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Manipulating ocular endothelial tight junctions: Applications in treatment of retinal disease pathology and ocular hypertension

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ABSTRACT

Protein levels of endothelial tight-junctions of the inner retinal microvasculature, together with those of Schlemm's canal, can be readily manipulated by RNA interference (RNAi), resulting in the paracellular clefts between such cells to be reversibly modulated. This facilitates access to the retina of systemically-deliverable low molecular weight, potentially therapeutic compounds, while also allowing potentially toxic material, for example, soluble Amyloid-β1-40, to be removed from the retina into the peripheral circulation. The technique has also been shown to be highly effective in alleviation of pathological cerebral oedema and we speculate that it may therefore have similar utility in the oedematous retina. Additionally, by manipulating endothelial tight-junctions of Schlemm's canal, inflow of aqueous humour from the trabecular meshwork into the Canal can be radically enhanced, suggesting a novel avenue for control of intraocular pressure. Here, we review the technology underlying this approach together with specific examples of clinical targets that are, or could be, amenable to this novel form of genetic intervention.

Contents

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1. Introduction

Endothelial cells lining the cerebral and inner retinal microvasculatures, together with those of the Canal of Schlemm, possess tight-junctions. In the brain and retina, tight junctions form an exceedingly tight seal, with very low rates of fluid-phase transcytosis. Such junctions, constituting the blood-brain and inner blood-retina barriers (BBB/iBRB), prevent potentially noxious materials, including for example, low molecular weight blood-borne enzymes, anaphylatoxins, antibodies etc., from entering and damaging neurological tissues (Hawkins and Davis, 2005; Abbott et al., 2010; Campbell et al., 2011, 2012; Campbell and Humphries, 2012). Additionally, in the anterior segment of the eye, Schlemm's Canal (SC) constitutes part of the conventional aqueous humour outflow pathway, aqueous produced by the ciliary body passing into the anterior chamber of the eye and then into the trabecular meshwork, from where it filters into the canal across its endothelial barrier (Stamer and Clark, 2017). While weaker and less well characterised than their counterparts of the inner retinal vessels, the tight junctions of canal endothelia are dynamically responsive to fluctuations in intraocular pressure (see Figs. 1 and 2) (Ye et al., 1997). Since it is well proven and within our capabilities to artificially modulate levels of transcript encoding tight-junctions, a means exists to enhance the permeability of the inner retinal vessels and the Canal for potentially therapeutic purposes.

Here, we provide an overview of the structure of tight junctions and of how levels of transcripts encoding tight junction components can be reversibly modulated both in retinal and SC endothelial cells. We provide examples of how low molecular weight compounds can be systemically delivered to the retina in its 'barrier modulated' state, and speculate on those degenerative retinal conditions in which oedema accumulates in the presence of an essentially intact iBRB and which could, in principle, be targeted using this approach. In the context of glaucoma, up to six percent of cases of open-angle disease are bilaterally sub-optimally responsive to standard topically-applied pressure-reducing medications (Kass et al., 2002) and for the most commonly used prostaglandin analogue, Latanoprost, between 25 and 50% of patients do not achieve greater than a 20% reduction in intraocular pressure (Scherer, 2002; Noecker et al., 2003). These topical formulations act largely by inhibiting aqueous secretion by the ciliary body or to increase aqueous outflow through the unconventional drainage pathway. Down regulation of SC endothelial tight junctions has been shown to increase aqueous humour outflow in experimental animal model systems (Tam et al., 2017) and could form the basis of a radical therapeutic approach targeting the major aqueous humour outflow system of the eye.

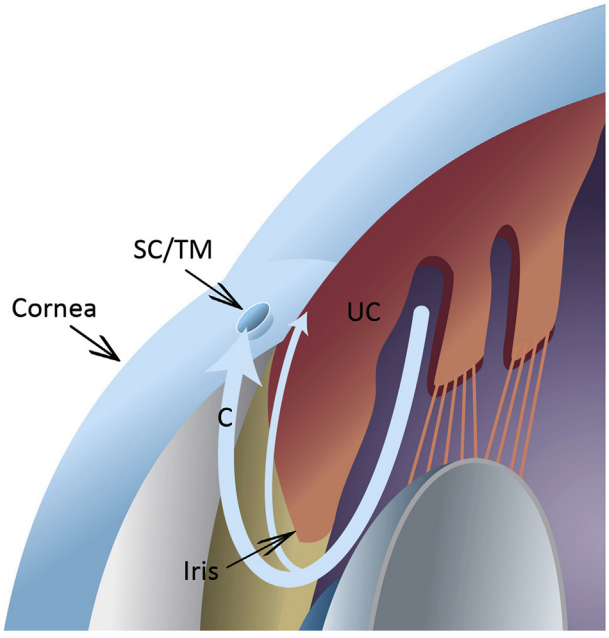

Fig. 1. Aqueous humour is secreted by the ciliary body and moves through the pupil, around the iris. A pressure gradient directs it toward the SC lumen, where most aqueous egresses (red arrow). This is termed the conventional pathway (C). The unconventional pathway (UC) involves the removal of aqueous through the fibres of the ciliary body into the supraciliary and suprachoroidal spaces.

2. Structure and organization of endothelial tight junctions of cerebral and inner retinal vasculatures

Tight junctions (TJs), are essentially contact points between the plasma membranes of adjacent cells, or indeed the point where one endothelial cell contacts itself in a microvessel. TJs are located at the apical periphery of the plasma membrane, and each TJ is paired to and associates with another TJ on the membrane of the adjacent cell. TJs have numerous functions and can act as sites for vesicle targeting, proliferation, transcription signals, mediating cell polarity. Given their molecular complexity, TJs also act as a physical barrier to limit paracellular diffusion of solutes across the BBB/iBRB and indeed the endothelium of Schlemm's canal.

Typically, each TJ consists of at least three different types of protein; 1) occludin, 2) junction adhesion molecule (JAM) family of proteins, and 3) claudin proteins (Ben-Yosef et al., 2003). However, the TJ itself can consist of over 40 individual proteins between support proteins, structural proteins, transport proteins, and other more unique proteins such as tricellulin, a homologue of occludin that concentrates when three cells come together (Anderson and Van Itallie, 2008). In addition, the actin cytoskeleton of the cell is critical for TJ formation, as actin strands bind the PDZ-domain containing scaffolding proteins ZO-1, ZO-2, ZO-3, MAGI-1, PatJ, PALS1 and MUPP1, which can subsequently bind to the TJ proteins that can mediate extracellular interaction with adjacent TJ proteins.

During the assembly stage of the TJ, the proteins Par3, Par6 and aPKC are critical and the TJ is also important for the function of the basolateral-located adherens junctions, which are largely cadherin based junctions. Indeed, the protein VE-cadherin can act as a mediator of intracellular signalling via its interaction with phosphatidylinositol-3-OH kinase or other growth factor receptors. For example, VE-cadherin has been shown to interact directly with β-catenin, which can subsequently regulate cellular homeostasis or responses to cellular stress (Taddei et al., 2008). Paracellular transport of molecules from blood to retina or across the Schlemm's canal endothelium is exclusively passive, driven predominantly by concentration gradients (Van Itallie and Anderson, 2004).

The molecular composition and indeed the overt permeability of TJs varies considerably amongst different tissue types. For example, some tissues display variable electrical conductance, charge selectivity, non-charged solute permeability, and size selectivity, and this is reflected to a large degree on the density of claudin proteins present in their TJs. Importantly, TJs are distinguished from adherens junctions (AJs) in that they are located at the apical periphery of the contact point of endothelial cells, with AJs expressed below them. Additionally, AJs have a different molecular composition, being enriched with cadherins, catenins and nectin amongst others.

Structurally claudins are integral membrane proteins with four transmembrane domains and two extracellular and one intracellular loop. In the first extracellular loop claudins have a common WGLWCC motif, and they also possess a C-terminus PDZ domain. This region binds to the PDZ domains of the TJ support proteins,

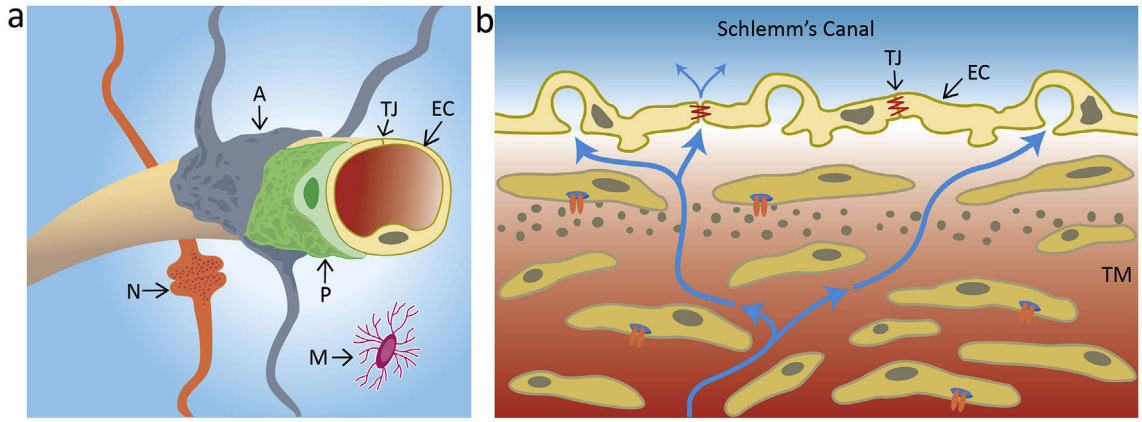

Fig. 2. a) Structure of the neurovascular unit of the inner blood retina barrier (iBRB) comprising endothelial cells (EC), pericytes (P) and atrocyte foot processes (A). The contacting point of an iBRB EC is where the tight junction (TJ) is formed. Neurons (N) and microglia (M) are located on the retina aspect of the iBRB. **b)** Schlemm's canal endothelial cells contain TJs but also manifest giant vacuoles to allow for aqueous humour movement (see arrows for directionality of flow) into Schlemm's canal via the trabecular meshwork (TM).

such as ZO-1, ZO-2, ZO-3 (Itoh et al., 1999) and MUPP-1 (Hamazaki et al., 2002). The C-terminus tail, is also the region which confers stability to claudins, and swapping the C-terminus tails has been found to coincide with a reversal of protein half-lives (Van Itallie et al., 2004).

