

# Altered Decorin Biology in Proliferative Vitreoretinopathy: A Mechanistic and Cohort Study

Ghazala Begum,<sup>1,2</sup> Jenna O'Neill,<sup>3</sup> Rishika Chaudhary,<sup>1,2,4</sup> Karen Blachford,<sup>5</sup> David R. J. Snead,<sup>6</sup> Martin Berry,<sup>1</sup> Robert A. H. Scott,<sup>1,2,7</sup> Ann Logan,<sup>1,2</sup> and Richard J. Blanch<sup>1,2,5,8</sup>

<sup>1</sup>Inflammation and Ageing, University of Birmingham, Birmingham, United Kingdom

<sup>2</sup>NIHR Surgical Reconstruction and Microbiology Research Centre, University of Birmingham, Birmingham, United Kingdom

<sup>3</sup>Ridgeway Research Ltd., St. Briavels, Gloucestershire, United Kingdom

<sup>4</sup>Ophthalmology Department, University Hospitals Birmingham NHS Foundation Trust, Birmingham, United Kingdom

<sup>5</sup>Academic Unit of Ophthalmology, Birmingham and Midland Eye Centre, Sandwell and West Birmingham Hospitals NHS Trust, Birmingham, United Kingdom

<sup>6</sup>Department of Pathology, University Hospitals Coventry and Warwickshire NHS Trust, Coventry, Warwickshire, United Kingdom

<sup>7</sup>SpaMedica, Birmingham, United Kingdom

<sup>8</sup>Academic Department of Military Surgery and Trauma, Royal Centre for Defence Medicine, Birmingham, United Kingdom

Correspondence: Richard J. Blanch, Neuroscience and Ophthalmology, Institute of Inflammation and Ageing, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK; blanchrj@bham.ac.uk.

Submitted: March 8, 2018

Accepted: September 11, 2018

Citation: Begum G, O'Neill J, Chaudhary R, et al. Altered decorin biology in proliferative vitreoretinopathy: a mechanistic and cohort study. *Invest Ophthalmol Vis Sci.* 2018;59:4929-4936. <https://doi.org/10.1167/iovs.18-24299>

**PURPOSE.** To determine if vitreous levels of the pro-fibrotic cytokine transforming growth factor beta2 (TGF- $\beta$ 2) and its opposing regulator decorin predict subsequent proliferative vitreoretinopathy (PVR) development in patients with rhegmatogenous retinal detachment (RRD).

**METHODS.** We examined the effect of TGF- $\beta$ 2 and decorin on epithelial-mesenchymal transition (EMT) and collagen expression in vitro using ARPE-19 cells, and we analyzed extracellular matrix marker expression in PVR membrane and internal limiting membrane patient samples. We performed a prospective noninterventional cohort study, recruiting 125 patients undergoing vitrectomy for RRD and macular hole surgery, measured vitreous levels of TGF- $\beta$ 2 and decorin by ELISA, and followed them up for 6 months. Patients who did not develop PVR were compared to those who did, in order to determine whether vitreous TGF- $\beta$ 2 and decorin levels predicted PVR development.

**RESULTS.** In vitro, TGF- $\beta$ 2 induced EMT and collagen production. Decorin strongly inhibited EMT and collagen production at high levels. PVR membranes expressed high levels of fibrosis-associated proteins, consistent with EMT. Vitreous TGF- $\beta$ 2 levels were unchanged between patients with macular holes and RRD who did or did not subsequently develop PVR. Average decorin levels were higher in the vitreous of RRD patients who subsequently developed PVR compared to those who did not, but at the measured vitreous concentrations (1–2  $\mu$ g/mL), decorin did not demonstrate an in vitro inhibitory effect on EMT.

**CONCLUSIONS.** In vitro, high concentrations of decorin inhibited EMT and fibrosis. At the levels seen in human vitreous, decorin did not prevent fibrosis or EMT in vitro, and higher initial vitreous decorin levels were associated with the development of postoperative PVR after vitrectomy to treat RRD, but did not reliably predict the outcome.

**Keywords:** proliferative vitreoretinopathy, vitreous humor, epithelial to mesenchymal transition, transforming growth factor beta 2, retinal detachment

Proliferative vitreoretinopathy (PVR) describes a retinal scarring process that occurs in 5% to 10% of rhegmatogenous retinal detachment (RRD) patients and up to 50% of open globe injury patients and is the main cause of surgical failure after retinal detachment surgery.<sup>1</sup> Clinically, PVR is characterized by the growth and contraction of cellular fibrotic membranes within the vitreous, retina, and subretinal space, which exert traction, open treated breaks, create new breaks, and cause redetachment or distort the macula. Although the clinical manifestations of PVR are wide, they share a common sequence of underlying cellular responses that are associated with RRD and vitreous changes, and eyes with existing PVR are at higher risk of increased retinal cell proliferation after repeat vitreoretinal surgery.<sup>2</sup>

The TGF- $\beta$  superfamily modulates cell growth, inflammation, matrix synthesis, and apoptosis.<sup>3</sup> TGF- $\beta$  exists in two main forms (TGF- $\beta$ 1 and TGF- $\beta$ 2), with TGF- $\beta$ 2 being the predominant form in the posterior segment of human eyes.<sup>4,5</sup> Both in vitro and in vivo, TGF- $\beta$  isoforms regulate synthesis and degradation of extracellular matrix (ECM) proteins, causing increased collagen accumulation and fibrosis,<sup>6</sup> which makes them key candidate prognostic biomarkers for PVR development and important therapeutic targets for prophylaxis and treatment of PVR. TGF- $\beta$  is secreted as part of a latent complex,<sup>7</sup> cleaved into its active form by RPE-derived thrombospondin-1. Activated TGF- $\beta$  causes RPE cells to undergo epithelial-mesenchymal transition (EMT) to become fibroblasts, to produce type 1 collagen, and to become



myofibroblast-like in the absence of normal cell-cell or cell-matrix interactions *in vitro*.<sup>8-10</sup> There are separate TGF- $\beta$ 1 and TGF- $\beta$ 2 receptors, though many of these are cross-reactive.<sup>11,12</sup> Several studies have found elevated TGF- $\beta$ 2 levels in vitreous samples from patients with PVR,<sup>5,13,14</sup> suggesting a role in pathogenesis and potential as a predictive biomarker for PVR development.

