Experimental Eye Research 176 (2018) 29–39

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Essential function of NHE8 in mouse retina demonstrated by AAV-mediated CRISPR/Cas9 knockdown[☆]

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ARTICLE INFO

Keywords:
NHE8
Retinal pigment epithelium
Photoreceptor cells
CRISPR/Cas9
pH regulation

ABSTRACT

We studied the role of sodium/proton exchanger 8 (NHE8) in retinal pigment epithelium (RPE) and photoreceptor cells of adult mouse retina by using the clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease (Cas)9 from *Neisseria meningitidis* (Nm). Specific single guide RNAs (sgRNAs) were designed to knockdown the *Slc9a8* gene, which encodes the NHE8. Nuclease null NmCas9 and sgRNAs were packaged respectively using adeno-associated viral vector (AAV), and delivered into mouse eyes *in vivo* by subretinal injection on wild-type mice of about four-week-old when mouse retina is fully developed. Eye samples were collected four weeks after injection for phenotype examination. Real-time PCR analysis demonstrated ~38% reduction of NHE8 transcripts in retinas injected with AAV-knockdown sgRNA and AAV-Cas9. Loss of photoreceptor cells was found in eyes injected with AAV-knockdown sgRNA and AAV-Cas9 under either the human rhodopsin promoter or the minimal chicken β-actin promoter, while normal morphology was observed in control eyes injected with AAV-Cas9 and AAV-control sgRNA; immunostaining data showed degenerating photoreceptor cells and RPE cells in eyes injected with knockdown sgRNA and Cas9 AAVs. We further determined that mutant M120K-NHE8 displayed altered intracellular pH regulation in human RPE and primary mouse RPE cells using genetically encoded pH sensor pHluorin and that primary cultured NHE8 mutant RPE cells showed different pH titration curves. These results indicate that NHE8 plays essential function in both RPE and photoreceptor cells. NHE8 dysfunction either in photoreceptor or RPE is sufficient to cause retinal degeneration in adult mice at any age.

1. Introduction

NHE8, encoded by the *Slc9a8* gene, is a member of a large group of monovalent cation/proton antiporters that predominately move Na^+ in exchange for H^+ and play diverse physiological roles, including the regulation of intracellular pH, absorption of sodium into epithelia, salt tolerance, cell volume, cell adhesion, cell proliferation, organelle biogenesis and protein trafficking (Orlowski and Grinstein, 2004). Although NHE8 is widely expressed in adult mouse tissues, the most obvious pathological phenotypes of its deletion or mutation are retinal degeneration and male infertility (Jadeja et al., 2015; Oberheide et al., 2017; Xia et al., 2015; Xu et al., 2015). We and others previously reported that mice with either a NHE8 knockout or mutant M120K-NHE8 developed impaired RPE cells and late-onset photoreceptor cell loss, and suggested that NHE8 plays an essential role in RPE cell polarity/function by possibly controlling the homeostasis of pH and sodium in protein trafficking of trans-Golgi network during development (Jadeja et al., 2015; Xia et al., 2015). It remains unclear whether the retinal degeneration is a consequence of earlier eye or retinal developmental defects due to systemic NHE8 knockout or mutation. This work intends to directly evaluate the function of NHE8 in mature mouse retina, and study whether NHE8 plays a role in intracellular pH regulation in RPE cells.

In mouse retina, NHE8 protein expression was observed in the RPE and the photoreceptor cells (Jadeja et al., 2015; Xia et al., 2015). It has been shown that NHE8 proteins co-localized with various intracellular vesicles, and especially with the trans-Golgi network marker in RPE (Xia et al., 2015) and in recycling endosomes (Jadeja et al., 2015). Using genetically encoded fluorescent pH sensor pHluorin (Miesenbock et al., 1998), we studied the intracellular pH homeostasis in human RPE cells and cultured mouse primary RPE cells. By infecting the cells with AAV-NHE8-mCherry-pHluorin, we monitored the fluorescent intensity

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[☆] Supported by a grant from the East Bay Community Foundation (XG) and a Core Grant for Vision Research (NIH P30EY003176).

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https://doi.org/10.1016/j.exer.2018.06.026
Received 9 February 2018; Received in revised form 19 May 2018; Accepted 22 June 2018
Available online 26 June 2018
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ratios of pH-sensitive ecliptic pHluorin and pH-insensitive mCherry. Our data suggest that NHE8 plays important roles in the pH homeostasis of RPE and photoreceptor cells.

