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# Extracellular matrix component 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

when cultured on Matrigel and photoreceptor markers when cultured on laminin coated surfaces respectively [40]. Matrigel and Laminin-111 also support the highest level of pigmentation frequency in iPSC-derived RPE cells [41].

Given that human retina develops largely *in utero*, it is imperative to understand the expression of ECM components during retinal ontogenesis, in order to use this knowledge to improve the differentiation of pluripotent stem cells to retinal organoids and understand the role of various ECM components in retinal development and disease [21,22]. To date, there has been no detailed analysis of ECM component distribution during human ophthalmic development and so the functional importance of ECM in retinal ontogenesis is poorly understood [40]. In this manuscript, a detailed expression study of Collagen IV, Fibronectin, Versican, Brevican, IMPG1, CD44 and IMPG2 was undertaken in adult mouse, monkey and human retina, developing human embryonic and foetal retina, and in retinal organoids developed from hESCs and hiPSCs. The role of CD44 and IMPG1 was further investigated by functional blocking experiments in developing retinal organoids *in vitro*.

## 2. Materials & methods

### 2.1. Preparation of retinal sections from mouse and macaque monkey

Eyes were removed post-mortem from macaque monkeys which had undergone experiments under terminal anaesthesia as part of an unrelated study into the neural control of movement. That study was carried out under the approval of the Newcastle University Animal Welfare and Ethical Review Board, and under appropriate licences from the UK Home Office. Mouse eyes were obtained from wild type mice (C57BL/6 background, 7 months old) from the Functional Genetic Unit, Institute of Genetic Medicine, Newcastle University. Mice were sacrificed humanely via cervical dislocation. For wax sections, mouse and monkey eyes were dissected from the orbit and immediately transferred to phosphate buffered saline (PBS, Sigma, UK). The anterior portion was removed using micro-scissors and forceps, and fixed in 4% (v/v) paraformaldehyde (PFA) at 4 °C overnight. Eye fixation was followed by dehydration steps through increasing concentrations of ethanol (50%, 70%, 95% and 100%) before clearing in histoclear and embedding in paraffin wax [42] (pastillated Gurr wax, VWR, UK). 6 μm thick sections were cut on a microtome and collected onto slides (Superfrost Plus, Thermo Scientific, UK) then stored at room temperature (RT) protected from light and dust.

### 2.2. Preparation of retinal sections from adult human eyes

Adult human eyes were obtained from Manchester Eye Bank, Manchester Royal Eye Hospital. Three eyes were collected from three adult donors 48 h after death (a female aged 84 years and two males aged 67 and 88 years). The tissue was stored in organ culture medium at 34 °C for 7 days before screening them for bacteria and fungi using blood agar plates and liquid microbiological media [43]. Organ culture medium was composed of Eagle's Minimum Essential Medium (MEM) containing 26 mM sodium bicarbonate, 20 mM HEPES buffer, 2% Fetal Bovine Serum (FBS; Australian origin, Life Technology Ltd, UK), 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 mg/mL) [43]. All consumables used for storage were from Sigma-Aldrich Company Ltd (UK) except for FBS. Adult human eyes were fixed in 4% PFA, cryoprotected in PBS containing 30% sucrose overnight and embedded in OCT (Cell Path Ltd., Newtown, UK) and 20 μm thick sections were collected.

### 2.3. Human developmental tissue

Human embryonic and foetal specimens aged between 6 and 19 weeks of post-conception (PCW) were obtained from the MRC/Wellcome-Trust funded Human Developmental Biology Resource at Newcastle University (HDBR, http://www.hdbr.org), with appropriate maternal written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee. HDBR is regulated by the UK Human Tissue Authority (HTA; www.hta.gov.uk) and operates in accordance with the relevant HTA Codes of Practice. Ready-prepared 10 μm paraffin wax sections were collected from the HDBR and stored at 4 °C in slide boxes prior to immunostaining.

### 2.4. Immunohistochemistry for ECM proteins

Expression of ECM proteins was determined using immunohistochemistry (IHC). Tissue sections were dewaxed in histoclear, cleared in 100% ethanol, then rehydrated through several incubations in decreasing concentrations of ethanol (90%, 70% and 50%) followed by two washing steps in PBS [42]. For antigen retrieval, the tissue was placed in citrate buffer (pH 6.3) and microwaved at high power for 10 min. Non-specific epitope binding was blocked by incubation at RT for 45 min with PBS containing 10% (v/v) FBS.

Primary antibodies were diluted in PBS containing 2% FBS (Table 1), applied on tissue sections and incubated overnight at 4 °C. After washing, appropriate fluorescently-labelled secondary antibodies (Table 2) diluted in PBS containing 2% (v/v) FBS were added to each tissue section and incubated at room temperature for 1.5 h in a dark humidified chamber. To quench the autofluorescence in samples, tissue sections were incubated in 0.3% (w/v) Sudan Black (prepared in 70% ethanol) for 15 min at room temperature [44], washed in PBS, then mounted with Vectashield medium containing Hoechst as a nuclear counter-stain. Tissue sections were air-dried, washed several times with PBS and blocked with PBS containing 10% normal goat serum or donkey serum and 0.3% Triton-X-100 for 1 h at room temperature. Primary antibodies (Table 1) were diluted in antibody diluent solution (ADS; PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton–X-100) and applied overnight. After several washing steps in ADS, secondary antibodies (Table 2) were diluted in ADS and sections were incubated for 2 h at RT. After washing with PBS, sections were mounted with VectaShield (Vector Laboratories, Burlingame, CA) containing Hoechst (Life Technologies).

To assess the specificity of primary antibody binding, these were omitted and replaced with isotype controls (Table 1). For all antibodies, no non-specific binding was observed (Fig. S1). To assess the specificity of secondary antibody staining, primary antibodies were omitted. Again no non-specific binding was observed (Fig. S2).

### 2.5. Human pluripotent stem cells differentiation to retinal organoids

Retinal organoids were generated from two different cell lines: hiPSCs derived from adult fibroblasts (SB-Ad3) [45] and human embryonic stem cells (H9, Wicell Inc.). Stem Cells were expanded in mTeSR™1 (StemCell Technologies, 05850) at 37 °C and 5% $CO_2$ on 6 well plates pre-coated with Low Growth Factor Matrigel (Corning, 354230). The retinal organoids were generated following the method described in Mellough et al., 2015 [7] with the addition of ROCK inhibitor (Y-27632 dihydrochloride) (10 μM) for the first 48 h of differentiation. Further modifications include the addition of 10% Fetal Calf Serum, T3 (40 ng/ml), Taurine (0.1 mM) & Retinoic Acid (0.5 μM) from day 18 of differentiation. Retinal organoids were collected on day 35, 90 and 150 for IHC. After collection,

**Table 1**
List of primary antibodies and isotype controls used for the immunohistological analysis.

| Antibody | Tissue | Cat. No. | Host | Source | Dilution |
|---|---|---|---|---|---|
| *AP2α* | Amacrine cells | sc-12726 | Mouse | Santa Cruz | 1:200 |
| *ARL13B* | Connecting cilium | ab83879 | Rabbit | Abcam | 1:500 |
| *BCAN* | ECM | ab111719 | Rabbit | Abcam | 1:50 |
| *Caspase-3* | Apoptotic cells | 9661S | Rabbit | Cell Signaling Technology | 1:200 |
| *CD44* | ECM | ab119348 | Rat | Abcam | 1:100 |
| *Collagen IV* | ECM | ab6586 | Rabbit | Abcam | 1:100 |
| *Cone Arrestin* | Cone photoreceptors | NBP2-41249 | Rabbit | Novus Biologicals | 1:100 |
| *CRALBP* | RPE & Muller cells | GTX15051 | Mouse | Genetex | 1:100 |
| *CRX* | post-mitotic photoreceptors | H00001406-M02 | Mouse | Abnova | 1:200 |
| *CRYAA* | Lens | – | Mouse | Provided by Prof. Roy Quinlan Durham University, UK | 1:50 |
| Donkey anti-goat IgG | *IMPG2*-Isotype Control | 705-005-147-JIR | Donkey | Jackson ImmunoResearch | 1:1300 |
| Donkey anti-rabbit IgG | *BCAN*-Isotype Control | 711-005-152-JIR | Donkey | Jackson ImmunoResearch | 1:65 |
| | *Collagen IV*-Isotype Control | | | | 1:130 |
| | *IMPG1*-Isotype Control | | | | 1:260 |
| *FN1* | ECM | sc-8422 | Mouse | Santa Cruz Biotechnology | 1:20 |
| Goat anti-mouse IgG1 | *FN1*-Isotype Control | NB7508 | Goat | Novus Biologicals | 1:100 |
| Goat anti-mouse IgM | *VCAN*-Isotype Control | NB7494 | Goat | Novus Biologicals | 1:100 |
| Goat anti-rat IgG2b | *CD44*-Isotype Control | NB7127 | Goat | Novus Biologicals | 1:100 |
| *HuC/D* | amacrine and retinal ganglion cells | A21271 | Mouse | Invitrogen | 1:200 |
| *IMPG1* | ECM | Ab113493 | Rabbit | Abcam | 1:100 |
| *IMPG2* | ECM | sc-103232 | Goat | Santa Cruz Biotechnology | 1:200 |
| *Ki-67* | Proliferating cells | ab15580 | Rabbit | Abcam | 1:200 |
| *Opsin red/green* (*OPN1LW/MW*) | L/M cone photoreceptors | AB5405 | Rabbit | Millipore | 1:200 |
| *PAX6* | retinal progenitors, amacrine and ganglion cells | ab78545 | Mouse | Abcam | 1:200 |
| *PROX1* | horizontal, bipolar and amacrine cells | AB5475 | Rabbit | Millipore | 1:1000 |
| *RBP3* | IPM | 14352-1-AP | Rabbit | ProteinTech | 1:100 |
| *Recoverin* | photoreceptors and midget OFF bipolar cells | AB5585 | Rabbit | Millipore | 1:1000 |
| *RetP1* (Rhodopsin) | Rod photoreceptors | O4886 | Mouse | Sigma | 1:200 |
| *Syntaxin* | presynaptic plasma membrane | S0664 | Mouse | Sigma | 1:200 |
| *VCAN* | ECM | MABT161 | Mouse | Millipore | 1:100 |
| *vGlut1* | synaptic terminals of photoreceptors and bipolar cells | AB5905 | Guinea Pig | Millipore | 1:1000 |
| *VSX2* | retinal progenitor cells & bipolar cells | HPA003436-100UL | Rabbit | Sigma | 1:50 |
| *WGA*[*] | inner/outer segments of rods/cones | W11261 | N/A | Invitrogen | 1:1000 |

