

Deconstructing Retinal Organoids: Single Cell RNA-Seq Reveals the Cellular Components of Human Pluripotent Stem Cell-Derived Retina

JOSEPH COLLIN,^a RACHEL QUEEN,^b DARIN ZERTI,^a BIRTHE DORGAU,^a RAFIQUH HUSSAIN,^c JONATHAN COXHEAD,^c SIMON COCKELL,^b MAJLINDA LAKO ^a

Key Words. Pluripotent stem cells • Single cell RNA-Seq • Retinal organoids

ABSTRACT

The rapid improvements in single cell sequencing technologies and analyses afford greater scope for dissecting organoid cultures composed of multiple cell types and create an opportunity to interrogate these models to understand tissue biology, cellular behavior and interactions. To this end, retinal organoids generated from human embryonic stem cells (hESCs) were analyzed by single cell RNA-sequencing (scRNA-Seq) at three time points of differentiation. Combinatorial data from all time points revealed the presence of nine clusters, five of which corresponded to key retinal cell types: retinal pigment epithelium (RPE), retinal ganglion cells (RGCs), cone and rod photoreceptors, and Müller glia. The remaining four clusters expressed genes typical of mitotic cells, extracellular matrix components and those involved in homeostasis. The cell clustering analysis revealed the decreasing presence of mitotic cells and RGCs, formation of a distinct RPE cluster, the emergence of cone and rod photoreceptors from photoreceptor precursors, and an increasing number of Müller glia cells over time. Pseudo-time analysis resembled the order of cell birth during retinal development, with the mitotic cluster commencing the trajectory and the large majority of Müller glia completing the time line. Together, these data demonstrate the feasibility and potential of scRNA-Seq to dissect the inherent complexity of retinal organoids and the orderly birth of key retinal cell types. *STEM CELLS* 2019;37:593–598

SIGNIFICANCE STATEMENT

The rapid improvements in single cell sequencing technologies have opened new opportunities for dissecting the complexity of organoids derived from stem or primary cells. To demonstrate the feasibility of this approach, single cell RNA-sequencing on retinal organoids was performed, which revealed the presence of multiple retinal cell types and their sequential emergence during the differentiation time course. Data show that this method has great potential for identifying multiple cell types arising within complex organoids, enabling detailed molecular and temporal systematic studies and close comparisons between in vitro derived tissues and in vivo organogenesis.

INTRODUCTION

It has been estimated that 285 million people are affected by visual impairment globally, with retinal diseases accounting for approximately 26% of blindness [1]. Many inherited and age-related retinal dystrophies culminate in the loss of photoreceptors [2, 3]. There are currently no treatments to reverse this degeneration, thus cell replacement has become a prerequisite on the path toward therapeutic transplantations. The derivation of human embryonic stem cells (hESCs) in 1998 [4] and induced pluripotent stem cells (hiPSCs) in 2007 [5] has provided the much needed breakthrough as both cell types can be expanded indefinitely in vitro as well as

being able to generate photoreceptors and retinal pigment epithelium (RPE) [2, 3]. The subsequent development of protocols to derive three-dimensional retinal organoids from hESCs/hiPSCs demonstrated that structures akin to the developing eye and laminated retina arise and many retinal cell types are produced [6, 7]. Despite these rapid advancements, methods to accurately define and characterize the cells that arise within these organoids over time have not been fully realized.

The improvements in next generation sequencing technologies and protocols for the application of these at a single cell level have broadened their application to multiple systems [8, 9]. A few pioneering studies have applied

^aInstitute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom; ^bBioinformatics Support Unit, Newcastle University, Newcastle upon Tyne, United Kingdom; ^cGenomics Core Facility, Newcastle University, Newcastle upon Tyne, United Kingdom

Correspondence: Majlinda Lako, Ph.D., Newcastle University, Institute of Genetic Medicine, International Centre for Life, Newcastle upon Tyne NE1 3BZ, United Kingdom. Telephone: 44-191-241-8688; e-mail: majlinda.lako@ncl.ac.uk

Received September 7, 2018; accepted for publication December 3, 2018; first published online in *STEM CELLS EXPRESS* December 12, 2018.

