Crosslinking of extracellular matrix scaffolds derived from pluripotent stem cell aggregates modulates neural differentiation

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Abstract

At various developmental stages, pluripotent stem cells (PSCs) and their progeny secrete a large amount of extracellular matrices (ECMs) which could interact with regulatory growth factors to modulate stem cell lineage commitment. ECMs derived from PSCs can be used as unique scaffolds that provide broad signaling capacities to mediate cellular differentiation. However, the rapid degradation of ECMs can impact their applications as the scaffolds for in vitro cell expansion and in vivo transplantation. To address this issue, this study investigated the effects of crosslinking on the ECMs derived from embryonic stem cells (ESCs) and the regulatory capacity of the crosslinked ECMs on the proliferation and differentiation of reseeded ESC-derived neural progenitor cells (NPCs). To create different biological cues, undifferentiated aggregates, spontaneous embryoid bodies, and ESC-derived NPC aggregates were decellularized. The derived ECMs were crosslinked using genipin or glutaraldehyde to enhance the scaffold stability. ESC-derived NPC aggregates were reseeded on different ECM scaffolds and differential cellular compositions of neural progenitors, neurons, and glial cells were observed. The results indicate that ESC-derived ECM scaffolds affect neural differentiation through intrinsic biological cues and biophysical properties. These scaffolds have potential for in vitro cell culture and in vivo tissue regeneration study.

Statement of significance

Dynamic interactions of acellular extracellular matrices and stem cells are critical for lineage-specific commitment and tissue regeneration. Understanding the synergistic effects of biochemical, biological, and biophysical properties of acellular matrices would facilitate scaffold design and the functional regulation of stem cells.

The present study assessed the influence of crosslinked embryonic stem cell-derived extracellular matrix on neural differentiation and revealed the synergistic interactions of various matrix properties. While embryonic stem cell-derived matrices have been assessed as tissue engineering scaffolds, the impact of crosslinking on the embryonic stem cell-derived matrices to modulate neural differentiation has not been studied.

The results from this study provide novel knowledge on the interface of embryonic stem cell-derived extracellular matrix and neural aggregates. The findings reported in this manuscript are significant for stem cell differentiation toward the applications in stem cell-based drug screening, disease modeling, and cell therapies.

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1. Introduction

In recent years, pluripotent stem cells (PSCs), including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), emerge as indefinite and non-invasive sources of neural progenitor cells (NPCs) due to their unique self-renewal ability
and the capability of multi-lineage differentiation [1–3]. Although NPCs could be derived from the sub-ventricular zone and the hippocampus of adult and fetal brains, the limited cell number and the related ethical issues become major hurdles for clinical applications [4]. Alternatively, PSC-derived NPCs and neural tissues have shown promises for tissue engineering, organogenesis, drug screening, and disease modeling [1–3]. During neural tissue development from PSCs, extracellular matrix (ECM) plays a critical role in regulating cell survival, proliferation, and differentiation [5].

NPCs can secrete a large amount of ECMs and soluble factors which regulate cell proliferation and/or differentiation by autocrine feedback loop [6,7]. Decellularized ECMs from tissues, organs, and in vitro cultured cells have been recently used as tissue engineering scaffolds or carriers for growth factor delivery [8–10]. For central nervous system repair, the decellularized ECMs derived from neural tissues have been demonstrated to possess neurosupportive functions [11,12]. Neural tissue-derived ECMs promote proliferation, migration, and neural differentiation of NPCs, possibly due to the presentation of adhesive ECM proteins and the sequestered neurotrophic factors (e.g., vascular endothelial growth factor, fibroblast growth factor (FGF)-2, and nerve growth factor) [13]. However, the use of these somatic cell-derived ECMs is limited by the availability of cell source.

PSCs are unlimited sources of cell-derived ECMs which can endogenously regulate the self-renewal and lineage commitment of stem cells [14–16]. These endogenous ECMs not only provide the cell adhesion sites, but also serve as the reservoirs for various exogenous and paracrine/autocrine factors secreted at different developmental stages, thus possessing the unique signaling capacity [17,18]. For instance, undifferentiated ESCs secrete various autocrine factors (i.e., Lefty, FGF-2) and ECMs (i.e., fibronectin) to regulate their self-renewal [19–21], while the ECMs derived from differentiated embryoid bodies (EBs) induce lineage-specific commitment [15,16]. Compared to adult tissue-derived ECMs, PSC-derived ECMs bear a broad spectrum of signaling molecules to regulate cellular differentiation and may partially recapitulate the microenvironment during tissue morphogenesis [22]. Recently, PSC-derived ECMs have been investigated as the scaffolds for cellular expansion and differentiation [23–25].