[*] WGA - conjugated antibody to Alexa Fluor 488.

**Table 2**
List of fluorescently labelled secondary antibodies used for the immunohistological analysis.

| Antibody | Cat. No. | Host | Source | Dilution |
|---|---|---|---|---|
| anti-mouse Alexa 488 | A11001 | Goat | Life Technologies | 1:800 |
| anti-rabbit Cy3 | 111-165-003 | Goat | Jackson ImmunoResearch | |
| anti-goat Cy3 | 305-165-003 | Rabbit | Jackson ImmunoResearch | |
| anti-donkey DyLight 488 | SA5-10062 | Rabbit | Life Technologies | |
| Anti-Guinea pig Cy3 | AP108C | Goat | Millipore | |

the organoids were fixed in 4% (w/v) PFA for 20 min, followed by three washes in PBS, then overnight cryoprotection in 30% sucrose in PBS before embedding in OCT medium (Cellpath, UK) and freezing at −20 °C. 10 μm cryostat sections were collected onto Superfrost Plus slides using a Leica Cm1860 cryostat and stored at −20 °C in slide boxes prior to immunostaining.

### 2.6. Blocking IMPG1 and CD44 cellular receptors in retinal organoids

The role of IMPG1 and CD44 in photoreceptor differentiation was investigated using blocking antibodies to IMPG1 (Abcam, ab113493) and CD44 (Abcam, ab119348). The antibodies were added (10 μg/ml) to retinal organoid culture media at day 90 and 150 of the differentiation process. After 14 days in culture, the effects of IMPG1 and CD44 receptor inhibition was assessed and compared to a control group which was incubated with (10 μg/ml) non-specific control IgG blocking antibody (Jackson ImmunoResearch, 209-005-088) by IHC and qRT-PCR.

### 2.7. Image capture and analysis

Images were captured using an Axio Imager upright microscope with Apotome (Zeiss, Germany) structured illumination fluorescence using 20× objective and 40× oil objective operated with AxioVision software. A range of fluorescent filters were used to cover several dyes including Hoechst, Cy3 and Alexa 488. Tissue sections of two biological replicates of developing and adult retina and three biological replicates of pluripotent stem cell derived retinal organoids were analysed. Cell image quantitation was performed using the MATLAB software. The data was presented as image segmentation through Hysteresis thresholding technique to segment the cells and filter out any noise background from

the images [46]. Using measuring property of MATLAB (regionprops), all the pixels of each image were represented as single region in order to extract the information about each cell including the size, average intensity value and length. After obtaining the final segmentation information, the total size and the percentage of the positive cells were calculated and exported as excel file for further analysis.

### 2.8. qRT-PCR

15–20 retinal organoids were homogenised using a Dounce Tissue Grinder (Sigma Aldrich, UK) and RNA was extracted using the Promega tissue extraction kit (Promega, USA) as per the manufactures instructions. 1 μg of RNA was reverse transcribed using random primers (Promega, USA). qRT-PCR was performed using a Quant Studio 7 Flex system (Applied Biosystems, USA) with SYBR Green reaction mixture (Promega, USA). Each primer (Table 3) was used at a concentration of 1 μM, and at a ratio of 50:50 for forward and reverse. The reaction parameters were as follows: 95 °C for 15 min to denature the cDNA and primers, 40 cycles of 94 °C for 15 s followed by primer specific annealing temperature for 30 s (60 °C), succeeded by a melt curve. A comparative Ct (cycle threshold) method was used to calculate the levels of relative expression, whereby the Ct was normalised to the endogenous control (*GAPDH*). This calculation gives the $\Delta^{Ct}$ value, which was then normalised to a reference sample (i.e. a positive control), giving the $\Delta\Delta^{Ct}$. The fold change was calculated using the following formula: $2\text{-}\Delta\Delta^{Ct}$.

### 2.9. Measurements of neuroepithelium thickness

Bright filed images of retinal organoids were taken using a bright field microscope AxioVert (Zeiss, Germany). The morphology of day 90 and 150 retinal organoids was assessed at day 7, 10 and 14 after incubation with the anti-IMPG1, anti-CD44 and control blocking antibodies. Measurements of neuroepithelium thickness in retinal organoids were performed using ImageJ. A total of three biological replicates (n = 3) were assessed from each hESCs- and hiPSCs- derived retinal organoids. Each biological replicate of day 90 and 150 retinal organoids included 25 measurements from each group (control, anti-IMPG1 and anti-CD44) and the neuroepithelium thickness were measured at three time point (day 7, 10 and 14) after the addition of blocking antibodies.

### 2.10. Statistical analyses

All statistical analyses were performed using Prism software (GraphPad, USA). The standard errors of all means (SEM) were calculated. Statistical significance of differences was tested with two-tailed Student's *t*-test for paired samples for neuroepithelium thickness, qRT-PCR data and cell image quantitation and Mann-Whitney *U* test (Wilcoxon Rank-Sum test) for RNA-sequencing data. $^{*}$p-value < 0.05, $^{**}$p-value < 0.01, $^{***}$p-value < 0.001, $^{****}$p-value < 0.0001.

## 3. Results

### 3.1. Fibronectin, Collagen IV, Versican, Brevican, IMPG1 & IMPG2 expression in adult retina

In order to understand retinal ECM composition during development, IHC was performed on adult mouse, monkey and human retinae. Fibronectin and Collagen IV were expressed in BrM in adult mouse, monkey and human retinae (Fig. S3). Fibronectin was also expressed in the ILM in all three species (Fig. S3). Fibronectin and Collagen IV expression around blood vessels was also observed in monkey (Fibronectin) and human adult retina (Collagen IV). Collagen IV expression was also observed in the OPL, INL, IPL, GCL and ILM of adult human retina (Fig. S3).

Versican expression was conserved across adult mouse, monkey and human retinae (Fig. S4) with strong expression around the OS of the photoreceptors, and partially co-localised with the IPM marker, Retinol Binding Protein 3 (RBP3) (Fig. S4). Versican was faintly expressed in other retinal layers of monkey and adult human retina. The expression of Brevican varied between all three species; showing expression in the RPE, around outer and inner segments of photoreceptors, OPL, INL and GCL in adult mouse retinae, all retinal layers except OS in adult monkey retinae, and the INL, IPL and ganglion cell layers in adult human retinae (Fig. S4).

The expression of IMPG1 & IMPG2 was conserved across the three species examined (Fig. S5). IMPG1 and IMPG2 were strongly expressed around the OS of the photoreceptors in the adult human and monkey retina (Fig. S5), however expression around both inner and outer segments was observed in the mouse retina (Fig. S5). In adult human retina, IMPG1 was expressed in the rod photoreceptor OS as shown by co-expression with the rod marker Rhodopsin (Fig. S5). We were unable to perform co-immunostaining of IMPG1 with the cone markers (OPN1LW/MW) because the antibodies were raised in the same species, however the widespread expression of IMPG1 immunostaining in the OS indicates that these may include cone photoreceptors too (Fig. S5). IMPG2 expression did not co-localise with Rhodopsin or opsin staining, thus indicating that IMPG2 is expressed around but not in the OS themselves.

