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Choroideremia: molecular mechanisms and development of AAV gene therapy

Maria I Patrício[1,2,3], Alun R Barnard[1,2,3], Kanmin Xue[1,2,3], Robert E MacLaren[1,2,3,*]

1. Nuffield Laboratory of Ophthalmology, Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, UK

2. National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC), Oxford, UK

3. Oxford Eye Hospital, Oxford University Hospitals NHS Foundation Trust, Oxford, UK

***Corresponding author:**

Robert E MacLaren

Telephone: +441865223701

Email: enquiries@eye.ox.ac.uk

Abstract

Introduction: Choroideremia is an X-linked inherited retinal degeneration that causes blindness in afflicted males by middle age. The causative gene, *CHM*, plays a key role in intracellular trafficking pathways, and its disruption impairs cell homeostasis.

Areas covered: The mechanism by which mutations in *CHM* cause choroideremia is still under debate. Here we describe the molecular defects in choroideremia cells regarding both the deficiency of prenylation and the involvement of Rab GTPases. Important *in vivo* and *in vitro* studies that contributed to the current knowledge are also discussed. Finally, the rationale for the development of a treatment strategy using AAV for gene replacement is presented, together with other treatment strategies under consideration.

Expert opinion: Despite ubiquitous expression of the *CHM* gene, the primary defect in choroideremia is driven by retinal pigment epithelium (RPE) and photoreceptors degeneration. Here we discuss how impairment of vesicular trafficking pathways in the RPE plays a major role in the molecular pathogenesis of choroideremia. Moreover, this defect is likely restored by subretinal delivery of a functional copy of *CHM* using AAV, as evidenced by clinical trial results. The surgical complexity of delivering the AAV vector to the target area remains as the main challenge to this therapy.

Keywords: AAV; choroideremia; clinical trials; gene therapy; prenylation; Rab GTPases

Abbreviations:

AAV: adeno-associated virus
BCD: Bietti's crystalline dystrophy
CHM: choroideremia
CHML: choroideremia-like
Dfp: days post-fertilization
EMA: European Medicines Agency
ERG: electroretinogram
ETDRS: Early Treatment Diabetic Retinopathy Study
FDA: Food and Drug Administration
FTase: farnesyl transferase
GFP: green fluorescent protein
GGPP: geranylgeranyl-PP
GGTase-I: geranylgeranyl transferase type-I
GGTase-II: geranylgeranyl transferase type-II
HMG-CoA: 3-hydroxy-3-methylglutayl-CoA
HMGCR: HMG-CoA reductase
iPSC: induced pluripotent stem cells
IRDs: inherited retinal diseases
KO: knockout
LCA: Leber congenital amaurosis
NMD: nonsense-mediated mRNA decay
OCT: optical coherence tomography
PMBCs: peripheral blood mononuclear cells
POS: photoreceptor outer segments
PTCs: premature termination codons
Rab GGTase: Rab geranylgeranyl transferase
REP1: Rab escort protein-1
RPE: retinal pigment epithelium
TRIDs: translational read-through inducing drugs
WPRE: woodchuck posttranscriptional regulatory element

1. Introduction

Inherited retinal diseases (IRDs) are a heterogeneous group of degenerative disorders of the retina, which can cause blindness in the young to working-age population, affecting at least 1:4000 people worldwide [1]. IRDs are commonly caused by mutations in a single gene (monogenic) crucial for the function of the photoreceptors and/or the retinal pigment epithelium (RPE) cells. They are generally classified based on the genetic defect (when identified), inheritance pattern (autosomal dominant, recessive or X-linked), main cell type affected (rod or cone photoreceptors, or RPE), age of onset, type of visual dysfunction, rate of disease progression, and clinical fundus phenotypes [2].

Choroideremia (CHM; OMIM Phenotype MIM #303100) is an IRD with X-linked inheritance pattern. It was first described by the Austrian ophthalmologist Ludwig Mauthner [3], and the name means "an area barren (*erēmia*) of choroid", a reference to the barren appearance of the fundus [4]. The spelling of choroideremia with the "ae" diphthong is therefore incorrect, because the word is not derived from "*aemia*", which means relating the blood, as in anaemia. Choroideremia is characterised by a progressive degeneration of the RPE and the photoreceptors, and ultimately the choroid. Afflicted males typically lose their night vision in the first decade of life. The disease progresses with a gradual loss of peripheral vision during the 20s and 30s, which can result in legal blindness by middle age [5,6]. Female carriers usually maintain a good vision throughout their life, although more severe phenotypes have also been reported [7].

Choroideremia has a prevalence of about 1:50,000 [4]. It is caused by mutations in the *CHM* gene (OMIM Gene/Locus MIM number: 300390), located on the X chromosome at position Xq21.2, which encodes Rab escort protein-1 (REP1). The cloning of the *CHM* gene was completed in 1990 [8], but it took a bit longer to uncover its function as an escort of Rab proteins [9]. The mRNA spans 15 exons with a coding sequence of 1962 bp. It is expressed ubiquitously and encodes a 653-amino acid-long protein, REP1. REP1 plays a key role as part of the prenylation machinery. Prenylation is a type of post-transcriptional modification necessary for membrane association and protein-protein interactions, in which REP1 acts as an escort of Rab proteins [10]. The mechanism how *CHM*

mutations affect the prenylation process and its impact on cell homeostasis are explored in detail in the next section. Below we discuss the clinical and genetic diagnosis of choroideremia, as well as the genotype-phenotype correlations evidenced to date.

1.1. Clinical diagnosis of choroideremia

Choroideremia was originally named after what it was thought to be a congenital absence of the majority of the choroid [4]. There is now mounting evidence to suggest that the primary disease pathogenesis occurs in the RPE and photoreceptors, and that the choroidal atrophy is secondary to outer retinal degeneration [5,11]. Clinically, autofluorescence (AF) imaging provides an indirect modality to visualise disease progression (Figure 1). In the early stages, patches of RPE loss, seen as complete loss of autofluorescence, appear in the mid to far peripheral retina. These atrophic areas expand and coalesce, causing peripheral visual field loss around the equator as the disease progresses over the first to second decade of life. Towards the end of the second decade, a central residual 'island' of functional retina remains, which undergoes exponential centripetal area shrinkage over many years, eventually encroaching on the fovea and causing blindness by the fifth decade. Optical coherence tomography (OCT) analysis showed that the degeneration of photoreceptors closely follows the degeneration of the underlying RPE at the advancing edge of degeneration [11]. However, some RPE mottling and ellipsoid zone disruption can be seen within the island of surviving retina, indicating low-grade (usually subclinical) degeneration of the outer nuclear layer centrally [5].

