). Quantification of live/dead cells was accomplished by conversing all individual (red and green) channel to grayscale and followed by analysis with Metamorph Image software.

Immunofluorescence staining and confocal imaging

The immunofluorescence staining protocol is identical to the on-chip immunofluorescence protocol described above except for the following. For blocking buffer, 10% donkey serum (Jackson Immuno) was used. Anti-rabbit 488 and 555 Alexafluor secondary antibodies (1:500, Invitrogen) was used. The only primary antibody used here is Cpeptide (1:100, Cell signaling). Z–stacks images were acquired using a Nikon A1 laser confocal system (5 μm steps).

Array microscopy imaging and quantification

Microscope images of each array were acquired at 4x using an Olympus IX81 microscope. Images were recorded using Metamorph 7.5.6.0 (Molecular Device) software. The arrays were imaged using phase-contrast, DAPI, GFP, Cy3 and Cy5 optics. Scan slide module from Metamorph software were utilized and array images were montaged. For quantification purposes except for live/dead staining, the slides were acquired using Odyssey image studio software, exported and analyzed in Excel (Microsoft). Each array (on glass or tissue culture

wells) were imaged using a focus height that gave the maximum signal for each wavelength channel (700nm and 800nm) at the center of the array.

Statistical analysis

Quantification data were expressed as mean ± SD. Significant differences among groups were determined by two-tailed Student's t-test for two-group comparisons or ANOVA followed by post-hoc analysis for multiple group comparisons. Probability values at $P<0.05$ (*) and $P<0.01$ (**) indicated statistical significance.

RESULTS

Preparation and characterization of organ-specific ECM extracts

Figure 1A illustrates the overall workflow used to create an ECM array using decellularized organ ECM extracts. Organ-specific ECM scaffolds were first derived by perfusion-decellularization of whole organs [29] using an in-house bioreactor, as previously described. Keeping the decellularization protocol consistent, we successfully decellularized whole organ pancreas, liver and heart. Figure 1A(i) depicts the translucent acellular organ scaffolds generated after the complete removal of the cellular materials from the native organs. This is followed by lyophilization and pulverization steps to obtain organ-specific ECM powder (Figure 1A(ii)). We then extracted the ECM using high molar of chaotropic agents (8M Urea and GuHCl), a commonly used protocol for ECM preparation [52-54]. The resulting extracts were dialyzed against PBS to yield pancreas-, liver- and heart-organ-specific ECM extracts (denoted as P-ECM, L-ECM and H-ECM respectively). We first characterized the organ-derived ECM extracts through sodium dodecyl sulphate (SDS)–PAGE for the presence of ECM proteins or other peptides (Figure 1B). We loaded the same concentration of organ-derived ECM extracts (4μg/well) and controls (collagen I and matrigel) on each well of the gel. SDS page analysis revealed that all three organ-derived ECM extracts contained a number of high molecular weight ECM proteins (~250 KD) and low molecular weight (<100 KD) protein components that were absent in commercial bovine collagen type I and matrigel (Figure 1B). There were also considerable differences in terms of the bands detected among the three organ-derived ECM extracts. Next, the organ-specific ECM extracts were analyzed for protein (BCA assay) and for ECM content such as collagen and glycosaminoglycans (GAGs). BCA assay revealed highest protein content in H-ECM (8.07 ± 4.7 μg/mg dry weight), followed by L-ECM (6.90 ± 3.1 μg/mg dry weight) and P-ECM (3.98 ± 1.5 μg/mg dry weight) but without statistical significance among the three extracts ($n=3$, $P>0.05$; Figure 1C). For the ECM content, sircol collagen assay demonstrated significant higher collagen content in H-ECM (0.78 ± 0.2 μg/mg dry weight, $n=3$) as compared to L-ECM (0.48 ± 0.05 μg/mg dry weight, $P<0.05$; Figure 1D) but not significantly higher than P-ECM (0.53 ± 0.07 μg/mg dry weight, $P>0.05$). There was also no significant difference between L-ECM and P-ECM in terms of the collagen content ($P>0.05$). While L-ECM was low on collagen content, it was high for sGAG content, as quantified by Blyscan assay to be 0.14 ± 0.02 μg/mg dry weight,) significantly higher than H-ECM and P-ECM (0.08 ± 0.01 and 0.03 ± 0.001 μg/mg dry weight respectively, $P<0.01$; Figure 1E). This observation indicated that P-, L-, and H-ECM preserved complex multi-faceted ECM proteins, suggesting better mimicry of the native organs' intricate ECM composition. It also indicates quantitative differences in the retained ECM composition across different organs.