### 3.2. ECM component expression in developing human retina

To get insights on the expression dynamics of ECM components during human retinal development, we took advantage of a recent RNA-seq study of retinal samples obtained from 4.6 to 18 post conception weeks (PCW) performed by our group [47]. This study defined three developmental windows comprising 4.6–7.2 PCW, 7.7–10 PCW and 12–18 PCW which were characterised by stage specific gene expression patterns corresponding to the emergence of optic cup/RPE/lens, retinal ganglion cells (RGCs), and photoreceptors/interneurons/Muller cells respectively. This analysis revealed two key patterns for ECM expression: high expression in the early developmental stages followed by a significant down-regulation towards 12–18 PCW (*COL4A1*, *FN1* and *VCAN*) and low expression in early developmental stages followed by a significant

**Table 3**
List of primers used for qRT-PCR study.

| Gene | Forward Primer (Sequence (5′–>3′) | Reverse Primer (Sequence (5′->3′) | NCBI Reference Sequence |
|---|---|---|---|
| *GAPDH* | TGCACCACCAACTGCTTAGC | GGCATGGACTGTGGTCATGAG | NM_001256799.2 |
| *OPSINLW* | GCCTACTTTGCCAAAAGTGC | GATGAGACCTCCGTTTTGGA | NM_020061 |
| *RBP3* | AGATCATGCACACGGATGCC | AGCCATAGCGTTCACCTACA | NM_002900.2 |
| *RCVRN* | TTCAAGGAGTACGTCATCGCC | GATGGTCCCGTTACCGTCC | NM_002903 |
| *RHO* | TTTGGAGGGCTTCTTTGCCA | CCTCGGGGATGTACCTGGAC | NM_000539.3 |

upregulation towards 12–18 PCW (*BCAN*, *IMPG1*, *IMPG2*, *RBP3*, *CD44*) (Fig. S6). These expression trends may suggest stage specific roles played by various ECM components during retinogenesis or changes from a more widespread to defined expression patterns during retinal development. To investigate this in more detail, we used IHC on embryonic and foetal retinal specimens from 6 to 19 PCW of human development as detailed below.

### 3.2.1. Distribution of basement membrane glycoproteins

Collagen IV and Fibronectin are two major components of the basement membranes and have been shown to play an important role in optic cup morphogenesis [20]. Collagen IV was expressed in the choroid and BrM during the 6–19 PCW (Fig. 1A). This result was further corroborated by adjacent expression of the RPE marker, RPE65 (Fig. S7A). Collagen IV was also expressed in the hyaloid artery and lens capsule during the 6 PCW suggesting that it might play an important role in their development. Faint expression of Collagen IV was also detected in the RPE, Outer Neuroblastic Zone (ONZ), IPL and Inner Neuroblastic Zone (INZ) across all stages (Fig. 1A). The expression pattern of Collagen IV was consistent with the expression observed in adult human retina described in the previous section.

Fibronectin was expressed in BrM during the 12–19 PCW (Fig. 1B). In addition, expression was detected in the ILM in the 17 PCW and in the Choroid in 14–19 PCW (Fig. 1B). The expression pattern of Fibronectin in BrM and ILM at 17 PCW was consistent with the expression observed in adult human retina. Double staining of Fibronectin and Collagen IV confirmed that both of these ECM components were expressed in BrM, although in different parts with Fibronectin localised more basally than Collagen IV (Fig. S7B).

### 3.2.2. Distribution of Brevican & Versican

The expression of Brevican was observed throughout the retina (ONZ, IPL and INZ) across all developmental stages (Fig. 2A). Strong expression of Brevican was detected around developing photoreceptors (Fig. S7C, inset) and in the emerging space between the RPE and ONZ often referred as the IPM during the 12–19 PCW (Fig. 2A). Brevican was also detected in the surface ectoderm at 6 PCW and in the neural retina at 6 and 8 PCW (Fig. 2A). Versican was expressed throughout the retina and strongly expressed around the developing photoreceptors and IPM during the 12–17 PCW (Fig. 2B). Co-expression studies indicated that Brevican and Versican were both expressed in the developing IPM; however their expression did not overlap entirely (Fig. S7C). Versican expression did, however, overlap with the IPM marker RBP3 and was located above Recoverin-positive labelled photoreceptors (Fig. S7D), thus corresponding with the expression observed in adult human retina.

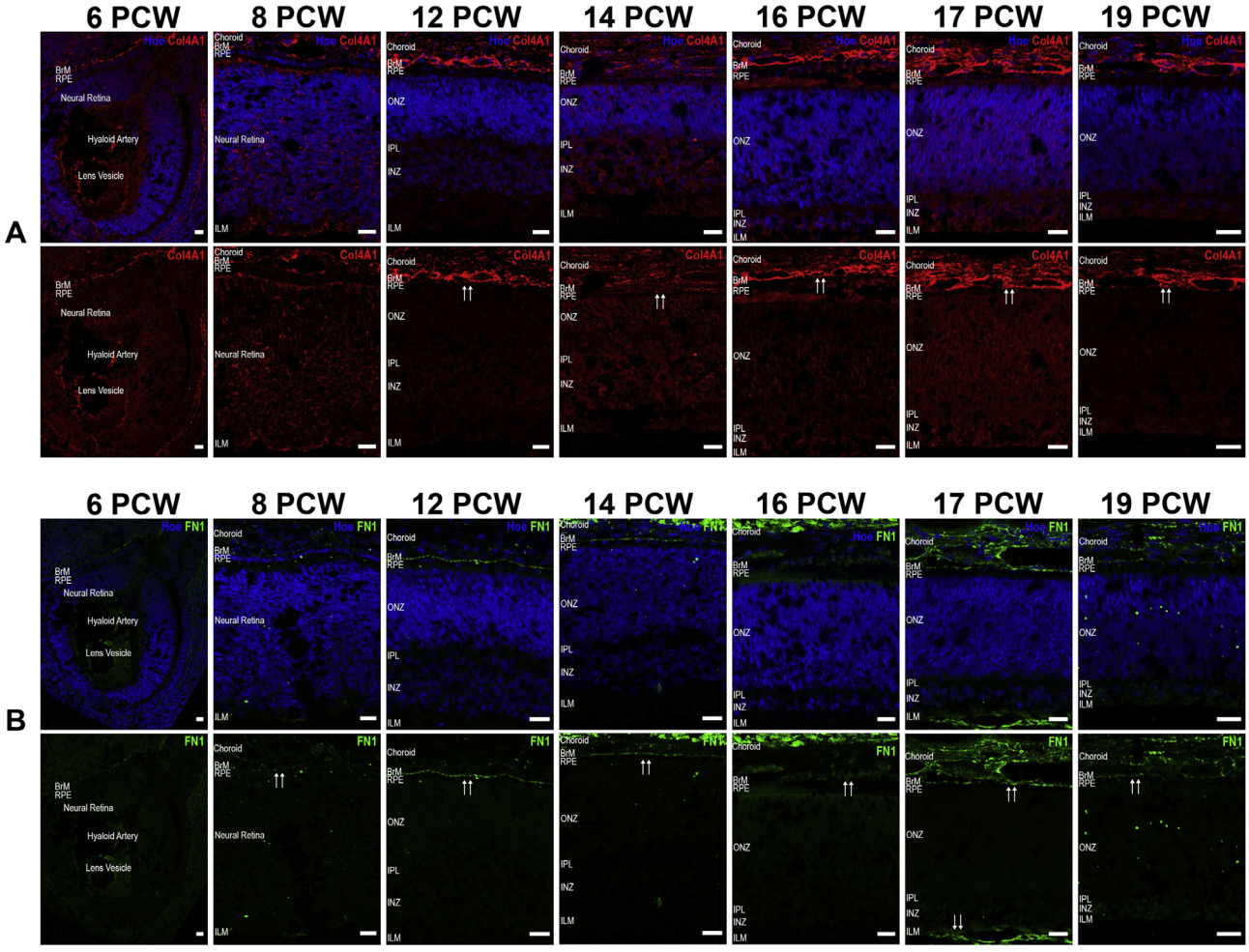


**Fig. 1.** Expression of Collagen IV (Col4A1; A) & Fibronectin (FN1; B) in the human embryonic and foetal eye. (A, B) Sagittal sections through the developing human eye showing antibody labelling with and without Hoechst (Hoe) during the 6–19 PCW. Scale bars = 20 μm. Arrows indicate Col4A1 expression in BrM and FN1 expression in BrM and ILM.