The retinal phenotype in choroideremia may be mimicked by other rare types of retinal dystrophies. Genetic testing and a clear X-linked family history in choroideremia usually allow clinical differentiation, helped by the subtle RPE mottling seen in female carriers. Interestingly, while generalised RPE mottling is often seen in female carriers due to random X-inactivation, it is not normally associated with clinically significant visual impairment. Occasionally, however, female carriers can be affected by patches of retinal degeneration, reminiscent of early choroideremia in affected males; these patches undergo gradual expansion, and may involve the fovea [7]. This most likely results from the effects of skewed X-inactivation during early retinal development.

1.2. Genetic diagnosis of choroideremia

The genetic characterisation of *CHM* plays a key role in the diagnosis process. The first report of *CHM* mutations was published in 1997, not long after the gene was described [12]. That paper reported predominantly single base-pair mutations, including nonsense, frameshift or splice-site. As technologies evolved, the mutational spectrum of *CHM* has increased significantly. Among the 280 unique pathogenic variants recorded in the Leiden Open Variation Database at www.lovd.nl/CHM (accessed December 2017), one can find insertions and deletions (some as large as the full gene), partial gene duplications, translocations, splice defects, and nonsense, frameshift and missense mutations. Missense mutations are the least common, and so far only three have been described as pathogenic, with reduced levels of REP1 expression reported in patient's fibroblasts [13–15]. Very recently two new types of mutations were reported for *CHM*: a synonymous mutation (S453S) [16] and one in the promoter region of *CHM* [17]. This first report of a mutation in the promoter region shed light onto the region that regulates *CHM* expression and extended further the inventory of molecular genetic changes causing choroideremia [17].

Nonetheless, it may happen that mutations in the *CHM* gene or promoter are not found in an individual. In these cases, other methods can be used to support the diagnosis, such as absence of detection of REP1 protein in peripheral blood mononuclear cells (PBMCs) [18]. As most *CHM* mutations are functionally null, i.e. REP1 is absent or inactive, an immunoblot assay can be effective at supporting the diagnosis of choroideremia.

1.3. Genotype-phenotype correlations in choroideremia

Choroideremia is one of few IRDs where a characteristic pale fundal appearance usually gives away the causative gene. However, despite several attempts to find genotype-phenotype correlations, it remains to be established why the retina appears to degenerate faster in some individuals than others while the majority of *CHM* mutations are functionally null [19–21]. Indeed, the very first report on *CHM* mutations in 1997 stated that no obvious correlation was found between the mutation type and the clinical manifestations of choroideremia [12]. Thus, we know that epigenetic factors also play a

role in the severity and the progression of the disease. Differential exposure to environmental insults was first suggested to possibly play a role in disease progression rate in 1996 [22], an idea that was recently reinforced with scientific data [20]. As for the degree of severity, additional genetic factors may contribute to the difference in phenotype found in patients carrying the same mutation.

2. Molecular defects in choroideremia cells

In this section we describe the studies and findings that uncovered a prenylation deficit to be the cause of choroideremia with insights from *in vitro* studies and animal models.

2.1. Prenylation deficit

A prenylation reaction involves the modification of the C-terminus of a protein by terpenoid lipids. Terpenoids, also known as isoprenoids, are organic chemicals formed from isopentenyl pyrophosphate (isopentenyl-PP), which contains the basic five-carbon (C5) building block named an isoprene unit. The isoprene units can be assembled and modified in many ways to generate an array of compounds, which play crucial roles in various metabolic pathways, for example the mevalonate pathway [23,24] (Figure 2). Starting from two molecules of acetyl coenzyme A, terpenoid synthesis continues to mevalonic acid via 3-hydroxy-3-methylglutayl-CoA (HMG-CoA); downstream in the pathway isopentenyl-PP is metabolised into and farnesyl-PP (three C5 units, or 15-carbon) and geranylgeranyl-PP (GGPP; four C5 units, 20-carbon). The covalent attachment of these farnesyl and geranylgeranyl isoprenoids, via thioether linkage, to cysteine (Cys) residues at or near the C-terminus of intracellular proteins is called prenylation [23,24]. The increment of hydrophobicity of the C-terminus of a cytoplasmic protein will modulate protein stability and favour its interaction with cellular membranes, therefore playing a key role in determining its localisation and protein-protein interactions [10,23].

Three enzymes are known to act as protein prenyl transferases: farnesyl transferase (FTase), geranylgeranyl transferase type-I (GGTase-I) and geranylgeranyl transferase type-II (GGTase-II) [23,25]. All three enzymes are heterodimers composed of a α- and β-subunits, but they differ in the type of substrates they modify. The first two constitute the class of CaaX prenyl transferases: they

recognise a CaaX tetrapeptide prenylation motif at the C-terminus of their substrates, where 'C' is a Cys residue, 'a' an aliphatic amino acid and 'X' any amino acid. The identity of the 'X' residue determines which enzyme, FTase or GGTase-I, will modify which protein. The prediction is that more than 200 prenylated proteins expressed by mammalian cells are CaaX-type [10,25].

The second class of prenyl transferases consists of the GGTase-II. This enzyme is also known as Rab geranylgeranyl transferase (Rab GGTase) because it attaches geranylgeranyl groups only to members of the Rab family of small GTP-binding proteins, with a CC, CXC, CCX, CCXX, CCXXX or CXXX termination sequence (or prenylation motif) [23,26]. Rab proteins are key players in all pathways of intracellular trafficking [27,28]. They act as molecular switches, cycling between 'inactive' GDP-bound and 'active' GTP-bound states, which determine their reversible association with their target membranes (Figure 2). The cell homeostasis is highly dependent on the spatial and temporal control of Rab activity, hence the importance of its regulation [27,28].