Fabrication of an array platform presenting the organ-derived ECM extracts

Having extracted the ECM from individual native organs, we next fabricated the patterning substrates to present the organ-derived ECM as individual microspots. We first coated glass slides with a thin nitrocellulose layer to immobilize the ECM proteins. Nitrocellulose coating rendered the surfaces more hydrophobic and resulted in water contact angles of 53.3º ± 0.2º, significantly higher than uncoated glass surface (36.9º ±1.1º, n=3, P<0.01; Figure 2A). This method allows adequate protein adsorption while providing sufficient optical clarity for microscopy and scanning applications [11]. To verify the efficiency of protein deposition, we used matrigel as our testing ECM solution and coated it on tissue culture plastic (TCP) pre-treated with nitrocellulose and compared with non-treated TCP for 5 min and 60 min. Immunostaining by Lectin peanut agglutinin (PNA) detected significantly higher intensity of relative fluorescence unit (RFU) on surfaces pre-coated with nitrocellulose (137500 ± 2210 RFU versus 58900 ± 3370 RFU for 5 min coating; 240000 ± 20405 RFU versus 110050 ± 28520 RFU for 60 min coating, P<0.01, n=3; Figure 2B-C), indicating efficient deposition of ECM-associated carbohydrate structures on nitrocellulose surface. After confirming the efficient adsorption of matrigel, we then evaluated our organ-derived ECM by microspotting them onto nitrocellulose-coated glass slide. All organ-derived ECMs (P-, L- and H-ECM) and control ECM (matrigel) were spotted at the same concentration of 100 µg/ml. Atomic force microscopy (AFM) of microspotted organ-derived ECM extracts showed significant higher surface roughness - root mean square (RMS) than nitrocellulose only surface (P<0.01, n=5; Figure 2D, Supplemental figure 1), confirming their immobilization onto the nitrocellulose coated surface. Furthermore, the RMS value were about the same for all the organ-derived ECM extracts and matrigel control, indicating similar amount of proteins were immobilized [55]. In order to reproducibly pattern protein microspots of similar amount of protein, we used a repeater pipette that dispenses constant volumes of ECM protein solution. Matrigel measuring in 1 - 3µl volume were spotted with a diameter ranging from 1500 µm – 2300 µm to yield spatially separated ECM microspots (Figure 2E). Each array contains up to 24 microspots for a glass slide (Figure 3A) or 25 microspots for a well in 6 well plate (Figure 6A). To render the glass surface in between the patterns resistant to unspecific protein and cell attachment, the remaining nitrocellulose layer were blocked with BSA. LI-COR slide scanning images of PNA lectin staining showed that each volume of microspot had similar diameter (<5% difference) and the diameter correlated linearly (R^2 = 0.95) with increasing volume (Figure 2F). The same trend was observed with LI-COR quantification of near-infrared (680 nm) PNA stain – it correlated linearly with increasing volume and there was narrow variability of RFU for each volume (Figure 2G), confirming the reproducible spotting quality that can be achieved by the repeater pipette dispersion. Taken together, these data suggest that this spotting technique is reproducible, and the platform can be efficiently patterned with organ-derived ECM proteins to generate an organ-specific ECM array.

ECM array combined with quantitative imaging permits analysis of ECM composition of organ-derived ECMs

Next, we characterized the individual matrix protein components of the organ-derived ECM spotted on the array by on-chip, immunofluorescence staining with IR-dye, and fluorescence quantification by LI-COR Odyssey imager (Figure 3A). As a validation for this assay, we microspotted matrigel to study the assay sensitivity and range for the detection of the two most abundant ECM protein contained in Matrigel – Laminin and Collagen IV proteins. LI-

COR image analysis detected increasing fluorescence intensity for both Laminin and Collagen IV staining with increasing concentration of spotted matrigel. The protein fluorescence intensity correlated linearly ($R^2 > 0.97$; Supplemental figure 2) with matrigel concentration for both proteins over a range of dilution factors (1:4 to 1:16). Having validated our array platform, we next used it to characterize the ECM composition in P-, L-, and H-ECM, testing for a range of ECM proteins including Collagen I, Collagen IV, Fibronectin and Laminin. These ECM molecules were chosen because they are known to be critical in determining stem cell fate [56] and are the primary ECM components in various organs, including the pancreas [57, 58]. We quantitatively determined the levels of individual ECM proteins and compared to matrigel, rat tail collagen I (positive controls) and gelatin (negative control) (Figure 3B-E). As expected, the on-chip immunofluorescence assay was able to detect the ECM protein profiles on both the positive ECM controls (high laminin and collagen IV for matrigel; high collagen I for rat tail collagen; Figure 3B-C). Negative ECM control - gelatin is a denatured form of collagen and resulted in lower collagen I fluorescence intensity (Figure 3B). Interestingly, despite the identical ECM preparation protocols and amount of protein being microspotted, the composition of the organ-derived ECM varied dramatically, illustrating the complex and different ECM compositional profiles among the three organs. There was no significant difference detected for Collagen I and Collagen IV ECM protein levels among the three organs ($P>0.05$, $n=3$; Figure 3B-C), consistent with the previous assessment by Sircol collagen assay (Figure 1D). However, laminin level was detected at much higher level in H-ECM (363.2 ± 15.8) than P- and L-ECM (84.9 ± 7.9; 153.5 ± 22.1 respectively, $P<0.05$, $n=3$; Figure 3D). In addition, fibronectin level was also detected to be significantly higher in L-ECM (16.9 ± 3.4) than P- and H-ECM (2.9 ± 1.2, 4.2 ± 1.2 respectively; $P<0.01$, $n=3$; Figure 3E). These data indicate that the ECM array combined with immunostaining and LI-COR analysis allows sensitive quantitative evaluation of different organ-derived ECM.

