Extracellular Matrix Expression in Human Induced Pluripotent Stem Cell-Derived Optic Vesicles

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ABSTRACT

Background: Human tissue/organ development is a complex, highly orchestrated process, regulated in part by the surrounding extracellular matrix (ECM). Every complex tissue, including the retina, has a unique ECM configuration that plays a critical role in cellular differentiation, adhesion, migration, and maturation.

Aim: To characterize ECM expression of human induced pluripotent stem cell-derived optic vesicles (iPSC-OVs).

Methods: A 3-dimensional (3D) in vitro suspension culture system was used to direct differentiation of human induced pluripotent stem cells (iPSCs) into optic vesicles (OVs). Stepwise differentiation of iPSCs into retinal progenitor cells was confirmed by sequential expression of OTX2, SOX1, SIX6, LHX2, PAX6, and CHX10. Expression of ECM genes in iPSC-derived OVs was analyzed by RT2 Profiler™ PCR Array, whereas immunofluorescence staining was performed to detect ECM proteins in the OVs.

Results: A number of cell adhesion molecules (CAMs) previously reported to be abundantly expressed in iPSCs such as E-cadherin, Intercellular adhesion molecule-1 (ICAM1), Integrin-α L, Integrin-α M, Integrin-α 6 were downregulated while neural and retina specific CAMs including neural cell adhesion molecule 1 (NCAM1), neural plakophilin-related armadillo repeat protein (NPRAP), Integrin-α 1 and Integrin-α 4 were upregulated. Several glycoproteins that have been reported to play key roles during retinogenesis, namely CD44, Tenasin C, Tenasin R, Neurocan, Neureglican C, Delta 2 Catenin, Vitronectin, and Reelin were also present.

Conclusion: We have identified an array of ECM proteins that were expressed during retinogenesis. Further characterization of these proteins will lead to a better understanding of retinal development.

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Introduction

Induced pluripotent stem cells (iPSCs) are derived from adult somatic cells that have been reprogrammed to become pluripotent. iPSCs share similar properties as embryonic stem cells (ESCs), including the plasticity to differentiate into any somatic cell [1, 2]. Several methods have been used to differentiate pluripotent stem cells into various cell types. One of the most common methods generates uniform and size-controlled 3-dimensional (3D) stem cell aggregates called embryoid bodies (EBs) that are further differentiated into organoids of interest [3, 4]. Studies have shown that 3D EBs allow directed iPSC differentiation into desired cell types in a more efficient and controlled manner by recapitulating the key events that occur during early embryogenesis [5, 6]. EBs help to create a microenvironment that is inducive to cell-cell interactions and endogenous autocrine/paracrine signaling essential for self-renewal and differentiation.
Embryogenesis is a highly orchestrated process consisted of stepwise differentiation of pluripotent stem cells. During this process, embryos also start to secrete laminin and other extracellular matrix (ECM) molecules as early as the two-cell stage by a process called exocytosis [7]. As these embryonic cells further divide, each population of cells differentiates into tissue specific somatic cells. An EB-based differentiation approach has often been applied to induce in vitro retinogenesis of iPSCs, in which the sequential development of neuroectoderm specification, along with eye field and optic vesicle (OV) formation have been observed [8, 9]. ECM is a complex, dynamic network of proteins and carbohydrate molecules within the interstitial space and basement membrane of tissue [10, 11]. It is composed of different macromolecules including glycosylated proteins (proteoglycans), integrins, fibrous proteins and polysaccharides [10, 12, 13]. These molecules constitute the cellular microenvironment that provides structural support and exert influences on cell behaviors in various ways [14-17]. By binding to transmembrane proteins, ECM can regulate the underlying intracellular signaling pathways [18]. ECM can also regulate cell migration and differentiation by virtue of its stiffness and has been shown to influence tissue morphogenesis [19, 20]. In addition, ECM can sequester growth factors thereby influencing cell growth by regulating growth factor bioavailability.

