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Extracellular matrix components expression in human pluripotent stem cell-derived retinal organoids recapitulates retinogenesis *in vivo* and reveals an important role for IMPG1 and CD44 in the development of photoreceptors and interphotoreceptor matrix

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ABSTRACT

The extracellular matrix (ECM) plays an important role in numerous processes including cellular proliferation, differentiation, migration, maturation, adhesion guidance and axonal growth. To date, there has been no detailed analysis of the ECM distribution during retinal ontogenesis in humans and the functional importance of many ECM components is poorly understood. In this study, the expression of key ECM components in adult mouse and monkey retina, developing and adult human retina and retinal organoids derived from human pluripotent stem cells was studied. Our data indicate that basement membrane ECMs (Fibronectin and Collagen IV) were expressed in Bruch's membrane and the inner limiting membrane of the developing human retina, whilst the hyalectins (Versican and Brevican), cluster of differentiation 44 (CD44), photoreceptor-specific ECMs Interphotoreceptor Matrix Proteoglycan 1 (IMPG1) and Interphotoreceptor Matrix Proteoglycan 2 (IMPG2) were detected in the developing interphotoreceptor matrix (IPM). The expression of IMPG1, Versican and Brevican in the developing IPM was conserved between human developing retina and human pluripotent stem cell-derived retinal organoids. Blocking the action of CD44 and IMPG1 in pluripotent stem cell derived retinal organoids affected the development of photoreceptors, their inner/outer segments and connecting cilia and disrupted IPM formation, with IMPG1 having an earlier and more significant impact. Together, our data suggest an important role for IMPG1 and CD44 in the development of photoreceptors and IPM formation during human retinogenesis.

Statement of Significance

The expression and the role of many extracellular matrix (ECM) components during human retinal development is not fully understood. In this study, expression of key ECM components (Collagen IV, Fibronectin, Brevican, Versican, IMPG1 and IMPG2) was investigated during human retinal ontogenesis.

Abbreviations: BCAN, Brevican; BrM, Bruch's membrane; CD44, cluster of differentiation 44; Col4A1, Collagen IV; ECM, extracellular matrix; FBS, Fetal bovine serum; FN1, Fibronectin; GCL, ganglion cell layer; HDBR, human developmental biology resource; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; Hoe, Hoechst; ILM, inner limiting membrane; IMPG1/2, interphotoreceptor matrix proteoglycan 1/2; INL, inner nuclear layer; INZ, inner neuroblastic zone; IPL, inner plexiform layer; IPM, interphotoreceptor matrix; IRBP, Interphotoreceptor retinoid binding protein; ONL, Outer nuclear layer, ONZ, Outer neuroblastic zone; OPL, outer plexiform layer; OS, outer segment; PBS, phosphate buffered saline; PCW, weeks of post-conception; PFA, paraformaldehyde; RBP3, Retinol Binding Protein 3; RGCs, retinal ganglion cells; RPE, Retinal pigmented epithelium; RT, room temperature; VCAN, Versican.

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Collagen IV and Fibronectin were expressed in Bruch's membrane; whereas Brevican, Versican, IMPG1 & IMPG2 in the developing interphotoreceptor matrix (IPM). Retinal organoids were successfully generated from pluripotent stem cells. The expression of ECM components was examined in the retinal organoids and found to recapitulate human retinal development *in vivo*. Using functional blocking experiments, we were able to highlight an important role for IMPG1 and CD44 in the development of photoreceptors and IPM formation.

1. Introduction

The final impact of many forms of blinding diseases is the loss of photoreceptors, a specialised type of photosensitive neurons that are capable of photo-transduction, and/or the underlying retinal pigment epithelium (RPE) [1]. No treatments currently exist to restore lost photoreceptor cells and the accompanying vision loss which occurs in many retinal diseases. Thus, there is a pressing need to generate a source of functional photoreceptors and RPE, the underlying tissue which is essential for photoreceptor function and viability, for disease modelling and transplantation. The feasibility of this approach is supported by the fact that replacement photoreceptors transplanted into the fovea need only a single synaptic connection with the inner retina [2]. The retina is a prime system within which to test the development of cell transplantation therapies due to the relative accessibility of the eye that permits local delivery of cells with minimum risk of systemic consequences. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) are an attractive solution for cell replacement therapies as they offer the prospect of generating unlimited quantities of desired cell types for transplantation [3]. In the last few years several groups, including ours, have demonstrated the generation of laminated retinal organoids from human pluripotent stem cells containing multiple retinal cells types which interact with each other, form synaptic connections, respond to electrophysiological stimuli [1,4–9] and engraft within the degenerate retinae [10]. Such organoid cultures provide an extremely useful tool for large scale pharmacology and toxicology testing, understanding human retinal development and dissecting the role of individual genes during this process. As such, it is important to ensure that *in vitro* pluripotent stem cell models are similar counterparts to the retinal tissue *in vivo*.