<http://dx.doi.org/10.1002/stem.2963>

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

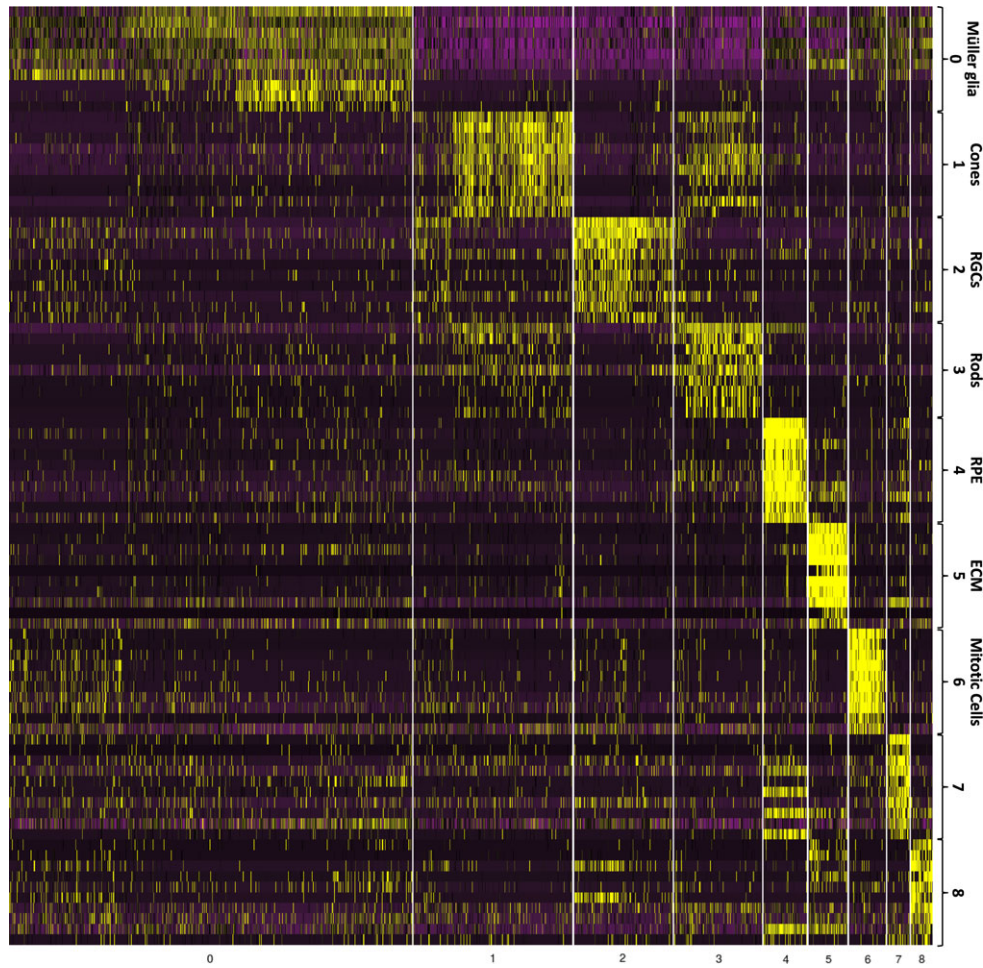


Figure 1. Clustering analysis reveals the presence of nine cell clusters. Seurat was used to align all time points to generate a combined data set. Clusters were then found and marker genes for each cluster identified and used to annotate them. The top 10 markers used for cluster annotation are shown in Supporting Information Table S1.