One critical issue for PSC-derived ECMs as scaffolds or carriers is their fast degradation (both in vivo and in vitro), which impacts their applications in regenerative medicine [26,27]. To improve the ECM stability, modifications by crosslinking using genipin, glutaraldehyde or other methods have been explored for adult tissue-derived ECMs [26,28]. Such modification would also increase the stiffness and change the biophysical properties of the ECM scaffolds [27]. The biophysical properties of various substrates or scaffolds were recently shown to differentially regulate lineage commitment of stem cells [29–31]. While PSC-derived ECMs have been assessed as tissue engineering scaffolds in several studies [15,16,32], the impact of crosslinked ECMs generated from PSCs at distinct developmental stages on neural differentiation has not been studied.

The objective of this study is to evaluate the crosslinking effects of ESC-derived ECMs on the scaffold stability and neural differentiation of the reseeded cells. Built on our previous work about ESC-derived ECM decellularization [16], the present study investigated the synergistic effects of the physical properties and the biochemical/biological properties of ESC-derived ECMs on neural differentiation through genipin-crosslinking [33,34]. Genipin is a natural crosslinking reagent and is reported to have lower cytotoxicity than commonly used glutaraldehyde [35]. This study reveals the intricate interactions of ECM scaffolds with the reseeded cells during neural lineage commitment and is important for ECM scaffold design and functional characterizations.

2. Materials and methods

2.1. Undifferentiated ESC cultures

Murine ES-D3 line (American Type Culture Collection, Manassas, VA) was maintained on 6-well culture plates coated with 0.1% gelatin (Millipore, Temecula, CA) in a standard 5% CO2 incubator. The expansion medium was composed of Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% ESC-screened fetal bovine serum (FBS, Hyclone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL) (all from Invitrogen), and 1000 U/mL leukemia inhibitory factor (LIF, Millipore). The cells were seeded at 2–4 × 10^5 cells/cm² and sub-cultured every 2–3 days. This culture was used to generate undifferentiated aggregates, spontaneous EBs, and neural progenitor aggregates.

2.2. Generation of Undifferentiated aggregates, EBs, and NPC aggregates

Undifferentiated aggregates were obtained by seeding 1 × 10^6 cells from ESC monolayer into Ultra-Low Attachment (ULA) 6-well plates (Corning Incorporated, Corning, NY) in 3 mL growth media containing LIF [36]. The aggregates were cultivated for 3–4 days and used to derive ECM scaffolds. For spontaneous EB formation, 1 × 10^6 ESCs were seeded in ULA 6-well plates in 3 mL of differentiation medium. The differentiation medium consisted of DMEM supplemented with 10% FBS and 0.1 mM β-mercaptoethanol. The EBs were cultivated for 3–4 days prior to decellularization. NPC aggregates were derived following the previously described procedure [37,38]. Briefly, ESCs were seeded at 1 × 10^6 cells into ULA 6-well plates in 3 mL of DMEM-F12 plus 2% B27 serum-free supplement (Invitrogen). At day 4, all-trans retinoic acid (RA, Sigma–Aldrich, St. Louis, MI) was supplemented at 1 μM to enrich neural lineage. The cells were cultivated for additional 4 days and the NPC aggregates were collected to derive ECM scaffolds. The day 8 NPC aggregates were also used for seeding various ECM scaffolds. The aggregates were also formed in ULA 96-well plates (3 × 10^4 ESCs per well) to achieve uniform aggregate size for some study.

2.3. Decellularization to generate ECM scaffolds

The decellularization of ESC-derived aggregates was performed as previously described [16]. Briefly, about 600–1000 undifferentiated aggregates, EBs, or NPC aggregates were distributed into each of 1.5 mL microcentrifuge tubes and treated with 1% Triton X-100 (Sigma) for 30 min. After the treatment, the samples were spun down at 18,000g for 2 min, rinsed with phosphate buffered saline (PBS), and incubated with 2000 unit/mL DNAse I (Sigma) for 30 min. The samples were centrifuged at 18,000g for 2 min again and rinsed with PBS prior to characterization or crosslinking.