Various post-translational modifications have also been implicated in claudin function. For example, phosphorylation has been shown to increase claudin1-4 permeability to chloride ions (Yamauchi et al., 2004), and loss of palmitoylation sites on claudin-14 results in reduced localization of the protein to the membrane (Van Itallie et al., 2005).

Some claudins, such as claudin-1, are widely expressed (Van Itallie and Anderson, 2004), whereas other claudin proteins are expressed only in certain cell types or during embryonic development (Turksen and Troy, 2001). This, suggests that claudins play various roles not necessarily limited to the TJs.

Various studies expressing combinations of different claudins in Madin-Darby canine kidney (MDCK) cells found that over-expression of claudin-7 increased paracellular permeability to cations, while reducing it to anions. Additionally, over-expression of claudin-4, -8 and -14 reduced permeability to cations but not anions, while over-expression of claudin-2 decreased barrier integrity without reducing the number of TJ strands (Ben-Yosef et al., 2003; Furuse and Tsukita, 2006).

The finding that claudin-14 reduces permeability to cations aligns with its abundant *in vivo* expression in the outer hair cells of the cochlea in the ear. Here TJs function to separate the K^+-rich endolymph and Na^--rich perilymph, a separation essential for optimal hearing. The importance of claudin-14 for this role is observed in the deafness observed in mice and humans with claudin-14 mutations (Ben-Yosef et al., 2003). Claudin-11-null mice also exhibit deafness, and this is due to a similar role of claudin-11 in maintaining the endocochlear potential in the ear (Kitajiri et al., 2004).

Indeed, mutations in the claudin-16 gene, expressed in the thick ascending limb of the nephron, cause Mg^{2+} reabsorption in humans and cattle, resulting in deficiency of the ion. Claudin-16 contains a number of negatively charged residues in its first extracellular loop, which appear to electrostatically interact with soluble ions, enabling or inhibiting their passage (Simon et al., 1999). Detailed in Table 1, are some key properties of the claudins listed in this section.

As well as mediating selective ion transport across the paracellular pathway, claudins also play a central role in determining the maximum size of molecules that diffuse across an endothelial cell layer. Claudin-1 knockout mice, for example, die within one day of birth due to excessive water loss across the skin. Additionally, mutations in claudin-1 are associated in humans with ichthyosis – a condition manifested by skin dehydration (Hadj-Rabia et al., 2004). These observed phenotypes in humans and other animals lacking claudin proteins demonstrate their importance in regulating TJ function and in controlling passive paracellular diffusion of materials across the TJ. Claudins are distinguished from other tight junction proteins such as occludin in that their extracellular domains have a certain degree of homology and mediate size selectivity and ion flux across the various barriers. Occludin appears to have a more regulatory role at the tight junction.

3. Structure and organization of Schlemm's canal endothelial tight junctions

The endothelial tight junctions of Schlemm's Canal differ from those seen in vascular endothelia in several ways. Early studies using electron microscopy showed electron dense junctions present between endothelial cells of Schlemm's Canal similar to those seen in vascular endothelia (Vegge, 1967). Subsequent freeze fracture studies showed that Schlemm's Canal endothelial cells TJs were composed of parallel junctional strands with minimal branching which did not form complex bi-dimensional networks. The lack of branching between TJ strands in SC endothelial cells leads to the formation of channels between junction strands that are continuous from the juxtacanalicular tissue to the SC lumen. These channels were identified as a potential paracellular route for AH outflow in to the SC lumen through intercellular clefts in the SC inner wall endothelium (Raviola and Raviola, 1981; Bhatt et al., 1995). Early tracer studies using cationised ferritin showed staining of cell membranes lining channels between tight junctions in the SC inner wall endothelium, showing therefore that these channels represented a paracellular pathway across the SC inner wall. Further, these channels were of greater size and number in eyes that were fixed at elevated pressure, (Epstein and Rohen, 1991). Additionally, endothelial tight junctions in perfused human donor eyes were shown by freeze fracture to exist in single and double stranded forms at the majority of TJs, with a minority of TJs having three or more junctional strands. The complexity of these junctions was shown to be responsive to applied pressure in the eye, with the number of junctional strands decreasing as perfusion pressure increased, (Ye et al., 1997). The above features are in

Table 1
Relevant claudins and their chromosomal and tissue expression pattern.

Gene	Protein name	Chromosome	Tissue expression (protein)
CLDN1	claudin 1	3q28	Heart, brain, lung, liver testis
CLDN2	claudin 2	Xq22.3	Liver, kidney
CLDN3	claudin 3	7q11.23	Lung, liver, kidney, testis
CLDN4	claudin 4	7q11.23	Lung, kidney
CLDN5	claudin 5	22q11.21	Brain, heart, lung, liver, kidney, testis, endothelial cells in general
CLDN6	claudin 6	16p13.3	Embryonic tissues
CLDN7	claudin 7	17p13.1	Lung, kidney, testis
CLDN8	claudin 8	21q22.11	Lung, liver, kidney, testis
CLDN10	claudin 10	13q32.1	Liver
CLDN11	claudin 11	3q26.2	Brain, testis
CLDN14	claudin 14	21q22.13	Liver, kidney, ear
CLDN16	claudin 16	3q28	Kidney

contrast to TJs of the inner retinal vascular endothelium, which are composed of complex networks of multiple TJ strands, and do not exhibit dynamic regulation in response to pressure, with no changes to TJ strand structure in response to perfusion pressure (Fujimoto, 1995; Schneeberger and Karnovsky, 1976).

Schlemm's canal endothelial cells also differ from inner retinal vascular endothelial cells in that they have remarkably high hydraulic conductivity, with Schlemm's canal having a hydraulic conductivity of 4000-9000 $\times$ 10^{-11} cm^2 s/g, significantly higher than other ocular barriers, and possibly one of the highest hydraulic conductivities of all body vessel linings (Johnson, 2006). The inner wall endothelium of SC is also permeable to higher molecular weight tracer molecules than other endothelial cells of the eye, with labelled ferritin, 450 kDa, staining the interior of paracellular pores, while the inner blood-retinal barrier (iBRB) excludes molecules as small as 500 Da (Epstein and Rohen, 1991; Campbell et al., 2009). It must be noted that these differences in endothelial permeability cannot be solely apportioned to differences in TJ structure and organization as, in addition to TJ mediated paracellular pores between cells, SCECs possess giant vacuoles containing large intracellular pores. These intracellular pores have an average pore size of approximately 1 μm in diameter, with pores greater than 3 μm reported (see Fig. 3 for an overview of the differences in iBRB and SC endothelia) (Sit et al., 1997).

Human SC endothelial cells have differing TJ protein expression profiles than is seen other endothelial cells. For example, at the iBRB, ZO-1, occludin and claudin-5 are instrumental in maintaining barrier function, with claudin-5 being particularly important in controlling paracellular permeability (Campbell and Humphries, 2012; Morita et al., 1999). Contrastingly, recent studies have shown a more simplistic TJ composition in human SC endothelial cells, with claudin-11 and ZO-1 being the major TJ proteins present, and claudin-5 and occludin being expressed at low levels only (Tam et al., 2017).