Decorin is a small leucine-rich proteoglycan matrix-constituent that opposes the actions of TGF- $\beta$  by interacting with TGF- $\beta$  and other cytokines. Decorin modulates matrix metalloproteinase (MMP) activity by increasing levels of plasminogen<sup>15</sup> favoring higher ECM turnover leading to ECM degradation. Decorin elevates MMP2 and MMP9 levels, reduces fibronectin and laminin deposition,<sup>16</sup> and attenuates fibrosis in animal models of many pathological conditions, including proliferative vitreoretinopathy,<sup>17</sup> renal fibrosis,<sup>18</sup> lung fibrosis,<sup>19</sup> juvenile communicating hydrocephalus,<sup>20</sup> and spinal cord injury.<sup>5,21</sup>

We hypothesized that TGF- $\beta$  would induce EMT in RPE cells *in vitro* and that decorin would oppose EMT. We also predicted that, in humans, high vitreous TGF- $\beta$  and low vitreous decorin levels would predict the subsequent development of PVR in patients with RRD and that RRD patients would have higher vitreous TGF- $\beta$  levels than patients with macular holes (MH).

We investigated the effects of TGF- $\beta$ 2 and decorin on ARPE-19 cell EMT *in vitro*, we looked for immunohistologic evidence of EMT in human PVR membranes, and we performed a prospective noninterventional cohort study of MH and RRD patients, following them up for 6 months to determine PVR development. Eyes with macula holes do not develop intraocular scarring despite having a retinal break and were used as a control for retinal detachment cases where there is a retinal break and a risk of intraocular scarring.

## METHODS

### Cell Culture

ARPE-19 cells (CRL-2302; ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium/F12 supplemented with 100 U/mL penicillin/streptomycin and 10% heat-inactivated fetal bovine serum. Cells were incubated at 37°C in a humidified environment containing 5% CO<sub>2</sub>. For real-time PCR (qPCR), 300,000 cells/well were seeded in a six-well plate, incubated overnight, and washed with PBS to remove FBS. Cells were then either treated with serum plus medium, 10 ng/mL transforming growth factor  $\beta$ 2 (TGF- $\beta$ 2) in serum-free (–serum) media or given –serum medium only. After 72 hours, cells were treated with decorin concentrations from 1 ng/mL up to 100  $\mu$ g/mL with and without the previous treatment of TGF- $\beta$ 2 in –serum medium and left for a further 72 hours, after which they were harvested. All cell cultures were performed in triplicate experiments, and each experiment was repeated on three independent occasions ( $n = 3$  repeats in total).

### Real-Time Quantitative qPCR Analysis

RNA was extracted from ARPE-19 cells using a purification mini kit according to the manufacturer's instructions (Qiagen RNeasy; Qiagen, Hilden, Germany). RNA was reverse transcribed using a cDNA synthesis kit (Sensifast; Bioline, London, UK). qPCR was carried out using a fluorescent dye (SYBR Green Master Mix; Applied Biosystems, Foster City, CA, USA) in 20- $\mu$ L reactions using a multicolor qPCR detection system (iQ5; Bio-Rad, Watford, UK). Primers were optimized using a seven-point standard curve (Supplementary Table S1). Genes of interest were normalized to human GAPDH using the  $\Delta\Delta$ Ct

method based on primer efficiencies (HK-SY-hu-1200; Primer-design, Camberley, UK). Data are presented normalized to TGF- $\beta$ 2-treated groups or the –serum group.

### Bromodeoxyuridine (BrdU) ELISA

The ELISA was performed as previously described.<sup>22</sup> Please see Supplementary Methods.

### Clinical Studies

This cohort study was approved by the Solihull National Research Ethics Service committee and the use of tissue for immunohistochemistry by Arden Tissue Bank. The study was conducted in accordance with the Declaration of Helsinki.

We included patients in the cohort study who were undergoing vitrectomy for new RRD, MH, and established PVR and who were capable of consenting to participation. We excluded patients who had any previous vitreoretinal surgery (except for the established PVR group) or were aged under 10.

Tissue for immunohistochemistry was collected with consent from patients who were undergoing vitrectomy with membrane peel for PVR or internal limiting membrane (ILM) peel for MH repair. Total number of samples collected for each group is represented in Supplementary Figure S1.

### Sample Collection

Vitreous was collected into a 5-mL syringe through an unprimed cutter at the start of surgery in the largest safe volume possible, ranging between 0.5 and 2 mL, before the infusion was turned on. Samples were refrigerated for up to 4 hours before being centrifuged at 12,300 *g* for 10 minutes and the supernatant collected and frozen at –80° until analysis.

### 3, 3-Diaminobenzidine (DAB) Staining of Human PVR and ILM Tissues

The membranes were removed from fixative and embedded in warm agar. Set agar blocks were processed into paraffin wax using a tissue processor (study reference: 17-0477) (Path-Center; Shandon Scientific, Runcorn, UK).