2.4. Crosslinking of ECM scaffolds

Crosslinking of the ECM scaffolds was performed using genipin or glutaraldehyde [26]. Decellularized ECMs from undifferentiated aggregates (DE-A), EBs (DE-E), and NPC aggregates (DE-N) were incubated with 0.3% genipin (Wako Chemicals USA, Inc, Richmond, VA) or 0.3% glutaraldehyde (Fisher scientific) for 6 h. After the incubation, genipin-crosslinked ECM scaffolds displayed blue color, which indicated the effective crosslinking as reported in literature [26,35]. The ECM scaffolds crosslinked with genipin (G) were referred as DE-AG, DE-EG, and DE-NG. Similarly, the ECM scaffolds crosslinked with glutaraldehyde (GL) were referred as DE-AGL, DE-
face roughness was measured by a Bruker Icon AFM (Digital Instru-
turer's instructions. The absorbance at 595 nm was read on a

All tests were conducted at 30°C.

The Young's modulus of ECM scaffolds was measured using a

2.6. Scanning electron microscopy (SEM) and atomic force microscopy
(AFM)

For SEM observation, the ECM scaffolds were washed with PBS,

2.7. Measurement of ECM scaffold modulus

The Young's modulus of ECM scaffolds was measured using a
dynamic mechanical analyzer (DMA Q800, TA Instruments) which

Table 1
A list of decellularized crosslinked and non-crosslinked ECMs derived from ESCs.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>ESC growth condition</th>
<th>Crosslinking</th>
<th>Growth factor treatment</th>
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<tbody>
<tr>
<td>DE-AC</td>
<td>Undifferentiated aggregates</td>
<td>None</td>
<td>LIF</td>
</tr>
<tr>
<td>DE-AG</td>
<td>Undifferentiated aggregates</td>
<td>Genipin</td>
<td>LIF</td>
</tr>
<tr>
<td>DE-AGL</td>
<td>Undifferentiated aggregates</td>
<td>Glutaraldehyde</td>
<td>LIF</td>
</tr>
<tr>
<td>DE-EC</td>
<td>Spontaneously differentiated EBs</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>DE-EG</td>
<td>Spontaneously differentiated EBs</td>
<td>Genipin</td>
<td>None</td>
</tr>
<tr>
<td>DE-EGL</td>
<td>Spontaneously differentiated EBs</td>
<td>Glutaraldehyde</td>
<td>None</td>
</tr>
<tr>
<td>DE-NC</td>
<td>NPC aggregates</td>
<td>None</td>
<td>RA</td>
</tr>
<tr>
<td>DE-NG</td>
<td>NPC aggregates</td>
<td>Genipin</td>
<td>RA</td>
</tr>
<tr>
<td>DE-NGL</td>
<td>NPC aggregates</td>
<td>Glutaraldehyde</td>
<td>RA</td>
</tr>
</tbody>
</table>

ECM: extracellular matrix; ESC: embryonic stem cell; LIF: leukemia inhibitory factor; RA: retinoic acid; NPC: neural progenitor cells.

EGL, and DE-NGL (Table 1). All ECM scaffolds were rinsed six times
in PBS prior to characterizations or reseeding with ESC-derived NPCs. The original non-crosslinked ECM scaffolds, denoted as DE-
AC, DE-EC, and DE-NC, were used as the corresponding controls (C).

2.5. Stability assay of ECM scaffolds

The crosslinked or non-crosslinked ECM scaffolds were incu-
bated with 100 μg/mL collagenase solution (Life Technologies)
for 2, 4, or 6 h. The morphology change of ECM scaffolds over time
was imaged under a light microscope. The remaining ECM scaffolds
after collagenase treatment were centrifuged at 800g for 5 min and
solubilized by sonication. The protein contents were then assessed
by the Bradford protein assay (Bio-Rad) according to the manufac-
turer's instructions. The absorbance at 595 nm was read on a
microplate reader (Bio-Rad). The protein contents were normalized
to the amount of proteins in the ECMs before collagenase treat-
ment and this ratio was used as an indicator for the stability of
ECM scaffolds.

2.6. Scanning electron microscopy (SEM) and atomic force microscopy
(AFM)

For SEM observation, the ECM scaffolds were washed with PBS,
fixed in 2.5% glutaraldehyde for 30–60 min and dehydrated in
graded ethanol solutions. The samples were dried by hexamethyl-
disilazane (Sigma) evaporation, mounted, and sputter-coated with
graded ethanol solutions. The samples were dried by hexamethyl-

ter nal to the amount of scaffolds in contact with the
aggregates against the total number of scaffolds. The size distribu-
tions of NPC aggregates and ECM scaffolds as well as the viability
were analyzed by the ImageJ software. Due to the red auto fluores-
cence of the crosslinked ECMs [39], ethidium homodimer-1 staining
was not used to determine cell viability (Supplementary Fig. 1).

