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Supplementary Information

Materials and Methods

Chimeric mouse production

Wild type ↔ Arf\(^{-/-}\) chimeras were generated as previously described.\(^1\) Briefly, 12 – 16 week old Arf\(^{-/-}\) females were super-ovulated with pregnant mare serum (4 IU) and human chorionic gonadotropin (4 IU) (both from Sigma, St. Louis, MO) and mated with Arf\(^{-/-}\) males. At E2.5, embryos were flushed from the uterus of pregnant females with medium.\(^2\) Morulae were subsequently aggregated to those obtained from similarly handled Arf\(^{+/+}\), Actin-Gfp (wild type) females. Aggregated morulae were cultured overnight at 37°C and then transferred to the uterus of 2.5 day pseudopregnant females. This fusion resulted in 30 chimeric pups from 4 different host females.

Histology Studies

Chimeric pups were euthanized on postnatal day 1 for histological analysis. Enucleated eyes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 hours at 4°C, perfused with 20% sucrose in dH\(_2\)O, and embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). Twelve micron thick sections, oriented in the anterior-posterior plane, were taken through the midline of both eyes. Following staining (see below), coverslips were applied using Vectashield Mounting Medium with DAPI (Vector Labs, Burlingame, CA) or TOPRO-3 (Molecular Probes, Eugene, OR) mixed in PPD mounting medium.\(^3\)

Morphology of chimeric eyes was assessed by hematoxylin and eosin staining and light microscopy. Serial sections from the midline through the vitreous cavity were examined for each eye to determine the presence or absence of a retrolental mass. The area of the retrolental mass was determined in a representative, midline photomicrograph (40x magnification) using ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Gfp expression in Arf wild type cells was assessed by direct fluorescent microscopy, except when dual staining for smooth muscle \(\alpha\)-actin (Sma), in which case it was
assessed by antibody-based detection. Expression of tissue-specific proteins was assessed by immunofluorescence staining using goat α-Pdgfrβ, rat α-CD31 or mouse α-smooth muscle α-actin (Sma) and rabbit α-GFP as described previously. Midline sections from three chimeric eyes were used to quantify the fraction of cells within the retrolental mass that expressed CD31 or Sma. Digital photomicrographs were obtained using an Olympus BX60 light/fluorescent microscope equipped with a SPOT RT Slider camera (Diagnostic Instruments, Sterling Heights, MI) or a Zeiss 510 NLO META multiphoton/confocal laser scanning microscope.

Quantification of degree of chimerism in the eye

The degree of wild type ↔ Arf−/− chimerism was assessed by quantitative analysis of PCR-amplified genomic DNA from tail biopsy in 30 pups and by fluorescence microscopy in 26 eyes taken from 18 mice. For the former, PCR products were resolved on a 2% agarose gel, detected by ethidium bromide staining and quantified by signal intensity using the GelDoc 2000 gel documentation system (BioRad, Hercules, CA). Amplification of wild type and knockout alleles from an Arf+/- mouse served as the internal standard for 50% chimerism. Because the wild type and Arf knockout alleles were not equally amplified, a correction factor was calculated such that the Arf+/- wild type and knockout PCR products represented 50% of the sum of their intensities. The signal intensity for each chimeric sample was similarly corrected and presented as a percent of the sum of the wild type and knockout band intensities.

To quantify chimerism in the retina, uncompressed TIFF digital photomicrographs (40x magnification) of midline sections were taken using an Olympus BX60 light/fluorescent microscope. Degree of chimerism in the retina was calculated from the green fluorescence density using ImagePro Plus software (Media Cybernetics, Silver Spring, MD) as follows: First, an area of interest (AOI) encompassing the entire retina was defined from the DAPI-stained grayscale images, converted from the True Color TIFF images. This AOI was transposed onto the grayscale conversion of the green fluorescence photomicrograph of the same field. A threshold value of 21 was chosen to convert the mostly black pixels (representing Arf−/− derived cells) in the gray image to
white, and to convert the mostly white pixels (representing Gfp-expressing, Arf wild type cells) to black. This threshold was applied to each image. The average optical density of the resulting black and white image was measured to quantify the representation of the Arf\(^{-/-}\) and wild type cells within the AOI. A similar analysis on ten samples was performed using Zeiss 510 NLO META multiphoton photomicrographs (200x magnification) from two central retina fields adjacent to the optic nerve and from two fields in the peripheral retina midway between the optic nerve and iris.

The degree of chimerism in the corneal epithelium and retinal pigment epithelium (RPE) layer was calculated using grayscale images generated from the green fluorescent images. The Image Pro Plus software was used to trace a line along the entire lengths of the corneal epithelium and the RPE. The dark areas along the same linear region were then measured and used to derive the Arf\(^{-/-}\) contribution to each structure.

Multiphoton photomicrographs (200x magnification) from 10 different eyes with adequate preservation of the architecture were used to calculate the percent chimerism within the retrolental mass. As for the retina, the AOI around the mass was defined using the DAPI-stained image. Areas within the mass determined from the DAPI image to be acellular were excluded from the quantitative analysis. In this case, a threshold value of 12 was consistently applied to each sample.

**Statistics**

Measurements were averaged across eyes within subjects prior to subsequent statistical analysis. A general linear model\(^6\) was used to determine whether the chimerism percentages were equal across five tissue types (retina, cornea, retinal pigmented layer, tail DNA, and retrolental mass). The model included subject as a random effect to account for intra-subject correlation and tissue type as a fixed main-effect. To perform pairwise comparisons of chimerism percentages between tissue types, we applied Wilcoxon’s signed-rank test to intra-subject differences between chimerism percentages across the tissue types of interest. Spearman’s rank-based correlation coefficient was used to measure the association of the degree of chimerism in pairs of tissues. Among subjects
developing a retrolental mass, Spearman’s correlation coefficient was used to measure the association of the size of a retrolental mass with the degree of chimerism in various tissues. The Wilcoxon rank-sum test was used to compare chimerism percentages between subjects with and without a retrolental mass. Analyses were implemented using SAS software, Windows version 9.1 (Cary, NC). No adjustments for multiple-testing were performed. Spearman’s correlation coefficient and Wilcoxon’s signed-rank and rank-sum tests are described. 

References