To disrupt NHE8 function in RPE and photoreceptors of adult mice respectively, we constructed AAV vectors to express nuclease null NmCas9 under the control of a minimal chicken β-actin promoter/CMV enhancer (smCBA) (Pang et al., 2008) or a photoreceptor cell specific human rhodopsin promoter (hRho) (Bennett et al., 1995). Nuclease null NmCas9 fused with transcription repressor KRAB (Agata et al., 1999; Larson et al., 2013; Qi et al., 2013; Urrutia, 2003) was constructed, and specific sgRNAs were selected and designed to knockdown NHE8 expression. Wild-type Cas9 has Ruv C and HNH nuclease like domains with nuclease activities; upon RNA-DNA-Cas9 binding, the Ruv C nuclease will cut the non-complementary DNA strand and the HNH nuclease will cut the complementary DNA strand, resulting in DNA double strand breaks (Jinek et al., 2012); DNA double strand breaks can trigger the cells to repair the DNA mainly by non-homologues end joining (NHEJ), which is error prone and may have the potential to induce photoreceptor damage via DNA insertion and deletion (InDel) as well as result in off-target DNA double strand breaks. Mutagenesis in both Ruv C and HNH domains produced nuclease null Cas9 that binds specific targeted DNA guided by sgRNAs without cutting double strand DNA but works as a “block” to suppress transcription, which is referred as CRISPR interference (CRISPRi) (Qi et al., 2013). In theory, nuclease null Cas9 can recognize the target gene but cannot make DNA double strand breaks, it can therefore reduce the off-target effect of wild-type Cas9 and subsequent nucleotide insertions or deletions (Qi et al., 2013; Larson et al., 2013). The sgRNAs and nuclease null NmCas9-KRAB were packaged into AAV9-2YF (Byrne et al., 2015; Dalkara et al., 2012; Petrs-Silva et al., 2009; Zhong et al., 2008) respectively, and used to knockdown NHE8 expression in RPE or photoreceptor cells by subretinal injection into adult mouse retina. Both the pan-retina knockdown of NHE8 by smCBA-Cas9 and photoreceptor-specific knockdown by hRho-Cas9 displayed severe retinal degeneration. Thus, NHE8 is essential for the survival of photoreceptors in adult animals, presumably due to its function in proton/sodium homeostasis of both RPE and photoreceptors. In addition, by using AAV packaged NHE8-mCherry-pHluorin and cultured human RPE cell line or mouse primary RPE cells *in vitro*, we have demonstrated that NHE8 is important in intracellular pH regulation.

2. Materials and methods

2.1. Mice

All experimental procedures were approved by the Animal Care and Use Committee (ACUC) at University of California, Berkeley, and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. RPE cell culture

For primary mouse RPE culture, a modified method was developed based on a published work (Fernandez-Godino et al., 2016). Briefly, freshly enucleated eyeballs were placed in ice cold HBSS-H- (HBSS- no calcium + 10 mM HEPES, Life Technologies) to dissect away the cornea and lenses, the remaining eyecups were incubated in 1 mg/ml hyaluronidase at 37 °C with 5% CO_2 for 45 min. Eyecups were then transferred to cold HBSS-H+ (HBSS with calcium and Magnesium + 10 mM HEPES, Life Technologies) and incubated on ice for 30 min. In the HBSS-H+, the neural retina was removed, eyecups were transferred to 0.05% Tryspin +0.02% EDTA and incubated at 37 °C with 5% CO_2 for 45 min, followed by transferring to 20% FBS in HBSS-H+. The RPE sheets were gently dissociated, and sheets from the same genotype of mice were pooled in 20% FBS/HBSS-H+ solution and centrifuged at 25 °C at 340 g for 2 min. The cell pellets were re-suspended in 1 ml of 0.05% trypsin +0.02% EDTA and incubated at 37 °C for 1 min, RPE medium was added and the cells were centrifuged at 340 g 25 °C for 2 min, the resulting RPE cell pellets were re-suspended in RPE medium, which including 1% Penicillin/Streptomycin, 1% GlutaMax, 1× B-27 (Gibco, cat# 17504-044), 2% fetal bovine serum, 2 μM SB431542 (Stemgent, cat# 04-0010-10) in Gibco Advanced DMEM/F12. Cells were plated into glass bottom dishes precoated with 10 μg/ml mouse Laminin (Corning, cat# 354232). Human ARPE-19 cells were cultured in DMEM/F12 medium with 10% fetal calf serum.