The viability was calculated by dividing the green intensity (live
cells stained with calcine AM) by the intensity of total cells (stained
with 4,6-diamidino-2-phenylindole (DAPI)). To determine the effect of ECM scaffolds on cell death, Live-Green Poly Caspase Detection kit (Molecular Probes) was used to detect caspase expression according to the manufacturer's instructions.

To evaluate cell proliferation, cell numbers were determined using
a hemocytometer after trypsin/EDTA dissociation. Fold expansion
was calculated as the harvested cell number divided by the seeded
cell number. Cell proliferation was further evaluated using bro-
modeoxuridine (BrdU) assay. The samples were also incubated with
5 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide (MTT, Sigma) solution. Afterward, the formazan crystals
were hydrolyzed with dimethyl sulfoxide (Sigma) and measured at
500nm using a microplate reader.

2.9. BrdU assay

Briefly, the cells were incubated in medium containing 10 μM
BrdU (Sigma) for 90 min. The cells were then fixed with 70% cold
ethanol, followed by a denaturation step using 2N HCl/0.5% Triton
X-100 for 30 min in the dark. The samples were reduced with
1 mg/mL sodium borohydride for 5 min and incubated with mouse
anti-BrdU (1:100, Life Technologies) in blocking buffer (0.5% Tween
20/1% bovine serum albumin in PBS), followed by Alexa Fluor® 488
goat anti-Mouse IgG (Molecular Probes). The cells were counter-
stained with Hoechst 33342 and analyzed by a fluorescent micro-
scope and the ImageJ software.

2.10. Immunocytochemistry

After 3 days in suspension, the NPC-ECM constructs were
replated in 24-well plates coated with Geltrex™ (Life Technologies)
for another 3 days. Neural differentiation was assessed by
immunocytochemistry for neural markers. Briefly, the cells were
fixed with 4% paraformaldehyde (PFA) and permeabilized
with 0.2–0.5% Triton X-100. The samples were then blocked and incu-
bated with mouse or rabbit primary antibody against: Nestin
(Sigma) or Musashi-1 (Abcam) for neural progenitors, β-tubulin
III (Millipore) for neurons, or GFAP (Millipore) for astrocytes. After
washing, the cells were incubated with the corresponding sec-
dary antibody: Alexa Fluor® 488 goat anti-Mouse IgG, for GFAP
and β-tubulin III, or Alexa Fluor® 488 goat anti-Rabbit IgG for
Nestin and Musashi-1 (Molecular Probes). The samples were then
stained with Hoechst 33342 and visualized under a confocal micro-
scope (Zeiss LSM 880).
2.11. Flow cytometry

To quantify neural marker expression, the cells on ECM scaffolds were harvested by trypsinization and analyzed by flow cytometry. Briefly, 1 × 10^6 cells per sample were fixed with 4% PFA and washed with staining buffer (2% FBS in PBS). The cells were permeabilized with 100% cold methanol, blocked, and then incubated with primary antibodies against Nestin, β-tubulin III,
GFAP, or Musashi-1 followed by the corresponding secondary antibody: Alexa Fluor® 488 goat anti-Mouse IgG (for GFAP and β-tubulin III) or Alexa Fluor® 488 goat anti-Rabbit IgG (for Nestin and Musashi-1). The cells were acquired with BD FACSCan™ II flow cytometer (Becton Dickinson) and analyzed against isotype controls using FlowJo software.

2.12. Statistical analysis

Each experiment was carried out at least three times. The average values of independent experiments were presented and the results are expressed as [mean ± mean absolute deviation (MD)] unless otherwise noted. In each experiment, triplicate samples were used. To assess the statistical significance, ANOVA followed by Fisher’s LSD post hoc tests were performed. A $p$-value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of decellularized ECM scaffolds