The GGTase-II was first purified from rat brain cytosol in 1992 [29]. In that study, two components, designated A and B, were found to be required for activity, although only one was purified successfully. Purified preparations of Component B contained two peptides of approximately 60 and 38 kDa [29], which were analogous to the α- and β-subunits of the FTase, and nowadays are known to correspond to the α- and the β-subunits of GGTase-II. Component A, however, was only partially purified at this point [29]. Not long after, component A of the rat GGTase-II was described as a single polypeptide with an apparent molecular mass of 95 kDa. This protein was proved to recognise a sequence motif remote from the C-terminus prenylated sequence shared by members of the Rab family [30]. The authors also showed that component A had similarities to parts of both human and mouse *CHM* gene products, which themselves resemble a Rab-binding protein. These observations led the authors to suggest for the first time that (1) component A of the GGTase-II may bind to Rab proteins, while component B transfers the geranylgeranyl groups; (2) the *CHM* gene may encode the human counterpart of component A of rat GGTase-II; and finally (3) a defect in this reaction may cause choroideremia [30].

More results supporting this hypothesis were published the following year: extracts from lymphoblasts from patients with choroideremia showed less GGTase-II activity than control extracts [31]. The authors proved that it was the deficiency in component A that was responsible for the low prenylation activity in choroideremia lymphoblasts. Nonetheless, the mutant lymphoblasts still showed residual component A activity. This finding was justified at the time by suggesting that the product of a gene distinct from *CHM* might have component A activity [31], possibly the *choroideremia-like* intronless gene (*CHML*) gene located in chromosome 1 [32]. The final piece of the puzzle was unveiled only a few months later when the same group demonstrated at last that component A is the homolog of the human protein that is defective in choroideremia, and it acts in the geranylgeranylation of Rab proteins by accelerating the transfer reaction catalysed by component B [9]. Furthermore, the authors suggested a change of name of component A to Rab escort protein (REP) due to its newly described role in escorting the unprenylated Rab protein to the catalytic component B (α/β heterodimer) and removing it once the isoprenoids transfer has taken place, therefore freeing the catalytic site. Together with the GTP/GDP switch [27,28], this 'REP cycle' is fundamental to regulate the activity of Rab proteins (Figure 2).

The authors who proposed the name change to REP also suggested that another gene product, namely *CHML*, could compensate for the low levels of REP found in certain tissues [9]. The product of the *CHML* gene was fully characterised not long after [33] and shown to share 95% of its amino acid sequence with REP, which confirmed the previous suspicions. The ubiquitously expressed *CHML* gene product was proved to be a second REP, and therefore designated REP2, capable of supporting the GGTase-II reaction, although with different substrate specificity than REP (onwards designated REP1) [33]. Most importantly, this study provided some justification as to why some Rab proteins were found prenylated in choroideremia cells [31], and suggested that REP2 may be only partially effective in the retina, though preventing cellular dysfunction in other cell types [33].

Around this time another study shed some light into the mechanism of action of REP1. In addition to its escorting role of unprenylated proteins, REP1 was reported to chaperone newly prenylated Rab proteins to their target donor membranes on its own [34].

2.2. Rab substrates and the cause of choroideremia

The observation that REP1 showed different activity with respect to different Rab proteins [31] led the scientists to hypothesise that one or more Rab proteins would accumulate unprenylated in choroideremia cells. In fact, they found a human homolog of Ram (a rat Rab protein) accumulating unprenylated in choroideremia cells [35]. This protein was renamed RAB27, and the authors speculated it would regulate intracellular vesicle transport as other Rab proteins did. These experiments detected RAB27 in the cytosolic fraction of choroideremia lymphoblast extracts, suggesting it had lower affinity for REP2 than for REP1, when compared with other Rab proteins. The direct implication of these results was that RAB27 would not be as efficiently prenylated in choroideremia cells, where REP1 is missing. RAB27 was also found to be expressed in several rat tissues, with a high level in the RPE and the choriocapillaris. Altogether these findings prompted the authors to propose for the first time a mechanism for the retinal degeneration in choroideremia: the inability to efficiently prenylate RAB27 in RPE and/or choriocapillaris would lead to a functional defect, i.e. disruption of intracellular vesicle transport, and ultimately cell death. The RPE dysfunction would then cause secondary degeneration of photoreceptors and visual impairment [35]. The slow progressive clinical course of choroideremia would therefore be explained by the fact that a fraction of RAB27 remains fully functional due to the activity of REP2 [22,35]. At the same time, the question 'why choroideremia symptoms were confined to the retina' if RAB27 is expressed in other tissues was raised again [30,35]. Although some explanations were offered, as (1) the remaining functional RAB27 may be enough, (2) RAB27 function may be compensated by a related Rab, or (3) the dysfunctional transport step may not be so crucial for the viability of those tissues [35], it still remains unclear why it happens.

Studies with regards to the function of RAB27 in the eye and beyond followed. In a report that came out not long after the authors renamed human RAB27 into RAB27A, due to discovery of another isoform, named RAB27B, in skin melanocytes and platelets [36]. Both isoforms displayed all the characteristic features of small GTP-binding proteins and constituted a novel subfamily on their

own [36]. The knockout (KO) mouse for Rab27a later created [37] identified *Rab27a* as critical for pigment granule transport. In fact, this model was named *ashen* due to its lightened coat colour. However, *ashen* retinas appeared normal, which supported the hypothesis that loss of RAB27A function is not the ultimate cause of choroideremia, at least in mice [37]. RAB27A was later shown to co-localise with melanosomes and to regulate their distribution within the cell by recruitment of myosin Va [38,39]. Naturally the authors discussed that the choroideremia phenotype could result from defects in pigment granules transport in the RPE and choroid [38]. A new study involving mouse models, *ashen* included, showed that functional Rab27a was required in the light-dependent movement of melanosomes into the apical processes of RPE cells [40], in a tripartite complex with myosin VIIa and effector proteins [40,41]. Another Rab protein, RAB38, was later implicated in melanosome biology in RPE cells, namely in coordinating the delivery of melanin-synthesising enzymes to them [42]. However, symptoms related to skin pigment, e.g. depigmentation, or tanning problems were never reported in choroideremia patients.