Organ-derived ECM array to probe cell–ECM adhesion

Cell adhesion to the ECM is essential for a coordinated morphogenesis and growth of functional tissue [59, 60]. In order to measure the adhesive properties of the organ derived ECM, we used pancreatic progenitor (PP) cells derived from hPSC as described in our previous published studies [50, 51], and seeded them onto the organ-derived ECM array. As depicted in step 1 of schematic in Figure 4A, we first generated definitive endoderm from human embryonic stem cells (hESCs) using activin A (100 ng/ml) and wnt3a (25 ng/ml) for 4 days (day 0-4). We then induced pancreatic progenitor cell fate by sonic hedgehog inhibition (KAAD Cyclopamine, 0.2μM) for 4 days (day 4-8) and retinoic acid from day 6-8. With this reported protocol, we were able to efficiently generate up to 60% of PP cells expressing the transcription factor pancreatic and duodenal homeobox1 (Pdx1), which is the master regulator giving rise to all pancreatic lineage cells [61]. The pre-differentiated PP cells were then seeded onto the arrays in serum-free media and allowed to adhere for 2 h at 37 °C (step 2 of schematic Figure 4A). To ensure uniform seeding, the slides were agitated every 15 min. We first tested this approach with matrigel spotted arrays. The PP cells adhered preferentially to matrigel spotted regions and did not attach to the nitrocellulose coated regions lacking ECM proteins. The cell patterning was robust over the array surface (3 x 3 ECM microspots), yielding near confluent cellular microspots as shown in Figure 4B. To quantify cells bound to each spot, cell nuclei were stained with DraQ5 DNA stain, a DNA-binding dye that fluoresces in the infrared spectrum and the slides were scanned and quantified with LI-COR to detect the fluorescent intensity of DraQ5. As shown in Figure 4C, the cell density correlated strongly with the cell seeding density of the

hPSC-PP cells, which confirms both the quantification technique and compatibility of the array with hPSC derived cell types. Live/dead images of the adhered cells demonstrated good viability 24 hours after cell seeding, indicating cytocompability of the ECM array (Figure 4D).

Using the same methodology, we next seeded the hPSC-PP cells on organ-derived ECM (P-, L- and H-ECM) spotted arrays and evaluated their adhesion (Figure 4E). Live/dead images after 24 hours showed most adhered cells were viable but there were considerable differences in cell adhesion between the three organ ECMs (Figure 4F). This was further confirmed by quantification using LI-COR analysis of DRAQ5 DNA stain. Adhesion of the hPSC-PP cells to L-ECM was the highest among the three organ-derived ECMs, while H-ECM exhibited the lowest hPSC-PP cell adhesion ($P<0.01$). Interestingly, in spite of having pancreatic tissue origin, P-ECM exhibited significantly lower adhesion of hPSC-PP cells as compared to L-ECM ($P<0.05$; Figure 4G). These results clearly suggest that adhesion of hPSC-PP cells was responsive to the composition differences between different organ-derived ECMs.

Fabrication and characterization of 3D organ-derived ECM array

While the ECM array is a useful tool to interrogate cell-ECM interaction under adherent culture, the increasing appreciation of the importance of three-dimensional (3D) spatial organization of cells has prompted the development of 3D cellular arrays [17, 18, 20, 21, 62] which better mimics the complexity of the native microenvironment. In addition, many ECM proteins don't have an adhesive role but are still critical for stem cell growth, survival, differentiation and morphogenesis [56, 63, 64]. Hence, it is important to develop a 3D system to globally present the ECM proteins while supporting 3D culture of hPSCs. Alginate is a suitable hydrogel matrix which adequately supports hPSC growth and differentiation while being inert to cell adhesion [49, 65]. Further, alginate hydrogels have been previously developed into a 3D cell on-a-chip array for mouse embryonic stem cell [17] and human neural stem cell [18] differentiation. Here, we modified this technique to incorporate our organ-derived ECM into alginate, while encapsulating pre-differentiated hPSC cells. Instead of using poly (styrene-*co*-maleic anhydride) (PS-MA)-treated surface, we used nitrocellulose coated surface, which has been shown to have a higher protein binding capacity than PSMA-treated surface (80-100 $\mu g/cm^2$ [66] versus 68.2 $\mu g/cm^2$ [67, 68]). After pretreating the glass slides with nitrocellulose, mixture of poly-*L*-lysine (PLL) and $BaCl_2$ were microspotted onto predetermined positions using the same repeater pipette microspotting technique as described above. Alginate was pre-mixed with specific organ-derived ECM. hPSC-PP cells were harvested and suspended within the alginate-ECM matrix material before micro-spotting onto the predetermined positions on nitrocellulose-coated glass slides. 3D cell-laden gel were formed by the deposition of the pre-mixed alginate and ECM solution containing hPSC-PP cells on top of the PLL/$BaCl_2$ bottom layer as shown in schematic Figure 5A. Under these conditions, the positively charged PLL serves as a substrate to bind Ba^{2+} ions and assist the attachment of the negatively charged alginate. The divalent Ba^{2+} ions bind preferentially to the G-blocks in the alginate and cause instantaneous gelation of the alginate chains to give rise to 3D cell-laden microspots (1µl) with a height and diameter of 250 µm and 1600 µm respectively (Figure 6B).

