CRB1 gene therapy coming of age: mechanistic insight and rAAV assays on mouse & human retinal organoid models

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Chapter 7

Summary

Nederlandse Samenvatting

Acknowledgements

Curriculum Vitae

List of Publications

Summary

Around one out of 4000 people worldwide suffer from monogenetic hereditary retinal dystrophies (RDs; around 4000-6000 Dutch patients). Hereditary retinal diseases result from variations (*mutations*) on the genetic code (DNA) on genes causing changes on the gene expression and protein function. Biallelic *CRB1* gene variations can cause retinitis pigmentosa (RP), Leber congenital amaurosis (LCA), or in some cases macular degeneration. *CRB1*-related RDs account for around 7-17% of all Leber congenital amaurosis cases (1 / 250.000 people or 60-90 Dutch patients) and 3-9% of all retinitis pigmentosa cases (1/90.000 people or 130-390 Dutch patients). On *CRB1* animal models or eye scans of *CRB1* patients, it was shown that *CRB1*-related RDs (a) degenerate the light sensitive photoreceptors (PRs; especially in the retinal periphery), (b) reduce cell adhesion between Müller glial cells (MGCs)-PRs, PRs-PRs, and MGCs-MGCs, and (c) sometimes disrupts the cell cycle during retinal development. The consequence is vision loss and blindness. It is believed that the significance of CRB1 in the neuroretina rests on the participation of CRB1 in apical polarity complexes in Müller glial cells and photoreceptors – such as the Crumbs and PAR protein complex – at the outer limiting membrane (OLM). CRB1 protein at the OLM can support adhesion/junction molecules, recruit actomyosin cytoskeletal proteins, and regulate the spatiotemporal retinogenesis via Notch, mTORC1 & Hippo pathways.

No treatments are available for monogenetic neuroretinal diseases. However, gene therapy is becoming a promising treatment option for many hereditary diseases. The development of gene supplementation therapies begins at (a) establishing relevant disease models, (b) generating & optimizing transgene sequences and vector cassettes, (c) screening of carefully selected viral capsids, and (d) testing gene therapy vectors in proof-of-concept studies in animals. Promising gene therapy candidates need to be screened on how efficient they can express the vector in the target cells while minimizing off-target effects. This can be illustrated on Luxturna® (voretigene neparvovec-rzyl), a gene therapy for biallelic gene variation on the *RPE65* gene causing LCA2 and RP. The RPE65 protein is expressed in the retinal pigment epithelium (RPE). Luxturna® was the first retinal gene therapy approved both in the USA and Europe. The gene supplementation therapy is based on packaging the cDNA of the *RPE65* gene under the control of a ubiquitous promoter (CBA) into recombinant adeno-associated virus serotype 2 (rAAV2) particles. The rAAV-particles are then injected in the subretinal space where they can infect RPE cells. The single-stranded *RPE65* cDNA is then unpacked and transported to the nucleus where it forms episomal stable circular DNA structures from which the *RPE65* transgene is stably expressed rescuing the RPE phenotype. Similar strategies have been developed – but not approved yet – for many hereditary retinal dystrophies including *CRB1*-related RD (see for example **chapter 1, 2** and **4**). A summary of the clinical trials for ocular gene supplementation therapies, the different rAAV vector

cassettes (promoters, genes, polyadenylations sequences), the different rAAV capsid (tropism) studies, and the transgene & bioactivity assays are described in **chapter 1**.

We describe relevant human and mouse disease models for *CRB1*-related RP in **chapter 2+4+5**. In **chapter 2**, we describe a new RP-*Crb1* mouse model (*Crb1*^{KO}*Crb2*^{LowMGCs}) where we ablated *Crb1* expression and reduced the levels of the *Crb1* homologue *Crb2* in Müller glial cells. We also compared the OLM cell protrusion phenotype between four different RP-*Crb1* mice and wildtype mice. We find a negative correlation of the number of OLM breaks and the CRB1/CRB2 proteins (CRB's) at the OLM. It demonstrates that the amount of CRB proteins is important and not specifically the CRB1 or CRB2 proteins. Also, it was not known how important CRB1 or CRB2 is in MGCs at the OLM. It was interesting to see that reduction of CRB's in MGCs compared to rod photoreceptors caused a more severe OLM-phenotype. It means that a gene therapy should preferably also infect MGCs and not only photoreceptors. We also find that the reduced CRB2-protein expression at OLM in the *Crb1*^{KO}*Crb2*^{LowMGCs} mice still relatively preserved the OLM over 12 months. This was in stark contrast to a previous publication where we completely ablated *Crb2* (*Crb1*^{KO}*Crb2*$^{\Delta MGCs}$), having a severe retinal (LCA-like) phenotype. Thus, the adhesion of neuroretinal cells is quite dependent on the level of CRB proteins in glial (meaning "*glue*" in Greek language) cells.