4. Induction of inner retinal microvessel permeability: validation of the concept as a potentially therapeutic modality

In the original experimental approach (Campbell et al., 2008, 2009) targeting both the BBB and iBRB simultaneously, 20 μg of siRNA targeting Claudin-5 was hydrodynamically injected into the tail veins of mice in a volume of 10% of the body weight of the animal, a procedure that was well tolerated given the overtly high volumes. Using this approach, maximum suppression of claudin-5 was observed approximately 48 h after each tail injection, levels of claudin-5 returning to normal by 72 h post inoculation. During this period, both the BBB and iBRB became permeable to the perfused nuclear stain, Hoechst H33342 (molecular weight, 563Da). In cryosections of the retina, staining of the inner nuclear

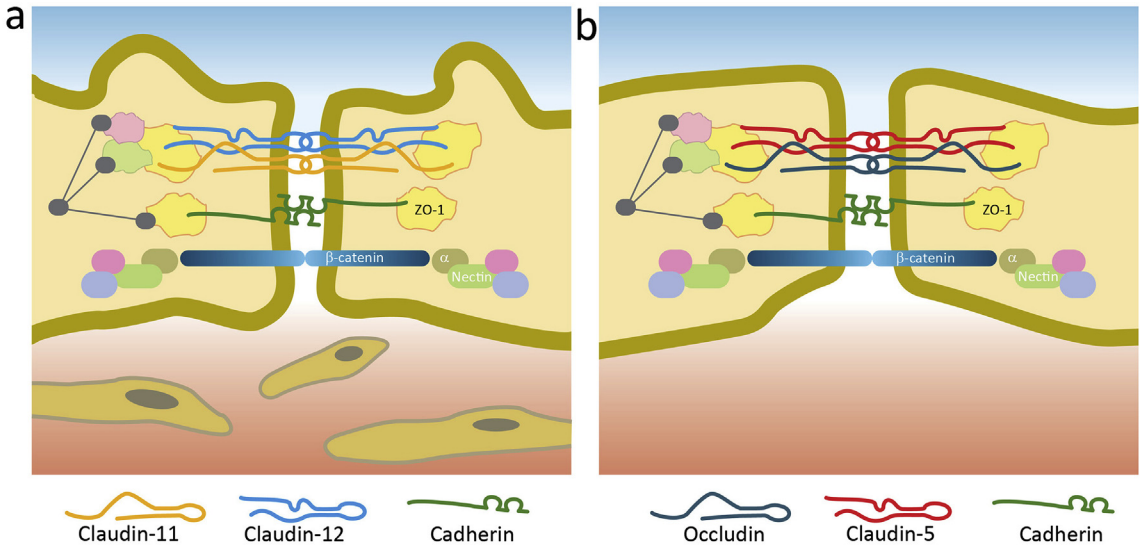

Fig. 3. a) Molecular architecture of the Schlemm's canal endothelial cells shows an enrichment of claudin-11 and claudin-12 at the tight junction. **b)** Tight junctions of endothelial cells associated with the inner blood retina barrier (iBRB) show an enrichment of claudin-5 and occludin at the tight junction.

layer (INL) was evident 24 h post-siRNA injection, while staining of the outer nuclear layer (ONL) became evident at 48 h. However, 72 h after siRNA inoculation, no staining could be detected in any of the nuclear layers (Fig. 4). Under the same conditions, systemically administered FITC-labelled dextran, FD-4 (MW 4,400Da), showed no evidence of being able to access any of the nuclear layers of the retina, these data indicating that down-regulation of claudin-5 facilitates a transient and size-selective enhancement of permeability at the iBRB to compounds of at least 563 Da, but not to those of higher molecular weight. In subsequent studies (Campbell et al., 2012), tail injections were undertaken with siRNA complexed with a clinically enabled polyethylene imine (PEI) carrier, *in vivo*-JetPEI (Polyplus Transfection). 20 μg of claudin-5 siRNA injected in a volume of 0.4 ml with this carrier was shown to be as effective as the hydrodynamic approach in down-regulation of claudin-5. While these initial experiments validated barrier modulation technology as an enabling system for enhancing drug delivery to neural tissues, it was unable to selectively target the retina while leaving the BBB intact.

5. Site-specific modulation of the iBRB

In order to selectively target the retina, claudin-5 shRNA was incorporated into an AAV-2/9 vector, inducible by doxycycline (Campbell et al., 2011). A single once off sub-retinal injection of this vector was required, provision of doxycycline in drinking water (2 mg/ml) being used to induce claudin-5 shRNA expression (viral constructs are now available that are capable of accessing the retina following intravitreal inoculation and there is no reason not to assume that such constructs will act in a similar manner, providing they have tropism for vascular endothelia). The efficacy of this system was initially validated in a light-induced murine model of apoptotic photoreceptor degeneration, in which it has been firmly established that death of photoreceptor cells is calpain-dependent (Perche et al., 2007). N-Acetyl-L-leucyle-L-methioninal; calpain inhibitor II (ALLM), molecular weight 401Da is a potent calpain inhibitor. Albino mice given doxycycline in drinking water, were treated IP with 20 mg/kg ALLM followed by exposure to 7900 lux of white light. After 24 h, photoreceptor cell death was assessed by TUNEL staining of retinal cryosections. As illustrated in Fig. 5, photoreceptor cell viability was extensively preserved in animals systemically treated with the drug which, under conditions where permeability of the iBRB had not been modulated, could not gain sufficiently effective access to the retina to provide therapeutic benefit.

Validating this approach, a murine model of the exudative form of age-related macular degeneration (AMD) was also used (Campbell et al., 2011). Wild type C57BL/6J mice were sub-retinally inoculated in one eye with an AAV-2/9 expressing claudin-5 shRNA and the other eye with an AAV-2/9 expressing a non-targeting shRNA as control. Animals were administered doxycycline (2 mg/kg) for 3 weeks prior to induction of laser burns to the RPE/Bruch's membrane, inducing localized expression of vascular endothelial growth factor (VEGF) and choroidal neovascularisation (CNV) at the site of laser burns. During an interval of 14 days subsequent to laser treatment, mice were administered two systemic (IP) doses either of 17-AAG (30 mg/kg), or sunitinib malate (20 mg/kg), both well characterised and potent inhibitors of VEGFR-2. As shown in Fig. 6, animals having received a claudin-5 targeting vector showed highly significant suppression of CNV compared to the contralateral eye receiving a non-targeting vector.

It is of interest also to note that endothelial tight junctions at the iBRB can be further manipulated in order to increase barrier permeability beyond that achievable using claudin-5 alone. In this regard, Keaney et al. (2015), demonstrated that co-suppression of transcripts encoding claudin-5 and occludin rendered the iBRB reversibly permeable to systemically administered compounds up to approximately 4 kDa in molecular weight. While manipulating permeability to this extent runs the risk of admission into the retina (or brain) of potentially damaging low molecular weight materials such as anaphylatoxins, or low molecular weight enzymes, no observable negative physiological consequences of such modulation were noted. The possible therapeutic implications of this observation will be considered later in this review.

6. On the potential therapeutic utility of manipulation of iBRB permeability

6.1. Experimentally enhancing macular pigment (MP) access to retina

The macular pigments, lutein, zeaxanthin and mesozeaxanthin, act protectively within the retina, acting as filters for short wavelength blue light and also as scavengers of reactive oxygen species (Whitehead et al., 2006). Potentially beneficial effects of dietary supplementation have been investigated in a number of scenarios, including for example, inherited retinal degenerations, specifically retinitis pigmentosa and Usher syndrome (Aleman et al., 2001) and in patients with AMD (Ma et al., 2012; Liu et al., 2014). In the study by Aleman et al. (2001), there was a trend toward more severe progression of disease in those patients who had lower retinal MP levels. In the study by Ma et al. (2012), abnormalities in central retinal function in early AMD were reported to be improved and in the study reported by Liu et al. (2014,2015), a meta-analysis of 1176 AMD patients, both visual acuity and contrast sensitivity were found to improve with supplementation. However, it is well

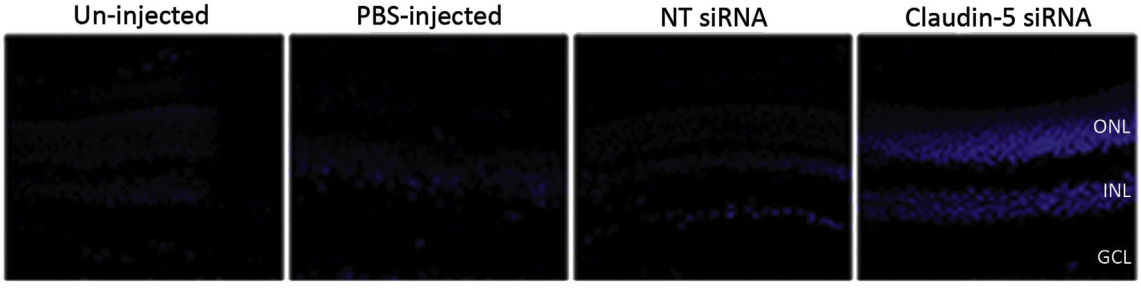

Fig. 4. Extravasation of Hoechst H33342 from the retinal microvessels was manifested by distinct staining of nuclei in the inner nuclear layer (INL) and outer nuclear layer (ONL) 48 h post delivery of claudin-5 siRNA when compared to control groups.
This is part of figure 6 from our paper, Campbell et al., J. Gene Medcine 2008 Aug; 10(8) 930–47