ECM also plays a crucial role in degeneration/regeneration of tissue including retina in various diseases and injuries [21]. Several ECM molecules are upregulated in response to retinal damage. For example, Neuroglycan-C, a brain-specific chondroitin sulfate proteoglycan, has been shown to inhibit optic nerve regeneration after nerve crush injury in rats [22]. Other glycoproteins like Neurocan and CD44 are expressed at the outer interface of abutted retinas and inhibit neurite outgrowth [23]. Tenascin-C is also abundantly expressed after nerve injury which is important for axonal regeneration [24]. ECM remodeling also occurs in retinal diseases like glaucoma and diabetic retinopathy [25, 26].

In this study, we used a 3D in vitro suspension culture system to direct differentiation of human iPSCs into OVs and examined their ECM composition during early events of retinogenesis. Based on the results described here, we report that iPSCs undergoing retinal differentiation in 3D produce retina-specific ECMs. Further characterization of selected iPSC-derived ECM proteins can lead to a better understanding of retinal development and shed light on the potential application of stem cell-derived retinal organoids as a source of autologous ECM materials for retinal repair and regeneration.

Materials and Methods

I Culture and Maintenance of iPSCs

Human iPSC line IMR90-1 was obtained from WiCell (Madison, WI). iPSCs were seeded on Matrigel-coated (Corning, NY) 6 well-plates and maintained in mTeSR1 medium (Stem Cell Technologies, Vancouver, BC, Canada) at 37°C with 5% CO2. Differentiated colonies were identified and removed via visual inspection using a dissecting microscope. Cells were manually passaged using Stempro EZPassage (ThermoFisher Scientific, Waltham, MA) when they reached 80% confluency, where approximately 40 to 50 colonies were seeded onto each well of the Matrigel-coated plates. iPSCs were tested for pluripotency via immunostaining for various pluripotency markers as previously described [27].

II 3D In Vitro Retinal Differentiation

A retinal differentiation protocol was adapted from previous studies with modifications [9, 28]. Briefly, iPSCs were treated with 10 µM Rock inhibitor, Y-27632 (Stem Cell Technologies, Vancouver, Canada) for two hours, rinsed with Phosphate Buffered Saline (PBS), and dissociated into single cells using 0.1% Trypsin for 10 minutes. The cells were washed with PBS and resuspended at a density of 90,000 cells/mL in retinal differentiation medium containing G-MEM (ThermoFisher Scientific, Waltham, MA) with 20% KnockOut Serum Replacement (KSR) (ThermoFisher Scientific, Waltham, MA). 0.2 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol, 10 µM Y-27632, 3 µM IWR-1-endo (Wnt inhibitor, Calbiochem, Billerica, MA) and antibiotic/antimycotic (ThermoFisher Scientific). These cells were then assembled into uniform and size-controlled EBs of 9000 cells by aliquoting 100 µL of the cell suspension into each well of the Sumilon PrimeSurface 96-well V-bottomed plates (Sumitomo Bakelite, Novi, MI). After 24 hours, 1 µL of Matrigel at 1% w/v was added to each well. Half the medium in each well was changed to fresh medium without Y-27632 on Day 6. Cells were further cultured until Day 12.

On Day 12, the EBs were collected, washed twice and resuspended in medium containing G-MEM with 20% KSR, 10% Fetal bovine serum (FBS), 1% Matrigel, 0.2 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol, and 1X antibiotic/antimycotic cocktail and transferred to 6-well ultra-low attachment plate (Corning, Corning, NY) at a density of 30-40 EBs/well and cultured in suspension. Three µM CHIR 99021, (Wnt agonist, R&D Systems, Minneapolis, MN) and 100 nM smoothed agonist (SAG, R&D Systems, Minneapolis, MN) were added to the medium on Day 15. The medium was changed to neuroretina medium containing DMEM/F12-Glutamax, N2 supplement (ThermoFisher Scientific, Waltham, MA), and 1X antibiotic/antimycotic cocktail on Day 18. Cell differentiation was continued until Day 24.

III Immunofluorescence Staining of EBs During Stepwise Retinal Differentiation

EBs were harvested on Day 8, 12, and 24 during stepwise differentiation and fixed in 4% paraformaldehyde (PFA). After overnight fixation, the EBs were immersed in 15% and 30% sucrose (in PBS) for 2 hours each and embedded in optimal cutting temperature (OCT) compound. Embedded molds were frozen gradually and then sliced into 20 µm sections.