With the platform now being 3D, we wanted to test the sensitivity of LI-COR imaging platform in detecting the protein expression throughout the thickness of the 3D gel microspots with the same on-chip, immunofluorescence staining method. Previous studies have shown that LI-COR was able to detect near infrared and infrared fluorescence signals of up to 2 mm thickness [69, 70], which is within the thickness range of our 3D gel microspots (250 µm). In

addition, we wanted to ensure that the ECM proteins were stably incorporated into the 3D gel microspots throughout the culture period (up to 10 days). To validate this, we microspotted premixed matrigel and alginate (cell free) at various concentration (0 µg/ml – 200 µg/ml) onto the PLL/$BaCl_2$ pre-spotted array and maintained it in maturation media for 10 days at 37 °C culture incubator. The range of matrigel concentration on the 3D gel array were stained with PNA lectin and quantified by LI-COR Odyssey imager at day 0 and day 10 (Figure 5B). We observed good linearity among the range of matrigel evaluated (Day 0, R^2 = 0.94; Day 10, R^2 = 0.98; Figure 5C), indicating sensitivity of the immunostaining and LI-COR imaging under 3D configuration. Furthermore, majority of the matrigel was retained within the alginate throughout the 10 day culture period with less than 20% loss of ECM protein at higher matrigel concentration (i.e. 100 µg/ml and 200 µg/ml; Figure 5D). Specific ECM antibody staining also detected similar protein levels (data not shown) compared to the quantitative evaluation by 2D ECM array as described above (Figure 3), indicating retained ECM after inclusion into alginate gel array, as would be expected.

Differentiation of hPSC-PP is sensitive to organ-specific ECM, as detected in the 3D array

Next, we used this miniaturized 3D alginate-ECM array to investigate the differentiation of encapsulated hPSC-PP cells. The pre-differentiated hPSC-PP were harvested with enzymatic digestion to yield single cell suspension (cell density of 2.5 x 10^6 cells/ml) and mixed with specific organ ECM-alginate solutions. Arrays of 25 alginate microspots (Figure 6A) (n=5 for each ECM condition) were prepared and cultured in maturation media with notch inhibition, a known inducer of beta cell phenotype [49, 51, 71] for 9 days. During this period, the cells remained confined within the microspots, with no spot breakage (Figure 6C) and no visible gel detachment from the slide, indicating the microspots were structurally stable. Confocal microscopy images also indicated that the encapsulated cells were evenly distributed inside the alginate-ECM microspots (Figure 6B). However, live/dead staining showed significant cell death (up to 80%) in the 3D cell-laden alginate-ECM microspots (Figure 6D-E). This is most likely due to cell-cell contact inhibition while harvesting the hPSCs, which is known to activate the apoptotic pathway in hPSCs [72]. While we added Rho-associated protein kinase (ROCK) inhibitor (i.e. Y-27632), which has been illustrated to boost cell survival in this process [72, 73], we still observed significant cell death.

In order to retain high viability of hPSC-PP cells in our 3D ECM array platform, we chose to encapsulate cell clusters instead of single cell hPSC-PPs. We hypothesize that the preserved cell-cell contact in the clusters will not activate apoptotic pathway and hence will retain high viability. In our previous study we have reported the excellent viability and pancreatic differentiation of hPSC-PP aggregates in alginate capsules [49]. hPSCs were encapsulated as single cells within the alginate capsules, where they formed small aggregates upon propagation and subsequently differentiated into pancreatic progenitor cells upon induction. These hPSC-PP aggregates can be easily harvested by mild treatment of EDTA to dissolve away the alginate capsules. Schematic in Figure 6F illustrates the differentiation protocol to generate 3D hPSC-PP aggregates and encapsulate into the 3D ECM gel array (Figure 6G). To ensure relatively homogenous-sized aggregates were encapsulated, we passed the hPSC-PP aggregates through a cell strainer (100 µm mesh size) to avoid inclusion of cell clusters larger than the microspot gel size. This will avoid cellular overgrowth and protrusion out of the alginate microspots as shown in our Supplemental figure 3. After culturing for 9 days in different organ ECM gel array, live/dead staining of encapsulated hPSC-PP aggregates showed excellent

viability (>90%, Figure 6H-I) much higher than what was experienced with the encapsulated single cell hPSC-PPs. In comparing across different organ-derived ECM, no significant difference was observed in the viability of PP aggregates ($P>0.05$, Figure 6I).

Having achieved high viability, we next investigated the differentiation outcome of hPSC-PP aggregates exposed to different organ-derived ECM in the 3D alginate microspots. We cultured the encapsulated hPSC-PP aggregates in different organ-derived ECM alginate microspots (n=5 for each organ) for 9 days in the presence of notch inhibition (DAPT). Parallel cultures on alginate with and without matrigel was used as controls. Immunostaining and LI-COR imaging was performed to assess pancreatic maturation using pancreatic and islet-specific transcription factors such as Pdx1, Nkx6.1, and insulin protein marker, C-peptide. The result showed that higher PDX1 expression was detected in all the alginate-ECM microspots compared to alginate only controls (Figure 6J-K). NKX6.1 did not show any differences between P-ECM, H-ECM, matrigel and the alginate only control. However, L-ECM elicited a substantial increase of all PP markers, including insulin (C-peptide) protein expression (Figure 6K). Interestingly, P-ECM only showed a modest increase in C-peptide level but did not show significant increase of PP markers or C-peptide when compared to H-ECM or controls ($P>0.05$, Figure 6K). Confocal sectioning also corroborated this finding and detected higher expression of C-peptide staining in L-ECM alginate microspots (Figure 6L). To ensure that this observation was attributed to the ECM-specific differences and not any physical parameters of the gel, like stiffness, we performed AFM stiffness measurement of the alginate microspots after incorporation of ECM proteins. Our result revealed no stiffness differences between the alginate microspots alone and alginate microspots incorporated with ECM proteins (Supplemental figure 4). Collectively, these data suggest that the hPSC-PP aggregates were responsive to organ-derived ECM differences and further matured into insulin-expressing cells, with highest differentiation phenotype observed in L-ECM matrix. Furthermore, such stem cell differentiation phenomena can be studied in a miniaturized 3D alginate-ECM array.