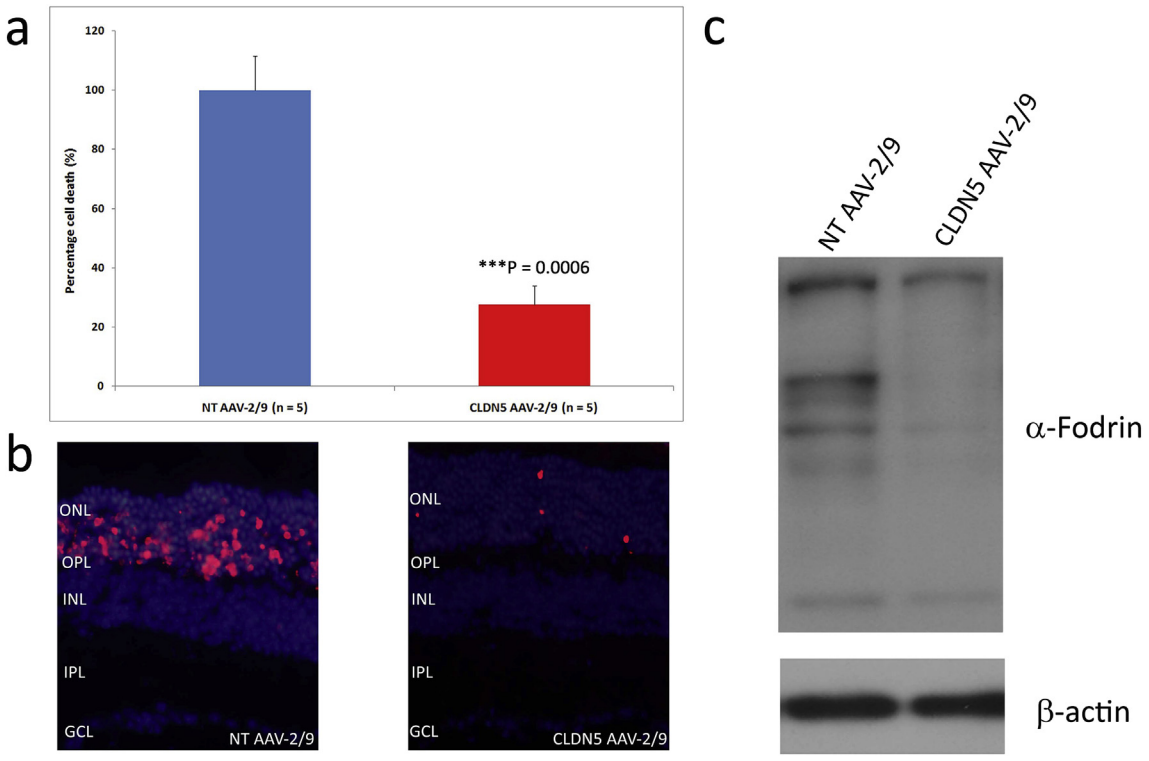

Fig. 5. (a) Albino BalB/c mice were inoculated sub-retinally with either the NT AAV-2/9 in their left eye or the CLDN5 AAV-2/9 in their right eye. Significant protection of photoreceptor cells was observed in the right eyes (CLDN5 AAV-2/9) of mice compared to the left eyes (NT AAV-2/9) (***P = 0.0006). (b) TUNEL positive cells were shown to be consistently localized in large numbers to the outer nuclear layer (ONL) of NT AAV-2/9 injected retinas compared to CLDN5 AAV-2/9 injected retinas. Outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL). (c) Extensive cleavage of the calpain substrate α-fodrin was observed in mice receiving the NT AAV compared to mice receiving the CLDN5 AAV with ALLM prior to light ablation.
This is figure 4 from our paper, Campbell et al., EMBO Molecular Medicine 2011, 3, 235–245.

recognised that not all on supplementation show increases in MP density within the retina, a probable reflection of variability in carotenoid transporter efficiency (40, 43, 44).

While mice do not possess a macula, they nevertheless could represent a highly cost efficient avenue for study of the physiological effects of MPs in normal and degenerating retinas. However, wild type mice do not accumulate these carotenoids within retinal tissues even when supplemented in their diet. Recent studies however, have shown that mice with targeted disruptions of β-carotene oxygenases 1 and 2 (*Bco2*−/− in particular) are able to accumulate MPs within the retina (Li et al., 2017). However, a very large amount of carotenoid ~2.6 mg per mouse per day, equivalent to an average human dose of about 635 mg per day, was required to achieve measurable levels of MP within these retinas. Controlled modulation of the permeability of the iBRB of *Bco2*−/− mice, would enable very much smaller systemic doses of MP to be required in order to achieve elevated MP levels within retinal tissues. Given the fact that lutein and zeaxanthin have molecular weights (569Da), well below the cut off for systemic delivery following transient down regulation of claudin-5, *Bco2*−/− mice would likely be rendered a more versatile and useful model for studies of the physiological or protective effects of MPs within the retina. The same system could be used to experimentally enhance MP uptake in primates.

6.2. Alleviation of retinal oedema

Previous studies from this laboratory (Campbell et al., 2012) have shown that siRNA-mediated down regulation of claudin-5 is highly effective in reducing pathological cerebral oedema in a murine model of traumatic brain injury (TBI). In this model, a small ultra-cold probe is placed for a short period of time onto the skulls of anaesthetised animals. This induces a focal necrotic cerebral lesion and breakdown of the BBB, with extensive extravasation of fluid from the cerebral capillaries into the parenchyma of the brain. The barrier then reforms, pathological oedema remaining within penumbral region of the brain, adjacent to the injury site. [It is of interest to note that TBI accounts for about 1% of all adult mortality worldwide and cerebral oedema induced by out-of-hospital cardiac arrest is similarly prevalent. Treatments involving injection of the osmotic diuretic, mannitol, are archaic and inefficient if oedema persists beyond 24 h. This treatment paradigm has hardly changed in over 80 years]. In the current approach, claudin-5 siRNA complexed with the carrier agent *in-vivoJetPEI* was systemically administered. The transiently modulated BBB allowed efficient fluid drainage from the brain, reducing lesion volumes and improving cognitive function (Fig. 7).

It is of interest to note in the above context, that a number of well-defined retinal conditions are characterised by a build-up of intra-retinal oedema and could be targetable using this approach. In general, retinal oedema as a co-morbidity of a range of retinal conditions will involve vasogenic oedema as the initial insult, *i.e.*, oedema derived from a vascular source, with extravasation of fluid from blood to neural tissues. Vasogenic oedema will lead to acute pressure changes within neural tissue and will lead to eventual cytotoxic oedema, where cells within the penumbral region of injury/damage will gradually decline. Bearing in mind that the retina is simply an extension of the central nervous system (CNS), we speculate that in such conditions, it may be possible to counterbalance fluid exudation into retinal tissues, by enhancing fluid

