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Influence of substrate composition on human embryonic stem cell differentiation and extracellular matrix production in embryoid bodies

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Abstract

Stem cells reside in specialized niches *in vivo*. Specific factors, including the extracellular matrix (ECM), in these niches are directly responsible for maintaining the stem cell population. During development, components of the stem cell microenvironment also control differentiation with precise spatial and temporal organization. The stem cell microenvironment is dynamically regulated by the cellular component, including stem cells themselves. Thus, a mechanism exists whereby stem cells modify the ECM which in turn affects the fate of the stem cell. In this study, we investigated whether the type of ECM initially adsorbed to the culture substrate can influence the composition of the ECM deposited by human embryonic stem cells (hESCs) differentiating in embryoid bodies, and whether different ECM composition and deposition profiles elicit distinct differentiation fates. We have shown that the initial ECM environment hESCs are exposed to affects the fate decisions of those cells and that this initial ECM environment is constantly modified during the differentiation process.

Introduction

Human embryonic stem cells (hESCs) are capable of differentiating into derivatives of all 3 germ layers and self-renewing indefinitely in culture. This unique plasticity allows for differentiation into many therapeutically relevant cell types. However, effectively controlling the differentiation of these cells into a single desired cell type or a spatially-organized tissue remains one of the principle barriers to the therapeutic use of pluripotent stem cells.^{1–4} The decision to differentiate down a specific lineage is highly complicated. The stem cell integrates soluble biochemical cues, nutrients, biophysical signals, as well as cues from the insoluble extracellular space to make its differentiation decision.^{4,5} Many of these cues are dynamic and change over time during development.^{1,6,7}

The interactions between hESCs and the insoluble, protein based extracellular matrix (ECM) that surrounds them govern many cell functions such as attachment and survival as well as

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informing and directing cellular decisions such as differentiation vs. self-renewal.^{1,2,8-11} For example, in mouse embryonic stem cells, the ECM protein fibronectin was shown to be necessary for attachment and self-renewal during *in vitro* culture but excess amounts of the protein also gave rise to increased cell spreading and subsequent differentiation.¹²

Matrix composition has also been shown to influence the self-renewal of hESCs. Within the growing body of recent literature on fully defined culture systems for hESCs, much attention has been given to culture substrates capable of supporting hESC self-renewal.¹³ A small number of ECM proteins, including vitronectin and laminins 511 and 521, have been shown to maintain hESC pluripotency and self-renewal, whereas other ECM proteins such as collagens and fibronectin are unsuitable for maintaining hESCs in their undifferentiated state.¹³⁻¹⁷

The interactions between stem cells and the ECM can additionally have profound effects during development. Laminins have been shown to be critical during neural development and loss of function studies in mice resulted in severe deformations and early embryonic lethality.¹⁸⁻²² The importance of these ECM interactions is not limited to just the neural lineages. Other work has shown that the ECM proteins fibronectin and laminin are required for endoderm differentiation in mouse ESCs.⁹

Studying and understanding the effects of specific ECM components on the differentiation of hESCs is complicated by the fact that the ECM composition is dynamic. hESCs deposit their own ECM proteins during culture and constantly remodel the ECM environment.^{14,16,23,24} ECM remodeling is primarily governed by a class of proteins known as matrix metalloproteinases (MMPs). MMPs cleave most major ECM proteins and allow cells to degrade and reorganize the ECM environment.²⁵ In addition to mediating this cell-driven ECM reorganization, MMPs also play important roles in cell-cell signaling. In recent work by Horejs et al., a MMP-generated fragment of the ECM protein laminin was shown to modulate the epithelial to mesenchymal transition in differentiating mouse ESCs.²⁶

Understanding how hESCs interact with and modify their ECM environment is important to achieving control over these differentiating cell populations. In this study we profiled ECM production by differentiating populations of hESCs and investigated how these ECM environments influenced the differentiation decisions of the cells. An embryoid body based differentiation approach allowed for differentiation of hESCs into cell types from all three embryonic germ layers and demonstrated that different ECM production profiles biased differentiation to distinct germ lineages.^{27,28} For example, laminin-enriched ECM generated neural-enriched populations and fibronectin-enriched ECM led to endoderm-enriched populations. During efficient directed differentiation to neural lineages, the cells produced ECM similar to that of EBs plated on a laminin ECM.

