Supplementary Information for

**Molecular taxonomy of human ocular outflow tissues defined by single cell transcriptomics**

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Supplementary text

Preparation of human eye for TM dissection

The whole globes were soaked in betadine solution for 2-3 minutes and then rinsed with PBS twice. Using scalpel blade incision was 1mm posterior of limbus and eye globe was cut open with curved scissors along the circumference keeping 1mm distance from limbus, sometimes requiring cutting through vitreous. Using forceps, anterior and posterior eye globes were separated and from anterior part of eye, lens, iris, ciliary body were gently pulled off. Iris/pigment, ciliary muscle, and other tissue debris were gently scrapped off using edge of a clean blade and brief rinse of tissue in PBS. TM was then dissected using blunt dissection approach. This was accomplished by lifting TM with forceps and teasing away continuous strand of TM tissue between the Schwalbe’s line and the scleral spur. The TM tissue was placed in digestion buffer containing 5 mg collagenase A (Worthington Biochemical Corporation, Lakewood Township, NJ) dissolved in human albumin (catalog#A9080, Sigma-Aldrich).

Preparation of DMEM media

Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen-Gibco Life Technologies, Grand Island, NY, USA) was supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA), penicillin (100 units/mL), streptomycin (0.1 mg/mL), and L-glutamine (0.292 mg/mL) (Thermo Fisher Scientific, Rockford, IL, USA)

Sample preparation for scRNAseq

At the end of incubation in DMEM media, TM single cell suspension and tissue debris were mixed with DMEM media to neutralize the enzymatic reaction. The single cell suspension solution was filtered through 70-micron filter to get maximum number of cells after tissue digestion, retaining tissue debris on filter and to exclude doublets which can interfere with further scRNAseq steps

NucleoCounter® NC-250™

The NucleoCounter® NC-250™ is an automated cell analyzer utilizing fluorescence imaging to characterize cell properties. We used the ‘Viability and Cell Count Assay’ with Solution 18 to determine single cell suspension viabilities through Acridine Orange (AO)/DAPI counterstaining (www.chemometec.com).

In situ hybridization using RNAscope

The expression pattern of TM single cell cluster specific gene expression in the human donor eye was determined by in situ hybridization using RNAscope® according to manufacturer’s specifications (Advanced Cell Diagnostics). RNAscope®
Technology is a novel in situ hybridization (ISH) assay for detection of target RNA within intact cells. Briefly, 10% NBF (neutral buffered formalin) fixed and paraffin embedded human donor eye cups were cut into 5 μm sections and mounted on SUPERFROST® Plus glass slides. For RNAScope, slides were baked and deparaffinized. Tissue sections then underwent series of pretreat steps including hydrogen peroxide treatment for 10 minutes, target retrieval buffer treatment for 20 minutes, protease plus treatment for 30 minutes and DNase I treatment for 15 minutes. Tissue sections were then washed five times with water, hybridized with RNAScope probes for target genes for 2 hours at 40°C and the remainder of the manufacturer’s assay protocol was implemented (ACD, 322360) from Amplified 1 to Amplified 6. The slides were washed twice with RNAScope wash buffer (ACD, 310091). Signal was detected by incubation with fast red working solution (1:60 ratio of Red B to Red A) for 10 minutes in the absence of light, followed by washing the slides in water several times, mounting the slides and viewing under bright-field microscope. In some experiments, fluorescent signals were visualized and captured using an open-field Nikon Eclipse Ti-E microscope and others using Keyence microscope (Itasca, IL, USA).

**Immunohistochemistry of human anterior segments**

Radial wedges cut from anterior segments of human donor eyes (Table S2) were paraffin-embedded, sagittally sectioned (5um), deparaffinized following xylene and 100% ethanol incubations, and antigen-retrieved in boiling citric acid buffer for 15 min. Blocking buffer was prepared with 5% normal serum, 1% bovine serum albumin, 0.025% triton-x in tris-buffered saline. Sections were incubated in blocking buffer at room temperature (RT) for 1 hour. Antibodies directed against protein targets (Table S3) were incubated with tissue sections in blocking buffer overnight at 4°C. Tissue on slides were coverslipped and merged z-stacks (~10μm) were imaged at different magnifications using Nikon Eclipse confocal microscope. All confocal stacks are 10 X 1 m. Controls were treated exactly as experimental except primary antibodies were omitted. Controls were imaged at identical confocal settings as experimental (Figure S10).

