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Conditional loss of *Kcnj13* in the retinal pigment epithelium causes photoreceptor degeneration

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ABSTRACT

The retina is the light sensing tissue of the eye which contains multiple layers of cells required for the detection and transmission of a visual signal. Loss of the light-sensing photoreceptors leads to defects in visual function and blindness. Previously, we found that mosaic deletion of *Kcnj13,* and subsequent loss of the potassium channel Kir7.1, in mice leads to photoreceptor degeneration and recapitulates the human retinal disease phenotype (Zhong et al., 2015). *Kcnj13* expression in the retinal pigment epithelium (RPE) is essential for normal retinal electrophysiology, function, and survival. Mice with homozygous loss of *Kcnj13* die at postnatal day 1 (P1), requiring a tissue-specific approach to study retinal degeneration phenotypes in adult mice. We used the CRISPR-Cas9 system to generate a floxed, conditional loss-of-function (cKO) *Kcnj13*^{flox} allele to study the pathogenesis of *Kcnj13* deficiency in the retina. To investigate if the *Kcnj13* is required in the RPE for photoreceptor function and survival, we used *Best1-cre*, which is specifically expressed in the RPE. We observed complete loss of *Kcnj13* expression in Cre-positive RPE cells. Furthermore, our findings show that widespread loss of *Kcnj13* in the RPE leads to severe and progressive thinning of the outer nuclear layer and a reduced response to light. Finally, to detect *Best1-cre* expression in the RPE of live animals without sacrificing the animal for histology, we generated a Cre-reporter-containing *Kcnj13* cKO mouse line (cKOR: *Kcnj13*$^{flox/flox}$*; Best1-cre; Ai9*) which can be rapidly screened using retinal fluorescence microscopy. These findings provide new tools for studying the roles of *Kcnj13* in retinal homeostasis.

1. Introduction

Leber Congenital Amaurosis (LCA) is the most severe inherited retinal dystrophy, affecting 2–3 in 100,000 births worldwide (Shah, 2016). Patients with LCA typically exhibit various visual abnormalities, including severe vision impairment or blindness within the first year of life, diminished response to light, nystagmus, and a habitual oculo-digital reflex (Shah, 2016; Weleber et al., 2013). LCA is a genetically heterogeneous autosomal recessive disease and is associated with mutations in 22 genes, to date. Mutations affect genes involved in numerous cellular pathways essential for visual function, including phototransduction, protein trafficking, the visual cycle, photoreceptor outer segment phagocytosis, and photoreceptor development.

Mutations in *Kcnj13* were identified and associated with the human retinal dystrophies LCA and Snowflake Vitreoretinal Degeneration (Hejtmancik et al., 2008; Kumar and Pattnaik, 2014; Pattnaik et al., 2015; Sergouniotis et al., 2011; Zhang et al., 2013). *Kcnj13* encodes an inwardly rectifying potassium (Kir) channel subunit, KCNJ13 or Kir7.1, which is abundantly expressed in the apical membrane of the retinal pigment epithelium (RPE) (Krapivinsky et al., 1998; Kumar and Pattnaik, 2014; Kusaka et al., 2001; Shimura et al., 2001; Yang et al., 2003). The RPE is a pigmented epithelial cell monolayer in the eye which is essential for nourishing and maintaining the neural retina (Strauss, 2005). The RPE is involved in numerous retinal functions, including 1) the phagocytosis and clearance of photoreceptor outer segments; 2) the recycling of all-trans-retinol into cis-11-retinal (the visual cycle); 3) formation of the blood-retinal barrier; 4) absorption of scattered light; and 5) the transport of nutrients and ions between the blood supply and the retina. Kir7.1 regulates potassium (K^+) transport between the choriocapillaris/RPE and the subretinal space by

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facilitating K^+ efflux into the extracellular microenvironment surrounding photoreceptors (Kumar and Pattnaik, 2014; Shahi et al., 2017). However, the molecular mechanisms underlying *Kcnj13*-associated retinal functions and how KCNJ13 dysfunction and/or loss promotes retinal degeneration are unknown.

