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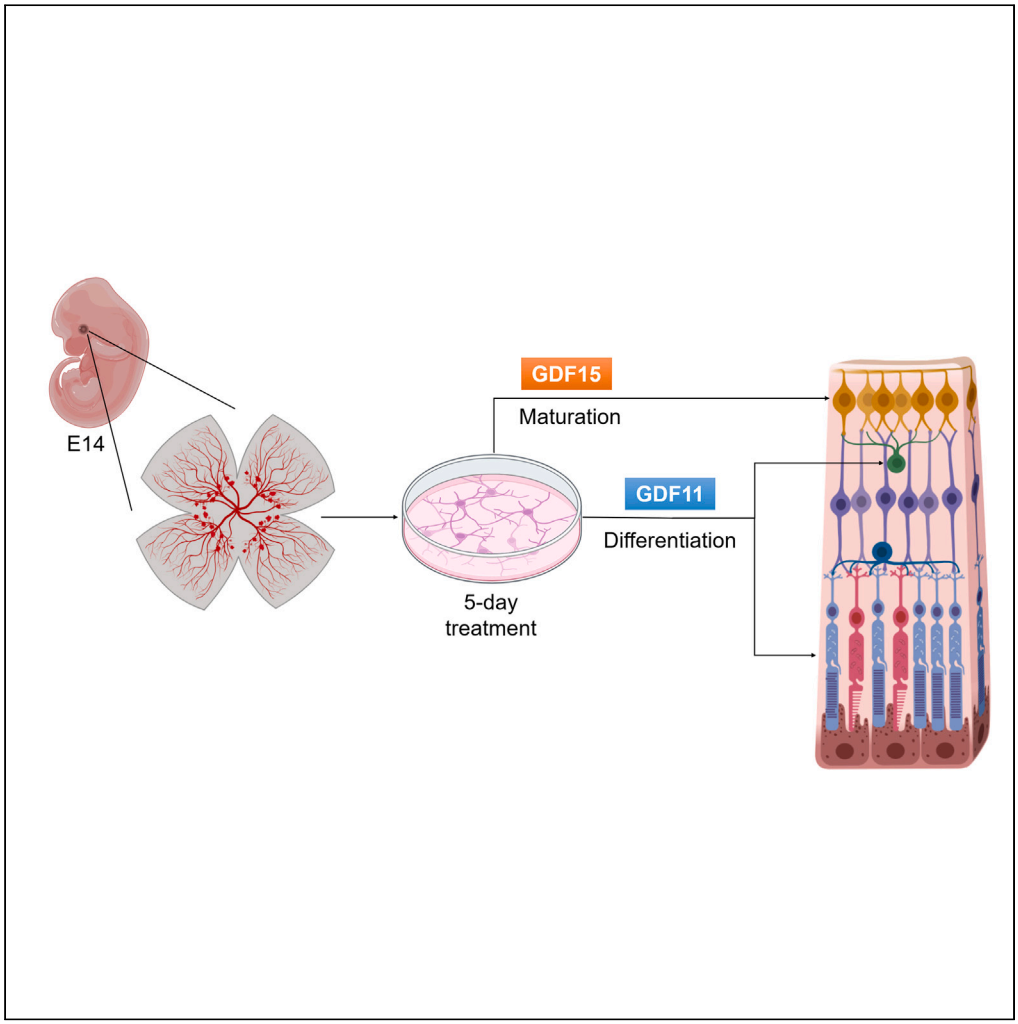

Ziming Luo, Sahil Shah, Bogdan Tanasa, Kun-Che Chang, Jeffrey L. Goldberg

luozm@stanford.edu (Z.L.)
kcchang@pitt.edu (K.-C.C.)

Highlights

GDF receptors are expressed in various cell types in the retina

GDF-11 and GDF-15 play different roles in RGC/AC development

GDF-11 induces photoreceptor and amacrine differentiation

GDF-15 promotes RGC maturation

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Gene regulatory roles of growth and differentiation factors in retinal development

Ziming Luo,[1,*] Sahil Shah,[1] Bogdan Tanasa,[1] Kun-Che Chang,[1,2,3,*] and Jeffrey L. Goldberg[1]

SUMMARY

Retinal ganglion cell (RGC) differentiation is tightly controlled by extrinsic and intrinsic factors. Growth and differentiation factor 15 (GDF-15) promotes RGC differentiation, opposite to GDF-11 which inhibits RGC differentiation, both in the mouse retina and in human stem cells. To deepen our understanding of how these two closely related molecules confer opposing effects on retinal development, here we assess the transcriptional profiles of mouse retinal progenitors exposed to exogenous GDF-11 or -15. We find a dichotomous effect of GDF-15 on RGC differentiation, decreasing RGCs expressing residual pro-proliferative genes and increasing RGCs expressing non-proliferative genes, suggestive of greater RGC maturation. Furthermore, GDF-11 promoted the differentiation of photoreceptors and amacrine cells. These data enhance our understanding of the mechanisms underlying the differentiation of RGCs and photoreceptors from retinal progenitors and suggest new approaches to the optimization of protocols for the differentiation of these cell types.