**Single cell RNA sequencing, read mapping**

Single cells suspended in PBS with 0.04% BSA were loaded on a Chromium Single Cell Instrument (10X Genomics). RNAseq libraries were prepared using Chromium Single Cell 3’ Library, Gel Beads & Multiplex Kit (10X Genomics). To minimize the presence of doublets in our population, approximately 6000 cells were loaded per lane and 2000-3000 cells per lane were recovered. Paired-end sequencing was performed on Illumina NextSeq500 (Read 1 26-bp for unique molecular identifier (UMI) and cell barcode, 8-bp i7 sample index, 0-bp i5, and Read 2 55-bp transcript read). Cell Ranger Single-Cell Software Suite (10X Genomics, v2.0.0) was used to perform sample de-multiplexing, alignment, filtering, and UMI counting. Human b37.3/ Mouse mm10 Genome assembly and UCSC gene model were used for the alignment.
Data analysis

We mainly used Seurat 2.3 software package developed by Satija lab for the single cell data analysis. Seurat object was created using the Digital gene expression (DGE) UMI data file for each of the 8 samples. Genes expressed in less than 5 cells in each sample were removed from analysis. Cells with number of genes detected in less than 500 or over 5000, or UMI ratio of mitochondria encoded genes vs. all genes over 0.20 were also removed. Data normalization and scaling for each cell were achieved by using Seurat global-scaling “LogNormalize” method, which normalizes the gene expression measurements by the total expression followed by multiplying a scaling factor of 10,000 and log-transformation.

To avoid potential sample-to-sample variation caused by technical variation at various experiment steps, we employed Seurat data integration method. First, top 1000 variable genes of each of the 8 Seurat objects were identified using “FindVariableGenes”. The union of these variable genes that were detected in at least 5 cells in each of the 8 samples were used for Seurat “RunCCA” function of canonical correlation analysis. Subsequently, 13 dimensions were used to run AlignSubspace to combine the 8 Seurat objects into 1. Cells were then grouped into clusters by using Seurat functions “RunTSNE” and “FindClusters” of resolutions 0.8-1.0. Marker genes for each cluster were identified using Seurat function “FindAllMarkers”. Parameters were used such that these genes were expressed in at least 25% of the cells in the cluster, and on average 1.28-fold higher than the rest of cells with a negative binomial test p value of less than 0.01. The expression of cluster marker genes as well as canonical cell type-specific genes were used to define the cell type for each cluster. The Seurat function “FeaturePlot” or “DotPlot” was used to examine the expression pattern of genes of interest. The similarity or dissimilarity among the identified cell types was examined by hierarchical clustering using Euclidean distance and complete linkage algorithm in R (R Core Team 2017, https://www.r-project.org/).
**Figure S1:** Blunt dissection of human outflow tissues. Hematoxylin and eosin stained human donor eye section showing intact TM before dissection (A) and after TM dissection (B). Magnification: 20x. TM: Trabecular meshwork; SC- Schlemm’s canal; CM- Ciliary muscle
Figure S2: Three steps of QC. Histograms of total UMI (A), gene count (B) and percentage of mitochondria read counts (C)
Supplementary figure 3

A)                                                B)                                            C)