DISCUSSION

Extracellular matrix (ECM) components are important physiological regulators of stem cell function and fate [6, 10, 56]. In order to facilitate the high throughput analysis of cell-ECM interaction to dictate stem cell fate and function, several groups have developed combinatorial ECM array platform using single purified proteins in 2D adherent [10, 14] and 3D hydrogel [19, 20, 74] configurations. However, recapitulating the native ECM microenvironment niche with single purified proteins has proved to be inadequate [14, 23], hence more complex ECM has also been investigated using cell-derived matrix *in-vitro* [26, 75] or decellularized tissue or organ [28, 29]. Herein, we have developed an ECM array using native-mimicry ECM from decellularized organs (namely pancreas, liver and heart). The system is compatible with quantitative imaging, and permits rapid evaluation of the matrix protein profiles of different organ-derived ECM, along with the sensitive measurement of the cell-ECM interaction both in 2D adherent cultures as well as 3D non-adherent culture. In this report, we evaluated the effect of ECM specifically on pancreatic differentiation of hPSCs, but the developed platform is versatile to be applied to characterize any lineage specific differentiation.

To generate organ-specific ECM components, we employed a protocol combining decellularization, lyophilization, pulverization, and protein extraction to create organ ECM-

derived solutions from pancreas, liver and heart. It is important to note that chaotropic agent extraction process does not completely solubilize the ECM. The residual ECM pellet is likely to contain mostly collagen as reported by Lin et al. [54]. When compared to the ECM extracted by urea (a chaotropic agent), they found that the SDS-PAGE profile of the collagenous ECM pellet was similar to that of purified collagen, and it did not exhibit high potency to support mesenchymal stem cell (MSC) function. In contrast, ECM extracted by urea contained multiple bioactive ECM components that accelerated MSC proliferation, attachment, spreading, migration and multi-lineage differentiation. Hence it was likely to contain majority of the bioactive ECM component. In addition, the same extraction technique using urea was also proven effective in the extraction of Matrigel from Engelbreth-Holm-Swerm (EHS) tumors. Matrigel contains multiple ECM components such as laminin, collagen IV and entactins, and is commonly used as a bioactive culture substrate to promote cell differentiation and function [76]. Hence we hypothesized that by using chaotropic agent extraction method, we will be able to extract the bioactive portion of organ-specific ECMs. Consistent with Lin et al.'s finding [54], our result from immunostaining, SDS-PAGE and biochemical characterization of the organ-derived ECM extracts revealed retention of multi-components ECM, distinctly different than purified rat tail collagen I (Figure 1F and Figure 3A-E). Even though we are preserving multi-component ECM, it is likely that we are not capturing the full spectrum of ECM proteins from native organs, and losing some fraction from the harsh treatments of decellularization and ECM extraction. Nevertheless, we have previously demonstrated the effectiveness of this ECM processing approach, and the resulting ECM have been shown to recapitulate essential components of native ECM from different tissues including retina [53, 77], embryoid bodies [75] and pancreas [29].

Quantitative comparison of cell-ECM interaction requires reproducible substrate preparation and a sensitive detection method. Using a nitrocellulose-coated surface in combination with repeater pipette microspotting, we were able to generate arrays of microspots with a highly reproducible protein content. There are several advantages of using nitrocellulose-coated surface for patterning ECM proteins. First, nitrocellulose coating is clear and permits excellent optical observation of the cells. Secondly, it is inert and non- toxic to cells [78]. When blocked only with BSA, nitrocellulose is a very poor substrate for cell attachment [46]. Lastly, nitrocellulose coating is well suited to bind a broad range of different ECM proteins via a combination of intermolecular forces, dominated by hydrophobic and van der Waals interactions [79], without compromising the protein structure of the immobilized ECM extracts. The protein binding capacity of the nitrocellulose coating is instantaneous, nearly irreversible, and quantitative to 80–100 $\mu g/cm^2$ [66], much higher than poly(styrene-co-maleic anhydride) (PS-MA) coating (another common surface coating for protein immobilization), which shows only 68.2ug/cm^2 of protein binding capacity [67, 68]. Several alternate substrate preparation have been developed for patterning protein microarrays [10, 20, 80], but the optimal substrate for patterning ECM proteins remains to be investigated. To develop an array platform for quantitative characterization, we utilized an on-chip, near IR-dye, immunofluorescence staining method with LI-COR Odyssey scanner. This method provides a robust high-throughput method for the quantitation of immunofluorescent staining and has advantages over microscopy-based screening approaches with regard to throughput and sensitivity. Acquisition and quantification of our generated arrays with 25 microspots can be accomplished in less than 10 min on the LI-COR odyssey scanner. This is in contrast to other reported studies involving hours-long acquisition time on a high-throughput microscope in the visible spectrum and similar scanned

area [10, 17, 18, 81]. In addition, the high sensitivity of the LI-COR scanner and the near-IR dyes provided robust signals at lower antibody dilutions than were possible using a microscopy-based system and dyes in the visible spectrum. With the increased sensitivity, however, there is an enhanced probability of artifact from unspecific antibody background staining. Hence, appropriate controls need to be included in the experimental plan to adequately remove the effect of background from acquired data.

