



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 PSC 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

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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 $2\text{--}4 \times 10^4$ 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-

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