Methods

Human embryonic stem cell culture

H9 hESCs (WiCell, Madison, WI) were maintained in E8 medium on Matrigel-coated tissue culture polystyrene (TCPS) plates (BD Falcon, Corning, Tewksbury, MA) as described.²⁹

Briefly, hESCs were cultured in 6 well plates (BD Falcon) coated with Matrigel (83 μg per well) in 2.5 mL of E8 medium per well. Cells were split 1:12 every 5 days with Versene solution (Life Technologies, Carlsbad, CA).

ECM Coating Preparation

TCPS plates were coated with 0.61 μg of ECM protein per cm^2 ECM (fibronectin (Sigma, St. Louis, MO), laminin 1,1,1 (Sigma), or collagen type 1 (Nutragen, Advanced Biomatrix, San Diego, CA)) in phosphate buffered saline (PBS) and placed in an incubator overnight at 37°C. For Synthemax plates, TCPS plates were coated with 3.3 μg per cm^2 Synthemax (Corning) in PBS and incubated overnight at 37°C. Prior to cell seeding, plates were washed with sterile PBS to remove any non-adsorbed coating.

EB Formation

Embryoid bodies (EBs) were generated from H9 hESCs according to established protocols (National Stem Cell Bank Cardiac differentiation from human ES cells – EB method, SOP-CH-203c³⁰). Briefly, H9 hESCs were harvested from E8 medium and expanded on MEF feeder cells (2×10^4 MEFs/ cm^2) (WiCell) for five days in UM/F+ medium (DMEM/F12 (Life Technologies), Knockout Serum Replacer (KOSR, 20%, Life Technologies), L-glutamine (2 mmol/L, Life Technologies), and MEM non-essential amino acid solution (1%, Life Technologies) supplemented with 4 ng/mL bFGF). After two days in culture, 2 μM BIO (6-bromoindirubin-3'-oxime, Sigma) was added to the culture medium. On day 5, colonies were enzymatically dissociated with pre-warmed dispase (1 mg/mL, Life Technologies) in DMEM/F12. Dissociated colonies were placed in an ultra-low attachment plate (Corning) for 5 days in EB20 medium (DMEM/F12 (Life Technologies), fetal bovine serum (20% Life Technologies), L-glutamine (2 mmol/L Life Technologies), and MEM non-essential amino acid solution (1%, Life Technologies)). After 5 days in suspension, EBs were plated onto TCPS plates coated with the indicated ECM protein and cultured for 4, 8, or 12 days in EB20 medium.

Monolayer Neural Differentiation

The directed neural differentiation procedure was adapted from Lippman et al.³¹ H9 hESCs were singularized with Accutase (Innovative Cell Technologies, San Diego, CA) and plated into 12 well TCPS plates coated with 0.61 $\mu\text{g}/\text{cm}^2$ human recombinant vitronectin in PBS at a density of 150,000 cells per well in the presence of 1 μM ROCK inhibitor (Y27632, Stemgent, Cambridge, MA) in E8 medium. After 24 hours, the culture medium was changed to E6 (E8 medium lacking bFGF and TGF- β) and replaced every 24 hr for 3 days. After 3 days, the partially-differentiated neural precursor cells were again singularized with Accutase and split 1:6 onto TCPS plates coated with Synthemax, fibronectin, laminin, or collagen type 1 and cultured for an additional 4 days in E6 medium.