While the function of RAB27A was being unveiled, others continued to investigate the interactions between Rabs and REP deeper. New results contradicted previous findings that RAB27A binds REP1 with higher affinity than REP2: RAB27A was found to bind equally well to REP1 and REP2 [43]. The difference in prenylation kinetics was instead due to the fact that RAB27A-REP1 complex has a higher affinity for GGTase-II than RAB27A-REP2. Additionally, RAB27A was shown to have the slowest rate of GTP hydrolysis among the Rabs tested [43]. These facts were corroborated by others who looked into the structure of the RAB7:REP1 complex [44]. Using innovative real-time monitoring of Rab prenylation, they demonstrated that the weaker interaction of REP2 with Rabs was not specific to RAB27A, but rather, was common to all substrates tested. Therefore, a new model was proposed for the accumulation of underprenylated RAB27A in choroideremia cells: when the total activity of REP is limiting, other Rab proteins are better positioned than RAB27A [44].

Almost ten years later a different approach to the query added a new piece of information: Rabs may be hierarchically processed by the GGTase-II based on their prenylation rates [45].

Following analysis of Rab prenylation rates *in vivo* and *in vitro*, the authors found that RAB27A displays one of the slowest prenylation rates, together with RAB42, RAB27B and RAB38 [45]. The authors suggested these proteins would be more prone to underprenylation in choroideremia cells, and possibly contribute to the phenotype.

2.3. Insights from animal models

Animal models of disease are valuable tools to better understand the underlying mechanisms and test potential treatment options. For choroideremia research, however, this is a limitation because there is no natural occurring model that resembles the human condition.

The first attempt to generate a choroideremia mouse model involved the disruption of the *Chm* gene to generate a premature stop codon [46]. The viable offspring exhibited ocular defects resembling the human choroideremia phenotype [46]. Researchers also found that, if the allele was of maternal origin, the null mutation was embryonically lethal in both males and heterozygous females. While unexpected at the time, this observation was explained by the preferential inactivation of the paternal X chromosome in murine extra-embryonic tissues, thereby preventing placental development [46]. This pattern of inactivation does not seem to be a problem for humans because males can transmit the disease (as carriers) to females. However, the absence of any natural occurring animal model of choroideremia identified to date might be explained by this embryonic mechanism.

A more sophisticated and successful gene targeting approach was presented in 2006: a conditional KO model of choroideremia was created using tamoxifen-inducible and tissue-specific Cre expression in combination with *Chm* alleles [47]. $Chm^{null/WT}$ females displayed a rapid and severe retinal degeneration. Moreover, RPE and photoreceptor cell layers were shown to degenerate independently of each other [47]. Further details into the dynamics of the degenerative process were revealed using layer-specific KO models [48]. The study showed that a diseased RPE accelerates the rate of photoreceptor degeneration, and it suggested functional defects would cause visual impairment, which would be worsened by the cell death that occurs later. This claim was sustained by

the complex interplay between the survival and function of photoreceptors, RPE status and aging [48]. Later, the loss of the *Chm* gene in pigmented cells (*Chm*^{flox}, *Tyr-Cre+*) was reported to cause premature accumulation of aging features in the RPE, namely accumulation of lipofuscin-containing deposits and disorganised basal infoldings [49].

The choroideremia conditional KO mouse also showed accumulation of pigment granules in the main cell body of the RPE [47]. This phenotype could be explained by a deficiency in Rab27a prenylation, in agreement with the well-described role of RAB27A in melanosome movement [40,41]. However, no visual defects were found in *ashen* retinas [37,40]. Similarly, although the KO model for GGTase-II, *gunmetal* (*gm*), featured alterations in pigmented tissues, the phenotype is quite different from choroideremia [50].

The identification of a zebrafish model of choroideremia was more straightforward than for the mouse. In search for genes involved in hair-cell function, Starr et al. identified a mutant line, *ru484*, which showed balance and hearing defects as well as disruption of retinal cell layers; however, these fish die at day 6 post-fertilization (dpf) [51]. Using a positional cloning strategy, the authors mapped the gene to the human homolog *CHM*, and named it *chm* [51]. The retinal defects were then analysed in more detail at 4.5 dpf: the outer segments were dishevelled and the RPE was hypertrophic and disorganized [52]. Later it was reported that not only fish have a single *chm* gene, but also *CHM* is the ancestral gene in the REP family [53]. *CHML*, on the other hand, is restricted to the mammalian lineage and represents a retrogene that was reinserted into the genome at some point during mammalian evolution. Therefore the lack of any compensation by REP2 in *chm* mutants determined their general organ failure and early lethality [53]. Although the choroideremia zebrafish was ruled out as a good model to study the human disease [53], it has its uses in drug screening programmes [54].

2.4. Insights from *in vitro* models

Given the unfeasibility in obtaining human retinal tissue to deeply study the mechanisms of the disease *in vitro*, alternative methods are very valuable. As REP1 is ubiquitously expressed, cells from choroideremia patients could be used for *in vitro* confirmation of diagnosis and testing of potential therapies. Unlike retinal tissue, peripheral blood cells can be harvested non-invasively and even cultured for a period of time. Such an approach was first tried with blood samples from choroideremia patients back in 1998 [55]. An immunoblot analysis on PBMCs obtained from a fresh peripheral blood sample showed absence of REP1 expression, confirming the diagnosis of choroideremia [55]. More families were screened using this approach and in most of them the diagnosis was supported by the immunoblot analysis [56]. The authors added, though, that this method would not necessarily exclude diagnosis, but could be used together with mutational screening of the *CHM* gene [56].