The fabricated ECM array was first applied to characterize the cell-ECM interaction for cell attachment, and its specificity to organ-derived ECM. Analysis of the decellularized extracts for individual ECM proteins revealed that pancreas, liver and heart each has differing levels of ECM proteins such as collagen I, collagen IV, laminin and fibronectin (Figure 3). When seeded with hPSC-PP cells, we observed differential adhesion profile responding to different organ-specific ECM with the L-ECM resulting in highest PP cells adhesion. Pancreatic progenitor (PP) cells display αvβ3 and αvβ5 integrins to mediate adhesion and migration both *in vitro* and *in vivo* model of pancreatic islet development [82]. These integrins are among the receptors that have been shown poised to receive signals from ECM components including fibronectin, collagen IV and vitronectin [83, 84]. Hence it can be postulated that the favorable adhesion of hPSC-PP cells to L-ECM can be attributed to the significantly higher fibronectin content found in L-ECM over P- and H-ECM.

While the 2D ECM array was efficient in analyzing the composition and adhesive properties of the ECM, it restricts us to analyze cell-ECM interactions for adherent cells only. However, ECM proteins are known to contribute beyond just adhesive function to dictate cell fate commitment [56, 64]. Hence to explore the cell-ECM interactions under non-adhesive cultures, we devised an encapsulation system in an inert hydrogel, which allows cell growth without adhering to the hydrogel. In our earlier work we have reported culture and differentiation of hESCs into islet-like cells in alginate capsules [49]. In addition to providing means for adhesion-free culture, it also provides a 3D environment to the cells, which is gaining increasing appreciation as a vital component of cell microenvironment, more so for human pluripotent stem cells [85]. While our previous report was in free suspension culture of encapsulated hPSCs, Fernandes et al. [17] have reported an alginate-based matrix array immobilized on glass slide to facilitate toxicity and drug screening applications [18]. With this immobilization technique, it is now possible to distribute nanoliter volumes of different samples in a spatially addressable footprint for high throughput applications. They applied this platform for screening of human neural stem cells toxicity [18] and mouse embryonic stem cells differentiation [17]. In this study, we adopted the alginate immobilization platform and modified it to introduce the organ-derived ECM to the encapsulated hPSCs. The incorporation of ECM was stable with near-complete retention of the ECM throughout the culture period (Figure 5D).The negligible ECM loss from the array spots further rules out the concern of possible influence from adjacent array spots. Inclusion of ECM also did not modify the alginate stiffness thus confirming the observed effect is not from likely physical hydrogel parameters (Supplemental figure 4).

Our alginate-ECM array is a versatile platform. It allows the feasibility of simultaneous multi-parametric modulation. In our study we modified the ECM composition of the array spot while keeping the physical gel parameters unchanged. It is possible to simultaneously change gel physical parameters, for example the array stiffness, by micro-spotting different divalent cation (e.g. Ca^{2+} or Sr^{2+}) or different concentration of $BaCl_2$ cationic solutions onto the nitrocellulose surface, which will give rise to different stiffness upon alginate gelation. In our

study, we microspotted only one concentration of $BaCl_2$ solution (10mM), which led to identical low stiffness (1kPA) across the different ECM conditions tested. This low stiffness was chosen mainly to be within the “soft” tissue range of native pancreatic tissue, which has been identified to be another critical parameter to modulate cell fate [44, 86]. In addition, the platform is also versatile enough to allow various encapsulation configurations, such as single cells or cell aggregates. Encapsulation of single cells and its propagation into colonies under encapsulation is the most common practice for most of the cell lines. A parallel study from our lab shows that hESC differentiation is sensitive multiple simultaneous parameters, such as the stiffness of encapsulating alginate and encapsulation configuration [87, 88]. Hence we demonstrated the feasibility of microspotting single cells of MIN6 beta cells and maintaining viable culture in the 3D alginate gel array for 10 days (supplemental figure 5). However such single cell encapsulation tends to be restrictive for hPSCs because hPSCs are prone to cell death after being made single cell. While there was significant survival of the single-cell encapsulated hPSC-PP in our array platform, the dissociation process still led to significant cell death, as expected. These dead cells remained entrapped in the alginate matrix which is an undesirable culture condition. While we observed good viability of hPSC-PP cells in the 2D cell-ECM adhesion assay, likely only viable cells were capable of integrin-ECM mediated adhesion. Loosely attached dead cells were expected to be washed away during the staining process. In contrast, the 3D cell-laden ECM array involved the encapsulation of all dissociated cells, which likely include the entrapment of significant dead cells. Hence we developed a methodology to generate hPSC-PP aggregates and encapsulate these aggregates without dissociation. The preserved cell-cell contact in the aggregates protect the cells from apoptosis, and furthermore, it results in stronger differentiated phenotype, as demonstrated by our previous study [49] and reports from other groups [89-92].