Although *Crb1*^{KO}*Crb2*$^{\Delta MGCs}$ mice compared to *Crb1*^{KO}*Crb2*$^{\Delta MGCs}$ mice had a mild phenotype, they still need natural *Crb2* protein levels for proper retinal function and normal morphology. The mice lost their vision function earlier compared to *Crb1*^{KO} littermates measured by optokinetic head-tracking response (OKT) and electroretinograms (ERGs). Also, early effects on morphology were seen such as outer/inner segments of photoreceptor loss and more retinal cell protrusions. Also, when the *Crb1*^{KO}*Crb2*^{LowMGCs} retina was stressed by a low dose of a Müller glial toxin (DL-AAA), causing a breakdown of the OLM, then the vision function (measured by ERGs and OKT) was reduced one month after the DL-AAA injection. It shows that the recovery capacity of the *Crb1*^{KO}*Crb2*^{LowMGCs} neuroretina after aggravation is much lower compared to wildtype and *Crb1*-knockout neuroretinas.

Also, when codon-optimized human *CRB1* or *CRB2* cDNA is provided to *Crb1*^{KO}*Crb2*^{LowMGCs} Müller glial cells by rAAV-vector therapy, then the morphological phenotype caused by the toxin can be prevented. Thus, rAAV-*CRB1* or rAAV-*CRB2* gene therapy can increase the OLM stability in *Crb1*^{KO}*Crb2*^{LowMGCs} mice. Interestingly, only the rAAV-*CRB2* treatment protected the retina from vision loss measured by OKT and ERG, whereas rAAV-*CRB1* treated eyes had an unwanted increase of activated microglial cells and neovascularization events in the ganglion cell layer(GCL)/nerve fiber layer(NFL) and ciliary body. This and other studies show that CRB1 and CRB2 proteins have compensatory and overlapping functions in the neuroretina and that rAAV-*CRB2* is a promising candidate for clinical trials.

The protein shell (capsid) of the rAAV particle determines what cells are infected (tropism), to what degree (potency), and how easily it breaks down by enzymes or escapes immune recognition (capsid stability and potency). The rAAV infection of a cell is mediated by surface receptors on the host cell (see **chapter 1**). What is more, the surface receptors can be quite different depending on the species requiring screening of the rAAV capsids on human cells or non-human primates. In **chapter 3**, we set up a new rAAV transgene expression assay on (a) how to screen the tropism of rAAV capsids in parallel on human donor neuroretina's *ex vivo* and (b) how to keep the overall morphology intact for 21 days. In **chapter 4**, we tested three different rAAVs (rAAV9, rAAV5 and rAAV6-derived ShH10^{Y445F}) on their potency to infect the target cells of a gene therapy for *CRB1*-related RP patients: Müller glial cells and photoreceptor cells. We show on human donor retinal explants and human induced pluripotent stem cell (hiPSC)-derived retinal organoids that rAAV5 and rAAV6-derived ShH10^{Y445F} can efficiently infect Müller glial cells. Also, both rAAVs infected photoreceptors on human donor retinal explants. Surprisingly, they also outperformed on potency the rAAV9 on three different titers measured. Previously, rAAV9 showed excellent infection profiles in mice *in vivo* for *CRB1* rAAV therapies. These results demonstrate that (a) species-differences can influence potency and tropism, and (b) rAAV5 is a strong AAV-capsid candidate for *CRB1* rAAV-based treatments.