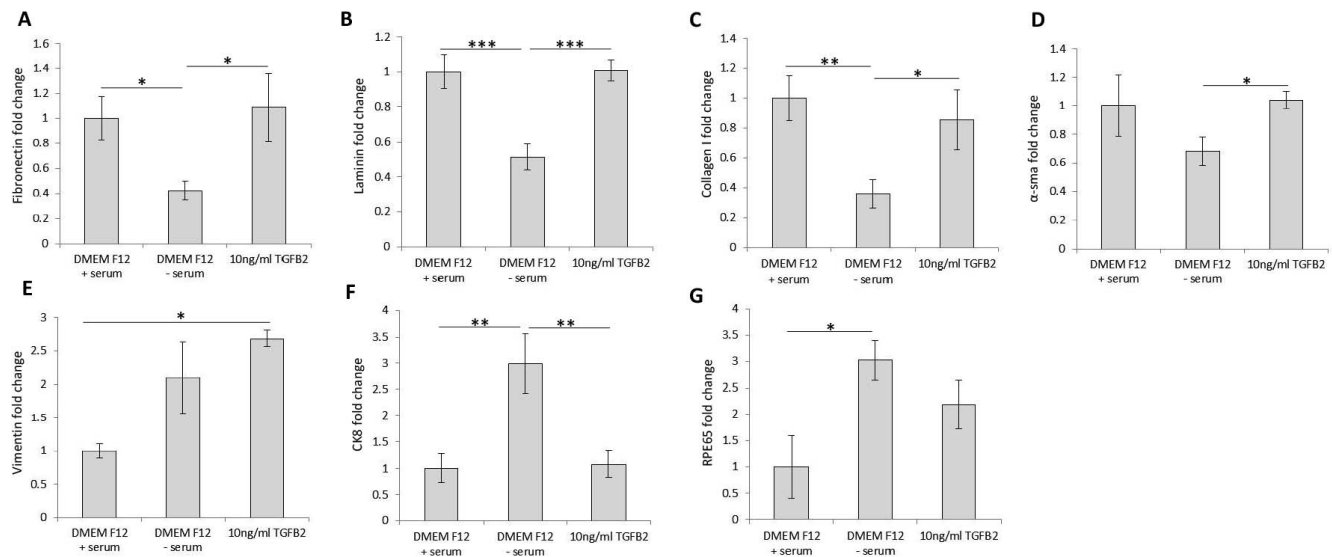
Blocks were sectioned at 5  $\mu$ m using a microtome (Bright 5040; Bright Instruments, Luton, UK) and placed onto slides (Superfrost; Thermo Fisher Scientific UK Ltd, Loughborough, UK) and dried overnight at 50°C. Slides were stained by DAB according to the manufacturer's instructions (Leica, Wetzlar, Germany). Please see Supplementary Methods.

### TGF- $\beta$ 2 and Decorin ELISA

Sandwich ELISAs (R&D Systems, Minneapolis, MN, USA) were used to measure TGF- $\beta$ 2 and decorin levels in human vitreous samples after vitreoretinal surgery. Total number of samples used for each assay is shown in Supplementary Figure S1. Vitreous samples were normalized according to volume as the protein content was below the level of sensitivity. TGF- $\beta$ 2 and decorin ELISAs were performed in accordance with manufacturer's instructions. Please see Supplementary Methods.

### Statistics

Averages are displayed as means plus or minus standard error in text and figures.



**FIGURE 1.** An in vitro model of EMT in ARPE-19 cells in the presence of serum, without serum, and 10 ng/mL TGF- $\beta$ 2 as characterized by qPCR. Removal of serum from cultures reduced the mRNA expression of EMT markers fibronectin (A), laminin (B), collagen I (C),  $\alpha$ -SMA (D) with no alteration in vimentin (E), and an increase in the expression of epithelial cell markers CK8 (F), RPE65 (G). The addition of 10 ng/mL of TGF- $\beta$ 2 led to increased expression of EMT markers and a reduction in epithelial marker expression, which is consistent with an EMT phenotype. Data are presented as a fold change over GAPDH. Each group is representative of  $n = 3$  with three experimental repeats. Data were analyzed using the 1-way ANOVA where \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Sample size calculation was performed with statistical power analysis software (G\*Power, v. 3.1.4; Kiel University, Kiel, Germany) for a two-sample  $t$ -test with an expected allocation of one PVR case per 10 retinal detachment cases. Pilot data from 46 patients with retinal detachment and 16 patients with MH indicated that vitreous levels of decorin were ( $1463 \pm 219$  vs.  $261 \pm 57$  pg/mL). Power calculation performed in G\*Power for a two-sample  $t$ -test indicated that we would require group sizes of 7 and 71 patients to have 80% power to detect the observed difference.

Our prespecified analysis was a  $t$ -test to compare vitreous decorin levels between patients with retinal detachment who did and who did not develop postoperative PVR; as variances were not homogeneous between groups, the PVR group was smaller and had a higher mean and a higher variance, a Mann-Whitney  $U$  statistic is also presented. For all other analyses of ELISA and PCR data, sample means were compared by 1-way ANOVA with Bonferroni-corrected post-hoc tests. For ELISA data, variances were again nonhomogeneous, with larger variances associated with smaller sample sizes (which tends to cause overly conservative  $P$  values), so Kruskal-Wallis results are also given.  $P$  values were Bonferroni corrected within experiments.

## RESULTS

### ARPE-19 Cell Cultures

To characterize the effects of decorin on RPE cells we developed an in vitro model of RPE EMT. Serum-free ARPE-19 cell cultures expressed low levels of mRNA for connective tissue molecules laminin, fibronectin, and collagen type I and higher levels of the RPE markers RPE65 and CK8. Consistent with not having undergone EMT and not being in a pro-fibrotic state, they also expressed low levels of vimentin and  $\alpha$ -SMA (Fig. 1). In comparison to -serum, cultures supplemented with serum (+serum) had increased expression of laminin ( $P < 0.001$ ), fibronectin ( $P < 0.05$ ), and collagen I ( $P < 0.004$ ) and reduced expression of the RPE markers CK8 ( $P < 0.013$ ) and

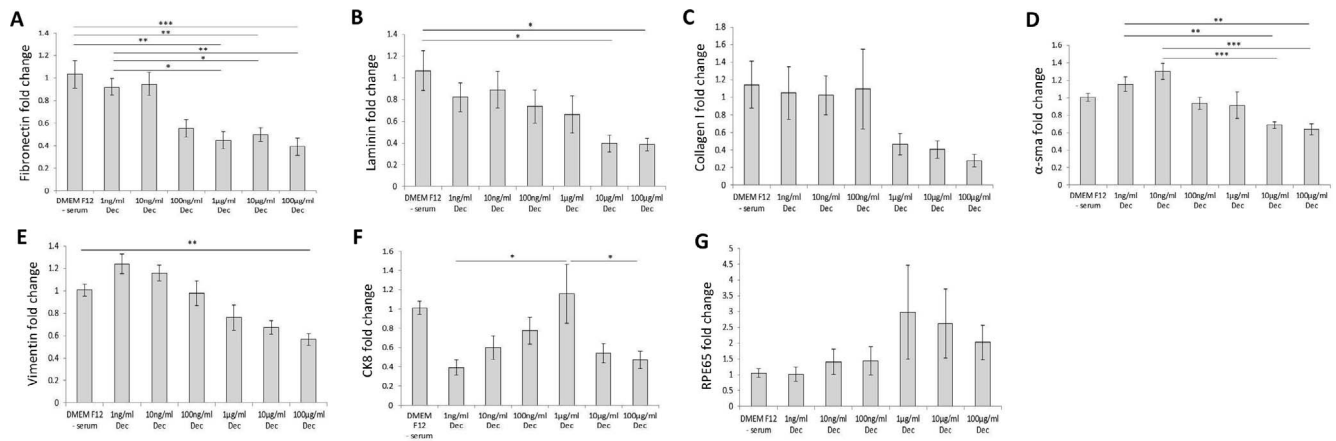
RPE65 ( $P < 0.035$ ). Vimentin and  $\alpha$ -SMA levels were not significantly altered with serum. Serum-free cultures were therefore used to examine the effect of TGF- $\beta$ 2 and decorin on ARPE-19 cells.