ELISA and Immunocytochemistry

The cellular deposition of ECM proteins onto uncoated and ECM-coated TCPS plates was assessed via ELISA and immunocytochemical staining. Samples were fixed in 10% formalin (Sigma) and permeabilized using 1% Triton-X100 (Sigma) in PBS. Primary antibodies

against human fibronectin (Santa Cruz Biotechnology, SC80559, Dallas, TX), laminin (Abcam AB30320, Cambridge, MA), and collagen type 1 (Sigma C2456) were used at a 1:500 dilution in a solution with 3% BSA in PBS and allowed to incubate overnight at 4°C. Secondary staining was performed using AlexaFluor 488 and 568-conjugated secondary antibodies (Life Technologies) in 3% BSA at room temperature for 1 hr. Fluorescent images were captured using a Nikon IX51 epifluorescent microscope and mean fluorescence intensities were measured using FIJI software.³²

For ELISA analysis, sample preparation and primary antibody staining were performed as described above for immunofluorescence analysis. HRP-conjugated secondary antibodies (goat anti-rabbit Millipore #12-348 and goat anti-mouse #12-349) were used at 1:250 dilution in 3% BSA at room temperature for 1 hr. Samples were washed 3 times with PBS and Turbo-TMB substrate (Thermo Scientific, Waltham, MA) was added to each well and incubated at 37°C for 1 hr. After incubation, the reaction was stopped by adding 10 µL of 1M HCl. Sample absorbance was measured at 450 nm on a TECAN Infinite M-1000 plate reader.

MMP Analysis

MMP activity and concentration were quantified from media supernatants during the first 4 days of EB culture using Anaspec Sensolyte kits (Generic MMP Activity #72095 and MMP9 ELISA #72105). For MMP activity assays, 300 µL of cell culture supernatant was collected after 24 hr in culture. 50 µL of supernatant was added to 50 µL of generic MMP substrate and incubated at room temperature for 1 hr. 50 µL of stop solution was added and absorbance was read at 412 nm on a TECAN Infinite M-1000.

For MMP ELISAs, 100 µL of cell culture supernatant was added to a well of the ELISA plate and incubated on a shaker for 2.5 hr at room temperature. Plates were washed 4x, then 100 µL of antibody was added and incubated for 1 hr on a shaker at room temperature. Plates were again washed 4x and 100 µL of HRP – streptavidin solution was added to each well and incubated for 45 min at room temperature. Plates were washed and 100 µL of TMB substrate was added and incubated for 30 min. 50 µL of stop solution was added and absorbance was read at 450 nm on a TECAN Infinite M-1000.

qRT-PCR

mRNA was isolated from cultured cells using the Qiagen RNeasy plus mini kit. cDNA was produced using the Applied Biosystems high capacity cDNA reverse transcription kit. Gene expression was assessed via qRT-PCR using the following human Taqman gene expression assays from Applied Biosystems: *OCT-4*, *Brachyury (T)*, *MESPI*, *GFAP*, *FOXA2*, *MAP2*, *NKX2.5*, *FLK1*, *CD34*, *MYOD*, *PPAR γ* , *RUNX2*, *SOX-9*, *PDX1*, and *AFP*. Samples were measured using a StepOne real time PCR system (Applied Biosystems) and data analysis was performed using the $\Delta\Delta C_t$ method relative to *GAPDH*. Abbreviations listed in Supplemental Table 4.

Flow Cytometry

Cells were singularized with Accutase and fixed using cold 90% methanol. Primary antibodies against nestin (Santa Cruz Biotech SC23927), β -3 tubulin (Sigma T2200), and an isotype control (Mouse IgG₁, κ ; BD 550878) were used at a 1:500 dilution in 0.3% BSA and incubated overnight at 4°C. Secondary antibodies (AlexaFluor 488 and 568-conjugated) were used at 1:500 in 0.3% BSA and incubated for 1 hr at room temperature. Samples were analyzed on a BD Canto flow cytometer using BD FACSDiva software.