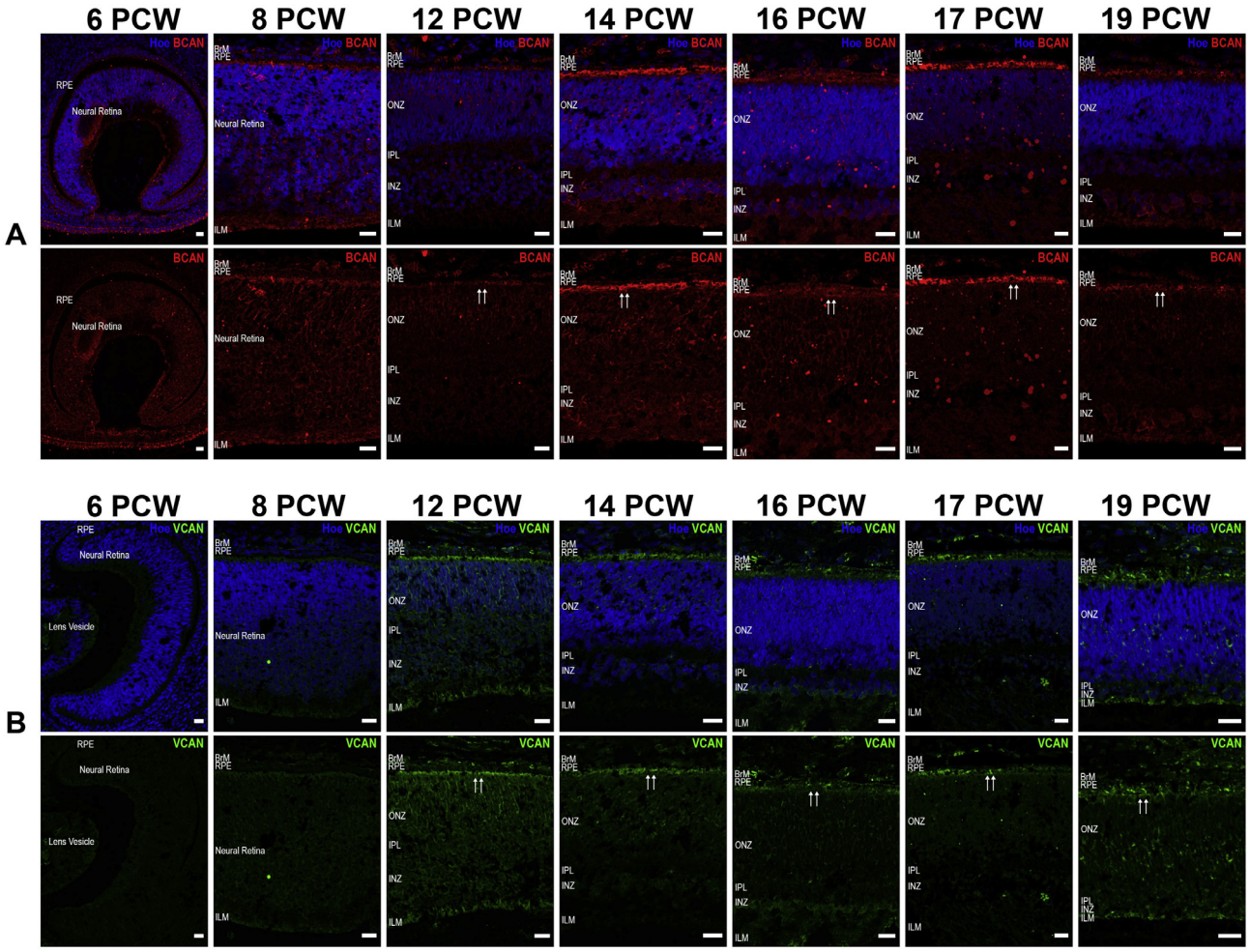


**Fig. 2.** Expression of Brevican (BCAN; A) & Versican (VCAN; B) in the human embryonic and foetal eye. (A, B) Sagittal sections through the developing human eye showing antibody labelling with and without Hoechst (Hoe) during the 6–19 PCW. Scale bars = 20 μm. Arrows indicate BCAN & VCAN expressions in IPM.

*3.2.3. Expression of IMPG1 & IMPG2 in the developing human retinae*

Similar to Versican, strong expression of IMPG1 was observed around the developing photoreceptors and emerging IPM during the 12–19 PCW (Fig. 3A). This was confirmed by the co-expression of IMPG1 with Versican (Fig. S7E). IMPG1 expression was also detected across the neural retina from the 6–19 PCW. Co-expression of IMPG1 with CRYAA (a lens-specific marker) was observed in the lens at 6 PCW suggesting that IMPG1 might be important for lens development (Fig. S7E). IMPG2 expression was not detected until the 17 PCW (Fig. 3B). Co-expression of IMPG2 with Recoverin in the ONZ was observed in 19 PCW suggesting that IMPG2 is expressed by the developing photoreceptors (Fig. S7F). Co-expression of IMPG2 with RBP3 was also found in the developing IPM, thus corroborating the expression observed in the adult human retina (data not shown).

*3.2.4. Distribution of cluster of differentiation 44 (CD44) in human developing retina*

CD44 acts as a receptor for Hyaluronic Acid and other ECMs such as Versican, Brevican and Aggrecan [48,49]. It interacts with the GAGs of IMPG1 and may participate in retinal adhesion [50]. It was, therefore, important to investigate CD44 expression in the developing human retina. CD44 was found to be expressed in the ONZ and INZ during the later stages of human retinal development studied including 16–19 PCW (Fig. 4A). No immunostaining of CD44 was detected in retinal sections from the 6$^{th}$ until the 14$^{th}$ PCW (Fig. 4A). Strong expression of CD44 was also observed in the developing IPM in 16–19 PCW as revealed by the co-localisation with RBP3 (Fig. 4B).

*3.3. ECM expression during differentiation of hESC and hiPSC to laminated retina*

Retinal organoids were generated from hESCs (H9) and hiPSCs (SB-Ad3) following a differentiation protocol which was previously described [7], with a few modifications highlighted in the methods section (Fig. S8). The development of retinal progenitors and their maturation over time was assessed using a combination of specific markers (Fig. 5). VSX2, a marker of retinal progenitor and bipolar cells, was expressed in the apical region of retinal organoids on days 35 and 90 of differentiation (Fig. 5A). Amacrine and RGCs (detected by the expression of HuC/D) were located in the basal layer (toward the centre of the retinal organoids) (Fig. 5A). CRX, a post-mitotic photoreceptor marker, was expressed throughout the retinal organoids with strong expression towards the apical region of the organoid as the photoreceptors developed over time (Fig. 5A). Strong Recoverin expression was observed at days 90 and 150 (Fig. 5A). At day 150, there was no overlap between VSX2 and CRX expression (Fig. 5A). PAX6 and VSX2 co-immunostaining showed that bright VSX2 and bright PAX6 expressing cells did not overlap, indicating that high VSX2 and high PAX6 expression in the putative inner nuclear layer of day 150 retinal organoids

