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SHORT COMMUNICATION

Age- and Gene-Dosage—Dependent Cre-Induced Abnormalities in the Retinal Pigment Epithelium

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To conditionally inactivate genes in the retinal pigment epithelium (RPE) transgenic mouse strains have been developed, in which Cre recombinase (Cre) expression is driven by an RPE-specific gene promoter. The RPE is a quiescent epithelium, and continuous expression of Cre could affect its function. Here, we tested the hypothesis that continuous postnatal Cre expression in the RPE may lead to cellular abnormalities, which may depend on both age and Cre gene dosage. We therefore examined the eyes of homozygous and heterozygous VMD2-Cre mice at various ages. In VMD2-Cre heterozygous mice variable progressive age-dependent RPE abnormalities were noticed, including attenuation of phalloidin and cytoplasmic active β-catenin staining, reduced cell size, and loss of the typical honeycomb pattern of RPE morphology in those RPE cells that stained for Cre. These morphological RPE abnormalities were not noticed in Cre-negative RPE cells in VMD2-Cre or age-matched control mice. In addition, an abnormal number and morphology of cell nuclei were noticed in a subset of Cre-expressing RPE cells in aged heterozygous VMD2-Cre mice, whereas more severe nuclear abnormalities were observed already in young homozygous VMD2-Cre mice. Thus, continuous postnatal expression of Cre causes abnormalities in the RPE in an age- and Cre gene dosage-dependent manner, which needs to be considered in the interpretation of gene targeting studies in the RPE. *(Am J Pathol 2014, 184: 1660—1667; http://dx.doi.org/10.1016/j.ajpath.2014.02.007)*

The retinal pigment epithelium (RPE) is a nonregenerative single layer epithelium in the adult eye that is essential for the visual cycle and photoreceptor function.[1] Degenerative changes in the RPE affect retinal function and cause several ocular diseases. The fact that this epithelium does not continuously regenerate postnatally could make this epithelium more susceptible to accumulative genetic alterations that affect its overall function and morphology.

Because of its critical role in vision and for the maintenance of photoreceptor function, understanding biological processes in the RPE are fundamentally important. Conditional gene inactivation studies have been undertaken to determine RPE-specific functions of certain genes. Several transgenic mouse strains have been developed that express Cre recombinase (Cre) in the RPE driven by a gene promoter that is active in this epithelium. These mice allow targeted inactivation of a gene in the RPE when crossed with mice that carry floxed alleles for the gene of interest.[2–7] Most RPE-specific Cre strains that have been reported show an incomplete Cre expression pattern in the RPE, with patches of Cre-positive RPE cells adjacent to Cre-negative RPE cells. Thus, it is important to correlate phenotypic changes in the RPE with the Cre expression pattern. Furthermore, the extent of Cre expression is variable, and it has been reported that mice of the same transgenic strain show a high degree of variability of Cre expression, with the observation of complete silencing of Cre expression in some mice that carry the Cre allele.[2] Cre-mediated RPE toxicity has been reported in Trp1-Cre transgenic mice,[3,8] raising the question whether this is a strain-specific observation or whether Cre expression in the RPE results in toxicity as a general phenomenon that may depend on the level and duration of cellular Cre

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expression in the RPE. The Trp1-Cre strain shows RPE-specific Cre expression already at embryonic day 10.5, which suggests that RPE-specific abnormalities in these mice may result from developmental abnormalities due to Cre expression at the onset of RPE morphogenesis. Whether Cre expression in the RPE postnatally would affect its viability and function is not known.

In several other cell types Cre-mediated cell toxicity has been reported, with degenerative changes that correlated to the degree of Cre expression.[9–11] This Cre toxicity may result from genetic alterations due to sequences in the genome that may be targeted by Cre. Cre catalyzes recombination between two loxP recognition sites, which is characterized by a short recognition sequence.[12,13] Reports have shown that Cre expression can lead to DNA cleavage in mammalian cells at sites with homology to the loxP recognition sites (pseudo-loxP sites), which results in genetic alterations in mammalian cells that can significantly affect cell function.[14,15]