### TGF- $\beta$ 2 In Vitro

Compared to -serum, 10 ng/mL of TGF- $\beta$ 2 increased mRNA levels of fibronectin ( $P < 0.05$ , Fig. 1A), laminin ( $P < 0.001$ , Fig. 1B), collagen I ( $P < 0.05$ , Fig. 1C),  $\alpha$ -SMA ( $P < 0.03$ ) and decreased CK8 ( $P < 0.017$ ), which is consistent with an EMT phenotype. However, RPE65 and vimentin expression were not significantly altered with TGF- $\beta$ 2 treatment when compared to -serum control. TGF- $\beta$ 2 at 10 ng/mL did not affect its own expression or that of decorin (Supplementary Fig. S2).

### Cell Culture Effects of Decorin

ARPE-19 cells -serum were treated with decorin in the range of 1 ng/mL to 100  $\mu$ g/mL. There was a strong overall effect of decorin supplementation on laminin, fibronectin,  $\alpha$ -SMA, and vimentin (Fig. 2). Bonferroni post-hoc testing found no evidence that mRNA expression levels of laminin, fibronectin,  $\alpha$ -SMA, and vimentin (Fig. 2) were affected by decorin treatment in the range of 1 to 100 ng/mL. However, decorin concentrations higher than 100 ng/mL reduced fibronectin expression (1  $\mu$ g/mL [ $P < 0.002$ ], 10  $\mu$ g/mL [ $P < 0.02$ ], and 100  $\mu$ g/mL [ $P < 0.001$ ]), laminin (10  $\mu$ g/mL [ $P < 0.048$ ], 100  $\mu$ g/mL [ $P < 0.029$ ]), and vimentin (100  $\mu$ g/mL [ $P < 0.009$ ]).  $\alpha$ -SMA levels were not significantly downregulated when compared to the -serum control, but high-concentration decorin supplementation did reduce  $\alpha$ -SMA expression compared to the low concentration ( $P < 0.001$ ). There was weak evidence that collagen I expression was reduced by an increasing concentration of decorin. CK8 levels were upregulated by 1  $\mu$ g/mL of decorin ( $P < 0.046$ ) and then reduced at 100  $\mu$ g/mL ( $P < 0.045$ ) decorin when compared to 1  $\mu$ g/mL. RPE65 levels showed a weak evidence of a trend similar to that



**FIGURE 2.** mRNA expression analysis of EMT markers in ARPE-19 cells in the presence of low- and high-dose decorin only. ARPE-19 cells were serum starved and treated with decorin in the range of 1 ng/mL to 100 µg/mL for 3 days. The addition of increasing concentrations of decorin led to significant decreases in the mRNA expression of fibronectin (A), laminin (B), collagen I (C),  $\alpha$ -SMA (D), vimentin (E). Decorin doses led to increases in the epithelial marker CK8 (F), which then decreased at the highest doses of 100 µg/mL. Decorin did not significantly alter the expression of RPE65 (G). Data are presented as a fold change over GAPDH. Each group is representative of  $n = 3$  with three experimental repeats. Data were analyzed by 1-way ANOVA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

seen with CK8. Decorin did not regulate its own expression, but did reduce TGF- $\beta$ 2 expression (Supplementary Fig. S2).

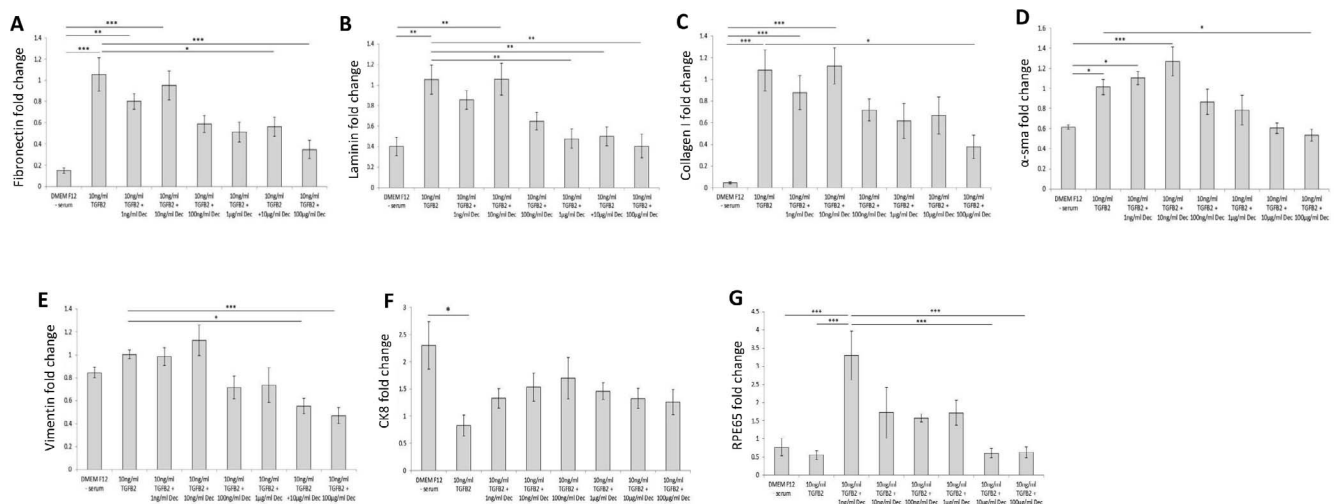
**Cell Culture Effects of TGF- $\beta$ 2 and Decorin**