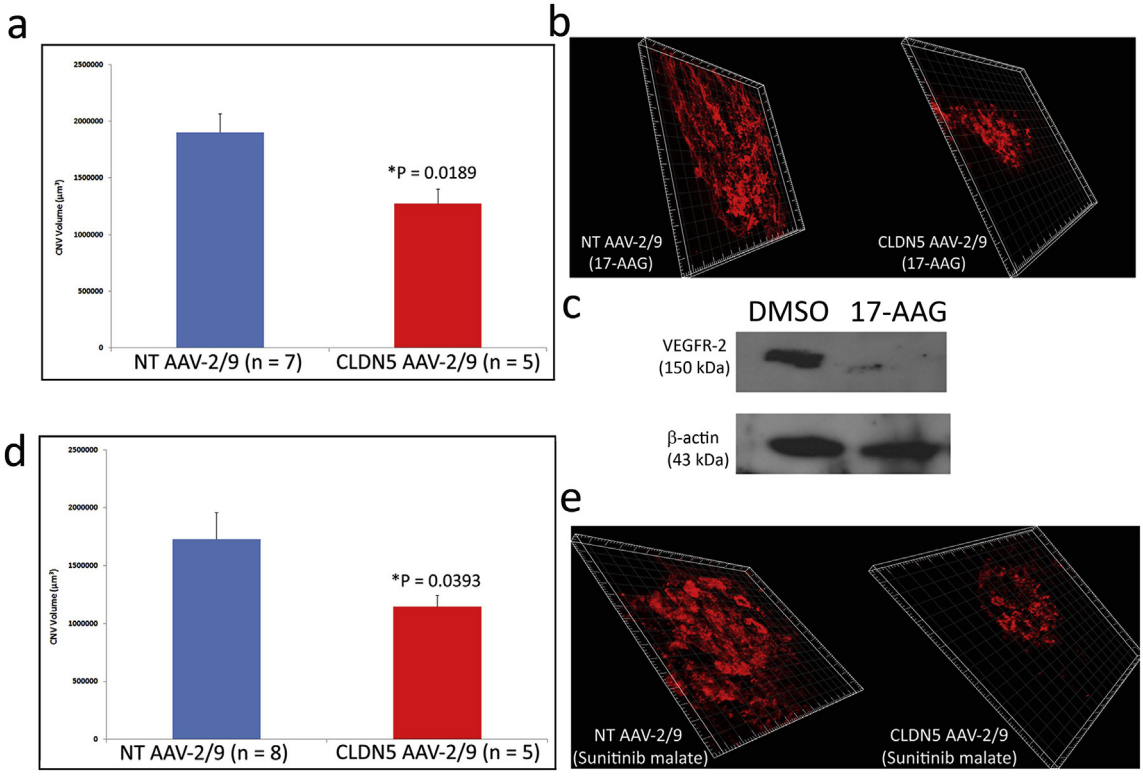

Fig. 6. (a) Albino BalB/c mice were inoculated sub-retinally with either the NT AAV-2/9 in their left eye or the CLDN5 AAV-2/9 in their right eye. Significant protection of photoreceptor cells was observed in the right eyes (CLDN5 AAV-2/9) of mice compared to the left eyes (NT AAV-2/9) (***P = 0.0006). (b) TUNEL positive cells were shown to be consistently localized in large numbers to the outer nuclear layer (ONL) of NT AAV-2/9 injected retinas compared to CLDN5 AAV-2/9 injected retinas. Outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL). (c) Extensive cleavage of the calpain substrate α-fodrin was observed in mice receiving the NT AAV compared to mice receiving the CLDN5 AAV with ALLM prior to light ablation.
This is part of Figure 5 from our paper, Campbell et al., EMBO Molecular Medicine 2011, 3, 235–245.

resorption into the inner retinal vasculature by manipulating iBRB permeability. While this may appear counter-intuitive, the approach is now validated in murine models of TBI, where BBB breakdown is a hallmark pathology. The same will apply to retinal oedematous conditions, where penumbral regions are at risk of perpetuating neural damage. Such conditions include, *inter alia,* Non-arteritic Anterior Ischaemic Opthic Neuropathy (NAION), characterised by axoplasmic flow stasis and extracellular oedema at the optic nerve head and X-linked Juvenile Retinoschisis, in which fluid-filled cavities develop as the retina splits, cavities largely forming in the outer/inner plexiform and inner nuclear layers of the retina (fundus images of these conditions, clearly revealing the presence of retinal oedema are shown in Fig. 8). NAION is caused by reduced blood flow in the posterior ciliary artery, which causes ischaemia in the anterior part of the optic nerve head, resulting in a vasogenic oedema and axoplasmic flow stasis in ganglion cell axons, causing them to swell and become damaged (Hayreh, 2013). It is a major cause of visual handicap in older people, with approximately 6000 cases per year in US (the prevalence is higher in diabetics). 25% of patients experience NAION in contralateral eye within five years of developing the disease and loss of vision is often permanent. Most importantly, there are no effective treatments (Hayreh, 2014). Animal models of NAION are controversial. However, given the impelling data that exist on efficient clearance of oedema from the brain, we suggest that claudin-5 suppression within the inner retinal endothelial vasculature could represent a plausible means of alleviation of optic nerve head swelling. In this regard, we tested the efficacy of intravitreal inoculation of GLP grade claudin-5 siRNA in the Vervet (African Green) monkey. Tissues at the optic nerve head were dissected and subjected to RT-PCR analysis. Clearly, claudin-5 levels can be reduced using this approach (see Fig. 9). In the current context it is of note that intravitreal inoculation is widely accepted in routine ophthalmic practice (some patients have now received several hundred intravitreal injections of Lucentis). Moreover, therapy could readily be packaged as lyophilized compound, reconstituted and administered in outpatient facilities and readout of efficacy over days/weeks following therapy would be rapid, using standard clinical techniques (fluorescein angiography, visual acuity, OCT, ERG).

X-linked juvenile retinoschisis, caused by recessive mutations within the RS1 (Retinoschisin) gene, is characterised by splitting of the retina, particularly in the region of the macula, largely occurring in the OPL, INL and IPL layers (Gerth et al., 2008; Yu et al., 2010; Gregori et al., 2009). Retinoschisin is an extracellular matrix (ECM) protein secreted from a number of retinal cell types including photoreceptors, bipolar, amacrine and ganglion cells, and binding strongly to the membranes of photoreceptors and bipolar cells, stabilizing retinal cellular architecture (Vijayasarathy et al., 2012; Molday et al., 2012). AAV-mediated therapeutic intervention involving either CMV or endogenous promoter-driven expression of the RS1 gene, has now progressed from murine models (Zeng et al., 2004; Bush et al., 2016; Byrne et al., 2014; Dalkara et al., 2013) into clinical evaluation (Clinicaltrials.gov.NCT02416622; NCT0231787). It is also of note that regression of retinal cysts in one patient with juvenile XL retinoschisis was induced by oral administration (500 mg/day over four days) of the carbonic anhydrase inhibitor, acetozolmide (Zhang et al., 2015). The latter is used as a topical medication for open angle

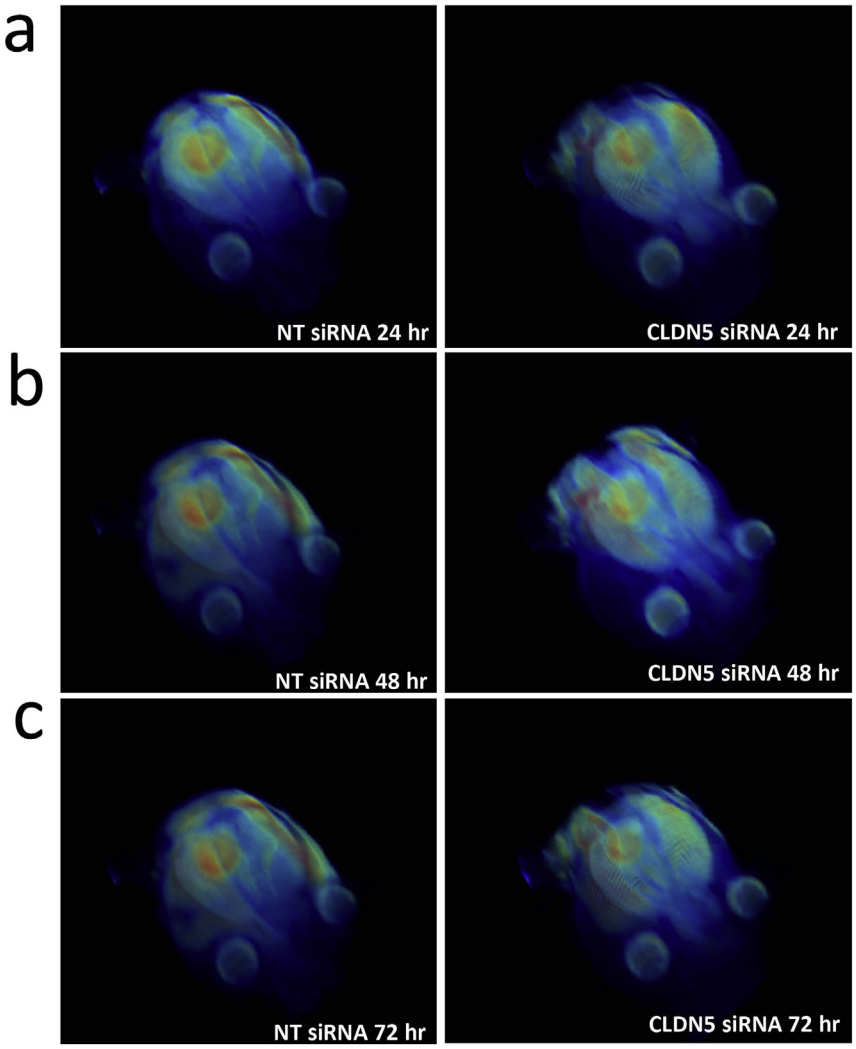

Fig. 7. Three-Dimensional volumetric rendering of MRI data showed lesion volume in red (pseudocolor) with NT siRNA injected mice in the left column and claudin-5 siRNA injected mice in the right column. **a)** NT siRNA and claudin-5 siRNA 24 h post injury. **b)** 48 h post injury. **c)** 72 h post injury.
This is a small part of figure 4 from our paper, Campbell et al., Nature Communications 2012 May 22; 3: 849.