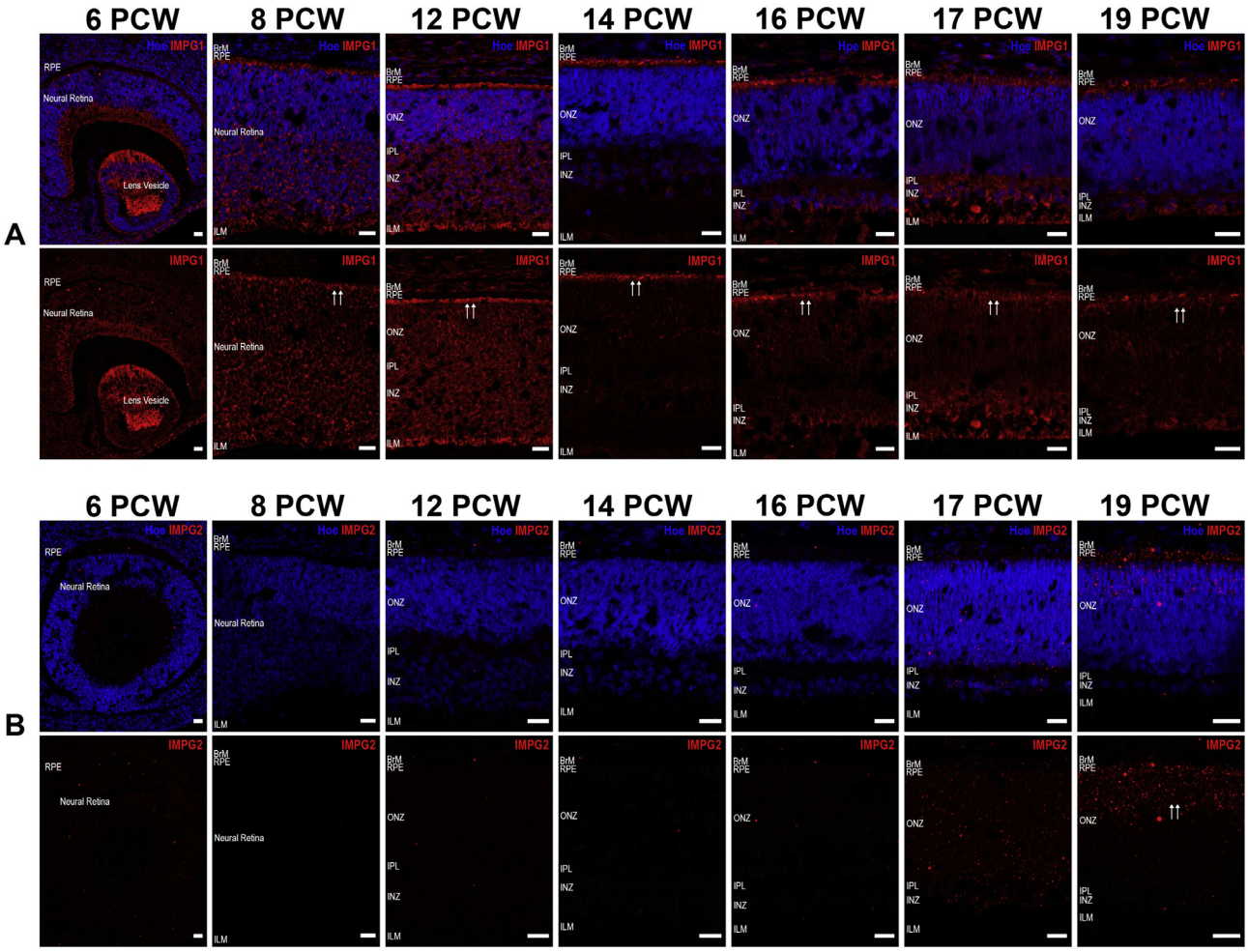


**Fig. 3.** Expression of Interphotoreceptor Matrix Proteoglycan 1 (IMPG1; A) & Interphotoreceptor Matrix Proteoglycan 2 (IMPG2; B) in the human embryonic and foetal eye. (A, B) Sagittal sections through the developing human eye showing antibody labelling with and without Hoechst (Hoe) during the 6–19 PCW. Scale bars = 20 μm. Arrows indicate IMPG1 & IMPG2 expressions in IPM.

are most likely to mark the emerging bipolar and amacrine/horizontal cells respectively, corroborating data published by Volkner *et al.* [51]. Nonetheless, within the retinal organoids, we could find cells with low PAX6 and low VSX2 expression which most likely mark the remaining proliferating retinal progenitor cells (Fig. S9), also shown by Ki67 expression (Fig. S9A). At day 150, other cell types including amacrine, horizontal, ganglion and Muller glial cells were observed through immunostaining with AP2α, Prox1, Cralbp (Figure S9A) in addition to the expression of presynaptic marker (Syntaxin), vGlut1 (expressed in photoreceptor and bipolar cell terminals) and photoreceptor connecting cilia marker, ARL13B. At day 150, we also observed expression of Rhodopsin in cell bodies and IS/OS like segments (Fig. S9A) in a similar pattern reported by Gonzalez-Cordero et al. recently [8]. The expression of cone markers (Cone Arrestin and long/medium wavelength Opsin) was assessed from day 150; however a typical expression pattern in cell bodies and IS/OS like segments was only observed from day 200 onwards (Fig. S9B).

Immunostaining with antibodies against the selected ECM components indicated that Fibronectin was expressed throughout the 3D retinal organoids during the entire period of differentiation in both hESC- and hiPSC-derived retinal organoids (Figs. 5B-D and S10A-C). The expression of Collagen IV was detected in the basement membrane on the luminal side of retinal organoids (Figs. 5B-D and S10A-C).

Versican was expressed throughout the retinal organoids with strong apical expression at all stages of differentiation. Furthermore, Versican co-localised with RBP3 in both hESC- and hiPSC-derived retinae at day 150 of differentiation (Figs. 5D and S10C), indicating expression in the emerging IPM, thus corroborating data obtained with developing and adult human retinae. A similar analysis in the hESC- and hiPSC-derived retinal sections showed highest expression of Brevican in the apical layer consisting of photoreceptors marked by the CRX expression at days 90 and 150 of differentiation (Figs. 5C-D and S10B-C).

The expression of IMPG1 in retinal organoids was first evident on day 90, with strong apical expression suggesting that IMPG1 might have a significant role in the development and maturation of photoreceptors *in vitro* (Fig. 5C and D). IMPG1 expression in hiPSC-derived retinal sections was only observed from day 120, suggesting potential differences between hESC and hiPSC in the kinetics of differentiation and/or IMPG1 expression (Fig. S10C). In contrast, IMPG2 was not detected at all stages of hESC and hiPSC retinal differentiation tested, suggesting that it might be expressed at later stages of the differentiation process. Similar to IMPG1, CD44 expression started at day 90 in the retinal organoids with broad expression throughout neural retinal tissue and a strong punctate-like pattern in the apical layer where Recoverin staining was strongly detected (Fig. S11A-C). Co-immunostaining showed CD44 co-expression with IPM marker RBP3 (Fig. S11D). However,

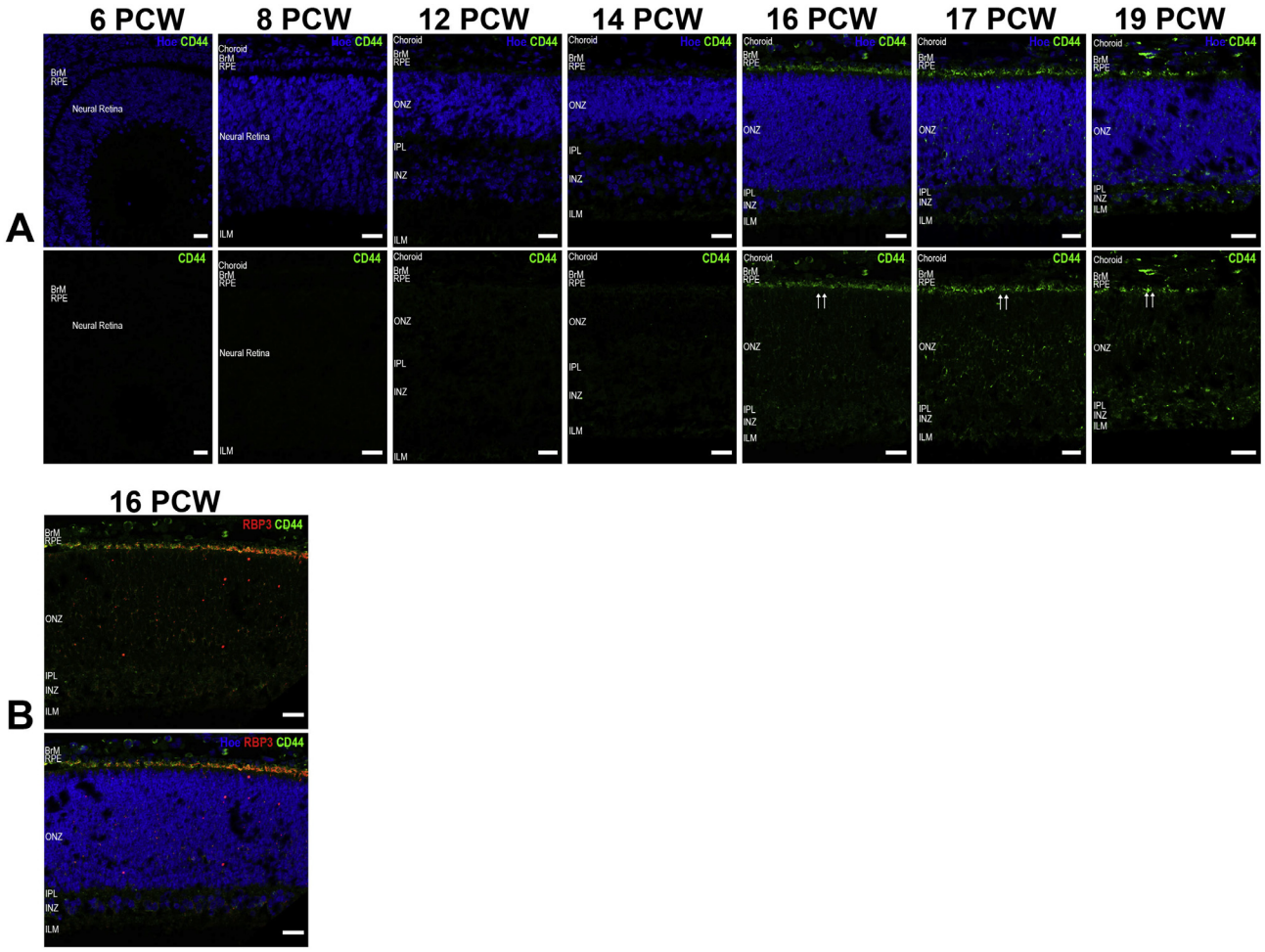


**Fig. 4.** Expression of cluster of differentiation 44 (CD44) in the human embryonic and foetal eye. (A) Sagittal sections through the developing human eye showing antibody labelling with and without Hoechst (Hoe) during the 6–19 PCW. (B) Tissue labelled with CD44 and RBP3 antibodies in 16 PCW. Scale bars = 20 μm. Arrows indicate CD44 expressions in IPM.

no overlap in expression was found with the cone and rod inner and outer segment marker (WGA, data not shown). Collectively, these data indicate that Versican, Brevican, IMPG1 and CD44 were expressed in the IPM of both human developing retina and human pluripotent stem cells derived retinal organoids.