Figure S3: Scatter plots of total UMI (A), gene count (B) and percentage of mitochondria read counts (C)
Figure S4: T-distributed stochastic neighbor embedding (tSNE) visualization of TM transcriptome heterogeneity of 8758 cells. Almost each of the human TM cell samples contributed uniformly to all clusters. Cells are color labeled by sample.
**Figure S5:** In situ hybridization mapping of cells from Schwann cell like cluster in human eye sections. A t-distributed stochastic neighbor embedding (tSNE) plot showing normalized expression of SCN7A gene in each cell (blue dots) is displayed on the left, and on the right is ISH stained human eye section showing mRNA signal (red fluorescence) from SCN7A gene. mRNA probe corresponding to SCN7A predominantly localized to the scleral spur and ciliary muscle regions, with some cells extending into the TM. DAPI staining (blue) counterstains cell nuclei. Magnification: 20x. TM- Trabecular meshwork; CM- ciliary muscle; SC- Schlemm’s canal; SS- Scleral spur. Scale in tSNE plot shows intensity of the natural log transformed scaled read counts. Scale bars: 50 µm
**Figure S6**: Localization of smooth muscle cell cluster gene candidates using in situ hybridization of human eye sections. On the left side is t-distributed stochastic neighbor embedding (tSNE) plots showing normalized TAGLN gene expression in each cell (blue dots) and on the right-side is ISH stained human eye sections showing specific mRNA signal as red dot fluorescence from TAGLN gene. mRNA probe to TAGLN was predominantly confined to ciliary muscle, and it also extended to a few cells in TM that were proximal to ciliary muscle. DAPI staining (blue) counterstains cell nuclei. Magnification: 20x. TM- Trabecular meshwork; CM- ciliary muscle; SC- Schlemm’s canal; SV- Scleral vessel. Scale in tSNE plot shows the natural log transformed scaled read counts. Scale bars: 50 µm.
**Supplementary Figure 7**

**Figure S7:** Mapping of macrophage genes in human outflow tissues using in situ hybridization. The left side is t-distributed stochastic neighbor embedding (tSNE) plot showing normalized TYROBP gene expression in each cell (blue dots) and right-side is ISH stained human eye section showing mRNA signal as red dot fluorescence of TYROBP gene. mRNA probe to TYROBP show that macrophages are present throughout the TM, ciliary muscle and around SC. DAPI staining (blue) counterstains cell nuclei. Magnification: 20x. TM- Trabecular meshwork; CM- ciliary muscle; SC- Schlemm’s canal. Scale in tSNE plot shows intensity of the natural log transformed scaled read counts. Scale bars: 50 µm.
Figure S8: Localization of lymphatic and vascular endothelial cell expression in human eye sections using in situ hybridization. The left side is a t-distributed stochastic neighbor embedding (tSNE) plot showing normalized FLT1 gene expression in each cell (blue dots) and the right-side is stained human eye section showing mRNA signal as red fluorescence of FLT1 gene. mRNA probe corresponding to FLT1 was predominantly found in the SC region. FLT1 was also localized more in SC, ciliary muscle and scleral vessel in human eye sections. DAPI staining (blue) counterstains cell nuclei. Magnification: 20x. TM- Trabecular meshwork; CM- ciliary muscle; SC- Schlemm’s canal. Scale in tSNE plot shows intensity of natural log transformed scaled read counts. Red circle in tSNE plot highlights lymphatic-vascular endothelial cell clusters. Scale bars: 50 µm
**Figure S9**: Mapping of pericyte, melanocyte, epithelium, and T/NK cell genes in human outflow tissues using in situ hybridization. A t-distributed stochastic neighbor embedding (tSNE) plot showing normalized expression of gene in each cell (blue dots) is displayed on the left, and on the right is ISH stained human eye section showing mRNA signal (red fluorescence) from gene of interest. mRNA probes to IL1RL1 (A), PMEL (B), (C) AQP5, and (D) KLRB1 show that pericyte, melanocyte, epithelium, and T/NK cell are not present throughout the TM, ciliary muscle and around SC, respectively. DAPI staining (blue) counterstains cell nuclei. Magnification: 20x. TM- Trabecular meshwork; CM- ciliary muscle; SC- Schlemm's canal. Scale in tSNE plot shows intensity of the natural log transformed scaled read counts. Scale bars: 50 µm
Figure S10: Control images of human conventional outflow pathway tissues in cross-section. Tissue sections were treated and imaged identically to those shown in Figure 10, except primary antibodies specific for candidate gene products were omitted. Left panels illustrate structures of the conventional outflow pathway at a low magnification. Right panels illustrate areas of interest (indicated with white box in left panels) at a higher magnification. All sections were counterstained with DAPI to show location of cells nuclei. Note tissue autofluorescence and secondary antibody precipitates that can be observed in some images. TM: trabecular meshwork, SC: Schlemm’s canal, CB: ciliary body, magnification bars left panel = 100 µm, and in right panels = 20 µm.
## Supplementary table 1: Sample description (sources, donor details)

