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Use of induced pluripotent stem cell models to probe the pathogenesis of Choroideremia and to develop a potential treatment

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ABSTRACT

Choroideremia (CHM) is a rare monogenic, X-linked recessive inherited retinal degeneration resulting from mutations in the Rab Escort Protein-1 (REP1) encoding CHM gene. The primary retinal cell type leading to CHM is unknown. In this study, we explored the utility of induced pluripotent stem cell-derived models of retinal pigmented epithelium (iPSC-RPE) to study disease pathogenesis and a potential gene-based intervention in four different genetically distinct forms of CHM. A number of abnormal cell biologic, biochemical, and physiologic functions were identified in the CHM mutant cells. We then identified a recombinant adeno-associated virus (AAV) serotype, AAV7m8, that is optimal for both delivering transgenes to iPSC-RPEs as well as to appropriate target cells (RPE cells and rod photoreceptors) in the primate retina. To establish the proof of concept of AAV7m8 mediated CHM gene therapy, we developed AAV7m8.hCHM, which delivers the human *CHM* cDNA under control of CMV-enhanced chicken β-actin promoter (CßA). Delivery of AAV7m8.hCHM to CHM iPSC-RPEs restored protein prenylation, trafficking and phagocytosis. The results confirm that AAV-mediated delivery of the REP1-encoding gene can rescue defects in CHM iPSC-RPE regardless of the type of disease-causing mutation. The results also extend our understanding of mechanisms involved in the pathophysiology of choroideremia.

1. Introduction

Choroideremia (CHM) is an X-linked disorder with an incidence of approximately 1:50,000 (McCulloch & McCulloch, 1948; L & CC, 2009). CHM is a progressive retinal degenerative disease that is characterized by childhood-onset night blindness (nyctalopia), loss of peripheral vision, and progressive loss of neural retina, RPE and choroid in a peripheral-to-central fashion. The cone photoreceptor-enriched region of fine visual discrimination, the fovea, is spared until late in the disease, but ultimately, total blindness results from destruction of this area, typically after the fifth decade of life (Aleman et al., 2017a; Sanchez-Alcudia et al., 2016). The disease-causing gene, *CHM*, encodes Rab Escort Protein-1 (REP1), a protein that enhances geranylgeranylation of ras-related GTPases, Rab proteins. The precise mechanisms by which lack of REP1 in the retina leads to disease are unknown, however a lack of prenylation of specific Rab proteins (Rab27a, Rab6a, Rab38) leads to defects in trafficking of motile small vesicles which is implicated in the disease pathogenesis of CHM (Bolasco et al., 2011; Kohnke et al., 2013). To date, >110 mutations have been found to result in CHM. In extra-ocular tissues, REP2 compensates for a lack of REP1 and so disease is limited to the retina (Kohnke et al., 2013; Larijani et al., 2003).

The primary retinal cell type leading to choroideremia is unknown, although evidence from engineered mice and zebrafish and cells from affected individuals indicate that the affected cells likely involve RPE cells, photoreceptors or potentially choroidal cells (Anand et al., 2003; Black et al., 2014; Cereso et al., 2014; Tolmachova et al., 2006; Tolmachova et al., 2013; Tolmachova et al., 2012; Vasireddy et al., 2013). Studies of Chm in mouse models are challenging in large part due to the fact that lack of Rep1 is lethal *in utero* in this species. Use of conditional mutants has helped confirm that RPE and photoreceptor cells are likely primary cell types causing the disease although these animals are not faithful models of the disease and are also difficult to access. Further, the fact that the earliest symptoms of choroideremia in

Abbreviations: CHM, Choroideremia; AAV, Adeno associated Virus; AAV2, AAV serotype 2 (Note: similar nomenclature for other AAV capsid serotypes); iPSC, induced Pluripotent Stem Cells; MEF, Mouse Embryonic Fibroblasts; REP1, Rab Escort Protein-1; TER, Transepithelial Electrical Resistance; RPE, Retinal Pigment Epithelium; iPSC-RPE, iPSC's that are differentiated into RPE cells *in vitro*; OS, outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; (CMV/CβA), cytomegalovirus (CMV) enhancer with Chicken β-actin promoter; *eGFP*, enhanced green fluorescent protein (eGFP) cDNA.