Fibroblasts grown from a skin biopsy cultured *in vitro* constitute an alternative source of cells obtained by minimally invasive methods. Immunoblot analysis of REP1 expression in cells from choroideremia patients, either PMBCs and/or fibroblasts, has been used to validate the diagnosis of choroideremia, regardless the mutation [13–15,18,19,55,57–63]. All these reports showed absence of REP1 expression in the cell type analysed, except for the three missense mutations, which showed reduced expression of REP1 [13–15], and an in-frame deletion (c.117_314del; R40_S105del), where a truncated protein would be predicted. Overall, this data confirm that most *CHM* mutations result in complete absence of REP1 expression, although exceptions exist.

Some of the aforementioned studies linked the expression of REP1 with its prenylation activity to confirm a functional defect in choroideremic PBMCs [45] and fibroblasts [15,19,57]. Moreover, induced pluripotent stem cells (iPSC)-derived RPE carrying a *CHM* mutation also showed a deficit in prenylation, reinforcing the idea that such a model could mimic the biochemical phenotype of patients *in vitro* [15,63]. One of these studies also investigated the distribution of RAB27A using differential centrifugation techniques: the authors found that RAB27A is more prevalent in the cytosolic than the membrane fraction of iPSC-derived RPE of choroideremia patients as compared to

controls. A different group has shown that RAB27A localised perinuclearly in choroideremic skin fibroblasts, as opposed to being trafficked to the cell membrane in control cells [58]. These findings are in agreement with the described role of RAB27A in intracellular vesicle movement [37].

The absence of REP1 in cells can also be recreated *in vitro* using gene-silencing strategies either with siRNA or shRNA technology. The successful inhibition of *CHM* by siRNA in human foetal RPE cells altered its phagocytic and secretory pathways, namely by delaying the clearance of photoreceptor outer segments (POS) [64]. A different approach using shRNA to knockdown (KD) *CHM* in HeLa cells proved equally efficacious [45]. In this study, the authors also showed a functional deficit regarding the prenylation levels of Rab proteins, which could be rescued by exogenous REP1, but not REP2. These results suggest the two REP isoforms have non-redundant functions in the cell, despite their degree of similarity [45].

2.5. Systemic manifestations of choroideremia

Until very recently, the choroideremia phenotype was described as restricted to the retina. A report by Zhang et al. shed light on the hypothesis of choroideremia being a systemic disease [65]. An X-linked family from Sri Lanka presented a severe choroidal degeneration that resembled the retinal phenotype of Bietti's crystalline dystrophy (BCD). Sanger sequencing analysis excluded mutations that would cause BCD and found a mutation in the *CHM* gene instead. However, because BCD also has systemic manifestations, the authors investigated further this family and found intracellular crystals in red blood cells, lymphocytes, and platelets. Additionally, they found consistent fatty acid alterations in a wider cohort of choroideremia patients and postulated its origin on the role that REP1 plays in vesicular trafficking. This was the first time choroideremia was illustrated as a systemic condition [65]. Recently another patient from Southeast Asia was reported initially to have X-linked retinitis pigmentosa caused by mutation in *RPGR*, but was subsequently found to have a second mutation in *CHM* which was disease causing [66]. These reports illustrate the difficulty in diagnosing choroideremia in heavily pigmented fundi because the pigment may mask exposure of the underlying white sclera.

3. AAV gene therapy approach to treat choroideremia

Choroideremia is considered a prime candidate for gene replacement therapy for the following reasons: (1) the disease burden is significant and the ratio benefit-risk is good, considering that no alternative therapy is available; (2) the gene locus has been identified, and there is extensive knowledge on how the disease progresses; (3) the target cells can be assessed for delivery of a drug product by subretinal injection. Particularly, a gene augmentation approach, where a working healthy copy of the gene is delivered, would be beneficial due to the fact that most *CHM* cases are functionally null, i.e. REP1 is predicted to be severely truncated or completely absent. Therefore, it exerts no dominant negative effects over a healthy protein. Moreover, it is highly likely that the phenotype will improve even with low expression levels of the gene. This fact is extremely important given the slow rate of disease and its long therapeutic window: a successful therapeutic intervention could preserve and potentially improve the remaining vision, even at later stages of the disease [4,67,68].

3.1. Design of an AAV vector

Of all possible delivery systems to be used for retinal gene therapy, adeno-associated viruses (AAV) are the most attractive, mainly because they cause a minimal immune response [68]. Furthermore, when a gene therapy approach for choroideremia was being planned, it has been already made public the safety profile of an AAV-mediated gene transfer to treat Leber congenital amaurosis (LCA). Three independent groups showed that the subretinal administration of an AAV carrying the sequence for *RPE65* in humans caused no significant immunogenicity, inflammation or toxicity [69–71].

AAVs are available in different serotypes, which confer different cellular tropism, i.e. preferential target of a specific cell. Serotype 2 was shown to transduce both the RPE and the photoreceptors after subretinal injection in non-human primates [72,73]. Moreover, expression of genes delivered by AAV2 is sustained for a very long time, more than 10 years in case of the dog

models of LCA [74] and in humans for more than three years [75–77]. AAV2 was therefore the selected vector to carry a gene expression cassette for choroideremia.

When designing an AAV expression cassette the goal is to achieve the highest efficiency possible, so the overall total dose needed is lower. The packaging capacity of the vector may constitute a problem, as it fits up to approximately 4.7 kb of DNA. However, this was not the case in choroideremia. The *CHM* gene is 1.9 kb long, which allows ample room to fit a few regulatory elements [4]. The first to be considered was a strong promoter. As concerns about diseased RPE cells being able to inactivate cell-specific promoters arose [68], the choice shifted towards a well-characterised ubiquitous promoter, the modified chicken β-actin (CAG) promoter. This is the same promoter used in two of the LCA clinical trials [70,71].

Regulatory elements, such as the woodchuck posttranscriptional regulatory element (WPRE) may be added downstream of the coding sequence [4]. This sequence acts on increasing mRNA levels by enhancing transcription termination, and it has been proven safe to use in gene therapy for Parkinson's disease [78]. Recently a new report fully supported the inclusion of the WPRE in the expression cassette of AAV vectors used in retinal gene therapy. The authors showed that the inclusion of WPRE led to significantly higher levels of green fluorescent protein (GFP) and REP1 expression, namely in all retinal layers following subretinal injection in wild-type mice [79].