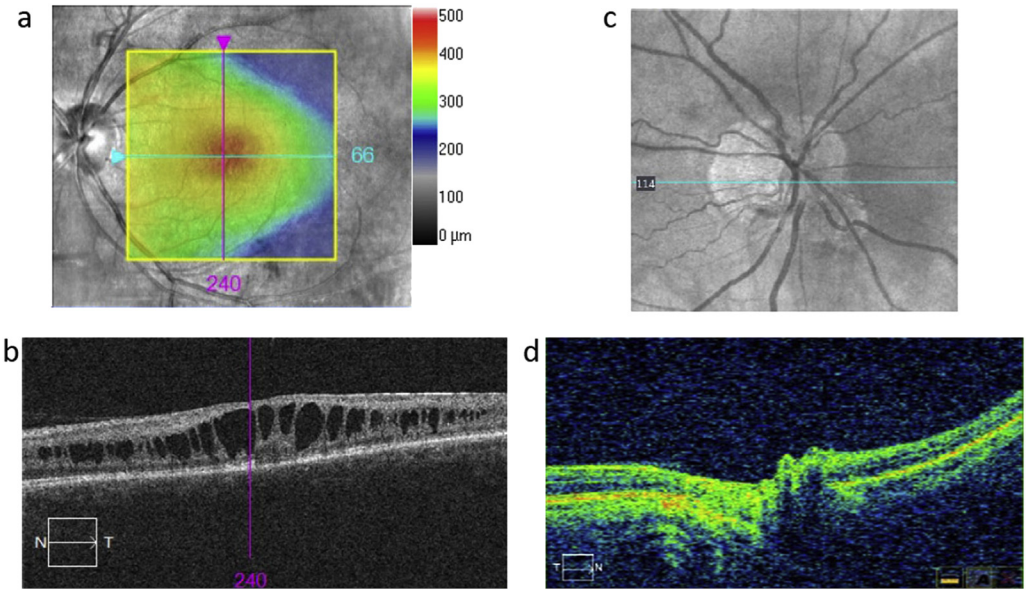

Fig. 8. a) and **b)** Optical coherence tomography (OCT) analysis of X-linked retinoschisis. **c)** and **d)** OCT analysis of non-arteritic ischemic optic neuropathy (NAION).

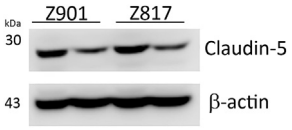

Fig. 9. Claudin-5 suppression in the vasculature associated with the optic nerve head in african green monkeys 48 h post injection of claudin-5 siRNA.

glaucoma and acts by enhancing aqueous outflow through the uveoscleral route. However, it also enhances the pumping activity of the RPE enabling excess fluid accumulating in intra-retinal cysts to be transported into the choroidal circulation. In view of the fact that the inner retinal microvessels innervate the layers of the retina in which schisis most frequently occurs, we speculate that modulation of the permeability of the inner retinal vessels may assist in facilitating fluid clearance from the large cysts that occur within these retinal layers. In this regard, it is noteworthy that intravitreal inoculation is an accepted mode of delivery of siRNA, the following trials serving as an indication: QPI-1007 (Quark): siRNA targeting caspase-2 for suppression of apoptosis in NAAION; AGN211745 (siRNA/Allergan): siRNA targets VEGFR1 (AMD); Bevasiranib (Opko Health): siRNA targets VEGF (diabetic macular oedema); PF-04523655 (Quark): siRNA targets RTP801/REDD1 apoptotic stress response gene; ALY040012 (Sylentis): siRNA targeting ocular hypertension. (Bevasiranib was initially withdrawn from trial owing to lack of efficiency in suppressing VEGF activity. Note however, that in the scenario described above, only partial knockdown of claudin-5 transcript is required to enhance iBRB permeability). This approach could also be envisaged as an adjunct to AAV-mediated therapeutic intervention, where AAV expressing an inducible claudin-5 shRNA is used in conjunction with gene replacement.

6.3. Enhancing clearance of soluble amyloid β1-40 from glaucomatous retinas

Evidence has accumulated over a number of years to suggest a pathological role for amyloid-β (Aβ) in degenerative retinopathies, including AMD and glaucoma. Co-localization of Aβ and drusen in post-mortem human AMD eyes has been observed in a number of studies, including those reported by Dentchev et al. (2003), Anderson et al. (2004) and Johnson et al. (2002), such studies suggesting Aβ as a putative activator of complement cascades. Further evidence for a pathological role for Aβ in AMD was reported by Ding et al. (2011) and Liu et al. (2015). In the former study, systemic anti-Aβ antibody treatment of APOE4 mice, fed on a high fat diet to induce AMD-like retinal symptoms, resulted in an alleviation of disease pathology, while in the latter, sub-retinal inoculation of Aβ into wild type mice produced pathological changes in RPE and photoreceptors. In optic nerve head post mortem tissue from individuals with glaucoma, Gupta et al. (2016) detected elevated levels of soluble Aβ, which has also been detected in aqueous humour from up to 40% of patients with glaucoma (Janciauskiene and Krakau, 2001).

These, and many other studies, strongly suggest that suppression of Aβ accumulation within retinal tissues may be protective in AMD, glaucoma and possibly in inherited retinal degenerations. In this regard, our own studies (Keaney et al., 2015) have shown that siRNA-mediated down regulation of endothelial tight junction transcripts encoding claudin-5 together with occludin, allowed paracellular transport of a soluble Aβ1-40 monomer (MW 4.3 kDa), modified with proline at position 19 and not prone to aggregation into higher molecular weight structures, to readily diffuse across brain endothelial cell monolayers in vitro. However, a dityrosine cross-linked Aβ1-40 dimer (MW 8.6 kDa) did not diffuse in such a manner, these data demonstrating that the paracellular spaces between brain endothelial cells can be widened sufficiently by down regulation of two tight junction components to allow soluble Aβ1-40 dimers to diffuse across them. These observations were confirmed *in vivo* in wild type mice and in a murine AD model (Tg2576) expressing a mutated form of APP, where it was shown that tail vein co-inoculation of claudin-5 and occludin siRNAs rendered the BBB reversibly permeable to biotinylated dextran of 3kD but not of 10kD. Periodic intravenous inoculation of claudin-5

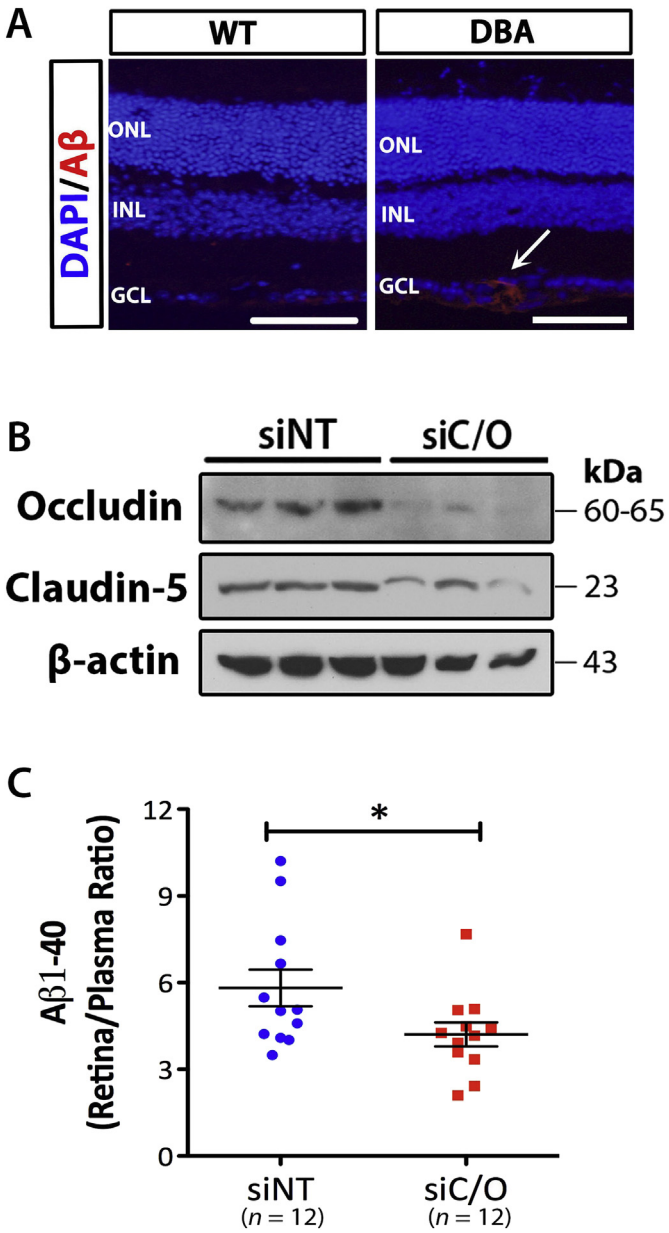

Fig. 10. Aβ in retinas of 8-month old siRNA-treated DBA/2J mice. **a)** Aβ immunostaining in the ganglion cell layer of wild-type (WT) and DBA/2J mouse retinas (ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer. Scale bar = 50 μm). **b)** Western blot analysis of claudin-5 and occludin in retinas of DBA/2J mice following systemic administration of non-targeting (NT) or claudin-5 and occludin (C/O) siRNAs. **c)** Retina/plasma ratios of Aβ(1–40) (pg/g soluble retina Aβ(1–40) to pg/ml plasma Aβ(1–40)) in DBA/2J mice after a single round of siRNA administration (unpaired Student's *t*-test, n = 12 animals per experimental group, $^{*}P = 0.045$. Data are means ± s.e.m.).
This is supplementary Figure 10 from our paper, Keaney et al., Sci. Adv. 2015 Sep; 1(8) e1500472.

and occludin siRNAs every 21 days into AD mice for a period of 9 months resulted during this period in a significant induction of movement of soluble Aβ1-40 from brain tissue into the peripheral circulation. Furthermore, in a murine model of pseudoexfoliative glaucoma, the DBA/2J mouse, in which Aβ1-40 was clearly detectable in retinal sections, a single intravenous injection of claudin-5 and occludin siRNAs resulted in retina:plasma ratios of Aβ1-40 being significantly reduced (Fig. 10).