DE-N scaffolds (i.e., ECMs derived from NPC aggregates) were used to characterize the effects of crosslinking with genipin (referred as G) on the physical properties of the decellularized ECMs. The ultrastructure analysis by SEM revealed changes in the morphology of ECM scaffolds upon crosslinking treatment. Non-crosslinked scaffolds (control, referred as C) displayed more porous structure while genipin-crosslinked scaffolds showed denser topography (Fig. 1A). The roughness was lower for the non-crosslinked scaffolds compared to the crosslinked scaffolds (167 ± 97 nm vs. 284 ± 30 nm or 331 ± 67 nm) as analyzed by AFM, but the difference was statistically insignificant ($p$-value = 0.15) (Fig. 1B). Crosslinking increased the Young’s modulus of ECM scaffolds from 4.9 ± 1.9 kPa for the DE-NC group (non-crosslinked) to 13.9 ± 0.7 kPa for the DE-NG group (after crosslinking) according to the stress and stain curve (Fig. 1C). In addition, stable scaffold size was observed for the DE-NG and DE-NGL (crosslinking with glutaraldehyde, referred as GL) scaffolds while the size of the DE-NC scaffolds decreased with collagenase treatment time (Fig. 2A and Supplementary Fig. 2). Consistently, the remaining protein content after collagenase treatment was 97% for the DE-NG group and 91% for the DE-NGL group, while the protein content decreased to 56% for the DE-NC group (Fig. 2B). Together, crosslinking treatment increased the stiffness and structural stability of decellularized ECMs.

3.2. Effects of the decellularized ECMs on NPC seeding and proliferation

The different types of decellularized ECM scaffolds were seeded with intact ESC-derived NPC aggregates and the interactions of NPC aggregates with ECM scaffolds were assessed (Fig. 3). NPC aggregates contacting ECM scaffolds were able to bridge with the scaffolds and some cells migrated and grew on the ECMs, especially for genipin-crosslinked scaffolds (Fig. 3A). The crosslinking treatment by genipin was found to increase the percentage of scaffolds that were occupied with NPC aggregates compared to the non-crosslinked scaffolds (40–60% vs. 5–30%) (Fig. 3B). Majority of the ECM scaffolds associated with the cells showed similar size range to the NPC aggregates (i.e., about 400 µm) (Fig. 3C).

Cell proliferation was evaluated by the fold increase after a 3-day culture on the scaffolds seeded with dissociated NPCs or intact NPC aggregates. Dissociated NPCs showed significantly lower
expansion fold than intact NPC aggregates (0.5–1.0 vs. 1.2–2.5) for all the groups (Fig. 4A). No significant difference was observed between the crosslinked and the non-crosslinked scaffolds in DE-A (i.e., ECMs derived from aggregates at undifferentiated state) or DE-E (i.e., ECMs derived from EBs) groups. The NPC aggregates reseeded on the DE-NC scaffolds had significantly lower expansion fold compared to other scaffolds (1.0-fold vs. 2.0–2.2-fold) except DE-NGL. The DE-NG scaffolds supported higher cell expansion fold than the DE-NC and DE-NGL groups, and was comparable to the DE-A and DE-E scaffolds (about 2.0-fold). Consistently, BrdU assay showed low percentage of positive cells for the DE-NC group (Table 2 and Supplementary Fig. 3). MTT assays for intact NPC aggregates grown on the DE-A and DE-E scaffolds showed no significant difference for the crosslinked or non-crosslinked scaffolds, similar to the observation for expansion fold (Fig. 4B). For DE-N scaffolds, cells in the DE-NG group had higher MTT activity compared to the DE-NC group at day 2 and 3, consistent with expansion fold data (Fig. 4C). Cells in the DE-NGL group also showed higher cell viability compared to other scaffolds (Fig. 4D). Thus, the DE-NC scaffolds enhanced neuronal differentiation compared to the DE-A and DE-E groups. Moreover, the crosslinking of DE-N scaffolds with genipin (i.e., DE-NG group) supported higher Nestin and lower β-tubulin III expression than the DE-NC scaffolds. GFAP expression was enhanced for the DE-NC group compared to the DE-NG group (30.3 ± 8.3% vs. 12 ± 0.1%), and for the DE-AC group compared to the DE-AG group (28.0 ± 1.0% vs. 12.3 ± 2.3%) (Fig. 6D). The ratio of β-tubulin III+ cells to GFAP+ cells was calculated and shown in 3.3. Effects of decellularized ECMs on neural differentiation

The expression of neural progenitor markers, Nestin and Musashi-1, was assessed for NPCs grown on all the scaffolds (Fig. 5). Nestin expression was comparable for the cells grown on the crosslinked or the non-crosslinked scaffolds in DE-A and DE-E groups. However, for DE-N scaffolds, the DE-NC group showed lower Nestin expression (61.0 ± 5.8% vs. 76.3 ± 3.3%) than the DE-NGL group while the difference compared to the DE-NG group was not significant (Fig. 5A–C). Musashi-1 expression was comparable for all DE-N scaffolds, while for DE-A groups, the DE-AGL group had lower Musashi-1 expression than the DE-AC and DE-AG groups (65.1 ± 5.8% vs. 77–80%) (Fig. 5D). Conversely, β-tubulin III expression was comparable for the cells grown on the scaffolds in DE-A and DE-E groups, while the DE-NC group showed higher β-tubulin III expression (30.2 ± 1.0% vs. 23 ± 1.6%) than the DE-NG group (Fig. 6). Thus, the DE-NC scaffolds enhanced neuronal differentiation compared to the DE-A and DE-E groups.
Table 2