| Donor ID | Sample ID | Age | Gender | Source              | Ethnicity/Race | Time of death to procurement |
|----------|-----------|-----|--------|---------------------|----------------|-----------------------------|
| 1        | 101, 102  | 55  | Female | Florida Lions Eye  | Black          | 7 hours                     |
| 2        | 103, 104  | 56  | Male   | Florida Lions Eye  | Black          | 9 hours                     |
| 3        | 107, 108  | 85  | Male   | Duke Eye           | White          | 6 hours                     |
| 4        | 109, 110  | 73  | Female | Duke Eye           | White          | 5 hours                     |
**Supplementary table 2: Cell number and percentage by cell type and sample**

| Cell Type                           | hTM_101 | hTM_102 | hTM_103 | hTM_104 | hTM_107 | hTM_108 | hTM_109 | hTM_110 | sum  | percent |
|-------------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|-------|---------|
| Schwann cell-like cell              | 394     | 334     | 270     | 208     | 241     | 519     | 315     | 242     | 2523  | 28.81   |
| TM1, fibroblast-like                | 189     | 207     | 242     | 138     | 338     | 570     | 278     | 192     | 2151  | 24.56   |
| smooth muscle cell                  | 44      | 105     | 385     | 68      | 76      | 356     | 64      | 90      | 1188  | 13.56   |
| TM2, myofibroblast-like             | 95      | 89      | 83      | 92      | 277     | 70      | 147     | 242     | 1095  | 12.50   |
| melanocyte                          | 9       | 3       | 15      | 7       | 128     | 246     | 118     | 66      | 592   | 6.76    |
| macrophage                          | 9       | 22      | 24      | 42      | 28      | 31      | 102     | 103     | 361   | 4.12    |
| pericyte                            | 29      | 29      | 13      | 25      | 48      | 42      | 28      | 24      | 238   | 2.72    |
| vascular endothelium                | 66      | 41      | 10      | 15      | 27      | 26      | 18      | 5       | 208   | 2.37    |
| T/NK cell                           | 33      | 22      | 9       | 23      | 14      | 14      | 18      | 13      | 146   | 1.67    |
| lymphatic-like endothelium          | 33      | 44      | 10      | 19      | 17      | 0       | 0       | 1       | 124   | 1.42    |
| myelinating Schwann cell            | 8       | 3       | 10      | 5       | 19      | 9       | 27      | 21      | 102   | 1.16    |
| epithelium                          | 2       | 0       | 15      | 6       | 6       | 1       | 0       | 0       | 30    | 0.34    |
| **sum**                             | 911     | 899     | 1086    | 648     | 1216    | 1884    | 1115    | 999     | 8758  | 100.00  |
### Supplementary table 3: Candidate gene-products evaluated for expression in sagittal sections of human outflow tissues by immunohistochemistry