Recent studies have shown the manifestation of enhanced stem cell functions and intricate tissue formation when organ-specific ECM scaffolds were employed [25, 93]. The ECM scaffolds were shown to have the capacity to direct tissue-specific stem cell lineage commitment and maintain the phenotype of mature cell populations. However, the organ specific effect observed from these studies cannot be solely attributed to the ECM composition difference as these studies employed decellularized whole-organ scaffolds that are complex and vastly different beyond biochemical ECM properties (e.g. difference in growth factors, biophysical properties such as stiffness and topology etc.). In our studies, we implemented a more level comparison between different organs by extracting only the bioactive ECM components devoid of any confounding biophysical factors. Despite the identical extraction protocol, organ-specific differences were detected in all three organs in terms of their ECM compositional profiles. These differences in turn led to different maturation outcome in the hPSC-PP cells, when complemented with chemical induction. Surprisingly, P-ECM, in spite of its pancreas origin, was not the most suitable in promoting the differentiation of hPSC-PP cells into insulin-expressing cells. In contrast, the liver derived ECM had the best performance in insulin-expression among the organs compared in this study. It is noteworthy that majority of the pancreas is composed of exocrine tissue with only 2% made up of islet cells. While the differences in the ECM composition between endocrine and exocrine pancreas is not obvious, majority of pancreas ECM could be of exocrine-origin and non-specific to the pancreatic endocrine cell population, which is of interest in this study. On the other hand, liver is developmentally close to pancreas (i.e. both from endoderm germ layer) with previous successful reports of hepatocytes trans-differentiation into insulin-producing cells [94]. Liver has

also been successfully used as a favorable transplant site to support islet insulin function during islet transplantation. In addition, liver has a very high density of vasculature and there is a substantial amount of interaction between islets and the ECM associated with vasculature. It has also been proposed that the vasculature basement membrane could be a favorable niche for promoting insulin gene expression and β cell proliferation [95]. In alignment with this, Citro *et al* [96] utilized decellularized lung scaffold to fabricate a functional islet organ, where the endothelialized acellular lung matrix provided adequate vascular support for islet engraftment. It should also be noted that there is likely a beneficial effect that can be attributed to the variety of matrix-bound growth factors and cytokines retained in the ECM extracts, which have been demonstrated effective for inducing differentiation and maintaining cellular phenotypes [3, 24, 93]. Future investigation is warranted to confirm the role of liver-ECM to identify the specific components or combinations of liver ECM that is conducive to pancreatic maturation.

CONCLUSION

In conclusion, we developed an array platform that allows culture of cells under both adherent 2D configuration and non-adherent 3D aggregate configuration. Our array system enables rapid and sensitive measurement of the differential effect of organ-specific ECM on hPSC differentiation. Our results strongly suggest that hPSC-cell maturation was responsive to different organ-derived ECM and L-ECM is more supportive of pancreatic differentiation of hPSCs than P- or H-ECM. The versatility of the platform could be easily adapted for other lineage-specific differentiation of hPSC to allow organ-specific interrogation of stem cell niches for tissue engineering applications.

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FIGURE LEGENDS

Figure 1. Fabrication and characterization of ECM extracts from decellularized pancreas, liver and heart. (A) Schematic illustrating the experimental workflow to generate organ-specific 2D ECM array. Step 1: Whole organ-decellularization of pancreas, liver and heart and schematic illustration of the ECM extraction process to yield organ-specific ECM. Step 2: Organ-specific ECM were patterned on the nitrocellulose slide using a repeater pipette. On-chip immunofluorescence characterization of ECM proteins followed by slide scanning with LI-COR imager. (B) Representative SDS-PAGE gel of P-ECM, L-ECM and H-ECM protein extracts contain abundant low molecular weight proteins (<100 KD) that are absent in collagen I and Matrigel. (C) Total protein content quantification for P-ECM, L-ECM and H-ECM by BCA assay. (D) Sircol collagen assay showed collagen content from P-ECM, L-ECM and H-ECM extracts. (E) sGAG content quantification for P-ECM, L-ECM and H-ECM by Blyscan assay. (Error bars indicate ± SEM and n=3 independent experiments, ** P<0.01).

Figure 2. Surface coating and immobilization of ECM proteins to generate 2D ECM array. (A) Water contact angles measurement showed increased hydrophobicity in nitrocellulose coated surface. (B) LI-COR images of PNA lectin staining of adsorbed matrigel protein on tissue

culture plastic (TCP) surface versus TCP surface coated with nitrocellulose after 5 and 60 min of matrigel coating time. (C) Corresponding quantification of PNA lectin fluorescence intensity showed higher amount of ECM proteins were immobilized on nitrocellulose coated surface. (D) AFM measurement of local root mean square (RMS) for surface roughness evaluation. Significant higher RMS value in microspotted organ-derived ECM than nitrocellulose only surface indicate organ-specific ECMs were immobilized onto the nitrocellulose coated surface. No significant difference were detected among different organs, indicating similar amount of proteins were immobilized. (E) PNA lectin stain visualized with LI-COR scanner demonstrated microspotted 2D ECM array with 1 µl, 2 µl and 3 µl of matrigel (triplicates). (F) Correlation plot shows the varying size of microspot can be achieved by varying the volume of matrigel droplets. (G) Quantification of PNA lectin stain shows the fluorescence intensity as a function of increasing volume of matrigel droplets. (Error bars indicate ± SEM and n=3 independent experiments, ** $P<0.01$).