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humans include difficulty in seeing in dim light (nyctalopia) and loss of peripheral visual fields together with the preservation of cone photoreceptor (foveal) function until late in the disease (Aleman et al., 2017a) suggest that rod (but not cone) photoreceptors are affected early in the disease course. Because of the difficulties with *in vivo* models for choroideremia and other retinal diseases, we and others have explored the approach of using induced pluripotent stem cell (iPSCs) models (Cereso et al., 2014; Vasireddy et al., 2013; Parfitt et al., 2016; Tucker et al., 2013).

Retinal pigmented epithelial cells are a critical cell type for active investigations for retinal developmental models, cell transplantation, and are increasingly used for preclinical assessment of therapeutics (Kamao et al., 2014; Bennett, 2017). RPE is a thin layer of pigmented epithelium that is adjacent to the photoreceptors of the retina and Bruch's membrane. The RPE monolayer provides numerous functions in the retina, such as protection against photo-oxidative stress, provision of nutrients (including secretion of growth factors and cytokines), separation from the blood supply (in part due to integrity of apical and basal polarity) and removal of waste products (due in part to elimination of outer segments of photoreceptors through phagocytosis) (Wavre-Shapton et al., 2013; Gordiyenko et al., 2010; Sun et al., 2016).

In this study, we explored the utility of iPSC-RPE to study disease pathogenesis and a potential gene-based intervention in four different genetically distinct forms of CHM which together, represent the most common disease-causing mutations: splicing defects, deletions and premature stop signals. iPSCs were derived from peripheral blood monocytes (PBMCs). Cells from normal-sighted individuals were used as control.

Current clinical studies for CHM focus on gene augmentation strategies using AAV2 and aim to prevent further degeneration and potentially also to enhance retinal function (Aleman et al., 2017a; MacLaren et al., 2014; Edwards et al., 2016; Aleman et al., 2017b). Progression to a human clinical trial was supported by the fact that AAV2-hCHM rescues prenylation and Rab27a protein trafficking in undifferentiated iPSC's derived from subjects with CHM mutations (Vasireddy et al., 2013). Recent clinical trials demonstrated the safety and efficacy of AAV2 mediated viral delivery (MacLaren et al., 2014; Edwards et al., 2016; Aleman et al., 2017b). In addition to delivering the transgene to RPE efficiently, high doses of AAV2 are required to target photoreceptors in non-human primates (Vandenberghe et al., 2011). In the present study, we further investigated the role of CHM mutations on cellular trafficking, phagocytosis, and Rab27a localization in patient-derived iPSCs differentiated into RPEs. We show that lack of REP1 leads to reduced and delayed phagocytosis, perinuclear accumulation of Rab27a, and inhibition of prenylation in three CHM patient-derived iPSC-RPEs. These defects are rescued by gene augmentation through the delivery with a vector that could be used in the future to deliver the transgene efficiently to the likely primary disease-causing cells, photoreceptors and RPE cells, in humans, AAV7m8-CHM.