Statistical Analysis

All statistical analysis was performed via one-way ANOVA with a Tukey HSD post-hoc test using the KaleidaGraph software package.

Results

ECM environment affects embryoid body cell composition

To determine if the initial ECM environment influenced the differentiation trajectory of hESCs, we first formed embryoid bodies (EBs) from hESCs via culture in ultra-low attachment plates and plated the day 5 EBs on different initial ECM matrices consisting of untreated tissue culture polystyrene (TCPS) plates or TCPS coated with collagen I, fibronectin, or laminin. Gene expression profiles of lineage-specific markers (Figure 1A) were then analyzed via qRT-PCR in EBs plated on different initial ECM substrates for 4, 8, and 12 days. The gene expression data are presented in Supplemental Tables 1-3, which show that many different markers in all three germ lineages were expressed in all differentiating cell populations throughout the culture period. Heat maps of the data show that expression of several lineage specific markers increased upon culture of EBs on different coatings relative to the uncoated TCPS condition (Figure 1B). For example, at day 12, expression of endodermal markers *FOXA2* and *PDX1* was greatest on the fibronectin-coated substrates while expression of the neural marker *MAP2* was greatest on the laminin-coated substrates ($p < 0.05$ relative to expression on TCPS), suggesting that these ECM environments were more conducive to EB differentiation down those specific lineage trajectories.

Initial ECM environment alters ECM deposition by differentiating hESCs

The observed differences in differentiation trajectories arising from exposure to different ECM environments raised the question of how differentiating hESCs alter their ECM environments. In order to evaluate the ECM produced by the differentiating cells, we quantitatively profiled the ECM at various time points throughout the culture period using an ELISA. The ECM composition of the substrate upon which the EBs were plated on day 0 influenced the composition of the ECM on days 4 and 12 (Figure 2). As seen in Figure 2A, we observed predominantly laminin, a small amount of fibronectin, and very little collagen I after culture of EBs on uncoated TCPS. Meanwhile, EBs cultured on fibronectin (Figure 2B) or collagen I (Figure 2C) exhibited a different ECM production profile, with cells depositing a significant amount of fibronectin and a small amount of laminin. EBs cultured on laminin

(Fig. 2D) exhibited minimal production of collagen, fibronectin or laminin. No measurable amount of vitronectin was detected in any condition (not shown).

Also of note, EBs plated onto collagen I-coated plates deposited large amounts of fibronectin and smaller amounts of laminin, but the amount of collagen I on the substrate decreased by 12 days ($p < 0.001$), suggesting that the collagen I was degraded by the cultured cells (Figure 2C). The laminin initially present on the laminin-coated plates was also degraded over 12 days of culture (Figure 2D).

Fluorescent staining of the deposited ECM proteins confirmed the general trends present in the ELISA data (Figure 3). No detectable collagen I was deposited by EBs cultured on TCPS or laminin. The amount of collagen I on the collagen I-coated plates was lower in areas where cells were localized (Figure 3G), suggesting that the collagen I degradation was caused by the cells. This degradation was likely due to the presence and activity of MMPs detected in the media supernatant (Supplemental Figure 1). In regions near the cells we observed the deposition of large amounts of fibronectin (Figure 3 C, D), consistent with the ELISA data presented in Figure 2. In all cases, the ELISA results closely matched the quantification of fluorescence intensities from immunochemistry (Supplemental Figure 2).

Differentiating hESCs produce an ECM that acts synergistically with other differentiation cues

EB differentiation is useful for identification of general effects of ECM on hESC differentiation fates, but the heterogeneity of EB differentiation complicates drawing conclusions about ECM effects on specific lineages. We therefore adopted a well-established monolayerbased neural directed differentiation protocol capable of generating high purity populations of neural progenitor cells in order to gain more specific insight into how interactions with the ECM may affect hESC differentiation. From the EB experiments, we expected that the ECM composition would affect neural differentiation efficiency, and that the differentiating neural cells would alter the composition of the ECM.