### *3.4. IMPG1 & CD44 receptors mediated effect on photoreceptors and IPM development*

In order to investigate the role of IMPG1 and CD44 in photoreceptor differentiation, blocking antibodies to IMPG1 and CD44 were added to retinal organoid culture media at day 90 and 150 of the differentiation process. IMPG1 antibody blocks the extracellular cell binding domain (SEA domain) of the receptor, while CD44 blocks the LINK domain on the cell membrane [49,52]. Treated groups were compared to a control group. After 14 days of incubation of retinal organoids at day 90 of differentiation with IMPG1 and CD44 blocking antibody, thinning of the phase bright neuroepithelium typically found at the periphery of retinal organoids, was observed in the group treated with IMPG1 antibody only (Fig. S12A). However, the same experiment performed at day 150, indicated thinning of the neuroepithelium in both CD44 and IMPG1 treated groups after 14 days of incubation with blocking antibodies (Figs. S13 and S14A). After 14 days in culture, the effects of IMPG1 and CD44 receptor inhibition at day 90 and 150 of differentiation were assessed using IHC and qRT-PCR with retinal markers including Recoverin (photoreceptor marker), Rhodopsin (rod marker), WGA (cone and rod inner and outer segment marker respectively), ARL13B (marker for connecting cilia) and RBP3 (IPM marker) to determine any effect on photoreceptors and/or IPM development (Figs. 6 and S12–S15). No cone markers for immunostaining analysis were included due to their emergence from day 200 onwards.

The expression of *RCVRN* (Recoverin; Fig. S12B) and the number of Recoverin positive cells (Fig. S15B) were significantly reduced upon blocking of IMPG1 at day 90 of differentiation. The expression of photoreceptor connecting cilia, ARL13B was also significantly reduced upon blocking of IMPG1 function (Figure S15B), however no changes in *RBP3* expression were observed (Figs. S12B and S15B), suggesting that IMPG1 plays a role in the development and emergence of photoreceptors, but not IPM formation at this developmental time point. Blocking of CD44 had no impact on the gene or protein expression of photoreceptor, connecting cilia or IPM markers at this time point (Figs. S12B, C and S15C). There were no significant changes in Caspase 3 immunostaining for control, IMPG1 and CD44 blocked groups as assessed by IHC and quantification at either day 90 or day 150 (data not shown). Blocking of IMPG1 action at day 150 led to a significant reduction in the expression of Recoverin and the number of Recoverin positive cells within the retinal organoids (Figs. 6B and S14B, C). The expression of the cone marker (*OPN1LW/OPN1MW*) was downregulated only in the IMPG1 blocked group (Fig. S14B), although we could not

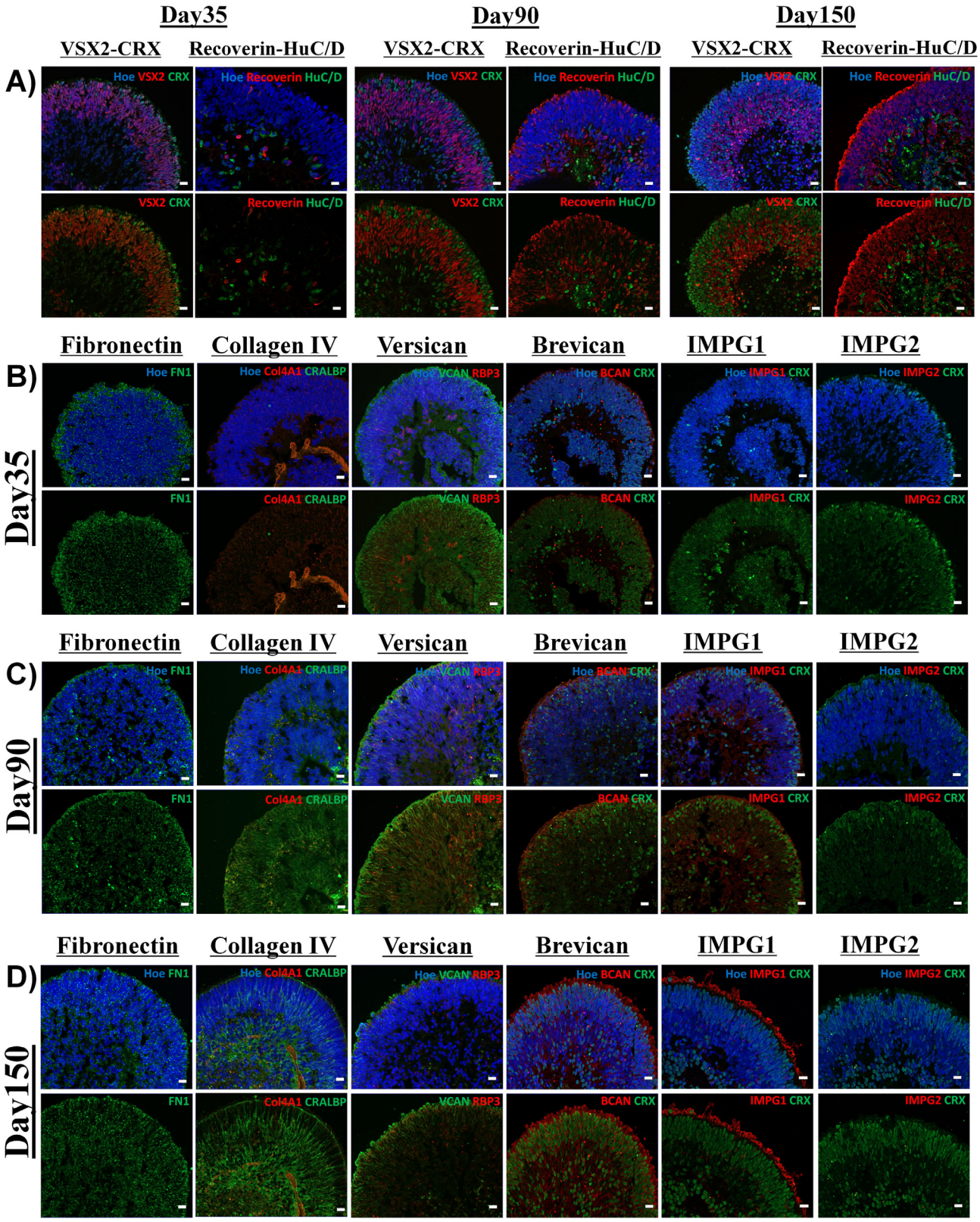


**Fig. 5.** Expression of selected ECM components during the differentiation of hESC to laminated retina. (A) H9-derived retinal organoid sections showing the development of retinal progenitors and their maturation over time using retinal markers: VSX2, HuC/D, CRX and Recoverin. (B-D) H9-derived retinal organoid sections showing Fibronectin, Collagen IV, Versican, Brevican, IMPG1 and IMPG2 antibody labelling with and without Hoechst on day 35 (B), day 90 and (C), day 150 (D). Scale bars = 20 μm.

assess its expression at the protein level given the later and typical emergence (from day 200) of this cell type. The expression of *Rhodopsin* (*RHO*) and the number of Rhodopsin immunopositive cells were reduced upon IMPG1 and CD44 blocking. In addition, the typical pattern of Rhodopsin immunostaining was lost in CD44 and IMGP1 blocked groups (Fig. 6B, C), instead a punctate pattern at