2.3. Generation of AAVs for pH titration measurement

We previously generated AAV5-smCBA-(wt, wild-type or r15, mutant M120K) NHE8-SE pHluorin with the pTR-smCBA-NHE8-SE pHluorin plasmids (Xia et al., 2015). To make AAV5-smCBA-NHE8-mCherry-SE pHluorin, we used BamHI to cut the SE pHluorin fragment from the pTR-smCBA-NHE8-SE pHluorin vector and dephosphorylated the remaining vector. We then used the following primers to amplify mCherry and SE pHluorin PCR fragments: mCherry forward (34-mer)- GTGGAGGTGGGGATCCGTGAGCAAGGGCGAGGAG and mCherry reverse (29-mer)- CCTTTACTCTTGTACAGCTCGTCCATGCC; pHluorin forward (36-mer)- GTACAAGAGTAAAGGAGAAGAACTTTTCACTGG AGT and pHluorin reverse (45-mer)- TATCATGTCTGGATCCTTAACCG GTTTTGTATAGTTCATCCATGC. The vector, the mCherry and the pHluorin PCR fragments were mixed together with molecular ratio of 1:1:1 in Cold Fusion reaction enzymes according to manufacturer recommended protocol (Cold Fusion cloning kit, System Biosciences), 2 μl of reaction mixture was used to transform Sure2 competent cells and pTR-smCBA-wt-NHE8 or r15-NHE8-mCherry-SE pHluorin colonies were selected and confirmed. The pTR-smCBA-wt-NHE8 or r15-NHE8-mCherry-SE pHluorin plasmids were packaged into AAV5 and an evolved AAV variant 7m8 (Dalkara et al., 2013), and the following AAVs were generated for transducing cultured cells: AAV5-smCBA-wt-NHE8-mCherry-SE pHluorin (1.72×10^{14} vg/ml), AAV5-smCBA-r15-NHE8-mCherry-SE pHluorin (5.87×10^{13} vg/ml), 7m8-smCBA-wt-NHE8-mCherry-SE pHluorin (3.30×10^{14} vg/ml), and 7m8-smCBA-r15-NHE8-mCherry-SE pHluorin (1.18×10^{14} vg/ml).

2.4. pH titration curves in RPE cells

Human ARPE-19 cells and mouse primary RPE cells expressing wt-NHE8-mCherry-pHluorin or r15-NHE8-mCherry-pHluorin were imaged at 37 °C with a 63x lens for pHluorin (488 nm laser) and mCherry (555 nm laser) fluorescence respectively. For each experiment, six to eight cells on a single plate were imaged for analysis. Cells were first imaged in Live Cell Imaging Solution (ThermoFisher, A14291DJ); for titration, cells were sequentially imaged in buffers at pH 7.5, 6.5, and 5.5 (ThermoFisher, P35379) containing 10 μM nigericin (an ionophore that equilibrates intracellular and extracellular pH), with a 5-min incubation period between each buffer. Titration curves were generated for each cell by plotting the pHluorin/mCherry intensity ratios against the buffer pH values.

2.5. Generation of AAV packaged sgRNA

Specific primers were designed to PCR amplify the two fragments for each sgRNA, using the template NmCas9 specific sgRNA backbone (M-NM-sgRNA, Addgene plasmid #48673, gift from George Church, www.addgene.com) (Esvelt et al., 2013) that includes a U6 promoter and a short form of NmCas9 sgRNA backbone in a TOPO vector. The following primers were used for PCR: 1) for sgRNA Nhe8 KO P1, fragment 1 forward- CGG TAC AAT TCA CGC GTG GAG CTC GGA TCC ACT AGT AAC, fragment 1 reverse- GCC CCG CCC CTG CCC GCA GCG GAA GTT GTA GCT CCC TTT CTC G, fragment 2 forward- TTC CGC TGC GGG CAG GGG CGG GGC GGT GTT TCG TCC TTT CCA C, and fragment

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