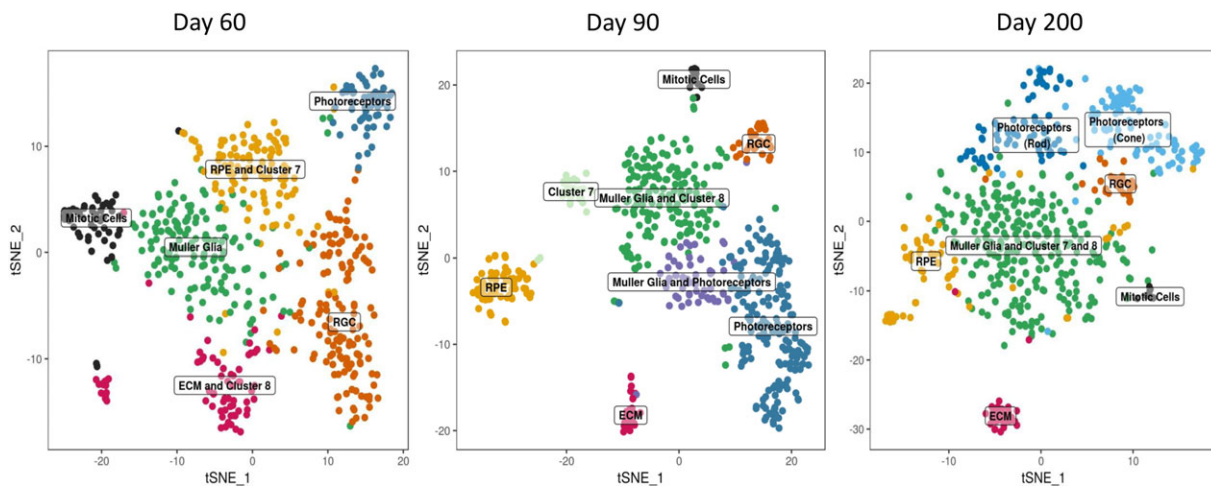


Figure 2. Comparison of individual clustering analysis at days 60, 90, and 200 of differentiation. Clusters for each time point were generated using Seurat and annotated using known retinal marker genes. The cells at different time points for each individual data set were down sampled to an equal number of 578 to ensure that the number of cells did not affect the number of clusters generated.

single cell RNA sequencing (scRNA-Seq) in human retinal tissue and organoids [10–12]. These have largely focused on enriched specific cell types through the use of reporters or bait genes. Despite these important studies, we postulated that the full

complexity of retinal organoids is yet to be fully resolved by scRNA-Seq. In this study we used high throughput Integrated Fluidic Circuits (IFC) for scRNA-Seq with a capacity of 800 capture sites to interrogate hESC-derived retinal organoids through a