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>BrdU+ cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE-AC</td>
<td>38.7 ± 13.8</td>
</tr>
<tr>
<td>DE-AGa</td>
<td>40.1 ± 2.8</td>
</tr>
<tr>
<td>DE-AGL</td>
<td>33.8 ± 9.7</td>
</tr>
<tr>
<td>DE-ECa</td>
<td>30.1 ± 3.7</td>
</tr>
<tr>
<td>DE-EC</td>
<td>30.2 ± 7.0</td>
</tr>
<tr>
<td>DE-EG</td>
<td>31.6 ± 7.0</td>
</tr>
<tr>
<td>DE-NC</td>
<td>22.2 ± 4.1</td>
</tr>
<tr>
<td>DE-Nc</td>
<td>43.1 ± 9.7</td>
</tr>
<tr>
<td>DE-NGLb</td>
<td>23.3 ± 6.5</td>
</tr>
</tbody>
</table>

a Indicates statistical difference (p-value < 0.05) compared to the DE-NC group.
b Indicates statistical difference (p-value < 0.05) compared to the DE-NG group.

3.4. Effects of retinoic acid-retinoic acid receptor (RA-RAR) signaling

The DE-N scaffolds, derived from NPC aggregates through RA treatment, were more responsive to genipin-crosslinking treatment during neural differentiation. Therefore, the effects of RA-RAR signaling were investigated through ±BMS 493 treatment for the DE-NC and DE-NG scaffolds (Fig. 7). DE-AC and DE-EC scaffolds were included as controls. BMS 493 is a pan RAR antagonist that blocks RA-RAR interactions. It was observed that NPC treatment with BMS 493 reduced cell proliferation on the DE-EC and DE-NG scaffolds (Fig. 7A). Nestin expression was increased upon BMS 493 treatment for the DE-EC and DE-NC groups (Fig. 7B). Consistently, β-tubulin III and GFAP expressions were decreased in cells grown on the DE-NC scaffolds (Fig. 7C and D). BMS 493 treatment also increased GFAP expression for the DE-EC condition (Fig. 7D). Together, these results indicated that the inhibition of RA-RAR signaling differentially modulated neural differentiation of NPCs on various types of ECM scaffolds.

4. Discussions

Dynamic interactions of acellular ECM scaffolds and stem cells are critical for lineage-specific commitment and tissue regeneration [40,41]. Understanding the synergistic effects of biochemical, biological, and biophysical properties of acellular ECM scaffolds would facilitate scaffold design and the functional regulation of stem cells [42–45]. The present study assessed the influence of crosslinked PSC-derived ECMs on neural differentiation and revealed the synergistic interactions of various ECM scaffold properties.

4.1. Crosslinking of decellularized ECM scaffolds modulates neural differentiation

Crosslinking improves the stability of ECM scaffolds and increases the elastic modulus, which affects the interactions of
the cells with ECM scaffolds. The decellularized ECMs (after high speed centrifugation and condensation during decellularization) from ESC aggregates showed Young’s modulus of 4.9 kPa, which is within the range (500 Pa–1.5 MPa) of various types of decellularized tissues and cells [27,46,47]. After crosslinking, the elastic modulus of ECM scaffolds was increased to 13.9 kPa. This crosslinking effect may regulate NPC seeding according to the differential interfacial tension hypothesis [48,49]. Based on this hypothesis, the cells tend to organize themselves according to cell-cortex tension, which results in the cells with low cell-cortex tension (softer) attach to the harder surface [48]. Because ECM crosslinking increases the scaffold’s stiffness [50], the cells from NPC aggregates (softer) have higher tendency to associate with the crosslinked ECMs (harder) compared to the non-crosslinked ECMs (softer) (Fig. 3A-b, A-d and A-f), leading to the enhanced reseeding of NPCs on the genipin-crosslinked ECM scaffolds.