Serum-free cultures of ARPE-19 cells were treated with 10 ng/mL of TGF- $\beta$ 2 for 3 days followed by 3 days with or without decorin in the range of 1 to 100 µg/mL. BrdU analysis did not show evidence of toxicity in terms of reduced proliferation with TGF- $\beta$ 2 and decorin supplementation in the dose-ranges studied (Supplementary Fig. S3). The addition of decorin at concentrations of 10 to 100 µg/mL abrogated TGF- $\beta$ 2-induced upregulation of fibronectin (Fig. 3A;  $P < 0.001$ ), laminin (Fig. 3B;  $P < 0.001$ ), collagen I (Fig. 3C;  $P < 0.001$ ),  $\alpha$ -SMA (Fig. 3D;  $P < 0.001$ ), and vimentin (Fig. 3E;  $P < 0.001$ ). No changes

were seen in CK8 expression; however, RPE65 expression increased at 1 ng/mL ( $P < 0.001$ ) and decreased at higher concentrations of decorin.

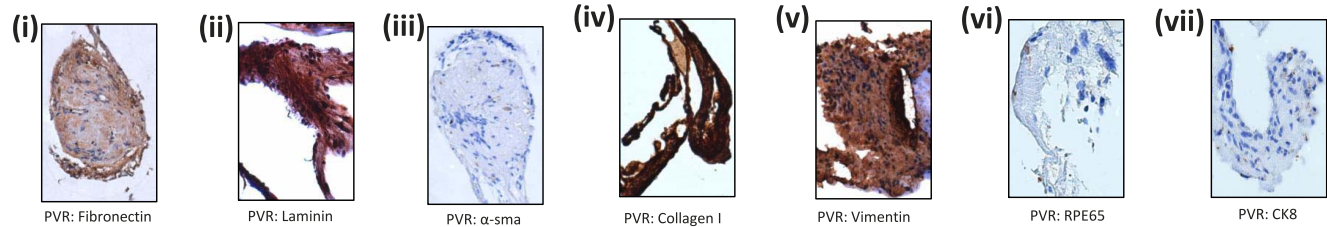
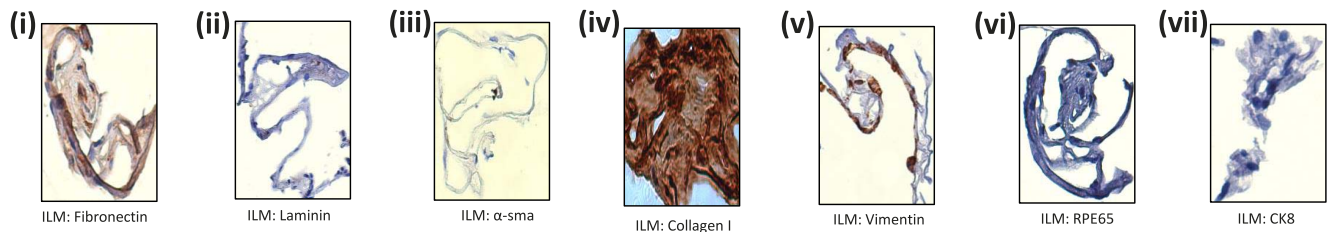
**PVR and ILM Staining**

Immunohistochemical analysis of human ILM and PVR membrane staining showed strong staining for fibronectin and collagen I (Fig. 4A[i], [iv] PVR tissue; Fig. 4B[i], [iv] ILM tissue). Laminin had greater levels of staining in the PVR tissue (Fig. 4A[ii]) with little staining observed in the ILM tissue (Fig. 4B[ii]). Vimentin is present in both tissues but is stronger in the PVR membranes (Fig. 4A[v], Fig. 4B[v]). Very low levels of  $\alpha$ -SMA, RPE65, and CK8 immunostaining were seen in the PVR and ILM (Fig. 4A[iii], [vi], [vii]; Fig. 4B[iii], [vi], [vii]).



**FIGURE 3.** Decorin inhibition of EMT induced in ARPE-19 cells with 10 ng/mL TGF- $\beta$ 2. ARPE-19 cells were serum starved and treated with 10 ng/mL of TGF- $\beta$ 2 for 3 days, after which they were treated with decorin in the range of 1 ng/mL to 100 µg/mL for 3 days. qPCR analysis for mRNA expression revealed an increase of EMT markers with TGF- $\beta$ 2 treatment, which was significantly downregulated with a dose-dependent increase of decorin treatment for fibronectin (A), laminin (B), collagen I (C),  $\alpha$ -SMA (D), vimentin (E), CK8 (F), and RPE65 (G) were then analyzed. Data are presented as a fold change over GAPDH. Each group is representative of  $n = 3$  with three experimental repeats. Data were analyzed by 1-way ANOVA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**A-PVR****B-ILM**

**FIGURE 4.** Representative images of EMT protein in human PVR tissue and human ILM. Tissue was collected from patients and paraffin embedded. DAB staining was carried out to measure protein in PVR tissue (**A**) (i) fibronectin, (ii) laminin, (iii)  $\alpha$ -SMA, (iv) collagen I, (v) vimentin, (vi) RPE65, (vii) CK8, and in ILM tissue (**B**) (i) fibronectin, (ii) laminin, (iii)  $\alpha$ -SMA, (iv) collagen I, (v) vimentin, (vi) RPE65, (vii) CK8 in ILM tissues. *Brown* staining is representative of positive protein expression with strong staining in the PVR and ILM for ECM markers such as fibronectin, collagen I, and vimentin and low levels of expression for the epithelial markers RPE65 and CK8. Hemotoxylin was used as a counterstain to stain the nuclei in *blue*.