It is likely that the RPE is particularly susceptible to Cre-mediated toxicity, because it is a nonregenerative epithelium that would accumulate genetic changes with progressive age. To test this hypothesis, VMD2-Cre mice (also named BEST1-Cre mice) that express Cre in the RPE after postnatal day 10 were examined for Cre-mediated RPE abnormalities.[2] If Cre expression by itself leads to phenotypic changes in the RPE, we hypothesized that these changes would increase with progressive age and the amount of Cre expression in the RPE. Indeed, we show that RPE abnormalities in VMD2-Cre mice progressed with age and were more pronounced in mice that were homozygous for the Cre allele than in heterozygous mice. These findings have important implications for conditional inactivation studies in the RPE, and they suggest that the RPE is susceptible to Cre-mediated cellular dysfunction. Phenotypes due to RPE-specific conditional inactivation of a target gene need to be interpreted in the context of Cre-mediated cellular abnormalities, and in all studies mice heterozygous for Cre with age-matched heterozygous Cre control mice should be used. The conclusions that can be made in RPE-specific gene inactivation studies that use these mice, and likely with other RPE-specific Cre strains as well, need to carefully consider the observed variability of RPE abnormalities even among Cre heterozygous mice.

Materials and Methods

Animals

The generation of VMD2-Cre mice (BEST1-Cre mice) was previously reported.[2] These mice were maintained on the original background and crossed with either C57BL/6J or VMD2-Cre mice to generate either homozygous or heterozygous VMD2-Cre mice. The *rd1* and *rd8* mutation was excluded in these mice. Mice heterozygous and homozygous for VMD2-Cre and control littermates were compared up to 13 months of age. Experimental groups of mice at ages 1, 4, or 7 months of age were used for quantitative analyses ($n = 3$ mice per group). In addition, aged wild-type C57BL/6J mice up to 20 months of age were examined. For all animal studies institutional approval by Massachusetts General Hospital was granted. Zygosity for Cre was determined by real-time quantitative PCR (Real Time Laboratories, Carrollton, TX).

Immunolabeling, Microscopy, and Quantification of RPE Abnormalities

Eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4°C or for shorter durations at room temperature. The observed RPE abnormalities occurred independently of the fixation protocol. Because Cre expression occurs in patches in VMD2-Cre mice, Cre-negative RPE cells served as an internal control for the observed abnormalities and the immunolabeling experiments. In addition, for each experimental mouse group littermate wild-type mice were used as controls. Eyes were dissected along the ora serrata to remove the anterior chamber, iris, and lens. The retina was removed, and the remaining posterior eyecup (choroid/RPE tissue) was used for subsequent analysis. For choroidal flat mount staining, posterior eyecups (choroid/RPE tissue without retina) were permeabilized in 0.5% Triton X and subsequently blocked with 5% goat serum. Primary antibodies were incubated overnight at 4°C. The following primary antibodies were used: mouse anti-Cre (clone 2D8; Millipore, Billerica, MA), monoclonal anti-mouse non-phospho (active) β-catenin (Ser33/37/Thr41; D13A1; Cell Signaling Technology Inc, Danvers, MA), and Alexa 488-conjugated phalloidin (Life Technologies). Secondary antibodies used were Alexa 555 or Alexa 647 (Life Technologies, Carlsbad, CA). Colabeling experiments as well as single-labeling experiments were performed, and experiments that omitted either the primary or the secondary antibodies were used to distinguish immunolabeling from autofluorescence. Nuclei were labeled with DAPI (Life Technologies). After immunolabeling posterior eyecups were flat mounted on cover slides with four radial incisions, and Prolong Gold mounting medium was used (Life Technologies). Images were acquired with a Nikon Eclipse confocal microscope (Nikon, Melville, NJ) or a Zeiss Axiovert epifluorescence microscope (Carl Zeiss, Thornwood, NY) at ×10 and ×20 magnification.

At least five images of choroidal flat mounts were acquired from each eye to cover the entire fundus. From representative microscopic images taken at ×20 magnification, 20 Cre-positive and Cre-negative RPE cells were randomly selected and analyzed for cell size and cytoplasmic active β-catenin immunolabeling intensity. Cell size was determined by outlining cell membranes of RPE cells by using Axiovision software version 4.8.2. Average cell size per mouse was measured, and statistically significant differences in each experimental group were determined with a two-tailed Student's *t*-test.

A scoring system was established to determine cytoplasmic active β-catenin levels. Normal cytoplasmic active