These data raise the distinct possibility for an avenue of clearance of neurotoxic Aβ1-40 from retinal tissues as a means of protecting against ganglion cell death in glaucoma. It will be of interest in this context to assess such an approach in additional models more accurately resembling the primary open-angle form of glaucoma, for example in transgenic mice expressing a C437H mutation within the human myocilin gene, a model mimicking primary open angle glaucoma in which there is an intracellular accumulation of ECM material within cells of the Trabecular meshwork, and a decrease in secretion of MMPs, favouring an extracellular accumulation of ECM materials, these animals classically displaying elevations in IOP and reduced outflow facility, with concomitant demise of retinal ganglion cells (Zode et al., 2011).

6.4. Targeting oxidative stress

The brain and retina account for approx. 20% of the body's oxygen consumption, which generates appreciable levels of reactive oxygen species and peroxides, contributing to neurological and retinal degeneration (Ott et al., 2007). For example, oxygen free radicals interact with membrane phospholipids generating MDA (malondialdehyde; HOCH=CH-CHO, or $C_3H_4O_2$). This compound is pro-inflammatory, and has been recently shown (Weismann et al., 2011) to be normally sequestered by complement factor H (CFH). CFH is an inhibitor of the alternative complement pathway and a mutation, His402Tyr within the SCR-7 domain of the protein was the first to be strongly associated with AMD. The risk variant, CC has been shown to have a very much lower affinity for MDA than the non-risk variant TT. People with AMD who are heterozygous for the normal and risk variants have a compromised ability to bind MDA, presumably increasing the risk of disease progression. The Authors conclude: 'We report the identification of CFH as a hitherto unrecognized innate defence protein against MDA, which is a ubiquitously generated pro-inflammatory product of lipid peroxidation'. Reactive oxygen species are also similarly important in contributing to disease pathology in IRDs and in diabetic retinopathy (Kiang et al., 2014).

Mitochondria are a major site of generation of oxygen free radicals in the retina. A recently developed oxygen free radical scavenger, XJB -5-131 (4- hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl nitroxide chemically linked to a mitochondrial targeting peptide, Leu-D-Phe-Pro-Val-Orn), enables the systemically-administered drug to become localized in the membranes of the mitochondria and it has been shown to be protective against neurological degeneration in a murine model of Huntington's disease (Xun et al., 2012). While this drug, with a molecular weight of 959 Da, can access the brain in the Huntington model, it's systemic access to the retina could be radically enhanced (with concomitant reduction in systemic dosage) by periodic manipulation of iBRB permeability, possibly representing a approach to slowing disease progression in a manner independent of primary mutations in IRDs or other retinopathies.

6.5. Targeting unique IRD molecular pathologies

With over 260 genes having so far been identified in IRDs, with 70 in retinitis pigmentosa, the molecular pathologies associated with these conditions are clearly of immense diversity, genes encoding proteins, or enzymes involved in transport processes, protein deglutamylation, membrane trafficking, ciliogenesis, calcium-sensitive chloride channels, proteins with protective function against apoptosis/oxidative stress, retinal neurotransmission, photoreceptor morphogenesis, fatty acid and steroid metabolism, extracellular proteins of the retina, retinal adhesion proteins, phagocytosis of rod outer segments, transcription factors and activators, semaphorins, protein ubiquitination, actin binding proteins, pre-mRNA processing, the rate-limiting step of the alternative pathway of guanine nucleotide biosynthesis, and about every gene encoding proteins or enzymes of the visual transduction and retinoid cycles, and indeed many more (Jane Farrar et al., 2017). In some cases, molecular pathologies specific to only one, or a limited number of genetic subtypes of disease may offer opportunities to target such uniqueness by systemic drug treatment.

The RP10 form of RP is arguably one of these, accounting for at least 5% of autosomal dominant cases of RP (perhaps 25–30,000 cases world-wide). It is an early onset, aggressive retinopathy, symptoms manifesting within the first decade. The disease is caused by mutations within the inosine monophosphate dehydrogenase 1 (IMPDH1) gene (Jordan et al., 1993; Kennan et al., 2002; Aherne et al., 2004), which, together with IMPDH2, are the rate-limiting enzymes of *de novo* guanine nucleotide biosynthesis. While each enzyme performs the same role in converting IMP to XMP, which is then converted to GMP, levels of expression between tissues vary considerably, the predominant enzyme within the murine retina being IMPDH1 (Aherne et al., 2004). IMPDH1−/− mice (hence having little or no IMPDH activity because of minimal expression of IMPDH2) have only a very slow deterioration in retinal function, the outer nuclear layer (ONL) of these animals remaining largely intact (Aherne et al., 2004). Since both IMPDH1 and IMPDH2 are minimally expressed in these retinas, retinal function appears to be maintained with a sufficient supply of guanine nucleotides generated by the salvage pathway of guanine nucleotide biosynthesis. Mutations identified in RP10, occur within the CBS domain of the IMPDH1 enzyme, a binding site for GDP/GTP inhibition, indicating that mutations within the IMPDH1 gene result in constitutive activation of the enzyme, disturbing guanine nucleotide pools within the retina and thus causing loss of photoreceptor function (Buey et al., 2015). Interestingly however, OCT imaging (unpublished data) of severely affected individuals reveals a remarkably intact outer nuclear retinal layer, indicating that while visual function is lost, photoreceptors appear to remain viable (Fig. 11), thus providing a potentially very robust window of opportunity for therapeutic intervention. These data suggest that suppression of IMPDH in dominant RP10 could result in a restoration of visual function. A number of FDA-approved IMPDH inhibitors (inhibiting both IMPDH1 and 2) are available, including CellCept, Mizorbine and VX-497 and these have been used as

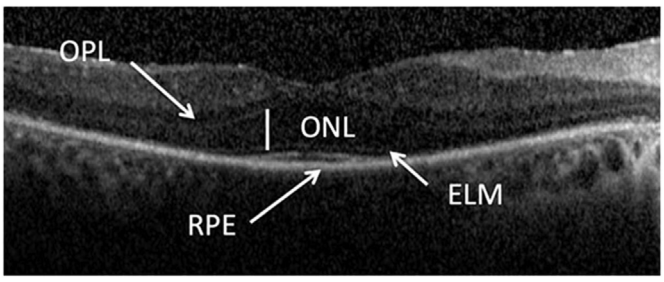

Fig. 11. Preserved outer nuclear layer (ONL) in a patient with the RP10 form of retinitis pigmentosa.

effective immunosuppressive agents (B and T lymphocytes are highly dependent upon the alternative, rather than the salvage pathway of guanine nucleotide biosynthesis). The concept of repositioning such agents as orally-available therapeutics for an IRD, delivery of which could be enhanced (thus reducing systemic dosage) by manipulating ocular endothelial tight junctions is a provocative but potentially realistic concept, dependent on long term tolerability. Given the ease with which systemically-deliverable compounds can access the retina (Fig. 3), tolerable doses may be achievable.

The gene encoding Bestrophin, a calcium-gated chloride channel located in the basolateral region of RPE cells, is mutated in the ciliopathy, autosomal recessive bestrophinopathy (Johnson et al., 2017). Mutated proteins have been shown to mis-localise and end up in proteasomes in the cytoplasm and are degraded (Uggenti et al., 2016). These authors have shown in vitro that proteasomal inhibitors, Bortezomib and 4-phenylbutyrate (4PBA), both approved proteasomal inhibitors, effectively restore the location of mutant bestrophins to the RPE and restore Cl-conductance of the channels in vitro. The Authors comment: "The functional rescue achieved with 4PBA is significant because it suggests that this drug, which is already approved for long-term use in infants and adults, might represent a promising therapy for the treatment of ARB and other bestrophinopathies resulting from missense mutations in BEST1". Again, the degree of enhancement of systemic drug uptake by claudin-5 modulated iBRB could render such compounds (MW 4-PBA 186Da; MW Bortezomib 384Da) systemically tolerable over prolonged periods.