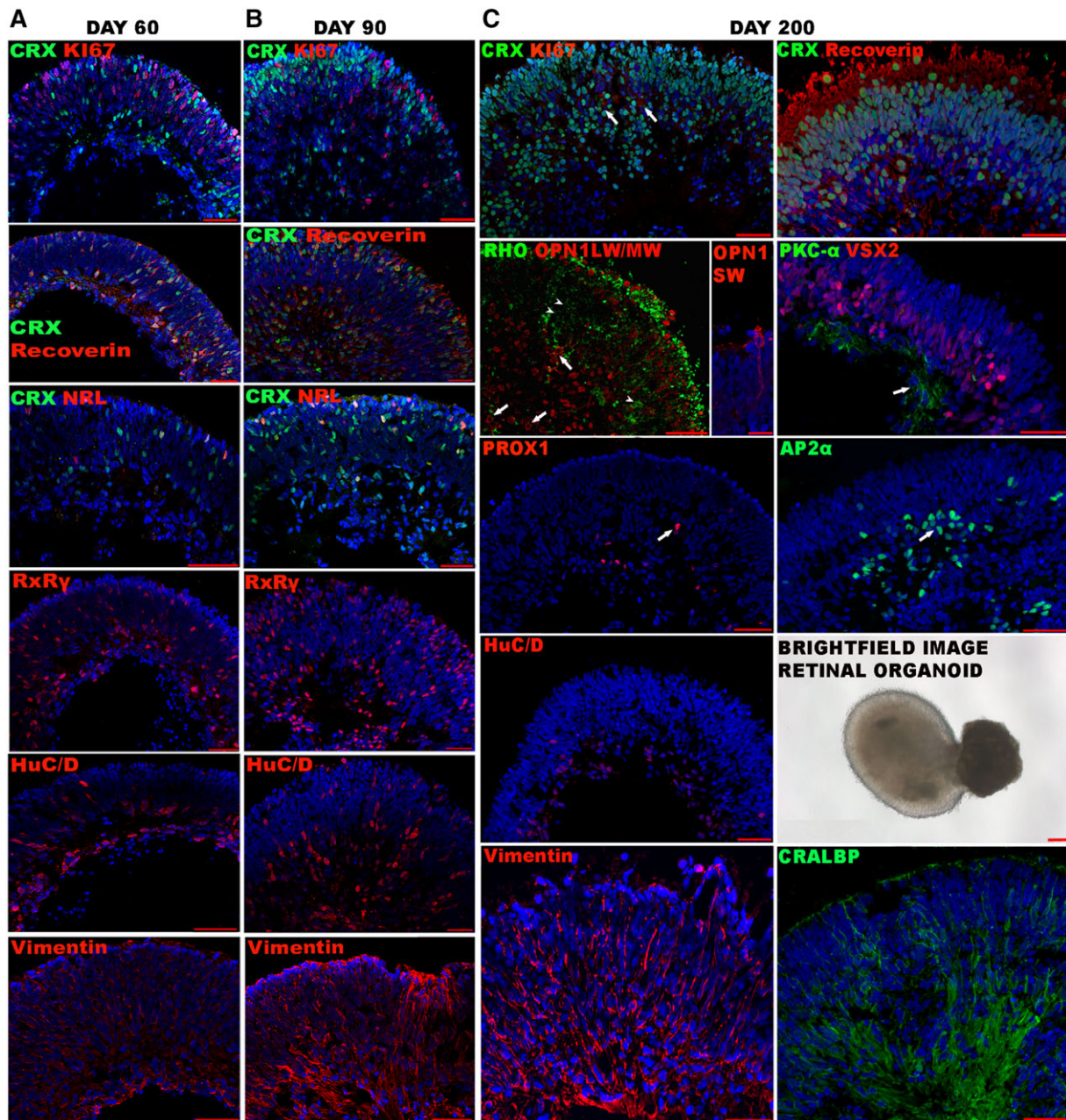


Figure 3. Immunohistochemical analysis of retinal organoids through the differentiation time course. Sections through retinal organoids at day 60 (A) and day 90 (B) using antibodies against: KI67 (red), CRX (green), Recoverin (red), NRL (red), RXR γ (red), HuC/D (red), and Vimentin (red) along with the nuclear stain Hoechst 33342 (blue). (C): Sections through retinal organoids at day 200 showing expression of selected retinal markers using antibodies against CRX (green), KI67 (red), Recoverin (red), rhodopsin (RHO; green, white arrowhead), OPN1LW/MW (red, white arrow), OPN1SW (red), PKC- α (green, white arrow), VSX2 (red), PROX1 (red, white arrow), AP2 α (green, white arrow), HuC/D (red), brightfield image of a retinal organoid, Vimentin (red), and CRALBP (green). Scale bars = 50 μ m except OPN1SW scale bar = 20 μ m and brightfield image retina organoid at day 200 scale bar = 100 μ m.

differentiation time course. Using a greater cell capture number and an unselected single cell population allowed us to perform cell typing of the organoid composition and to follow the development of cell type emergence through the differentiation.

MATERIALS AND METHODS

A detailed description of all experimental procedures is presented in the Supporting Information.

www.StemCells.com

RESULTS AND DISCUSSION

A hESC (H9) cell line was differentiated to retinal organoids. Samples were collected at 60, 90, and 200 days, dissociated, partitioned into single cells using the Fluidigm C1 Single-Cell mRNA-Seq HT IFC and processed for scRNA-Seq. Following quality control and filtering (Supporting Information Fig. S1), data from scRNA-Seq of each time point were normalized (Supporting Information Fig. S2) and then merged using the Seurat package to allow analysis of a higher cell number