The last but not least piece of the expression cassette is a polyadenylation signal sequence. With the addition of the bovine growth hormone polyA sequence, the design of the AAV vector for choroideremia gene therapy was complete [4]. And preclinical testing followed.

3.2. Pre-clinical testing

A research-grade preparation of the construct described above was tested *in vitro* and *in vivo*, and a similar construct containing GFP was created as a reporter for successful transduction [80]. Both constructs were first tested in fibroblasts derived from a choroideremia patient, where REP1 was absent. Following transduction with AAV2-REP1, the authors reported an increase in both

expression of REP1 and prenylation activity, when compared to AAV2-GFP or untransduced cells. *In vivo* studies then involved subretinal administration of the GFP vector in wild-type mice, where transduction of both the outer nuclear layer of the photoreceptors and the RPE was achieved. Treatment of human retinal explants with AAV2-GFP *ex vivo* showed transduction of the neural retina. The subretinal administration of AAV2-REP1 in wild-type mice produced human REP1 in the RPE as confirmed by immunoblot analysis at 5 weeks post-injection and showed no signs of toxicity when overexpressed. Finally, administration of AAV2-REP1 in $Chm^{null/WT}$ resulted in a dose-related effect in preservation of retinal function as assessed by electroretinography 6 months post-injection [80]. This data was key to apply for ethical approval to run a phase 1/2 AAV gene therapy clinical trial for choroideremia.

3.3. Results from human clinical trials

The first surgery to deliver AAV2-REP1 was performed in October 2011 in Oxford as part of a phase 1/2 dose escalation clinical trial to investigate gene therapy as a potential treatment of the retinal degeneration and blindness caused by choroideremia (ClinicalTrials.gov Identifier: NTC01461213). Although subretinal gene therapy had already been performed in *RPE65*-associated LCA, this was the first time that the central fovea (the area responsible for fine vision) was routinely targeted for treatment in patients with near normal levels of visual acuity. Since traumatic retinal stretch could adversely affect visual acuity, the surgical technique of vector delivery became an important factor in determining clinical outcome. Using a two-step approach, first detaching the macula with balanced salt solution then injecting the vector into the subretinal fluid bleb, the trial has shown that recovery to baseline acuity and retinal sensitivity generally occurred within one month, and that visual acuity gains are possible despite the iatrogenic foveal detachment.

The phase 1/2 clinical trial was primarily designed to assess the safety of the vector and the effective dosage. Early results from patients who received the low dose proved that choroideremia gene therapy was safe, and led to improvement in visual acuity in some patients: treated eyes gained on average more than one line (>5 letters) of vision on the ETDRS (Early Treatment Diabetic Retinopathy Study) acuity chart, compared to untreated fellow eyes [81]. A longer-term follow-up of

the same cohort showed that the initial gains in visual acuity had been sustained for 3.5 years after treatment [82]. Improvements in the surgical delivery technique [83,84] were required before the dose escalation arm of the trial, which has now finished. The final results show that visual acuity had been improved in treated eyes compared to untreated eyes [85]. The same vector was administered in similar trials run in Alberta (NCT02077361), Miami (NCT02553135) and Tübingen (NCT02671539); the outcomes so far are of a good safety profile [86]. A phase 2 clinical trial, REGENERATE (REP1 Gene Replacement Therapy for Choroideremia) was initiated in 2015 aiming to treat patients at an earlier stage of the disease, and it is still ongoing (NTC01461213).

Although the programme outlined above was the earliest, and has progressed the furthest, another independent team also investigated choroideremia gene therapy at around the same time. The vector design differs to that already described above in not having the WPRE sequence, which will reduce expression levels at sub-saturation doses [79], and proof-of-concept work has been published [58]. A phase 1/2 trial has begun (NCT02341807) with safety and tolerability as primary outcomes.

4. Alternative treatments for choroideremia

This section summarizes other strategies currently under development with a potential to treat choroideremia.

4.1. Small molecule translational read-through inducing drugs (TRIDs)

Around 30% of all IRD patients carry a nonsense mutation [87] resulting in premature termination codons (PTCs) which promote mRNA destabilization by nonsense-mediated mRNA decay (NMD) [88]. However, a functional protein may be produced if either its mRNA decay rate or extent of premature termination is altered. This possibility led researchers to investigate the use of molecules that could mimic aminoglycosides, a class of drugs that promotes read-through. Such molecules, named TRIDs, could in principle alleviate the pathologies of nonsense-mediated diseases [88].

Studies involving the only animal model of choroideremia with a nonsense mutation, the *chm*^{ru848} zebrafish, showed that aminoglycosides treatment was able to prevent mutant ocular phenotypes [89]. Later on also PTC124 (ataluren) and its chemically optimised-derivative PTC414 were tested in two independent disease models of choroideremia [57]. The authors found that both TRIDs could functionally rescue REP1 in both the *chm*^{ru848} model and in choroideremia fibroblasts harbouring a nonsense mutation [57].

PTC124 has also been shown to restore protein levels in two models of retinitis pigmentosa. Fibroblasts from a patient with a mutation in the *RP2* gene were reprogrammed to RPE using iPSC technology. The treatment of iPSC-derived RPE with PTC124 restored up to 20% of endogenous levels of RP2, which was enough to reverse the phenotype in this model [90]. A similar approach was used for MERTK deficiency: in this case, the treatment of iPSC-derived RPE with PTC124 was able to restore 12% of the phagocytic function of the cells [91]. However, *in vitro* results will be challenging to translate to *in vivo* because the equivalent plasma levels of PTC124 are likely to be highly toxic and probably lethal at the dose required for translational read-through in the retina of choroideremia patients. Furthermore the effects would be non-specific and translation would be impaired throughout the genome by read-through of normal stop codons in other genes.

4.2. Prenylation inhibitors

Pharmacological compounds able to inhibit prenylation have been studied as antiviral agents to treat infections by the hepatitis delta virus, and shown to potentially have broad-spectrum activity [92].