Finally, in **chapter 4** and **chapter 5**, we explore how *CRB1*-related retinitis pigmentosa can be modelled in a human cell system. First, we show that we can generate retinal organoids from hiPSCs of three different healthy donors (control retinas). We find back a laminated retina with all major types of retinal cells present (PRs, MGCs, amacrine cells, horizontal cells, ganglion cells, RPE cells). We then show that CRB2 protein precedes CRB1 protein expression at the OLM during retinogenesis in organoids and fetal retinal material. In the mature retinal organoid, CRB1 is found in the subapical region of photoreceptors & MGCs and CRB2 in photoreceptors and apical MGC-villi. In the mouse retina, CRB2 is found in PRs and MGCs. The protein location studies on immune-electronmicroscopy shows that CRB1 has a more prominent role in the human neuroretina when compared to the mouse retina where CRB2 is believed to compensate for the loss of *Crb1* in *Crb1*-null mouse models.

Second, we generated RP-*CRB1* organoids of three RP-*CRB1* patient donor hiPSC lines. They all developed a retinal volcanic-like cell protrusion phenotype as similar seen in *Crb1* and *Crb2*-RP mouse models. Interestingly, the OLM protrusions in RP-*CRB1* organoids do not only show a loss of the subapical & adherens junction proteins including variant CRB1 protein but also a strong decrease of apical NOTCH1. In control organoids, we find an extracellular domain (ECD) interaction of CRB1 and NOTCH1, which is largely lost in RP-*CRB1* organoids. This indicates that CRB1 is important for the recruitment of NOTCH1 or the stabilization of NOTCH1 at the OLM. Previously, it was shown that the neuroretina of

CRB1 LCA-like mice have an increase in the Notch pathway activation. The ECD interaction of CRB1/NOTCH1 could play an important role here.

Lastly, CRB1 and NOTCH1 need to be transported and continuously recycled (turned over) by the endolysosomal system at the OLM. We find much more early endosomes and an increase of the degradative cellular vesicles which is linked to decrease of RAB11A-postive recycling endosomes in RP-*CRB1* organoids. We hypothesize that the loss of CRB1 at the OLM, inhibits the maturation of early endosomes to recycling endosomes increasing the number of degradative vesicles. We also found an increase of WDFY1 proteins (especially in endosomes) in RP-*CRB1* organoids and an increase in *Wdfy1* mRNA expression in a *CRB1* LCA-like mouse model (*Crb1*^{KO}*Crb2*$^{\Delta RPC}$). The WDFY1 and NOTCH1 proteins could be interesting biomarkers for *CRB1* gene therapies. Yet, little is known on the function of WDFY1 and the endolysosomal system in the neuronal retina. Especially, controlling the endolysosomal system to inhibit intracellular disease processes is an exciting treatment approach. But first more research into the endolysosomal system is needed to delineate (sub-)populations of endosomes in the neuroretina (and characterize RP- and control retinal organoids) to make such treatments more predictable.

In conclusion, we describe the generation and analysis of RP-*CRB1* mouse and human retinas: C*rb1*^{KO}*Crb2*^{LowMGCs} (**chapter 2**), human RP-*CRB1* organoids (*CRB1*$^{M1041T/\ M1041T}$; *CRB1*$^{Y631C/E995*;}$ *CRB1*$^{M1041T/C948Y}$; **chapter 4** and **5**). The data indicates that the human RP-*CRB1* disease can be studied in mice and human organoids. Then, we show that rAAV-*CRB* gene supplementation therapy to Müller glial cells of the *Crb1*^{KO}*Crb2*^{LowMGCs} mouse retina can protect it from stress-induced vision loss, and that human *CRB2* cDNA was superior to human *CRB1* cDNA (**chapter 2**). We then developed an improved rAAV tropism assay on human donor eyes (**chapter 3**). This assay shows that rAAV5 can efficiently infect Müller glial cells and photoreceptors, the target cells of a RP-*CRB1* gene therapy. Also, rAAV5 infection studies outperformed rAAV9 on human retinal organoids and human donor retinas (**chapter 4**). Thus, this thesis on both human and mouse models provides new insight into retinal degeneration and rAAV gene supplementation therapies.