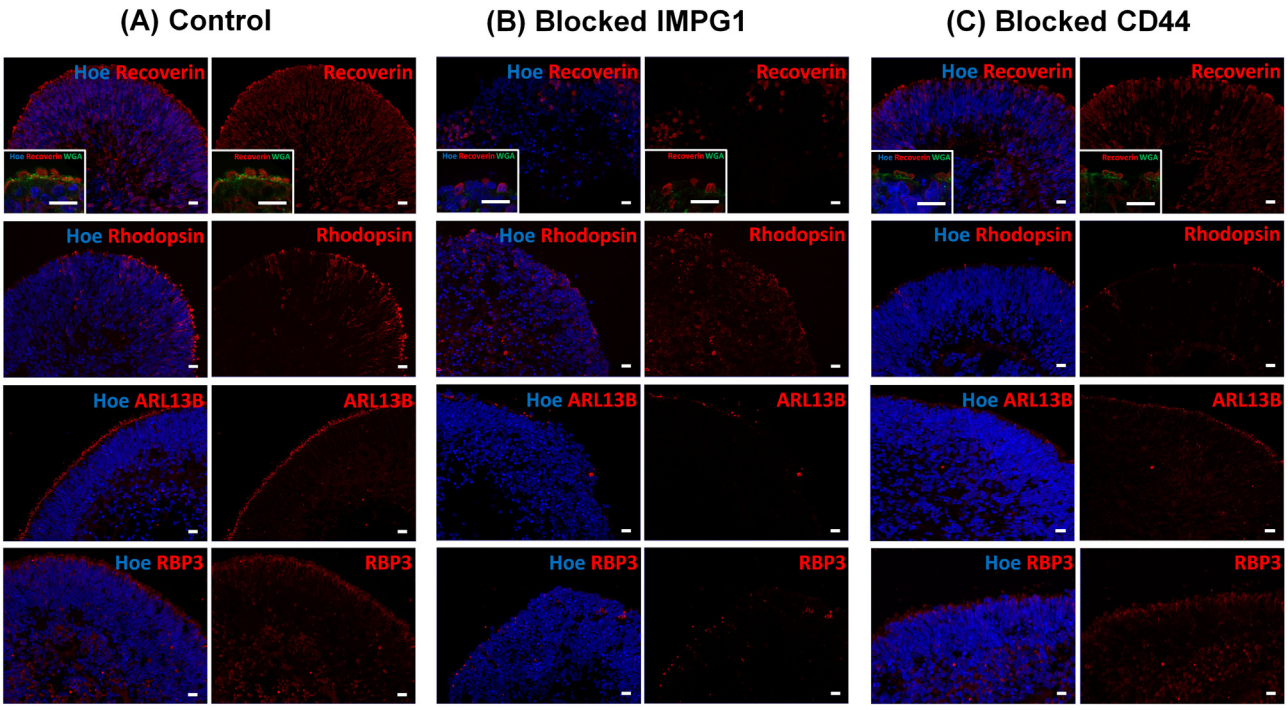


**Fig. 6.** Blocking of IMPG1 & CD44 action affected the development of photoreceptors and interphotoreceptor matrix. Sections of retinal organoid derived from H9s showing antibody labelling with and without Hoechst (Hoe) at day 164 in three different treatment groups: cultures treated with a non-specific IgG antibody (control, A), cultures blocked with an IMPG1 antibody (B) and cultures blocked with a CD44 antibody (C). Antibody labelling was performed with Recoverin, Rhodopsin, ARL13B and RBP3. Small insets show higher magnification images of organoid sections double labelled with Recoverin and WGA. Scale bars = 20 μm.

the surface of retinal organoids was observed. Immunostaining with the cone and rod inner/outer segment marker WGA showed a significant reduction in the IMPG1 and less so in the CD44 blocked group, suggesting that blocking the function of these two ECM components interferes with the development of photoreceptor inner/outer segments at different extent (Fig. 6B, C). Furthermore, the expression of photoreceptor connecting cilia (ARL13B) and IPM (RBP3) markers was significantly reduced in the IMPG1 treated group (Fig. 6B), indicating an important role for this protein in cilia and IPM formation. ARL13B and RBP3 immunostaining were also reduced in the CD44 blocked group, but less so when compared to the IMPG1 treated group. Collectively, these data suggest that IMPG1 and CD44 play an important role in the development of photoreceptors, their inner and outer segments, connecting cilia and IPM, with IMPG1 having an earlier and more significant impact.

## 4. Discussion

In this study the expression of key ECM components in adult mouse and monkey retinae, developing and adult human retinae and retinal organoids derived from hESC and hiPSC was investigated (for summary of ECM expression, please see Figs. 7 and 8). The results of this study demonstrate that different ECM components have distinct distribution patterns throughout the adult retina of different species [21]. Collagen IV was expressed in BrM, while Fibronectin was expressed in BrM and ILM, consistent with the expression previously observed in adult rat [53]. The current study also indicated that the expression of Versican, Brevican, IMPG1 and CD44 in the developing IPM was similar between native human developing retina and pluripotent stem cell-derived retinal organoids. IMPG2 was not expressed in retinal organoids (up to day 150) included in this study. The same analysis of developing human retina indicated that expression of IMPG2 only starts from 17 PCW. It may therefore be possible, that IMPG2 is expressed in later stages of retinal organoid formation (after day 150); however, this remains to be investigated further. The blocking experiments indicated that inhibition of CD44 and IMPG1 had a cell-type specific impact on the development of photoreceptors, their inner and outer segments and IPM.

The expression data obtained from this study are largely immunocytochemical and as such validation of antibody specificity is an important consideration. To address this, we employed widely used controls for immunocytochemistry including: (*i*) omission of primary antibodies; (*ii*) replacement of primary antibodies with isotype controls; (*iii*) usage of antibodies that have been widely published and validated in other studies. For example, the IMPG2 antibody used in our study was validated through incubation with pre-immune serum and shown to be expressed in the IPM of human retina by Acharya *et al.* [54]. In addition, the Collagen IV antibody used in our study showed a similar expression in BrM of pig retina [55] and the size of CD44 protein detected with the same antibody as us was confirmed by western immunoblotting by Enkhjargal *et al.* [56]. For the rest of antibodies (*BCAN, VCAN, FN* and *IMPG1*) where we felt that not enough literature evidence was found, we performed RNAi studies which showed in all cases a corresponding decrease in gene and protein expression by qRT-PCR and immunocytochemistry (data not shown).

Since the seminal discovery of Eiraku and Sasai in 2011 on generation of 3D retinal organoids from mouse ESC, multiple protocols have been developed and optimised for both human and mouse pluripotent stem cells [57]. The initial stages of differentiation protocols that encompass formation of neural progenitors vary between different published methods and include manipulation of IGF-1 and Wnt and TGFβ/activin signalling pathway [7,57]. The second and longer stages of differentiation protocols that encompasses the formation of retinal progenitors, photoreceptor precur-

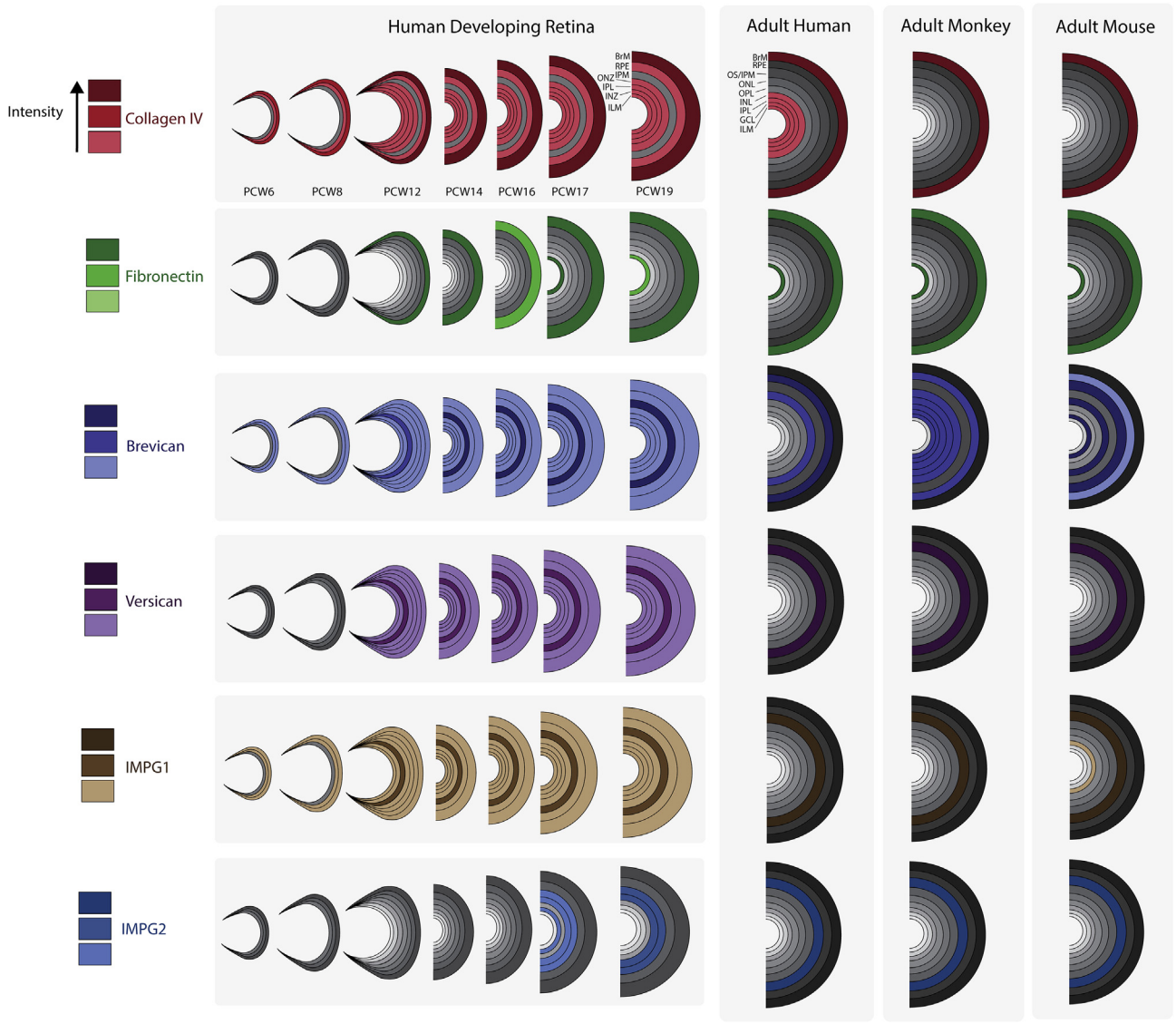


**Fig. 7.** Illustrated diagram showing a summary of ECM expression in human developing retina and adult human, monkey and mouse retinae.

sors and mature photoreceptors within a laminated structure are more similar to each other and in most cases include addition of Fetal Calf Serum, Taurine and Retinoic Acid. Our differentiation method was based on our previous publication which highlighted enhanced formation of eye and optic cup like structures from hESC by the addition of IGF-1 [7]. To obtain laminated retinal organoids with high efficiency from both hESC and hiPSC, we have optimised this protocol through the addition of Fetal Calf Serum, Retinoic Acid, Taurine and Tri-iodothyronine (T3) from day 18 of differentiation. Our data (Dorgau and Felemban, under preparation) indicate that this optimised differentiation protocol results in formation of laminated retinal organoids which contain all key retinal cell types, form synaptic connections and respond to light. In view of these results, this differentiation protocol was used for the ECM studies described herein.