**Vitreous Decorin**

We included 85 patients with retinal detachment (of whom 14 developed PVR), 33 patients with MH, and seven with PVR at presentation (Supplementary Fig. S1). Primary analysis showed there was some evidence that vitreous decorin levels were higher in patients with retinal detachment who did develop postoperative PVR ( $2391 \pm 708$  pg/mL, SD 1873 pg/mL) than in those who did not ( $1232 \pm 201$  pg/mL, SD 1694 pg/mL) develop postoperative PVR (*t*-test  $P = 0.038$ ; Mann-Whitney *U* test = 0.075). There was weak evidence that total vitreous decorin levels increased between MH ( $962 \pm 252$  pg/mL), RRD, and those patients who went on to develop postoperative PVR ( $P = 0.09$ , 1-way ANOVA;  $P = 0.075$ , Kruskal-Wallis; Fig. 5). Patients with established PVR ( $1352 \pm 669$ ) had vitreous decorin levels similar to that of RRD patients ( $1231 \pm 201$  pg/mL) and MH ( $962 \pm 252$  pg/mL) patients. The weak association between higher levels of decorin in detachment and those patients who developed postoperative PVR suggests that vitreous decorin levels do not reliably predict the development of PVR but does support the hypothesis that vitreous decorin biology is altered in RRD and PVR, even if the increase in expression is variable.

**Vitreous TGF- $\beta$** 

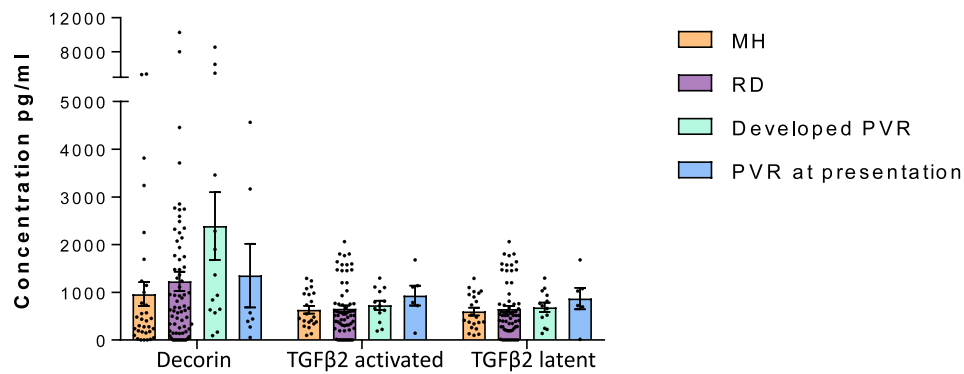
Vitreous TGF- $\beta$  levels were measured in the same patients as was decorin. Vitreous levels of TGF- $\beta$ 1 were undetectable by ELISA. Total vitreous TGF- $\beta$ 2 in patients operated for RRD ( $651 \pm 69$  pg/mL) and MH ( $598 \pm 81$  pg/mL) was not different between any of the groups (Fig. 5), including between RRD patients with an uncomplicated postoperative course, those who went on to develop PVR ( $685 \pm 99$  pg/mL), and those with established PVR ( $869 \pm 224$  pg/mL). Active TGF- $\beta$ 2 levels were low and similar for all groups (RRD  $33 \pm 12$  pg/mL, MH  $38 \pm 14$  pg/mL, developed PVR  $45 \pm 14$  pg/mL, established PVR  $62 \pm 24$  pg/mL).

**DISCUSSION**

In vitro TGF- $\beta$ 2 causes EMT and fibrosis, while decorin is an antifibrotic agent that drives ARPE-19 cells away from a fibrotic phenotype, with downregulation of the same markers of EMT expressed by human PVR membranes. In vivo, we assessed vitreous decorin and TGF- $\beta$ 2 levels in patients with retinal detachment who went on to develop PVR and those who did not and compared these to patients undergoing MH surgery. There was weak evidence of a relationship between the subsequent development of PVR and vitreous decorin levels, with higher levels in RRD compared to MH patients and levels higher still in patients who subsequently developed PVR. The weak relationship and high variability suggest that vitreous decorin at the time of retinal detachment repair would be an unreliable biomarker for PVR development, although the finding may be more mechanistically important, and it may be that a larger study would have had greater power to robustly demonstrate this effect. The absence of a reliable causative relationship from a single cytokine is perhaps not surprising given that PVR is a complex and multifactorial condition and probably represents the end point of a number of different disease processes. As an antifibrotic cytokine, the altered decorin levels may represent a reaction to the exaggerated profibrotic response to retinal detachment seen in PVR.

Before PVR occurs, a retinal break usually exposes RPE cells to vitreous. The subsequent RPE fibrosis with epiretinal, subretinal, and vitreous membrane formation suggests the presence of EMT activators within the vitreous.<sup>23</sup> While the source of these activators is not known, the TGF- $\beta$  superfamily of growth factors plays an important role as an EMT inducer in PVR.<sup>24–26</sup> All three isoforms are present in the eye<sup>25,27</sup>; however, TGF- $\beta$ 1 levels are low or undetectable in the vitreous in comparison to TGF- $\beta$ 2 levels, which are much higher.<sup>4,5,26</sup> This is supported by our results.

Altered TGF- $\beta$ 2 levels have been demonstrated in established PVR and in patients with RRD who subsequently



**FIGURE 5.** Decorin and TGF- $\beta$ 2 levels in the vitreous in patients who had MH and retinal detachment, and those who went on to develop PVR and those with PVR at presentation. TGF- $\beta$ 2 levels were not significantly different between the groups, with active levels in the lower range of 33 to 63 pg/mL. Latent levels of TGF- $\beta$ 2 were higher, 598 to 869 pg/mL, but with no significant changes between the groups. Decorin levels were higher and showed a nonsignificant increase in the developed PVR group. Number of patient samples for the decorin ELISA: MH,  $n = 33$ ; RRD,  $n = 71$ ; developed PVR,  $n = 14$ ; PVR at presentation,  $n = 7$ . Number of patient samples for the latent and active TGF- $\beta$ 2 ELISA: MH,  $n = 21$ ; RRD,  $n = 62$ ; developed PVR,  $n = 13$ ; PVR at presentation,  $n = 6$ . Data were analyzed using the *t*-test and the Mann-Whitney *U* test with a Levine's test for homogeneity of variances.

developed PVR, but this does not reliably and independently predict subsequent PVR development.<sup>5,13,28</sup> In our patients, neither latent nor active vitreous TGF- $\beta$ 2 levels separated RRD from MH patients or patients with established PVR and did not predict subsequent PVR development at the time of initial RRD. The narrow confidence intervals for TGF- $\beta$ 2 levels suggest that this negative finding is robust, and our study is not underpowered with respect to this outcome. Additionally, active TGF- $\beta$ 2 levels were significantly lower than latent TGF- $\beta$ 2 levels in all groups. Therefore, given that TGF- $\beta$ 2 is associated with fibrosis, our data are consistent with RPE EMT caused by increased RPE exposure to vitreous TGF- $\beta$ 2 after migration into the vitreous cavity, rather than elevated vitreous levels per se. It is also possible that TGF- $\beta$ 2 levels vary during the course of PVR development, and our relatively low measurements preceded a rise in TGF- $\beta$ 2 levels in our RRD patients, observed after its effect in the established PVR cases.