Cryosections were permeabilized with 0.1% Triton X-100 and incubated with antibodies against OTX2, SOX1, SIX6, LHX2, PAX6, or CHX10 to detect markers of retinal development (Table 1). Fluorophore-conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used for immunofluorescence detection. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Fluorescent images were acquired using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Inc. Buffalo Grove, IL) and processed using the Image J software.
Table 1: Primary antibodies used for immunofluorescence assay.

| Antibody   | Type       | Source                | Dilution |
|------------|------------|-----------------------|----------|
| CD44       | Mouse monoclonal | Novus               | 1:100    |
| CHX10      | Rabbit polyclonal | Novus            | 1:100    |
| LHX2       | Goat polyclonal     | Santa Cruz         | 1:25     |
| Neurocan   | Mouse monoclonal     | ABCAM             | 1:50     |
| Neurocan C | Rabbit polyclonal     | ABCAM             | 1:300    |

IV RT² Profiler™ PCR Array Assay

Total RNA was extracted from iPSCs (day 0) and iPSCs-derived OVs on day 24 using the RNeasy mini kit (Qiagen, Valencia, CA). RNA was purified with RNAse-free DNase (Qiagen). The purified RNA was reverse transcribed to cDNA using the RT First strand kit (Qiagen). cDNA was mixed with the RT SYBR Green ROX qPCR mastermix and aliquoted into the Human Extracellular Matrix and Cell Adhesion Molecules RT² Profiler™ PCR Array (Qiagen). Applied Biosystem 7300 RT PCR system (Applied Biosystems, Foster City, CA) was used for thermal cycling: segment 1 – 1 cycle: 95°C for 10 minutes, segment 2 – 40 cycles: 95°C for 15 seconds followed by 60°C for 1 minute. Data were analyzed using the RT² Profiler™ PCR Array Data Analysis Webportal (Link) according to the manufacturer’s instructions. Fold changes in gene expression were calculated using the ∆∆Ct method. Expression of house-keeping genes was used to normalize the data and calculate ∆∆Ct values.

V Immunofluorescence Staining of ECM

EBs on Day 24 post-differentiation were fixed and processed for immunofluorescence staining following the general protocol as described above. Antibodies against Neuroglycan C, Neurocan, Tenascin C, CD44, Tenascin R, Reelin, NCAM, Delta 2 Catenin, and Vitronectin were used to detect expression of ECM proteins. Fluorophore-conjugated secondary antibodies were used for immunofluorescence detection. DAPI was used to counterstain cell nuclei. Images were acquired using a confocal microscope as previously described. All images were processed using the Image J software.

VI Statistical Analysis

Each experiment was performed in triplicates. Data were analyzed by Student’s t test and presented as the mean ± standard deviation (SD). A value of p < 0.05 was considered statistically significant.

Results

I In Vitro 3D Differentiation of OVs

We applied a well-established retinal differentiation protocol to direct differentiation of iPSCs into OVs with some modifications [9, 28]. IMR90-1 cells were dissociated into single cells and aggregated to form uniform and size-controlled EBs of 9000 cells/EB (Figure 1A). We observed that IMR90-1 cells underwent stepwise differentiation into retinal progenitors. They expressed neuroectodermal marker OTX2 and SOX1 by Day 8 (Figure 1B) and acquired eye field specification markers, SIX6 and LHX2 by Day 12 (Figure 2). These EBs further developed into OVs as marked by co-expression of neuroretina markers PAX6 and CHX10 (Figure 3). Here we have shown that 3D in vitro differentiation of iPSCs into OVs recapitulates in vivo retinogenesis via sequential expression of the neuroectoderm, eye field and neuroretina markers.