The IPM is a highly organised structure that surrounds the photoreceptor cells and lies between the photoreceptors and the RPE [18]. It has several prominent functions including regulating retinoid transport, maintenance of the photoreceptor-specific microenvironment, cellular interactions, and retinal adhesion to the RPE [18]. A major component of the IPM, is Hyaluronic Acid, a large non-sulfated linear polysaccharide of (1-β-4) D-glucuronic acid and (1-β-3) N-acetyl-D-glucosamine [18]. Hyaluronic Acid can bind to a number of proteins in the IPM such as hyalectins, IMPG1 and IMPG2, forming a scaffold that fills the IPM [3,18,24,58]. Preceding any experimental work to mimic the specific IPM environment, it is essential to understand the composition of the IPM during human retinal organogenesis. Expression studies were performed to map the differential distribution of proteoglycan core proteins and their associated GAGs chain in sections of developing human retina and retinal organoids obtained from pluripotent stem cells. Versican was previously reported in the IPM in adult chick [59]. The data obtained in the current study corroborate this published report and also demonstrate Versican expression in the IPM of adult human, mouse and monkey retina. In the developing human retina and organoids generated from pluripotent stem cells, Versican expression surrounded the developing photoreceptors between the RPE and ONZ, an area which likely develops into the future IPM. The localisation of Versican in the IPM was confirmed by co-expression with interphotoreceptor retinoid binding protein (IRBP/ RBP3), the most abundant protein in the IPM [60].

IMPG1 and IMPG2 are two specific ECM components that are secreted by the photoreceptors into the IPM [34,54]. The results

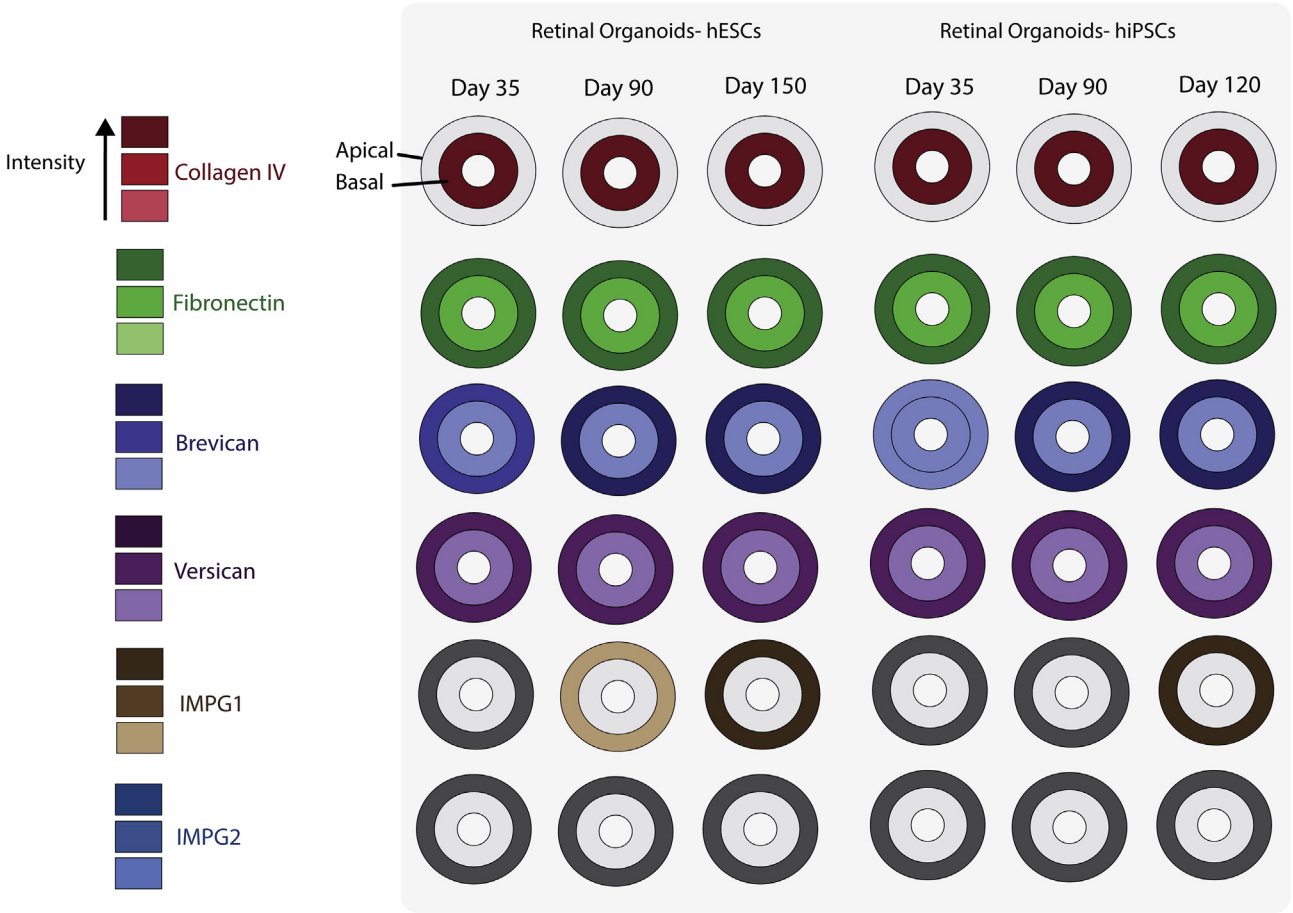


**Fig. 8.** Illustrated diagram showing a summary of selected ECM expression during the differentiation of hESCs and hiPSCs to retinal organoids.

of this study demonstrated expression of these two ECMs in the IPM in human developing tissue, with IMPG1 detected earlier and IMPG2 expressed at later stages of development, which may suggest a role for IMPG1 in early photoreceptor development and IMPG2 in photoreceptor maturation. This was corroborated by function blocking experiments which indicated that IMPG1 plays an important role in the development of photoreceptors as well as formation of their inner/outer segments, connecting cilia and IPM. Furthermore these data support the clinical phenotype of Vitelliform Macular Dystrophy caused by *IMPG1* mutations and is characterised by impaired metabolism of photoreceptors in the macula region due to the loss of IPM [36].

Several studies have demonstrated that CD44 is a multi-domain protein that spans the plasma membrane of the cell and acts as a receptor for Hyaluronic Acid and other ECMs such as Versican, Brevican and Aggrecan [48,49]. CD44 can also interact with the GAGs of IMPG1 and may participate in retinal adhesion [50]. The results obtained in this study indicated that CD44 was expressed in the IPM from the 16 PCW of human development. In retinal organoids, strong expression of CD44 was observed in the apical layer where the IPM develops, suggesting that CD44 may play an important role in the development of the photoreceptors. Blocking of the CD44 receptor resulted in a reduction in the number of rod photoreceptors within the retinal organoids as well as the expression of connecting cilia marker (ARL13B) and IPM marker (RBP3). Collectively these data suggest that CD44 may play an important role in the development of photoreceptors, in addition to being involved in formation of photoreceptor connecting cilia and IPM.

## 5. Conclusion

In summary, this study highlights a well conserved expression for Versican, Brevican, IMPG1 and CD44 in the IPM of developing human retina and retinal organoids. Furthermore, the study highlights an essential role for IMPG1 and CD44 in the development of photoreceptors, their inner and outer segments, connecting cilia and IPM, with IMPG1 having an earlier and more significant impact. Future work will endeavour to investigate whether manipulation of the ECM can accelerate and enhance retinal development *in vitro* so that effective retinal cell therapy may be realised.

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## Disclosures

The authors disclose no conflict of interest.

## Author contributions

MF and BD performed research, data acquisition and analysis and wrote the manuscript; YD, DZ and DH performed research, data acquisition and analysis; NH, JC, DS, JAA, CM contributed to study design, data analysis and interpretation; SL oversaw the donation of human embryonic and foetal samples; JC, DS, JAA, CM, NK and SL contributed to fundraising; ML designed and performed research, data analysis, manuscript writing and fundraising. All authors contributed to the final approval of manuscript.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2018.05.023.

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