Decorin antagonizes TGF- $\beta$  and has independent antifibrotic properties. It presents itself as a strong therapeutic candidate to inhibit TGF- $\beta$ -induced RPE fibrosis.<sup>29</sup> It prevents fibrosis by directly binding and sequestering TGF- $\beta$  and by inhibiting cell migration, proliferation, and extracellular matrix production.<sup>30,31</sup> Intravitreal injections of decorin reduced macroscopic fibrosis in a rabbit PVR model.<sup>17</sup> It is therefore surprising that increased (not reduced) vitreous decorin levels were associated with PVR. We characterized the role of decorin in RPE EMT by developing an *in vitro* model of TGF- $\beta$ -induced ARPE-19 cell EMT. TGF- $\beta$  induced expression of a wide selection of ECM and EMT markers combined with reduced epithelial marker expression in ARPE-19 cells. The mRNA changes *in vitro* reflected the human protein changes seen by immunohistochemistry, supporting the validity of this model.

The rate of PVR in our cohort was 11% (14/125; 95% confidence interval: 6.5%–18.4%), which is toward the upper end of published rates (5.1%–11.7%).<sup>32</sup> The Birmingham and Midland Eye Centre is a regional tertiary referral center for vitreoretinal surgery and it may be that the case mix in our institution has a high risk of PVR. As the study was not designed to evaluate PVR rates, we cannot comment on the extent of preoperative risk factors that may affect these rates in our cohort.

We treated cells with decorin at a range of concentrations to compare physiologic vitreous levels and those that would occur after therapeutic supplementation. At low, physiologic concentrations, decorin did not inhibit the production of ECM markers induced by TGF- $\beta$ 2 but did induce the expression of RPE65, a specific RPE marker. Decorin supplementation at higher concentrations (>1–10  $\mu$ g/mL) significantly abrogated TGF- $\beta$ -induced upregulation of ECM and EMT markers, confirming the feasibility of decorin as an antifibrotic agent. The effects of decorin on CK8 expression after TGF- $\beta$ -induced upregulation of ECM were less pronounced. The variable effects of decorin at different concentrations suggest a biphasic dose-response, which may be related to steric hindrance for some effects at concentrations above 1  $\mu$ g/mL<sup>33</sup> or to a decorin-independent TGF- $\beta$  pathway.<sup>34</sup>

In burn patients, we have found that serum decorin correlates with injury severity,<sup>35</sup> suggesting that its expression may be an injury response, with more severely damaged retinæ expressing more decorin, though not at levels sufficient to prevent intraocular fibrosis. Unfortunately, we do not have sufficient clinical data to test this hypothesis. The most effective antifibrotic concentrations were in the microgram per milliliter range, suggesting that any therapeutic intervention to prevent or treat PVR should use decorin aiming for vitreous concentrations >1  $\mu$ g/mL.

An important feature of PVR is the proliferation of RPE cells to produce epiretinal membranes. Our results suggest that this proliferation is not initiated by elevated vitreous TGF- $\beta$ 2, consistent with current literature suggesting that TGF- $\beta$ s are not involved in the initiation of proliferation.<sup>9,28</sup> Additionally, decorin, which antagonizes TGF- $\beta$ , did not affect RPE proliferation. Decorin has a variable antiproliferative effect, which differs between cell types.<sup>29,36</sup> As the study was restricted in sample collection by clinical treatment, it was not possible to carry out more relevant serial assays that may have highlighted variations in decorin levels over time.

The data presented show that vitreous levels of TGF- $\beta$ 2 and decorin in isolation are unlikely to be useful predictors of disease at the stages that we assayed, although elevated vitreous decorin may represent a response to injury. Our *in vitro* analysis shows that TGF- $\beta$ 2 induces EMT in human RPE cells and that decorin reverts the cells to a more RPE-like

phenotype, meaning that decorin may have potential as part of a clinical treatment strategy for PVR.

### Acknowledgments

The authors thank Jon Bishop, MD, (NIHR Surgical Reconstruction and Microbiology Centre) for statistical advice and the following surgeons who provided vitreous samples (in addition to R.A.H. Scott): Peter Cikatricis, Arijit Mitra, Kim Son Lett, Ash Sharma, and Ajai Tyagi.

Supported by the National Institute of Health Research Surgical Reconstruction and Microbiology Centre (NIHR SRMRC), with funding from the Ministry of Defence, NIHR, University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham.