II Expression Profile of ECM Genes in iPSC-Derived OVs

Figures 3 showed that OVs derived from iPSCs in 3D culture expressed markers of the neuroretina. These results suggest that iPSC-derived OVs can be used as a model to study the expression of retina-specific ECM. To characterize the ECM, gene expression profiles of iPSCs and iPSC-derived OVs were analyzed with a commercially available RT² Profiler™ PCR Array. iPSCs-derived OVs showed significantly downregulated expression of CAMs including E-cadherin, Intercellular adhesion molecule-1 (ICAM1), Integrin-αL, Integrin-αM, and Integrin-α 6 (Figure 4) and upregulated expression of Neural cell adhesion...
molecule 1 (NCAM1), Neural plakophilin related armadillo protein (NPRAP), Integrin-α 1 and Integrin-α 4 (Figure 4). Furthermore, ECM expression of glycoproteins Tenascin C, CD44, Vitronectin and Fibronectin I was significantly upregulated in iPSCs-derived OVs (Figure 5).

**Figure 1:** Stepwise differentiation of iPSCs towards a retinal lineage. A) 9000 iPSCs were aggregated to form uniform-sized EBs and differentiated towards a retinal lineage. B) Differentiating EBs expressed neuronal marker OTX2 and SOX1 by Day 8 (scale bar=200µm).

**Figure 2:** Differentiating EBs expressed eye field specification markers SIX6 and LHX2 by Day 12 (scale bar=200µm).
Figure 3: Optic vesicle formation from iPSCs. Developing EBs from iPSCs co-expressed neuronal markers PAX6 and retina progenitor cell marker CHX10 (scale bar=25µm).

Figure 4: Expression of CAMs and glycoproteins in iPSC-derived OVs on Day 24 of differentiation. iPSC-specific CAMs including E-cadherin, ICAM1, Integrin-α L, Integrin-α M, Integrin-α 6 were downregulated while neural and retina specific CAMs NCAM1, NPRAP, Integrin-α 1 and Integrin-α 4 were upregulated.

Figure 5: Expression of glycoproteins in iPSC-derived OVs on Day 24 of differentiation. Genes for ECM proteins important for retinogenesis namely Tenascin-C, CD44, Fibronectin I and Vitronectin were upregulated.
III Expression of ECM Proteins in iPSC-Derived OVs

The data from the RT² Profiler™ PCR array showed that iPSCs-derived OVs expressed several ECM genes associated with cells of the retinal lineage. Immunofluorescence analysis was performed to further verify the expression and localization of ECM proteins. CD44, a glycoprotein expressed in retinal Müller cells was significantly higher in iPSC-derived OVs as detected by RT-PCR microarray (Figure 4) and via immunofluorescence staining (Figure 7) [29]. Tenascin R, a known modulator of retinal axon growth, was also detected in iPSC-derived OVs (Figure 8) [30]. Additional neural and retinal ECM proteins were found to be expressed in the iPSC-derived OVs, include Reelin, Vitronectin, Delta 2 Catenin, Neuroglycan C, Neurocan, and Tenascin C (Figures 6-8) [31-33].

Figure 6: Protein expression of Neuroglycan C, Neurocan, and Tenascin C in iPSC-derived OVs. Neuroglycan C, Neurocan, and Tenascin C were abundantly expressed in the developing iPSC-derived OVs (scale bar=25µm). DAPI (middle column), merge view (right column). These ECM proteins are known to be expressed in the developing eye in vivo.

Figure 7: Expression of NCAM, CD44 and Vitronectin in iPSCs-derived OVs. Immunofluorescence analysis showed that OVs expressed NCAM, CD 44 and Vitronectin in the developing OVs (left column). Nuclear staining/DAPI (middle column), merge view (right column), scale bar=50µm.
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Figure 8: Expression of Reelin, Tenascin R and Delta 2 Catenin in iPSC-derived OVs. Retina abundant proteins like Reelin, Tenascin-R and Delta 2 Catenin were expressed in developing iPSC-derived OVs during early stage retinogenesis. Nuclear staining/DAPI (middle column), merge view (right column), scale bar=50µm.

Discussion

Intracellular and extracellular changes occur during tissue/organ development and provide necessary cues for differentiation to ensure cells can acquire a tissue-specific phenotype [17, 19, 27, 34]. Engineering iPSCs into 3D EBs provides a unique environment that mimics the environment of early embryogenesis [29, 30]. Pluripotent stem cells have shown stepwise acquisition of developmental phenotypes during differentiation towards a defined lineage [3]. During human retinogenesis, the appearance of eye field cells (SIX6+ and LHX2+) marks the initiation of the neuroretina development (with CHX10+ and PAX6+ retinal progenitor cells). The formation of OVs followed by subsequent invagination of the vesicles results in morphogenesis of the optic cup [8, 31, 32]. Similar sequences of retinal morphogenesis were reported in 3D differentiation of human ESCs and iPSCs in vitro [9, 28]. Even though cellular and phenotypical changes that occur during specification towards a retinal lineage have been well-documented in literature, little is known about the dynamics of ECM formation during retinal development.