The crosslinked ECM scaffolds can affect neural differentiation of ESC-derived NPCs and alter the populations of neuronal and glial cells due to the increased elastic modulus, especially for the DE-N groups in this study [51,52]. Soft substrates (with elastic modulus of 1–7 kPa) have been reported to enhance neural differentiation of NPCs and PSCs compared to the substrates with higher elastic modulus (10 kPa–1 GPa) [29,53,54]. NPCs encapsulated in alginate hydrogels also showed gradual decrease of neuronal and glial differentiation when increasing the hydrogel modulus [55]. Consistently, in the present study the DE-NG group (higher modulus) had the lower percentages of neuronal cells (by 6%) and glial cells (by 15%) and the higher percentage of Nestin + cells (by 15%) compared to the DE-NC group (lower modulus).

Crosslinking of decellularized ESC-ECM scaffolds enriches the proportion of neurons over astrocytes (Table 3), which could be attributed to the culture conditions that confound the effects of substrate stiffness on NPC fate decision. In the neuronal differentiation medium, increasing modulus from 1 kPa to 1 GPa was reported to have little effect on the percentage of neuronal cells [56], while glial cell purity was reduced during neural differentiation of adult neural stem cells [29]. In the culture medium that supported both neuronal and glial cells, stiffer surfaces were observed to reduce the percentage of neuronal cells while promoting glial cell population through the regulation of Rho GTPases [29,53,57]. In the present study, the retinol in B27 medium served as a source of RA and the differentiation medium promoted neuronal cell growth [58]. So, the increased stiffness via crosslinking had less effect on b-tubulin III + cells, but reduced the percentage of GFAP + cells, resulting in the enriched proportion of neurons over astrocytes.

The effects of crosslinking by glutaraldehyde are less pronounced, but in similar trend to the crosslinking by genipin in general. Moreover, genipin is a natural crosslinking reagent which can result in low cytotoxicity [27]. So crosslinking with genipin is a preferred method.

4.2. The aggregate properties prior to ECM decellularization modulates neural differentiation

ECM crosslinking did not affect NPC proliferation and neuronal differentiation for the cells grown on the DE-A and DE-E scaffolds but affected the cells grown on the DE-N scaffolds, suggesting

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**Fig. 6.** Neural differentiation of the reseeded ESC-NPCs grown on different ECM scaffolds. (A) Representative confocal fluorescent images of b-tubulin III and GFAP expression; Green: b-tubulin III or GFAP; Blue: Hoechst 33342. Scale bar: 100 lm. (B) Representative flow cytometry histograms of b-tubulin III and GFAP expression. (C) Percentage of the cells positive for b-tubulin III. (D) Percentage of the cells positive for GFAP. *p-value < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the synergistic effects of biophysical properties and biochemical/biological properties of ECMs. The differential cellular response can be attributed to both the heterogeneous populations of ESC-derived NPC aggregates and the properties of acellular ECM scaffolds.

ESC-derived NPC aggregates are heterogeneous and contain a mixture of primitive and definitive NPC populations [59,60]. Primitive ESC-NPCs (mimicking neural precursors at E5.5–E7.5 of embryonic development) are LIF-dependent [61] and prone to endogenous RA-mediated apoptosis [62]. In contrast, definitive NPCs generated from ESCs display the features of adult NPCs and are prone to neuronal and glial lineage commitment [63]. The response of heterogeneous NPC populations can be affected by the properties of ECM scaffolds.

The present study indicates that specific intrinsic cues exist for the three types of ECM scaffolds, i.e. DE-A, DE-E, and DE-N. The DE-NC scaffolds supported higher percentages of β-tubulin III* cells and GFAP* cells and were influenced by genipin-crosslinking to a greater extent compared to the DE-AC and the DE-EC scaffolds. To elucidate the specific cues in different scaffolds, the treatment with BMS 493 to inhibit retinoid signaling was performed in this study. Our previous study indicated the possible role of retinoid signaling in the cells grown on the ECMs derived from RA-treated EBs (similar to the DE-N scaffolds) [16]. The RA-RAR regulation in retinoid signaling has recently been found as a critical pathway during neural tissue development [64,65]. For example, RA was reported to regulate NPC proliferation and GFAP expression of definitive NPCs through the activation of RARα and RARγ [66]. By blocking RA-RAR interactions with BMS 493 (Fig. 7), differential cellular responses to different ECM scaffolds revealed possible interactions of ECMs with exogenous or autocrine/paracrine factors.