First, we quantified the efficiency of hESC differentiation to nestin/ β -3 tubulin-expressing neural progenitors on different ECM substrates. In order to better ascertain ECM deposition by differentiating hESCs we adopted a fully defined culture substrate, Synthamax, in addition to fibronectin, laminin, and collagen I. Surprisingly, the differentiation efficiency of hESCs to nestin or β -3 tubulin-positive neural progenitor cells was not affected by the initial ECM substrate, reaching greater than 90% on all substrates tested (Figure 4 A, B). Next, we monitored the ECM composition during neural differentiation by immunofluorescent analysis. The ECM profiles produced by the differentiating cells on all initial ECM substrates tested were similar to those observed culturing EBs on the laminin substrate (Figure 4 C-N). By day 4 of differentiation on the Synthamax, collagen I, and fibronectin-coated substrates we observed significant deposition of fibronectin and laminin, while on the laminin-coated substrates we observed the presence of predominantly laminin. Again similar to EB differentiation, we observed the degradation of the collagen coating in regions of cell attachment (Supplemental Figure 3). These results indicate that hESCs undergoing directed differentiation to neural lineages modify their ECM environment, but that the differentiation

fate is independent of the initial ECM composition, suggesting that the differentiating cells may deposit an ECM supportive of neural differentiation.

Discussion

In this study we profiled the compositions of ECM constituents produced by hESC-derived EBs differentiating into derivatives of all 3 embryonic germ layers. Our results show that in an unbiased EB differentiation scenario, the ECM that cells are initially exposed to can influence the long term composition of that population. The data demonstrate that different ECM composition profiles correspond to differences in hESC lineage specification, specifically that the laminin-rich ECM substrates correlated with a population enriched in neural (*MAP2* positive) cells while the fibronectin substrates correlated with an enriched population of endodermal (*FOXA2* and *PDX1* positive) cells. This finding is in agreement with recent work by Taylor-Weiner et al., who showed a strong correlation between mouse ESC expression of endodermal markers (*GATA4* and *Sox 17*) and fibronectin production and localization in EBs.⁹ Gene expression data (Figure 1) indicate that the initial ECM coating can preference a differentiating EB population toward a specific germ layer. Further, these germ layer-specific enrichments of differentiating populations correspond to ECM deposition profiles from plates initially coated with fibronectin or laminin. Thus, not only was the initial coating capable of exerting an influence on differentiating hESCs, but the matrix produced by the differentiating cells was also indicative of the enrichment of specific cell types.

This ECM-driven enrichment of specific lineages could be caused by one of two modes. It is possible that the ECM environment directly affects the differentiation fate of the cells as described using hESC-derived progenitor populations where these lineage-restricted cells differentiated more efficiently to an osteogenic fate when exposed to specific combinations of fibronectin and laminin in 3D hydrogels.³³ Alternatively, it is also plausible that the ECM environment selectively biases the growth of specific progenitor populations. A recent study by Selekmán et al. demonstrated differential proliferation rates of hESC-derived epithelial progenitor populations with altered ECM composition.³⁴

By supplying EBs with an environment rich in laminin, similar to what exists during early neural development, we observed elevated expression of neural proteins relative to EBs cultured on uncoated TCPS substrates.^{22,35-37} Similarly, EBs cultured on fibronectin substrates expressed greater levels of endodermal makers. While we noted that both fibronectin and laminin were produced by EBs during differentiation on most matrices tested, it is difficult to determine what populations of cells in the heterogeneous EBs synthesized which matrix components. Therefore we analyzed effects of ECM and ECM production in a more homogeneous, defined neural differentiation protocol.