Disclosure: **G. Begum**, None; **J. O'Neill**, None; **R. Chaudhary**, None; **K. Blachford**, None; **D.R.J. Snead**, None; **M. Berry**, None; **R.A.H. Scott**, None; **A. Logan**, None; **R.J. Blanch**, None

### References

- Sadaka A, Giuliari GP. Proliferative vitreoretinopathy: current and emerging treatments. *Clin Ophthalmol*. 2012;6:1325-1333.
- Bonnet M. The development of severe proliferative vitreoretinopathy after retinal detachment surgery. Grade B: a determining risk factor. *Graefes Arch Clin Exp Ophthalmol*. 1988;226:201-205.
- Taipale J, Saharinen J, Keski-Oja J. Extracellular matrix-associated transforming growth factor-beta: role in cancer cell growth and invasion. *Adv Cancer Res*. 1998;75:87-134.
- Pfeffer BA, Flanders KC, Guérin CJ, Danielpour D, Anderson DH. Transforming growth factor beta 2 is the predominant isoform in the neural retina, retinal pigment epithelium-choroid and vitreous of the monkey eye. *Exp Eye Res*. 1994; 59:323-333.
- Connor TB Jr, Roberts AB, Sporn MB, et al. Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. *J Clin Invest*. 1989;83:1661-1666.
- Verrecchia F, Mauviel A. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. *J Invest Dermatol*. 2002;118:211-215.
- Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci*. 2003;116:217-224.
- Dvashi Z, Goldberg M, Adir O, Shapira M, Pollack A. TGF-beta1 induced transdifferentiation of rpe cells is mediated by TAK1. *PLoS One*. 2015;10:e0122229.
- Tamiya S, Liu L, Kaplan HJ. Epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell-cell contact. *Invest Ophthalmol Vis Sci*. 2010;51:2755-2763.
- Kimoto K, Nakatsuka K, Matsuo N, Yoshioka H. p38 MAPK mediates the expression of type I collagen induced by TGF-beta 2 in human retinal pigment epithelial cells ARPE-19. *Invest Ophthalmol Vis Sci*. 2004;45:2431-2437.
- Chen YS, Hackett SE, Schoenfeld CL, Viores MA, Viores SA, Campochiaro PA. Localisation of vascular endothelial growth factor and its receptors to cells of vascular and avascular epiretinal membranes. *Br J Ophthalmol*. 1997;81:919-926.
- Memon MA, Anway MD, Covert TR, Uzumcu M, Skinner MK. Transforming growth factor beta (TGFbeta1, TGFbeta2 and TGFbeta3) null-mutant phenotypes in embryonic gonadal development. *Mol Cell Endocrinol*. 2008;294:70-80.
- Kon CH, Occlleston NL, Aylward GW, Khaw PT. Expression of vitreous cytokines in proliferative vitreoretinopathy: a prospective study. *Invest Ophthalmol Vis Sci*. 1999;40:5-12.
- Hinton DR, He S, Jin ML, Barron E, Ryan SJ. Novel growth factors involved in the pathogenesis of proliferative vitreoretinopathy. *Eye*. 2002;16:422-428.
- Davies JE, Tang X, Denning JW, Archibald SJ, Davies SJ. Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. *Eur J Neurosci*. 2004;19:1226-1242.
- AKH A. Matrix metalloproteinases and their inhibitors in kidney scarring: culprits or innocents. *J Health Sci*. 2009;55: 473-483.
- Nassar K, Lüke J, Lüke M, et al. The novel use of decorin in prevention of the development of proliferative vitreoretinopathy (PVR). *Graefes Arch Clin Exp Ophthalmol*. 2011;249: 1649-1660.
- Isaka Y, Brees DK, Ikegaya K, et al. Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. *Nat Med*. 1996;2:418-423.
- Giri SN, Hyde DM, Braun RK, Gaarde W, Harper JR, Pierschbacher MD. Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis. *Biochem Pharmacol*. 1997;54:1205-1216.
- Botfield H, Gonzalez AM, Abdullah O, et al. Decorin prevents the development of juvenile communicating hydrocephalus. *Brain*. 2013;136:2842-2858.
- Davies JE, Tang X, Bournat JC, Davies SJ. Decorin promotes plasminogen/plasmin expression within acute spinal cord injuries and by adult microglia in vitro. *J Neurotrauma*. 2006; 23:397-408.
- Hawker JR Jr. Chemiluminescence-based BrdU ELISA to measure DNA synthesis. *J Immunol Methods*. 2003;274:77-82.
- Parapuram SK, Chang B, Li L, et al. Differential effects of TGFbeta and vitreous on the transformation of retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2009; 50:5965-5974.
- Hirayama K, Hata Y, Miura M, et al. The involvement of the rho-kinase pathway and its regulation in cytokine-induced collagen gel contraction by hyalocytes. *Invest Ophthalmol Vis Sci*. 2004;45:3896-3903.
- Saika S. TGFbeta pathobiology in the eye. *Lab Invest*. 2006; 86:106-115.
- Kita T, Hata Y, Arita R, et al. Role of TGF-beta in proliferative vitreoretinal diseases and ROCK as a therapeutic target. *Proc Natl Acad Sci U S A*. 2008;105:17504-17509.
- Paquale LR, Dorman-Pease ME, Luty GA, Quigley HA, Jampel HD. Immunolocalization of TGF-beta 1, TGF-beta 2, and TGF-beta 3 in the anterior segment of the human eye. *Invest Ophthalmol Vis Sci*. 1993;34:23-30.
- Ghrably IE, Powe DG, Orr G, et al. Apoptosis in proliferative vitreoretinopathy. *Invest Ophthalmol Vis Sci*. 2004;45:1473-1479.
- Neill T, Schaefer L, Iozzo RV. Decorin: a guardian from the matrix. *Am J Pathol*. 2012;181:380-387.
- Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature*. 1990;346:281-284.
- Winnemoller M, Schmidt G, Kresse H. Influence of decorin on fibroblast adhesion to fibronectin. *Eur J Cell Biol*. 1991;54: 10-17.
- Kwon OW, Song JH, Roh MI. Retinal detachment and proliferative vitreoretinopathy. *Dev Ophthalmol*. 2016;55: 154-162.
- Dugan TA, Yang VWC, McQuillan DJ, Hook M. Decorin modulates fibrin assembly and structure. *J Biol Chem*. 2006; 281:38208-38216.
- Wu Z, Horgan CE, Carr O, Owens RT, Iozzo RV, Lechner BE. Biglycan and decorin differentially regulate signaling in the fetal membranes. *Matrix Biol*. 2014;35:66-275.

35. Al-Tarrah KS, Lee KC, Bamford AL, et al. Circulating biomarkers may predict burn scar severity: a pilot study (Poster). 8th World Congress on European Paediatric Burns, Birmingham, UK 21-23rd 2017.
36. Xu G, Guimond MJ, Chakraborty C, Lala PK. Control of proliferation, migration, and invasiveness of human extravillous trophoblast by decorin, a decidual product. *Biol Reprod.* 2002;67:681-689.