More widely known are statins, potent lipid-lowering agents that act by inhibiting the HMG-CoA reductase (HMGCR) therefore affecting the mevalonate synthesis, and ultimately cholesterol levels [10]. In fact, mevastatin, a HMGCR inhibitor, was tested in a "prenylation block-and-release" *in vitro* assay to study the membrane targeting mechanisms of Rab GTPases [93]. As the reduction of the downstream product of this pathway, GGPP, would affect the prenylation of Rab proteins, the

potential effects of statins on the retinal function in mice were assessed [94]. This study found changes in the full field electroretinogram (ERG) of wild-type mice treated with simvastatin for 4 weeks [94], and prompted further investigations. An internet-based health survey on the co-morbidities of choroideremia patients found patients taking statins had poorer vision [95]. However, after the obvious age correction, there were no significant differences between the groups of males with and without functional vision [95]. Although the use of statins may influence retinal function, this is, for the moment, another less understood factor that may contribute to the choroideremia phenotype, in the way environmental factors do [20].

5. Conclusion

This review highlights as much what we do know as what we still do not know about the molecular pathogenesis of choroideremia. Choroideremia is a degenerative condition, that (heavily) impacts the quality of life of afflicted males, and for which there is no treatment yet. AAV gene replacement therapy is the most promising candidate to be a successful treatment strategy in the long term. In fact, the AAV2-REP1 vector (part of Nightstar Therapeutics plc leading programme) was granted both US Food and Drug Administration (FDA) and European Medicines Agency (EMA) Orphan Drug Designation in 2015. This construct has been extensively studied pre-clinically [79,80] and has held promising safety and efficacy profiles following administration into patients [81,82,86]. However, as each choroideremia patient is unique, the challenge remains to further improve this treatment strategy and successfully target as many patients as possible.

6. Expert opinion

It took 25 years after the identification of the gene responsible for choroideremia for the scientific community to see improvements in vision following gene correction in a clinical trial [81].

The AAV gene therapy strategy for the treatment of choroideremia holds a high chance of success for the reasons abovementioned, when compared to the potential alternatives available. The use of TRIDs is limited to nonsense mutations, and the side-effects of their systemic use are yet to be determined. Upcoming clinical trials will hopefully shed light on these questions. Statins, on the other side, are safe to use systemically, but their potential impact on human RPE cells remains unknown.

The primary defect in choroideremia is retinal degeneration. The fact that *CHM* function is well established prevails as the key finding in choroideremia research. Nevertheless, it remains to be seen why the eye is the most affected organ of the body, if REP1 expression is ubiquitous. When RAB27 was first implicated in choroideremia, the authors addressed this question by suggesting that the dysfunctional transport step in which RAB27 is involved may not be so crucial to other tissues' viability [35]. In agreement with this argument, we discuss below the reasons that lead us to believe that a scenario of vesicular trafficking defects throughout the RPE better defines the choroideremia molecular pathology.

Prenylated proteins comprise 2% of total cellular proteins [23] and evidence shows that prenyl groups are key for protein-protein interactions in cells. The RPE is a highly organised cell layer, where an intricate balance of vesicular trafficking pathways is required for proper function (Figure 2). Besides the normal intracellular trafficking pathways of a cell, RPE has two specialised pathways that work to fulfil its demands. One of them is the phagocytosis of shed POS, which makes RPE one of the most actively phagocytic cells of the human body. The impact of *CHM* mutations on this mechanism was sustained by the delay in clearance of POS in response to *CHM* silencing observed *in vitro* [64] and the phenotype of *chm* zebrafish *in vivo* [51,52]. Furthermore, several Rab proteins are involved in this pathway (Figure 2) [28]; a deficit in the overall levels of Rab prenylation affects the shedding of POS, and contributes for accumulation of undegraded products in the RPE [49,96,97].

The RPE-choroid complex contains the highest melanin concentration of all human tissues [98]. This pigment is essential to absorb scattered photons, therefore protecting the photoreceptors

from environmental light damage. In response to light, melanosomes relocate to the apical microvilli of the RPE, a process also regulated by Rab GTPases [40–42]; this is the second specialised pathway to RPE cells (Figure 2). Recently a study reported that RAB6 controls the trafficking of melanosomal cargoes, and adds a new player to the mechanism by which melanosomes mature [99]. Evidence shows that most classical melanogenesis occurs pre-natally [42], although others show that melanin synthesis can also occur in adult mammalian RPE via an alternative pathway [98,100], in which case choroideremic RPE will be affected. The fact that the RPE appears slightly depigmented with redistribution of pigment granules to the main cell body in $Chm^{null/WT}$ and Chm^{flox}, *Tyr-Cre+* [47,48] supports this observation. Conclusively, a deficit in prenylation in the RPE impairs both melanosome maturation and movement, causing retinal degeneration in the long term.

Loss of *CHM* gene in choroideremia reduces the total amount of total REP activity. REP2 does compensate to an extent, but other multiple contributing factors also play a role in the process [43], namely the prenylation rate of Rab GTPases [45] (Figure 2). Ultimately, absence of REP1 does cause a disruption in both standard and specialised vesicular trafficking pathways. From this perspective, the retinal degeneration in choroideremia results from a lifetime accumulation of insults to the highly-demanding RPE cells and the photoreceptors. This suggests that delivery of a healthy, functional copy of REP1 could restore some cell functions and slow down the degeneration process. This can be achieved by subretinal administration of AAV2-REP1 to target a surviving area of the retina, which may improve patients' visual acuity if applied at a later stage when visual acuity is in decline [81,82,84]. The challenge that this therapy now faces is in the timing of intervention, given that choroideremia manifests itself within a range of progression rates. Recent data shows that the majority of choroideremia patients undergo retinal degeneration with a similar rate of exponential decay [101]. However, the question remains as to what determines the time of onset of retinal degeneration. The approval of LUXTURNA® (voretigene neparvovec-rzyl) by the FDA in the end of 2017 made history as the first directly administered gene therapy product to obtain market authorisation in the US. This follows the publication of the results of a phase 3 clinical trial where patients injected with AAV2-hRPE65v2 showed improved functional vision [102]. The future for

choroideremia gene therapy must include a larger, phase 3 trial, where efficacy can be assessed in a wider range of disease manifestations. All the evidence to date suggests that the restoration of both RPE and photoreceptors function, due to AAV mediated delivery of functional REP1 will improve, and sustain visual acuity in choroideremia patients.