During neural directed differentiation, altering the initial ECM substrate did not affect the efficiency of neural progenitor differentiation, and the ECM profiles produced by the differentiating cells on all 3 different initial ECM substrates were similar. Under these defined conditions it is possible that the soluble differentiation cues in this protocol dominated ECM effects. Alternatively, the differentiating cells remodeled the initial ECM

environment to one that was conducive to the neural differentiation. Differentiating neural cells specifically produced laminin on all ECMs tested (Figure 4). This remodeling is clearly evident in the degradation of the coated ECM as seen in the ECM deposition images from both the monolayer neural differentiation and EB differentiation, especially on the collagen I substrates (Figure 3G and Supplemental Figure 3) and is likely to involve MMP degradation of the existing matrix in addition to ECM synthesis. During hESC differentiation MMPs can drive the EMT transition by cleavage of a fragment of the ECM protein laminin that then signals back to the differentiating cells²⁶. Given both the presence and activity of MMPs during EB differentiation (Supplemental Figure 1), it is likely that some similar ECM-mediated feedback signaling mechanism was active during our observed differentiations.

The ECM components studied in this work have been shown to play important roles in regulating stem cell behavior both *in vitro* and *in vivo* during development^{9,19,22,38-40}. However, they are by no means the only ECM components produced by differentiating cells. In fact, given the complexity of the ECM produced during mammalian development, the endogenous ECM is likely to be significantly more complicated than just the four components studied here.⁴¹

From this study we conclude that the ECM initially presented to differentiating hESCs can impact the dynamic feedback cycle involved in ECM production and that specific ECM environments correlate with the composition of the differentiating cell population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

qRT-PCR of lineage-specific differentiation markers. (A) Represents differentiation trajectories and differentiation markers analyzed to profile embryoid body differentiation on different ECM coatings. (B) Fold change in expression of select differentiation markers in day 12 EBs on the indicated ECM coatings (uncoated: TCPS, fibronectin: FN, collagen I: Col 1, Laminin: Lam) and corresponding heat maps of trajectories shown in panel A. Gene expression values were normalized to expression levels on TCPS plates. n=3, Error bars represent standard deviations, * indicate expression levels statistically significant from

TCPS ($p < 0.05$). On the heat maps red indicates increased expression relative to EBs cultured on TCPS at the same time point ($p < 0.05$) while yellow indicates increased expression relative to undifferentiated hESCs ($p < 0.05$).

Figure 2.

Relative quantification of ECM concentrations by ELISA after 4 and 12 days in culture. EBs were plated onto (A) uncoated TCPS plates or TCPS plates coated with (B) fibronectin (FN), (C) collagen type I (Col 1), or (D) laminin (Lam) and cultured for 4 or 12 days. Day 0 data represent the amount of ECM protein initially adsorbed onto the plate prior to EB seeding (n=6). Error bars represent s. d. * indicate p<0.05 relative to D0 coatings, # indicate p<0.001 relative to D4 measurements.

Figure 3.

Immunofluorescent images of ECM proteins. EBs were plated onto TCPS plates coated with the indicated ECM proteins (uncoated: TCPS, fibronectin: FN, collagen I: Col 1, Laminin: Lam) and cultured for 4 days. The ECM present after the culture period was analyzed by immunofluorescent staining. Green = Fibronectin, Red = Collagen I, Yellow = Laminin, Blue = DAPI. Scale bars on all images are 100 μ m.

Initial Surface Coating

Figure 4.

Effect of ECM on directed differentiation to neural lineages. Flow cytometric quantification of the fraction of cells expressing (A) β III tubulin and (B) nestin when cultured on Synthemax coated TCPS plates (Synth), TCPS plates coated with fibronectin (FN), collagen type I (Col 1), or laminin (Lam) at day 7. (C-N) Immunofluorescent staining of ECM protein deposition after 4 days of culturing neural precursor cells on the indicated ECM substrates during neural differentiation. The indicated region in panel I is expanded in Supplemental

Figure 3 A,B. Green = Fibronectin, Red = Collagen I, Yellow = Laminin, Blue = DAPI.
Scale bars on all images are 100 μ m.