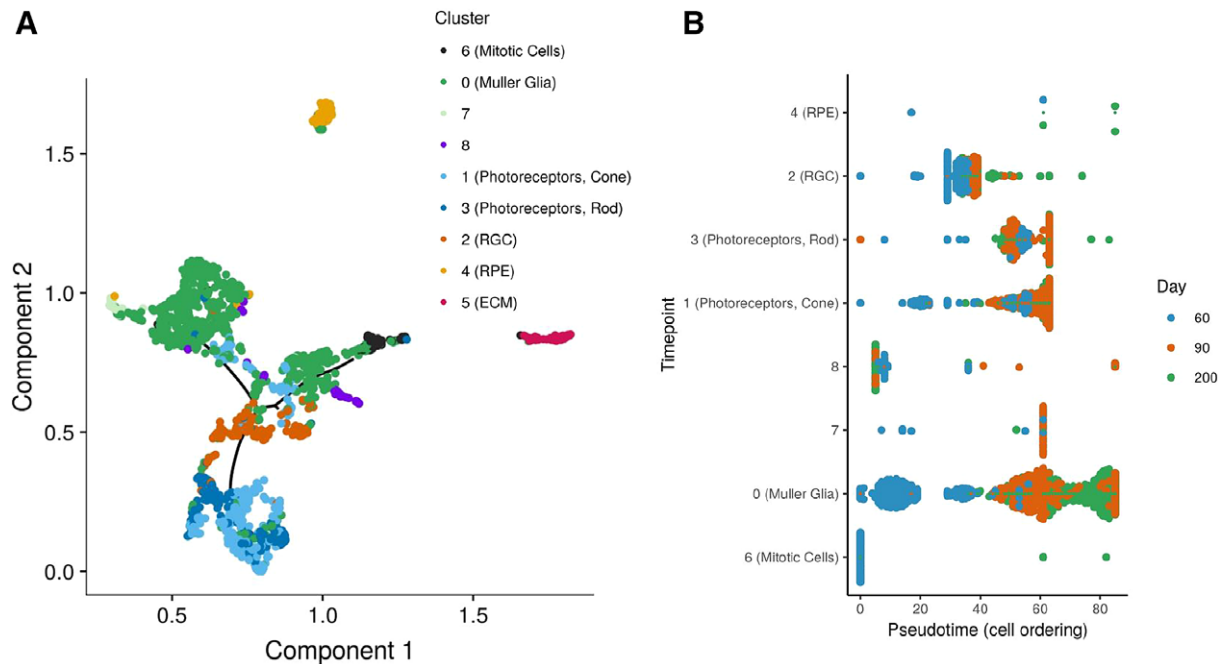


Figure 4. Pseudo-time analysis reveals the emergence of various retinal cell types during the differentiation process. **(A):** A pseudo-time trajectory from the RPE, RGC, Müller glia, cone, and rod photoreceptor clusters was constructed using monocle. **(B):** Order of cell emergence is shown by day and cell type.

(1,976 single cells). The findCluster function revealed nine distinct clusters (Fig. 1; Supporting Information Table S1 and Fig. S3). The findMarkers function found the marker genes for each cluster [13]. The top 10 marker genes were used to manually identify each cluster. Five of the clusters could be assigned to a certain cell type, with cluster 0 identified with genes commonly expressed in Müller glia, cluster 1 to cone photoreceptors, cluster 2 to retinal ganglion cells (RGCs), cluster 3 to rod photoreceptors and cluster 4 to RPE cells (Fig. 1). Cluster 5 expressed a number of extracellular matrix (ECM) genes shown to be expressed in retinal cells [14, 15], whereas cluster 6 expressed genes associated with mitosis, indicating a progenitor population. The remaining minor clusters 7 and 8 were comprised largely of ECM components, signaling molecules and metabolites, the majority of which have been proposed to play a role in retinal homeostasis (for example [16–19]).

Furthermore, clustering analyses were then performed on each time point (Fig. 2). This was compared with the combined data by superimposing the original clusters (identified in Fig. 1 and Supporting Information Fig. S3) to those identified at each individual time point (Fig. 2). This analysis revealed changes over the timeline of differentiation. For example, mitotic cells and RGCs decreased over time, photoreceptors resolved into cones and rods, a distinct RPE cluster formed and Müller glia cells increased toward day 200, recapitulating retinal development where Müller glia are the last retinal cell type to become postmitotic [20–22]. To confirm this increase in complexity the percentage of the cell types at each time point were compared. Mitotic cells decreased from 7.7% at day 60 to 2.4% at day 200, RGCs decreased from 21% at day 60 to 6.6% at 200 and Müller glia increased from 43.7% at day 60 to 53% at day 200. This was corroborated by immunohistochemical analysis, which indicated the decreased presence of mitotic cells and RGCs from