7. Targeting Schlemm's canal endothelial tight junctions: a novel process for enhancement of aqueous outflow through the conventional outflow pathway

Glaucoma in its various forms represents the second most common blinding disease on a global basis after cataract, elevations in IOP being the greatest risk factor (Quigley and Broman, 2006; Tham et al., 2014; Actis et al., 2016). Aqueous humour leaves the eye through the so-called conventional and un-conventional outflow pathways located close together in the periphery of the anterior chamber, at the apex of the narrow angle formed between the iris and the cornea. The conventional outflow pathway comprises the trabecular meshwork, which, as the name suggests, is an interlaced structure of cells embedded in a pressure-responsive ECM through which aqueous flows into the Canal of Schlemm both through paracellular gaps or through cellular pores in the canal's endothelial lining. While a number of genes have been firmly associated with open-angle glaucoma susceptibility, a robust understanding of the molecular pathology of the disease remains to be determined. It should be highlighted, that a balance in the production of matrix metalloproteinases (MMPs) and their tissue inhibitors within the trabecular meshwork is a significant factor in the maintenance of physiological IOP at between 12 and 22 mmHg (De Groef et al., 2013). From the canal, fluid flows through collector channels and eventually into the episcleral veins. Some aqueous also leaves the eye through the bundles of the ciliary muscles – the so-called un-conventional or uveoscleral route. Interestingly, currently used topical medications (including prostaglandin analogues, carbonic anhydrase inhibitors and β-blockers) either slow up aqueous production by the ciliary body, or enhance its clearance via the uveoscleral route, none of these formulations directly and primarily targeting the conventional pathway. Since an appreciable number of those with open angle disease do not respond adequately to currently available pressure-reducing drugs, a large amount of research in recent years has gone into the development of medicines capable of enhancing aqueous outflow through the conventional route. These include Rho kinase inhibitors (Li et al., 2016; Ren et al., 2016), adenosine receptor agonists (Laties et al., 2016; Myers et al., 2016), marine macrolides (Ethier et al., 2006), prostanoid receptor agonists (Kalouche et al., 2016) and AAV-mediated gene therapies involving enhanced MMP expression to remodel the ECM within the Trabecular meshwork (Spiga and Borras, 2010; Gerometta et al., 2010; Borras et al., 2016; O'Callaghan et al., 2017).

The bulk of outflow resistance in the conventional pathway is generated within the juxtacanalicular tissues and the endothelial cells of the canal itself. Aqueous enters Schlemm's canal either through the formation of intra-endothelial fluid-filled vacuoles (so called 'giant' vacuoles) or through the paracellular route, where it passes through pores left between the endothelial tight junctions. Less is known of the nature of the tight junctions joining SC endothelia than those of the cerebral and inner retinal vasculatures. Interestingly claudin-5, a major component of the tight junctions of the cerebral and inner retinal vascular endothelia, is absent from human SC endothelial cells (Tam et al., 2017). However, as outlined earlier, ZO-1, tricellulin and claudin-11 are prominently expressed TJ proteins. In order to explore the hypothesis that down-regulation of SC endothelial TJs might result in an increase in the permeability of the canal, Tam et al. (2017) intracamerally injected siRNA validated against tricellulin and ZO-1 into wild type mice. Injected material followed the natural flow of aqueous through the trabecular meshwork and into the canal's endothelial cells. As illustrated in Fig. 12, transmission electron microscopy revealed reversible opening of the paracellular clefts between endothelial cells as a result of such down-regulation. This was accompanied by a significant elevation in outflow facility in treated eyes *ex vivo*.

In translational terms, a major factor to be considered in this approach is the mode of siRNA delivery. Recent reports have indicated that siRNA can be effectively delivered to the eye in the form of topical drops to the cornea. However, in this approach, while the siRNA (SYL040012, targeting the β2-adrenoceptor) was able to efficiently access the ciliary body which was the primary site of action, little siRNA was found in the aqueous humour using this technique (Martinez et al., 2014; Moreno-Montanes et al., 2014). While periodic intracameral injection of siRNA as indicated by IOP is a realistic option, an alternative and less invasive procedure would involve retrograde introduction of siRNA into Schlemm's canal via the episcleral veins and a device facilitating this approach has been manufactured (Retroject Inc, NC). An alternative would be the use of AAV as a delivery vehicle for shRNAs. While single stranded AAV particles will not transfect tissues of the trabecular meshwork, self-complementary AAV have been reported to do so with high efficiency (Buie et al., 2010). However, it is unclear from this work as to whether AAV directly transfects SC endothelial cells. If this can be demonstrated, an AAV expressing shRNAs under the control of a promoter inducible by a topical eye drop could in principle be used. In this regard, it is of interest to note in a recently reported experimental system, that AAV transfecting the corneal endothelium can be activated to express MMP3 via the use of doxycycline applied to the cornea, the enzyme then being secreted from the corneal endothelia in to the TM, where re-modelling of the ECM resulted in increased outflow facility and decreased IOP in wild type mice (O'Callaghan et al., 2017). Since an inducing agent will travel with the natural flow of aqueous toward Schlemm's canal, there is reason to believe that AAVs transfecting SC endothelia could be periodically activated using such a procedure. A combinatorial AAV-mediated approach, targeting ECM remodelling together with incrementing SC permeability, is a realistic possibility based on observations to date.

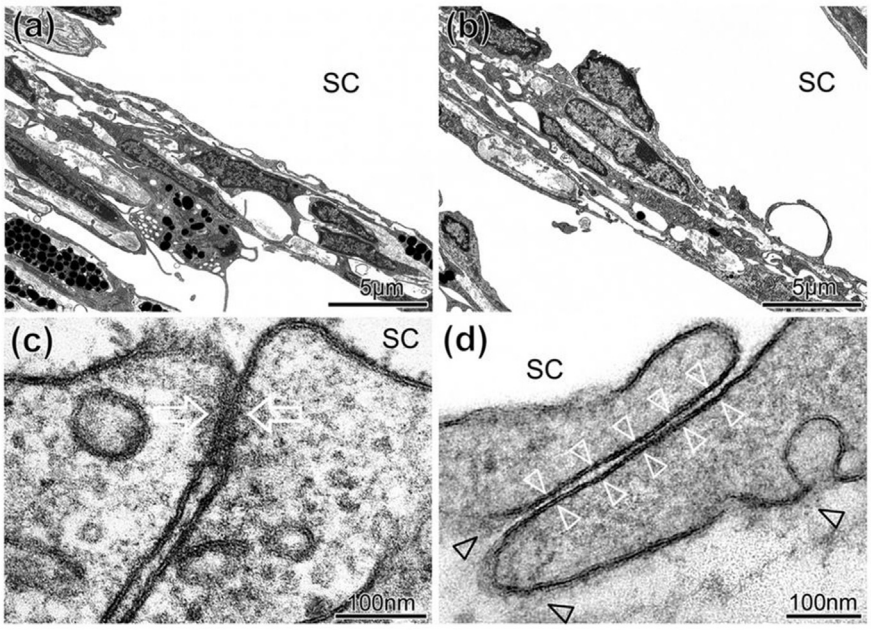

Fig. 12. Representative sagittal sections through the inner wall of Schlemm's canal (SC) and outer trabecular meshwork (TM) of a mouse eye treated with **(a)** non-targeting (NT) or **(b)** targeting (T) siRNA illustrating intact cells and an intact and continuous inner wall endothelium that appeared similar in both cases. The inner wall endothelium is connected to the underlying ECM so that no ballooning was visible. (**c**, **d**) High magnifications of sagittal sections through intercellular clefts along the inner wall endothelium of SC showing examples for junctions quantitatively evaluated as closed **(c)** with fusion between the neighbouring cell membranes (arrows) or open clefts **(d)** where the cell membranes of adjacent endothelial cells were clearly separated along the entire cleft length (white arrowheads). Despite the open clefts, adhesions to sub-endothelial matrix (black arrowheads) were preserved.
This is Figure 6 from our paper Tam et al., Scientific Reports 2017; 7: 40717 (pbl on line Jan 16 2017).

8. Future prospects

Retinal endothelial barrier modulation technologies are, in principle, deployable in a stand-alone sense, or in combination with gene and other molecular therapeutic approaches. There is a significant need for a therapy for one of the more common idiopathic retinopathies, NAION, which is currently incurable and can lead to irreversible blindness. Clearance of oedema from the optic nerve head by intravitreal injection of siRNA targeting claudin-5 could well be effective in this scenario. It is also of note that as yet there are no medicines available for the most common (non-exudative) form of AMD, and yet, clearance of Aβ, or drug-mediated suppression of oxidative stress within the retina, could have utility in slowing down disease progression in this, and indeed other forms of retinal degeneration including glaucoma. It will also be of interest to assess the efficacies of gene therapy for XL-retinoschisis *vis-à-vis* direct facilitation of fluid egress from intraretinal cysts, perhaps the two approaches, each using AAV could be used in a combinatorial sense. Repositioning approved drugs for use as therapies in early stage IRDs where the iBRB is essentially intact, is an interesting possibility, several scenarios having been outlined, including the possibility of IMPDH1 suppression in the RP10 form of RP and the use of proteasome inhibitors for at least one form of bestrophinopathy, but there will be many other examples based on the very extensive range of molecular pathologies that are involved, low molecular weight drug-mediated inhibition of protein misfolding for example, being one. Improved treatment regimens for open-angle glaucoma continue to be a priority. As outlined, much research is currently being directed toward the development of topical formulations targeting the major (conventional) outflow pathway. The fact that siRNA could periodically be delivered in a retrograde fashion into Schlemm's canal endothelia through the episcleral veins, obviating a requirement for intracameral inoculation, is an interesting concept which, in principle, could be deployed as an outpatient procedure. In summary, we suggest that direct manipulation of permeability of both the inner retinal microvasculature and the Canal of Schlemm, may have significant potential clinical utility.

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