Article highlights:

- Choroideremia is a degenerative inherited retinal disease for which there is no treatment yet.
- The product of the causative gene, REP1, has a well-described function: it regulates intracellular trafficking pathways by prenylation of Rab GTPases.
- In choroideremia, the impairment of trafficking pathways in the RPE specialised cell layer disrupts cell homeostasis and causes retinal degeneration.
- Targeting the RPE and photoreceptors of choroideremia patients using an AAV to deliver a healthy copy of *CHM* has shown promising results.
- The surgical complexity of delivering the AAV vector to the target area remains as the main challenge for the future of AAV gene therapy for choroideremia.

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Reviewer disclosures

A reviewer on this manuscript has disclosed that they are a co-principal investigator of one of the ongoing gene therapy trials for choroideremia.

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*** of considerable interest*

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Figure legends

Figure 1: Fundal appearance of choroideremia – optomap (Optos, Dunfermlin, UK) wide-field photography (top panel) and autofluorescence imaging (Heidelberg Spectralis BluePeak, Heidelberg, Germany; bottom panel) for three patients (A-B, C-D and E-F).

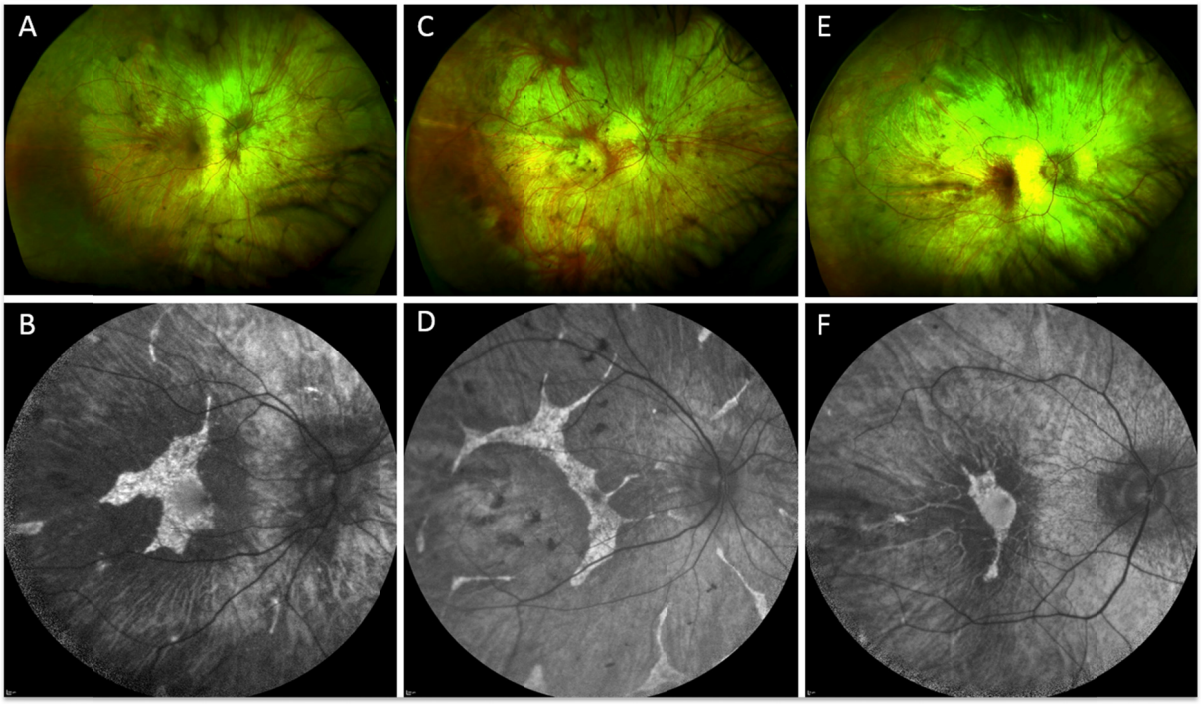

Figure 2: (A) Isoprenoid metabolism simplified. The production of geranylgeranyl-diphosphate (GGPP) share the early steps of cholesterol metabolism (the mevalonate pathway), which starts with HMG-CoA reductase, a target of statin drugs. (B) REP and Rab GTPase cycles. Rab proteins require a post-transcriptional covalent attachment of isoprenoids for membrane association. Rab escort protein (REP) binds newly synthesised Rab proteins in the cytosol and presents them to the GGTase-II heterodimeric complex (α and β subunits) to be prenylated. After attachment of GGPP to the C-terminal of the Rab protein, REP delivers the prenylated Rab to the membrane of the donor compartment, and recycles to bind another Rab. In the absence of REP or GGTase-II, Rab proteins remain in the cytosol. Once in the donor membrane, Rabs are activated into the GTP-bound state by a guanine nucleotide exchange factor (GEF); they can then recruit other effectors that direct the vesicle to the acceptor compartment [97]. During or following fusion, Rab-GTP is converted to the GDP-bound form by a GTPase activating protein (GAP), and returns to the cytoplasm, where it is free to bind a REP. (C) Representation of the main intracellular trafficking pathways in RPE cells, with a subset of Rab proteins involved in each (Note: the Rab proteins listed is not extensive but rather representative). Abbreviations: BM: Bruch's membrane; Ch: choroid; EE: early endosome; ER: endoplasmic reticulum; GDP: guanidine diphosphate; GGPP: geranylgeranyl-diphosphate; GTP:

guanidine triphosphate; HMG-CoA: 3-hydroxy-3-methylglutayl-CoA; E/Ly: late endosome/lysosome; Mel: melanosome; Mit: mitochondria; Nu: nucleus; RPE: retinal pigment epithelium; ROS: rod outer segments; SV: secretory vesicle; TGN: trans-Golgi network; TJ: tight junction.