day 60 to day 200, stepwise photoreceptor differentiation (shown by the presence of Recoverin photoreceptor precursors, NRL rod precursors and RxRy cone precursors at days 60 and 90 and separation of rod and cone photoreceptors at day 200) and increased Müller glia presence from day 60 to 200, shown by Vimentin immunostaining from day 60 to 200 and the expression of CRALBP at day 200 (Fig. 3A, 3C). It is interesting to note that although horizontal (PROX1), rod bipolar (PKC- α), and amacrine (AP2 α)-like cells were detected by immunohistochemical staining these were not depicted by scRNA-Seq analysis. Therefore, to be able to resolve such clusters it may be necessary to increase the cell numbers analyzed. Although the decreased presence of mitotic cells during the differentiation time course was expected, that of RGCs was not and could be due to either culture conditions, which are not able to maintain this cell type for prolonged periods, or programmed cell death as seen during development [20, 23].

Interestingly, this analysis also depicted cell types with transcriptional profiles shared by several clusters (Fig. 2). For example, at day 60, RPE shared transcriptional similarity with cluster 7 and ECM with cluster 8. At day 90, cell clusters with transcriptional profiles shared by Müller glia and photoreceptors, corroborating findings reported in adult murine retina [24] but not transcriptionally documented in human retinal development as yet, and Müller glia and cluster 8 were identified. Over time, the shared transcriptome diminished and distinct clusters emerged, with the exception of Müller glia cells, which retained a shared transcriptional profile with clusters 7 and 8.

As the complexity of the organoids increased and distinct cell types could be resolved over time additional pseudo-time analysis was conducted. Monocle [25] was used to analyze the highly variable genes from the mitotic, RPE, RGC, Müller glia,

cone, and the rod clusters (Fig. 4A). This analysis identified the mitotic cluster as the proliferating population from which the rest of the cells emerged. A Müller glia subpopulation resides next along this branch. This is potentially due to the expression of genes commonly expressed in retinal progenitor cells (RPCs) within the Müller glia cluster (Supporting Information Fig. S4), corroborating published data for murine Müller glia cells [24,26]. Continuing down this branch, a few cone-like photoreceptors begin to arise, here the pseudo-time trajectory forked with an upper branch bearing the majority and the remaining Müller glia cells along with minor cluster 7, which was characterized by genes that have been shown to be involved in retinal homeostasis, in which Müller glia play an important role. The lower branch is initially populated with RGCs and then terminates with two closely related clusters containing the rod and cone photoreceptors. This pseudo-time trajectory resembles the order of retinal cell development with a progenitor population giving rise firstly to RGCs, followed by cones, then rods and finally the majority of Müller glia, which are situated on a separate branch, potentially indicating their unique role in retinal development and homeostasis, and thus their transcriptome. The RPE and ECM clusters reside outside of this trajectory. RPE are known to differentiate relatively early in development during optic cup formation [27] and thus have a more distinct transcriptome. The genes from the ECM cluster are not exclusive to the retina, being common ECM components expressed throughout many tissue types and thus by ontology are unlikely to be associated with the retina. This is likely to explain why these two clusters are not associated with the time line (Fig. 4A). The cell ordering plot (Fig. 4B) further corroborates the order of cell type emergence within the organoids with mitotic cells, some Müller glia and cluster 8 present initially at the first time point. Cone photoreceptors and RGCs are next, arising at day 60, followed with diminishing RGC birth and the majority of cones appearing on day 90, alongside

the emergence of rods and more Müller glia. A large number of Müller glia cells (along with some rods) were present at day 200 as the later cell types to mature.

CONCLUSION

Our data demonstrate the feasibility and potential of scRNA-Seq to dissect the inherent complexity of retinal organoids and the orderly birth of key retinal cell types therein, which recapitulates the order of retinal development.

ACKNOWLEDGMENTS

We are grateful to ERC (#614620), RP Fighting Blindness (#GR593), BBSRC (#BB/I02333X/1), and MRC (#MR/M008886/1, MC_PC_15030 & MR/M008886/1) for funding this work.

AUTHOR CONTRIBUTIONS

J. Collin: study design, performed research, data collection and analysis, figure preparation, manuscript writing; R.Q.: data analysis, figure preparation, manuscript writing; D.Z., B.D.: performed research, data collection and analysis, contributed to manuscript writing; R.H., J. Coxhead: performed research, data collection; S.C.: data analysis; M.L.: study design, data analysis, figure preparation, manuscript writing, fund raising; J. Collin, R.Q., D.Z., B.D., R.H., J. Coxhead, S.C., and M.L.: approved the final version of the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

All authors indicated no potential conflicts of interest.