It is postulated that the DE-AC scaffolds bound LIF during the culture of undifferentiated aggregates prior to decellularization [67]. When blocking the RA-RAR signaling, no changes in neural marker expression was observed for the DE-AC group, while the possible LIF bound to the DE-AC scaffolds would result in higher proliferation of NPCs [64]. DE-NC scaffolds (derived from NPC aggregates) may bind endogenous neurotrophic factors (i.e., NGF, BDNF, GDNF, FGF-2 etc.) that can enhance neural differentiation compared to the DE-EC and DE-AC scaffolds [63,68]. In addition, due to RA treatment before decellularization, cells grown on the DE-NC scaffolds contain higher retinoid signaling which can induce NPC differentiation toward neuronal and glial lineages [16,69]. Therefore, by blocking RA-RAR interactions, neuronal and glial differentiations were reduced for the DE-NC group. DE-EC scaffolds, derived from spontaneous EBs, displayed less signaling specificity compared to the DE-AC and DE-NC scaffolds, but may still have endogenous retinoid signaling [70]. Conversely, the RA-RAR inhibition could favor the survival of primitive NPCs as indicated by the enhanced Nestin expression. Finally, NPCs reseeded on the DE-NG scaffolds showed little change in Nestin and GFAP expression but

### Table 3
The ratio of β-tubulin III* cells to GFAP* cells in various ECM scaffolds.

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>Ratio of β-tubulin III*/GFAP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE-AC</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>DE-AG^a</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>DE-AGL^2</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>DE-EC</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>DE-EG</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>DE-EGL</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>DE-NC</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>DE-NG^a</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>DE-NGL</td>
<td>1.7 ± 0.8</td>
</tr>
</tbody>
</table>

^a Indicates statistical difference (p-value < 0.05) compared to the corresponding control (C) scaffolds.

Fig. 7. Effects of BMS 493 treatment on the cells grown on different ECM scaffolds. (A) Fold change in the proliferation of the reseeded ESC-NPCs after BMS 493 treatment (+BMS) compared to untreated cultures (−BMS). Fold change in the expression of (B) Nestin, (C) β-tubulin III, and (D) GFAP. The fold change was calculated as the ratio of the expression after BMS 493 treatment (+BMS) compared to that of untreated cultures (−BMS). * p-value < 0.05.
lower proliferation rate and β-tubulin III expression with BMS 493 treatment, which was slightly different from the response for the cells grown on the DE-NC group. This observation indicates that the change in biophysical properties of ECMs due to crosslinking may affect their interactions with biological molecules, displaying the synergistic effects of various ECM properties.

PSC-derived ECMs are undefined materials containing numerous proteins and their characterization provides important information to elucidate the signaling molecules. Our previous study determined the key ECM proteins including fibronectin, laminin, Collagen IV and vitronectin [16]. For more detailed analysis, proteomics is a powerful tool to delineate the shared and distinct components in ECMs from different stages of stem cell development [71]. Our subsequent study uses proteomic analysis to characterize the three types of ECM scaffolds, i.e. DE-A, DE-E, and DE-N. DE-N group has higher spectra count of several laminin peptides and heparan sulfate core proteins [72]. It also has neutrophic proteins such as neuron-derived neurotrophic factor. These differences may partially explain the differences in cellular response to different types of ECMs. More in-depth analysis of cell-synthesized ECM microenvironment should provide further understanding on the natural signaling networks in PSC-derived ECMs and the associated growth factors to modulate stem cell fate decisions.

5. Conclusion

This study indicates that decellularized ECM scaffolds derived from different types of ESC aggregates display specific cues regulating the behavior of reseeded ESC-derived NPCs. Crosslinking modulates the structural and biophysical properties of ECM scaffolds, which enrich the neuronal cell population over the glial cell population. Decellularized ECM scaffolds derived from PSC aggregates at different developmental stages can regulate neural differentiation of the reseeded cells through the tunable biophysical and biological properties. The bioactive scaffolds derived from PSC-derived ECMs are suitable for the applications of in vitro cell culture. Since ECMs derived from murine ESCs cannot be used directly as the clinically relevant biomaterials in human, the following up step is to evaluate human induced pluripotent stem cell (hiPSC)-derived ECMs. The present study should help the further investigations on the ECM microenvironment generated from hiPSCs for in vivo applications.

6. Disclosures

No competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.11.016.

References

V. Tropepe, S. Hitoshi, C. Sirard, T.W. Mak, J. Rossant, D. van der Kooy, Direct...

A. Banerjee, M. Arha, S. Choudhary, R.S. Ashton, S.R. Bhatia, D.V. Schaffer, et al.,...