Figure 3. On-chip, near IR Dye, immunofluorescence staining and LI-COR quantification. (A) Representative LI-COR image of stained 2D organ-specific ECM array. Matrigel, Gelatin and Rat tail collagen I were microspotted as controls. (B-E) Quantification of ECM proteins, Collagen I (B), Collagen IV (C), Laminin (D) and Fibronectin (E) and normalized to rat tail collagen I. Specific composition of matrigel, rat tail collagen I and gelatin (denatured collagen I) were detected consistent with reported literature composition, suggests the sensitivity and validation of the assay to semi-quantify the ECM composition of organ-derived ECMs.

Figure 4. hESC-derived PP cells on 2D ECM array to investigate Cell-ECM adherence. (A) Schematic representation of cell-ECM adherence assay on fabricated organ-specific ECM array. Step 1: differentiation protocol of hESC to pancreatic progenitor cells. Step 2: Cell seeding of harvest hESC-PP onto ECM array. (B) Different cell seeding density were tested to seed PP cells on matrigel microspotted surface. (C) Corresponding DNA DraqQ5 quantification showed detection of fluorescence intensity increase linearly with increased cell seeding density. We determined 1 x 10^6 million cells/ml to be the optimal density as it gave rise to a uniform confluent cellular microspot under matrigel condition (positive control). (D) Live/dead staining of one of the confluent cellular microspot showed high viable cells indicating the cytocompatibility of the platform. (E) Phase-contrast images of short term cell seeding (5 min) on different organ-derived ECM on the 2D ECM array. Each organ ECM showed different cell adherence profiles. (F) Live/dead staining of washed 2D ECM array after long term cell seeding (24 hours). Similar adherence profile was observed with the short term cell seeding. (G) Quantification of DraQ5 stained 2D ECM array after cell seeding. L-ECM showed the highest PP cells adherence, followed by P-ECM and H-ECM. (n = 4-5, * $P<0.05$)

Figure 5. Fabrication and characterization of 3D organ-specific ECM array. (A) Schematic representation of alginate microspots with cells and ECM extracts on nitrocellulose glass slide pre-spotted with PLL/$BaCl_2$. (B) PNA lectin staining on matrigel alginate microspots (without cells) on 3D ECM array on day 0 and day 10 on a range of matrigel quantification (0-200 µg/ml) (C) Corresponding correlation plot of PNA lectin fluorescence signals at day 0 and day 10 showed good correlation of fluorescence intensity (RFU) over the range of matrigel concentration evaluated, indicating the good linearity and sensitivity of the assay. (D) Comparison of PNA lectin stain fluorescence signal at day 0 and day 10 over a range of different incorporated matrigel concentration. The incorporated matrigel after 10 days in culture showed less than 20% decrease in fluorescence signals, indicating released or degraded ECM

components was nominal. At higher concentration of matrigel (100-200 µg/ml), the difference between day 0 and day 10 was smaller and insignificant (P>0.05). (Error bars indicate ± SEM and n=3 independent experiments, * P<0.05).

Figure 6. hESC-derived PP cells on 3D ECM array to investigate differentiation. (A) Photograph of the organ-specific 3D ECM array, 25 microspots were generated in each well (6 well plate) (n=5 for each ECM). (B) Confocal images showing the three-dimensional distribution of hESC-PP cells after encapsulated onto the 3D ECM array. Shown in each panel is the top view and side view of the microspot, from a z-stack of 5 µm sections. Each spot is 1 µl resulting in a diameter of 1600 µ and a height of 250 µm. (C) Corresponding phase-contrast image showing one of the 3D microspot encapsulated with PP cells. (D) Representative live/dead images of hESC-derived PP cells encapsulated in organ-ECM microspots on the 3D array. High number of dead cells were detected. (E) Live/dead quantification confirmed high percentage of dead cells across all the ECM conditions. However, there was no significant difference observed between each ECM condition. (F) Step-wise differentiation protocol in alginate capsules to generate PP aggregates. After decapsulation with treatment of 100 mM EDTA, PP aggregates were filtered through 100 µm mesh-sized cell strainer to avoid larger clumps inclusion in the 3D ECM array. Right panel showed the phase contrast image of relatively smaller and more homogenous sized PP aggregates used in the 3D ECM array. (G) Representative phase contrast image of PP aggregates inside the 3D alginate-ECM microspot. (H) After cultured inside 3D ECM array for 9 days, live/dead images showed good viability in all the organ ECM conditions. (I) Quantification of live/dead images confirmed the viability >90% in all ECM conditions including the controls (matrigel and alginate only). (J) LI-COR image showed the immunostaining characterization of the differentiation of PP cells in 3D ECM array. PP aggregates were encapsulated in organ-specific 3D ECM array and cultured in maturation medium with notch inhibition for 9 days, fixed and stained for analysis on the LI-COR Odyssey instrument. DraQ5 (red) was used as a counter stain for cell number and the Pdx1, Nkx6.1 and C-peptide (green) antibodies followed by an IRDye-800CW secondary antibody for quantitation of pancreatic maturation. (K) The PP aggregates were responsive to different organ ECM and gave rise to different levels of Pdx1, Nkx6.1 (pancreatic progenitors) and C-peptide (insulin) markers. (L) Representative stacked confocal images showed the specific C-peptide staining on the PP aggregates and consistent with the expression level found with LI-COR. L-ECM microspots demonstrated the highest number of insulin-expressing cells. (Error bars indicate ± SEM, 5 microspots for ECM condition, n=3 independent experiments, * P<0.05).