Previously, we showed that somatic, mosaic loss of *Kcnj13* in the mouse retina leads to photoreceptor degeneration phenotypes, including thinning of the outer nuclear layer (ONL), rhodopsin mislocalization, and reduced visual function (Zhong et al., 2015). We found that RPE cells without KCNJ13 protein survive and wild-type RPE cells can rescue nearby photoreceptors cells underneath mutant RPE cells, suggesting a non-cell-autonomous and indirect role in maintaining photoreceptor function and survival. We also observed that homozygous *Kcnj13* null mice die postnatally, thus requiring a conditional approach to study *Kcnj13* functions in the retina of adult mice.

Here we report the generation of an RPE-specific *Kcnj13* loss-of-function (*Kcnj13* cKO) mouse model which mimics LCA pathology. The $Kcnj13^{flox}$ allele was generated using the CRISPR/Cas9 system to insert *loxP* sites flanking exons 2 and 3 of the *Kcnj13* gene, which include the entire coding region of the gene. We achieved RPE-specific deletion of *Kcnj13* using the Cre recombinase mouse line *Best1-cre* (Iacovelli et al., 2011). *Best1-cre* drives Cre expression using the Bestrophin-1 promoter, which is variably expressed in 40–70% of RPE cells (Iacovelli et al., 2011). This approach enables mice to survive beyond the P1 lethal phase observed with null homozygotes, permitting the study of *Kcnj13* in a tissue-specific manner. In this study, we used *Kcnj13* cKO mutant mice ($Kcnj13^{flox/flox}$; *Best1-cre*) to investigate retinal phenotypes associated with loss of *Kcnj13* in the RPE. We observe thinning of the outer nuclear layer by 15 days after birth (P15) in *Kcnj13* cKO mutant mice with broad *Best1-cre* expression, with severe thinning by 3 months of age. Photoreceptor degeneration observed in *Kcnj13* cKO mutant mice coincides with strong defects in visual function, consistent with previously reported findings (Zhong et al., 2015). Our results demonstrate the utility of new genetic tools for studying *Kcnj13* gene function in a tissue-specific manner.

2. Materials and methods

2.1. Generation of a Kcnj13 conditional allele using the CRISPR-Cas9 system

To achieve Cre-mediated deletion of *Kcnj13*, we generated a floxed *Kcnj13* allele ($Kcnj13^{flox}$) by introducing *loxP* inserts flanking exons 2 and 3 using the CRISPR-Cas9 system. As shown in Fig. 1A and Suppl. Fig.1A, we selected the two gRNA sites (target sites #1 and #2) in the mouse *Kcnj13* locus using an online CRISPR design tool (http://crispr.mit.edu/). We synthesized two 185-bp single-stranded donors (Suppl.Fig.1A, ssDonor#1 and ssDonor#2) to introduce *loxP* elements by homology-directed repair. The gRNAs were synthesized and cloned into pDR274 (Addgene, Cambridge, MA), a T7 promoter gRNA expression vector (Hwang et al., 2013). Following DraI digestion, the linearized expression vectors were purified using a QIAquick Gel Purification Kit (QIAGEN) and used as DNA templates to produce gRNAs with a MAXIscript T7 kit (Life Technologies). For Cas9 mRNA production, we used the T7 promoter-containing pX330 vector (Cong et al., 2013; Zhong et al., 2015), which was digested with NotI and then purified using a QIAquick Gel Purification Kit (QIAGEN). Subsequently, the linearized and purified vector was used as a DNA template to synthesize Cas9 mRNA using the mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies). Cas9 mRNA and gRNAs were purified using RNA Clean & Concentrator-25 (ZYMO Research) and dissolved in RNase-free water. RNA concentrations were measured using a NanoDrop ND1000. Finally, the gRNAs and Cas9 mRNA were mixed with an equal volume of formamide, respectively, and the denatured mixtures were run on an agarose gel to evaluate RNA quality (not shown).