| Target     | Antibody                  | Predicted labeling | Identification numbers of donor eye tissues tested                                                                 | Antibody dilutions tested | Frozen/Paraffin/Both | Staining Results |
|------------|---------------------------|--------------------|------------------------------------------------------------------------------------------------------------------|--------------------------|---------------------|------------------|
| CD31       | ab28364                   | SC                 | 19000420-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS), 1900-0369 (OS) | 1:100, 1:50, 1:25          | B                  | Positive         |
| MYOC       | PMID: 9727403             | TM                 | 19000420-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1900-0369 OS | 1:1000, 1:500, 1:250, 1200 | B                  | Positive         |
| RSPO2      | EMD Millipore (MABS1709)  | TM                 | 19000420-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS), 1900-0369 (OS) | 1:500, 1:250, 1:100 | B                  | Positive         |
| Lyve-1     | abcam ab14917             | Macrophage         | 1900-0369 (OS)                                                                                                                                                  | 1:200, 1:100, 1:50       | P                  | Positive         |
| RSPO4      | LS-C162784/143465         | TM                 | 19000420-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS) | 1:500, 1:250, 1:100, 1:25, 1:15, 1:10 | B                  | Negative         |
| Chromogranin B | ab1242            | TM                 | 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS), 1900-0369 (OS)                                                                                           | 1:1000 -1:100            | P                  | Negative         |
| VEGFR3     | AF349                     | SC                 | 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS), 1900-0369 (OS)                                                                                         | 1:500 -1:25              | P                  | Negative         |
| eNOS       | Novus NB300-500           | SC                 | 1900-0369 (OS)                                                                                                                                                  | 1:50-1:250               | P                  | Negative         |
| eNOS       | abcam5589                 | SC                 | 19000420-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS) | 1:100 -1:15              | B                  | Negative         |
| Trefoil Factor 3 | Origene AM20949PU-N | SC                 | 19000420-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS) | 1:500 -1:15              | B                  | Negative         |
| TMEM88     | PAS-21164                 | SC                 | 19000420-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS) | 1:500 -1:25              | B                  | Negative         |
| Marker | Source | Tissue | Staining | Dilution | Format | Result |
|--------|--------|--------|----------|----------|--------|--------|
| **DAP12** | Origene AM20949PU-N | NK cells | 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS), 1900-0369 (OS) | 1:500 - 1:25 | B | Negative |
| **DAP12** | BP1-85313 | NK cells | 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS) | 1:25 - 1:250 | P | Negative |
| **Myelin PLP** | ab28486 | Schwann | 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS), 1900-0369 (OS) | 1:500 - 1:25 | P | Negative |
| **Myelin PLP** | ab183493 | Schwann | 1900-0830-82 (OS and OD), 1900-0380-67 (OD and OS), 1900-1328-72 (OD and OS), 1900-1737 (OD and OS), CR1900-1971-38, CR1900-2037-50, 1900-0830 (OD only), 1800-2144 (OS), 1900-0731 (OS), 1900-0369 (OS) | 1:500 - 1:25 | B | Negative |

**TM:** trabecular meshwork; **SC:** Schlemm’s canal; **CD31:** PECAM1; **MYOC:** myocilin; **F:** frozen sections; **P:** paraffin sections; **B:** both.
**Supplementary table 4:** Human donor eye tissue tested for expression of candidate gene products

| Human Donor ID | Age | Race | Sex | Ocular history                  | Death to preservation |
|---------------|-----|------|-----|---------------------------------|-----------------------|
| 1900-0420     | 82 YO | W    | F   | No ocular history               | 6 hrs                 |
| 1900-0380     | 67 YO | W    | F   | No ocular history               | 6 hrs                 |
| 1900-1328     | 72 YO | AA   | F   | No ocular history               | 6.5 hrs               |
| 1900-1737     | 57 YO | W    | M   | No ocular history               | 6 hrs                 |
| 1900-1971*    | 38 YO | W    | M   | No ocular history               | 2.5 hrs               |
| 1900-2037*    | 50 YO | W    | F   | No ocular history               | 8.5 hrs               |
| 1800-2144     | 72 YO | W    | F   | Pseudophakic                    | 7 hrs                 |
| 1900-0731     | 58 YO | W    | M   | No ocular history               | 6 hrs                 |
| 1900-0731     | 58 YO | W    | M   | No ocular history               | 6.20 hrs              |
| 1900-0369     | 84 YO | AA   | M   | Pseudophakic, dry eye           | 5.3 hrs               |

*corneal rims, W: white, AA: African American; F: female; M: male