REFERENCES

- Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. *Br J Ophthalmol* 2012;96:614–618.
- Mellough CB, Collin J, Sernagor E et al. Lab generated retina: Realizing the dream. *Vis Neurosci* 2014;31:317–332.
- Chichagova V, Hallam D, Collin J et al. Cellular regeneration strategies for macular degeneration: Past, present and future. *Eye* 2018;32:946–971.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145–1147.
- Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131:861–872.
- Nakano T, Ando S, Takata N et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell* 2012;10:771–785.
- Eiraku M, Takata N, Ishibashi H et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* 2011;472:51–56.
- Macosko Ez Satija R, Nemes J, Shekhar K et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell* 2015;161:1202–1214.
- Kiselev VY, Kirschner K, Schaub MT et al. SC3: Consensus clustering of single-cell RNA-seq data. *Nat Methods* 2017;14:483–486.
- Welby E, Lakowski J, Di Foggia V et al. Isolation and comparative transcriptome analysis of human fetal and iPSC-derived cone photoreceptor cells. *Stem Cell Rep* 2017;9:1898–1915.
- Phillips MJ, Jiang P, Howden S et al. A novel approach to single cell RNA-sequence analysis facilitates in silico gene reporting of human pluripotent stem cell-derived retinal cell types. *STEM CELLS* 2018;36:313–324.
- Langer KB, Ohlemacher SK, Phillips MJ et al. Retinal ganglion cell diversity and subtype specification from human pluripotent stem cells. *Stem Cell Rep* 2018;10:1282–1293.
- Butler A, Hoffman P, Smibert P et al. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* 2018;36:411–420.
- Li M, Jia C, Kazmierkiewicz KL et al. Comprehensive analysis of gene expression in human retina and supporting tissues. *Hum Mol Genet* 2014;23:4001–4014.
- Kimoto K, Nakatsuka K, Matsuo N et al. p38 MAPK mediates the expression of type I collagen induced by TGF- β 2 in human retinal pigment epithelial cells ARPE-19. *Investig Ophthalmol Vis Sci* 2004;45:2431.
- Kerr NM, Johnson CS, de Souza CF et al. Immunolocalization of gap junction protein connexin43 (GJA1) in the human retina and optic nerve. *Investig Ophthalmol Vis Sci* 2010;51:4028.
- Zhang Q, Mashima Y, Noda S et al. Characterization of AOC2 gene encoding a copper-binding amine oxidase expressed specifically in retina. *Gene* 2003;318:45–53.
- Rice DS, Nusinowitz S, Azimi AM et al. The reelin pathway modulates the structure and function of retinal synaptic circuitry. *Neuron* 2001;31:929–941.
- Asahina H, Masuba A, Hirano S et al. Distribution of protocadherin 9 protein in the developing mouse nervous system. *Neuroscience* 2012;225:88–104.
- Vecino E, Acera A. Development and programmed cell death in the mammalian eye. *Int J Dev Biol* 2015;59:63–71.

21 Rapaport Wong DH, Wong LL, Wood ED et al. Timing and topography of cell genesis in the rat retina. *J Comp Neurol* 2004;474:304–324.

22 Young RW. Cell differentiation in the retina of the mouse. *Anat Rec* 1985;212:199–205.

23 Yamaguchi Y, Miura M. Programmed cell death in neurodevelopment. *Dev Cell* 2015;32:478–490.

24 Roesch K, Jadhav AP, Trimarchi JM et al. The transcriptome of retinal Müller glial cells. *J Comp Neurol* 2008;509:225–238.

25 Trapnell C, Cacchiarelli D, Grimsby J et al. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol* 2014;32:381–386.

26 Rowan S, Cepko CL. Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. *Dev Biol* 2004;271:388–402.

27 Fuhrmann S, Zou CJ, Levine EM. Retinal pigment epithelium development, plasticity, and tissue homeostasis. *Exp Eye Res* 2014;123:141–150.



See www.StemCells.com for supporting information available online.