According to the literature (Singh et al., 2015; Wang et al., 2013; Yang et al., 2013) and our previous injection experience (Zhong et al., 2015), a mixture of gRNAs, Cas9 mRNA, and single-stranded donor DNAs (12.5 ng/μl for each gRNA, 50 ng/μl Cas9 mRNA, and 25 ng/μl for each donor) were prepared using RNase-free water. The mixture was microinjected into the cytoplasm of C57BL/6 inbred zygotes. After injection, surviving zygotes were immediately transferred into oviducts of ICR albino pseudo-pregnant females. To confirm if the two *loxP* inserts were introduced into the intended sites, we performed genotyping using PCR on CRISPR founder mice, and selected founders carrying both *loxP* inserts. Next, we checked for germline co-transmission of the two *loxP* inserts by crossing selected founders to *Best1-cre* animals, to identify animals with *Kcnj13* alleles containing cis-inserted *loxP* sites. To check if the *loxP* inserts affect *Kcnj13* expression and if the *Best1-cre* induced excision could cause *Kcnj13* loss-of-function, we performed Cre and *Kcnj13* immunofluorescent staining on $Kcnj13^{wt/wt}$; *Best1-cre*, $Kcnj13^{flox/flox}$; *Best1-cre* and $Kcnj13^{flox/flox}$ mouse retinal paraffin sections. Subsequent generations of mice were genotyped for the $Kcnj13^{flox}$ allele using the following sets of primers: LoxP1 (Fwd: 5′-AAAATTTT ACTTCTCTCAACTTCT-3'; Rev: 5′-AAACATTTTTGGTTTTGTTTT-3′) and LoxP2 (Fwd: 5′-CAACTTAGATTTATGCTTGAAA-3'; Rev: 5′- AAAT AGACATTGATGATGTTGTT-3′). *Kcnj13* cKO mice are maintained with a mixed C57BL/6 and 129S6/SvEvTac background. All mice were maintained under 12-h light and 12-h dark cycles. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine.

Animals used in this study were screened post-mortem using KCNJ13/Cre immunostaining to assess extent of KCNJ13 and Cre expression. Animals with widespread RPE-specific Cre expression and loss of KCNJ13 immunoreactivity were selected for analyses. Animals with patchy Cre and KCNJ13 immunoreactivity were excluded from analyses. All histological and electroretinographic analyses were performed using the *Kcnj13* cKO mice without fluorescent Cre-reporter allele. Number of animal used per group and analysis are included in the figure legends.

2.2. Generation and in vivo screening of Kcnj13 conditional knockout reporter (cKOR) mice

We crossed *Kcnj13* cKO mice to *Ai9* (tdtomato fluorescent protein) Cre-reporter mice to generate the *Kcnj13* cKOR mouse mice with Cre-dependent retinal fluorescence (Suppl.Fig.3) (Madisen et al., 2010). Genotyping on subsequent generations was done using PCR with $Kcnj13^{flox}$, *Best1-cre*, and *Ai9* primers. *Ai9* and *Best1-cre* alleles were genotyped as previously described (Iacovelli et al., 2011; Madisen et al., 2010). *Kcnj13* cKOR mice are maintained with a mixed C57BL/6 and 129S6/SvEvTac background. TdTomato fluorescence was used as a proxy for *Best1-cre* expression. TdTomato fluorescence in the retina was evaluated in live animals using the Micron IV retinal fluorescence microscope (Phoenix Research Labs). A custom TRITC-optimized BrightLine BasicTM single-band exciter filter (25 mm housed, Cat# FF01-542/20–25, Semrock) and barrier (12.5 mm unhoused, Cat# FF01-620/52-12.5-D, Semrock) were installed on the Micron IV camera to allow visualization of TdTomato fluorescence. Mice were anesthetized by intraperitoneal injection of 22 mg/kg ketamine, 4.4 mg/kg xylazine and 0.37 mg/kg acepromazine. Both pupils were dilated, corneas anesthetized, and moistened for 1 min with a drop of tropicamide (1.0%), proparacaine (1.0%), and hypromellose (2.5%), respectively. The objective of the Micron IV camera was gently placed onto the cornea of the animal. The fundus was imaged using bright-field (10 gain; 6 FPS) and TdTomato (10 gain; 2 FPS) exciter and barrier sets.

2.3. Histological analysis and immunofluorescence

We performed hematoxylin and eosin (H&E) staining to examine retinal morphology and immunofluorescence (IF) staining for Cre and KCNJ13 to examine expression and localization in the RPE. Mice were