The extracellular matrix (ECM) is the non-cellular component of all tissues and organs. It consists of proteins, water, polysaccharides and glycosaminoglycans (GAGs), and varies in composition from one tissue to the next [11]. The ECM acts as a structural support that facilities cell adhesion via integrins and other adhesion molecules which mediate signalling pathways essential for cell proliferation, migration and differentiation [12–14]. It also provides biochemical and biomechanical cues that are essential for tissue morphogenesis [11].

In the retina, the ECM is divided into two distinct entities, namely: 1) the interphotoreceptor matrix (IPM) which surrounds the inner and outer segments of the photoreceptors and plays a significant role in retinal adhesion [15], retinoid transport [15], photoreceptor differentiation [16] and intercellular communication [17] and 2) the retinal ECM that surround other cells of the retina [18]. Various studies of rat, mouse, chicken and human retina [19–24] have shown that the localisation of ECM molecules varies between developmental stages [22] and species [21]. During retinal development, different ECM molecules including Fibronectin, Collagen IV and Laminins are required for optic cup morphogenesis [20]. For example, in chick embryos, removing the surface ectoderm and disrupting the surrounding ECM with collagenase results in the curvature of the optic vesicle at early stages of invagination and inhibition of subsequent invagination and optic cup formation [25]. Blocking the interaction of rat retinal progenitor cells with laminin β2 *in vitro,* reduces the fraction of cells that express rhodopsin, suggesting that this laminin could be important for rod photoreceptor differentiation [26]. Knockdown of laminin β2 also results in elongation of photoreceptor outer segments (OS), abnormal electroretinogram, and atypical synapse formation of rod photoreceptors and apoptosis in mice [27], whilst double knockout of laminin β2 and laminin γ3 in mice leads to the disruption of the inner limiting membrane (ILM) and retinal dysplasia [28].

Collagen IV, Laminin and Fibronectin are the main ECM components of the basement membranes including Bruch's membrane (BrM) and the ILM [19,20]. Basement membranes are essential for structural support, cellular adhesion, differentiation, proliferation, migration and intercellular communication [29]. A recent study has shown a direct link between increased expression of Fibronectin at the mRNA level and the thickening of BrM in animal models of diabetic retinopathy [30]. Inhibition of Fibronectin assembly *in vitro* prevents Collagen IV accumulation and thus could be a contributor to the development of fibrotic disease in age-related macular degeneration [31]. Mutations in Collagen IV genes (*COL4A3*, *COL4A4*, or *COL4A5*) were also reported in Alport syndrome patients and mice with ocular anterior segment dysgenesis [32].

Brevican and Versican are members of the hyalectin family which has been shown to play an important role in maintaining adhesion between the RPE and neurosensory retina as well as BrM [22]. Brevican and Versican bind to Hyaluronic Acid from their N-terminal region and are involved in the development of the central nervous system [23]. Hyaluronic Acid is expressed in the IPM and creates a scaffold that fills the matrix as well as binding to several proteins of the IPM such as Versican, IMPG1 and IMPG2 [3,24]. IMPG1 & IMPG2 are proteoglycans found in the IPM and shown to be involved in the development and maintenance of the photoreceptors in mice and human [33–35]. *IMPG1* mutations were reported in Vitelliform macular dystrophies leading to impaired metabolism of the IPM and the accumulation of Vitelliform deposits in the sub-retinal space [36]. A splice mutation in the Versican gene (*VCAN*) was reported in Wagner syndrome patients with retinal detachment [37], whilst mutations in *IMPG2* cause progressive degeneration of the photoreceptors, leading to autosomal dominant retinitis pigmentosa [36,38].

Most studies of the ECM expression accompanying retinal histogenesis have been performed in animal models due to the ethical issues associated with and scarcity of human embryonic and foetal retina. Pluripotent stem cells encompassing hESC and hiPSC provide a valuable platform for modelling retinal disorders, understanding human foetal ontogenesis and providing an unlimited cell source for cell based replacement therapies [1,3,39]. The process of cell lineage differentiation can be directed and functionally enhanced by supplementation with various ECM components. For example, hESC derived neural progenitors express RPE markers

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