2. Materials and methods

2.1. Human pluripotent stem cells generated from CHM probands

Primary peripheral blood mononuclear cells (PBMCs) were collected from four CHM probands carrying mutations in the *CHM* gene. The *CHM* mutations were 1) CHM-1 (JB-415, c.1327_1328delAT), 2) CHM-2 (JB-500, exon 2–4 deletion), 3) CHM-3 (JB-527, exon 2–4 deletion), and 4) CHM-4 (JB-588, Arg555STOP, AGA → TGA). Mutation validation was performed on patient-derived iPSC gDNA using primers in Supplementary Table 1. All human protocols were approved by the institutional review board (IRB) at the University of Pennsylvania and each donor provided signed informed consent (IRB 808828). PBMCs were expanded in SP34 media containing IL-3, IL-6, SCF, and FLT3 plus penicillin/streptomycin and glutamine. Reprogramming of expanded PBMCs was performed as previously described (Maguire et al., 2016). Cells showing iPSC morphology were collected and expanded in iPSC medium (iPSC·CM; DMEM/F12 (50:50; corning) containing glutamine, penicillin/streptomycin, 15% Knockout serum replacement (KSR), 1× NEAA, 0.1 mM β-mercaptoethanol (2-ME), and 5 ng/mL of bFGF (R&D Systems) on 0.1% gelatin coated dishes with irradiated mouse embryonic fibroblasts (iMEFs). Cell lines were expanded and characterized for morphology, pluripotency gene expression (cMYC, DNMT3B, Nanog, OCT3/4), surface markers expression (SSEA3-488; Biolegend 1:100, SSEA4-647, Biolegend 1:100), Sendai transgene clearance, and all g-banding analyses were performed by Cell Line Genetics, Inc. CHM iPSCs were cultured on 1:100 matrigel coated dishes in StemFlex media (Invitrogen, Carlsbad CA) to perform germ layer specific analysis; mesodermal cultures (days 0–2: RPMI media plus 1 μM Chir99021, 5 ng/mL BMP4, and 50 ng/mL VEGF), days 3–7: serum free media (SFM) containing (5 ng/mL BMP4, 50 ng/mL VEGF, and 20 ng/mL bFGF), Endodermal (Day 0–2: RPMI media plus 2 μM Chir99021, 50 ng/mL Activin-A), days 3–7: SFD media containing (0.25 ng/mL BMP4, 10 ng/mL VEGF, 5 ng/mL bFGF, and 50 ng/mL Activin-A), Ectodermal (days 0–7: DMEM/F12 media containing 2% B27, 1% N2, 100 nM LDN193189, 10 mM SB431542, and 2 μM XAV939). Cultures were collected after 7 days and analyzed for lineage specific genes and compared to iPSC RNA. The primers for pluripotency and germ layer are provided in Supplementary Table 2. The unaffected control iPSCs (JBWT2 (PBWT2), JBWT3 (PBWT3), JBWT4 (BMC)) were previously characterized and published (Sullivan et al., 2014; Mills et al., 2013).

2.2. Retinal pigmented epithelium (RPE) differentiation

iPSCs were cultured on 0.1% gelatinized plates containing iMEFs in 37 °C 5%O_2 5% CO_2 until initiation of RPE differentiation. Cell lines were placed in feeder independent growth conditions by passaging IPSC from iMEFs to matrigel-coated dishes using TrypLE. Once placed on matrigel dishes, cells were maintained in mTESR medium until cells reached a confluence of 50–60%. At this confluence, cells were transferred to 37 °C 5% CO_2 incubator and RPE induction was initiated. The base media for days 0–14 was DMEM/F12 plus 2% B27, 1% N2, 1× P/S, and 1× Glutamax. On days 0–2 (48 h), cells were treated with noggin (50 ng/mL), DKK1 (10 ng/mL), nicotinamide (10 mM), and IGF-1 (10 ng/mL) feeding daily. On days 2–4 (48 h), cells were treated with noggin (50 ng/mL), DKK1 (10 ng/mL), nicotinamide (10 mM), IGF-1 (10 ng/mL), bFGF2 (5 ng/mL). On day 4–6 (48 h), activin-A (100 ng/mL), IGF-1 (10 ng/mL), and DKK1 (10 ng/mL). On days 6–14 (9 days), activin-A (100 ng/mL), SU5402 (5 μM), and VIP (1 nM). On day 15, media was switched to RPEM media DMEM/Ham's F12 (70:30), 2% B27, 1× Glutamax, and 1× P/S. Cells were maintained in RPEM media for 21 days. At day 35, cells were passaged using accutase plus DNASE1 using a two-step approach. Cells were initially treated for 20 min with accutase + DNASE1 to eliminate no-RPE cells, washed 2× with PBS, and then treated with accutase/DNASE1 for 20 min. Cells were detached using p1000 and centrifuged at 1500 rpm for 5 min. Cells were suspended in X-Vivo 10 media containing ROCK inhibitor, Thiazovivin (2 μM). All subsequent expansion of RPE included addition of Thiazovivin (2 μM), which allows for extended passaging of iPSC-RPEs.

2.3. Phagocytosis assays

*pH*rodo *Escherichia coli* BioParticles were purchased through Invitrogen, Carlsbad, CA. iPSCs grown on 8-well chambers were maintained until all monolayer cultures had a cobblestone appearance (approximately 3–4 weeks post-passaging). iPSC-RPEs were incubated with 300 μL of pHrodo™ BioParticles® conjugate (1 mg/mL) for 2 h at 37 °C; thereafter, the cultures were washed 3× with 1× PBS to remove any residual *pH*rodo bioparticle (designated as 0 h). RPE at 4, 8, and 24 h time points were fixed with 4% PFA for 15 min at room temperature, then washed 3× with PBS. After washes, cultures were stained with Hoechst (1:2000, nuclear stain), washed and mounted using Prolong