In this study, we have demonstrated that iPSC-derived OVs expressed several CAMs/ECMs that have previously been shown to play key roles in retinogenesis in vivo, specifically NCAM1, NPRAP, Tenascin C, CD44, Fibronectin I, and Vitronectin. The iPSCs-derived OVs also expressed Tenasin R, Neurocan, Neuroglycan C, Reelin, Laminin S, and Delta 2 Catenin. Neuroglycan C is a neural tissue specific transmembrane chondroitin sulfate proteoglycan that regulates the formation of the retinal neural network [35, 36]. Another glycoprotein, Tenascin C works in conjunction with Tenascin R to regulate axonal growth during retinogenesis [24, 37]. Furthermore, Tenascin C has also been shown to interact with Neurocan, another glycoprotein that is expressed in neuronal tissue [38, 39]. CD44, another cell surface glycoprotein that was upregulated in this study, is known to be expressed by Müller cells in the retina in vivo [40, 41]. Finally, Fibronectin, and Vitronectin are integral components of the human retina which were also found to be significantly overexpressed in OVs derived from iPSCs [38-41].

The application of biological ECM scaffolds for tissue repair and regeneration is a rapidly advancing interdisciplinary field [33, 42]. A recent study showed that decellularized bovine retinal ECM can support attachment and growth of human retinal progenitor cells [43]. ECM scaffolds derived from CNS tissue have also been reported to support migration and proliferation of neural stem cells [44, 45]. Furthermore, another study demonstrated that ECM scaffolds derived from skin promoted cell engraftment, proliferation and wound healing without scar formation [46, 47]. Many groups have confirmed that tissue-specific ECM promotes maturation and integration when seeded with corresponding cell types [7, 20, 48-54]. These studies indicate that native ECM is a good candidate as a biomaterial for tissue engineering applications.

ECM can be acquired from different sources including human (allogeneic) or animal (xenogeneic) donor tissues [48]. However, it is important to note that animal-derived ECM products pose risks of immunoreactivity and zoonotic disease transmission, whereas human donor tissues are often in short supply [50, 55-60]. Since patient-derived
materials are generally preferred for tissue replacement therapy, generating ECM from iPSC-derived organoids could provide autologous materials ideal for tissue repair and regeneration. Using stem cells as a potential source for tissue-specific ECM is a novel approach. There are a few reports on stem cell-derived ECM applications in the literature, including a recent study that described the ability of ECM derived from undifferentiated EBs to support ESC proliferation and differentiation [7]. Several studies have shown that while undergoing chondrogenic differentiation, MSC-derived ECM can induce chondrogenic differentiation of seeded stem cells without exogenous growth factors [61, 62]. These studies have demonstrated the feasibility of obtaining chondrogenic ECM for cartilage regeneration from MSC aggregates differentiating towards a chondrogenic phenotype.

In summary, we have successfully used human iPSCs as a tool to recapitulate early stages of retinal development in this study. We have shown that developing OVs expressed several ECM proteins that are known to play critical roles during retinogenesis in vitro. We have also demonstrated that iPSCs in 3D suspension culture can generate native retina-specific ECM. Future work will include examining the potential of iPSC-derived OVs as an autologous source of ECM materials that can support axonal survival, growth, and guidance for retinal regeneration. Further studies will also be needed to validate the regenerative potency of autologous retina-specific ECM in retinal disease/injury.

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Disclaimers

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

Author Contributions

Kaini RR performed data collection, analysis and wrote the manuscript. Rettinger CL performed data analysis and wrote the manuscript. Wang HC and Kaini RR designed the experiments. Wang HC obtained funding, supervised the study, and revised the manuscript. All authors approved the manuscript.

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