INTRODUCTION

Glaucoma and other optic neuropathies are leading causes of blindness[1] and no therapy is currently available to restore vision once retinal ganglion cells (RGCs), the sole output neurons of the retina, are lost.[2] RGC death leads to monocular or bilateral blindness in as many as 14% of diagnosed patients.[3] Like most neurons of the central nervous system (CNS) in mammals, RGCs do not regenerate once lost, and therefore, vision restoration through stem cell replacement therapies has emerged as an attractive solution.[4] However, such an endeavor requires a thorough understanding of the molecular signals that govern endogenous RGC specification. We and others have shown that many intrinsic transcription factors (TFs) such as P*ax6*,[5] Sry-related high mobility box (Sox) superfamily genes,[6–8] *Atoh7* (Math5),[9–12] *Pou4f1*(Brn3a),[13] *Pou4f2* (Brn3b),[14–17] and Dlx1/2[18] are required for early or late RGC fate determination or differentiation during retinal development. Although a recent study claimed that *Atoh7* and *Brn3b* together can reprogram mature mouse Müller glia (MG) into RGCs,[19] confirmatory evidence and translation of such important findings to guide human stem cell differentiation into RGCs *in vitro* remain sparse. Thus, there is still an unmet need to significantly advance the cell replacement therapy field by providing a stable and well-characterized source of donor RGCs.

Growth and differentiation factor (GDF)-11 and GDF-15, two members of the transforming growth factor beta (TGFβ) superfamily, play opposing roles in RGC differentiation.[20] GDF-11 is reported to induce RE1-silencing TF (*Rest*) and repress *Atoh7* expression, which suppresses RGC differentiation *in vivo*.[21] GDF-15, on the other hand, promotes RGC differentiation through the activity of *Sox4*,[20] and has also been reported to increase RGC neurite outgrowth[22] and regulate hippocampal neurogenesis.[23] Somewhat surprisingly, GDF-15 is upregulated in RGCs following optic nerve injury as part of a putative neuroprotective response,[24] as well as in RGCs of glaucomatous rodents[25] and human glaucoma patients.[25,26] In light of the contrasting actions of GDF-11 and GDF-15 on RGC differentiation, we aim to investigate the single-cell profile of these two family members in an *ex vivo* culture model.

Both GDF-11 and GDF-15, members of the TGFβ superfamily with different molecular weights (GDF-11 dimer is 24 kDa vs. GDF-15 is 34 kDa), have been noted for their distinct interactions with Alk1-7 and other receptors. GDF-11 typically engages with activin receptors I/II (Alk 4, 5, and 7), a characteristic shared by most TGFβ proteins.[27] GDF-15 also interacts with GDNF family receptor alpha-like (GFRAL).[28] The different residues of GDFs were also reported to contribute to their ligand-receptor interaction. For instance, in GDF-11, Tyr347, Met348, and Phe349 are predicted as the crucial amino acids in the helix and N-terminal regions for engaging with the palm Alk5,[29] whereas Val283 and Ile285 are essential for GDF-15's binding to GFRAL.[30] Prior investigations have demonstrated that while GDF-11 activates both Smad1/5 and Smad2/3 signaling pathways, GDF-15 predominantly activates Smad1/5 signaling within retinal progenitor cell cultures.[20] This observation suggests the plausible existence of additional receptors, beyond GFRAL, that facilitate the interaction between GDF-15[31] and contribute to retinal development. Moreover, the study revealed that GDF-15 inhibits GDF-11-induced Smad2/3 phosphorylation,[20] suggesting a potential competitive relationship between the two ligands for downstream signaling pathways.

[1]Spencer Center for Vision Research, Byers Eye Institute, Stanford University School of Medicine, Palo Alto, CA 94304, USA
[2]Department of Ophthalmology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219, USA
[3]Lead contact
*Correspondence: luozm@stanford.edu (Z.L.), kcchang@pitt.edu (K.-C.C.)
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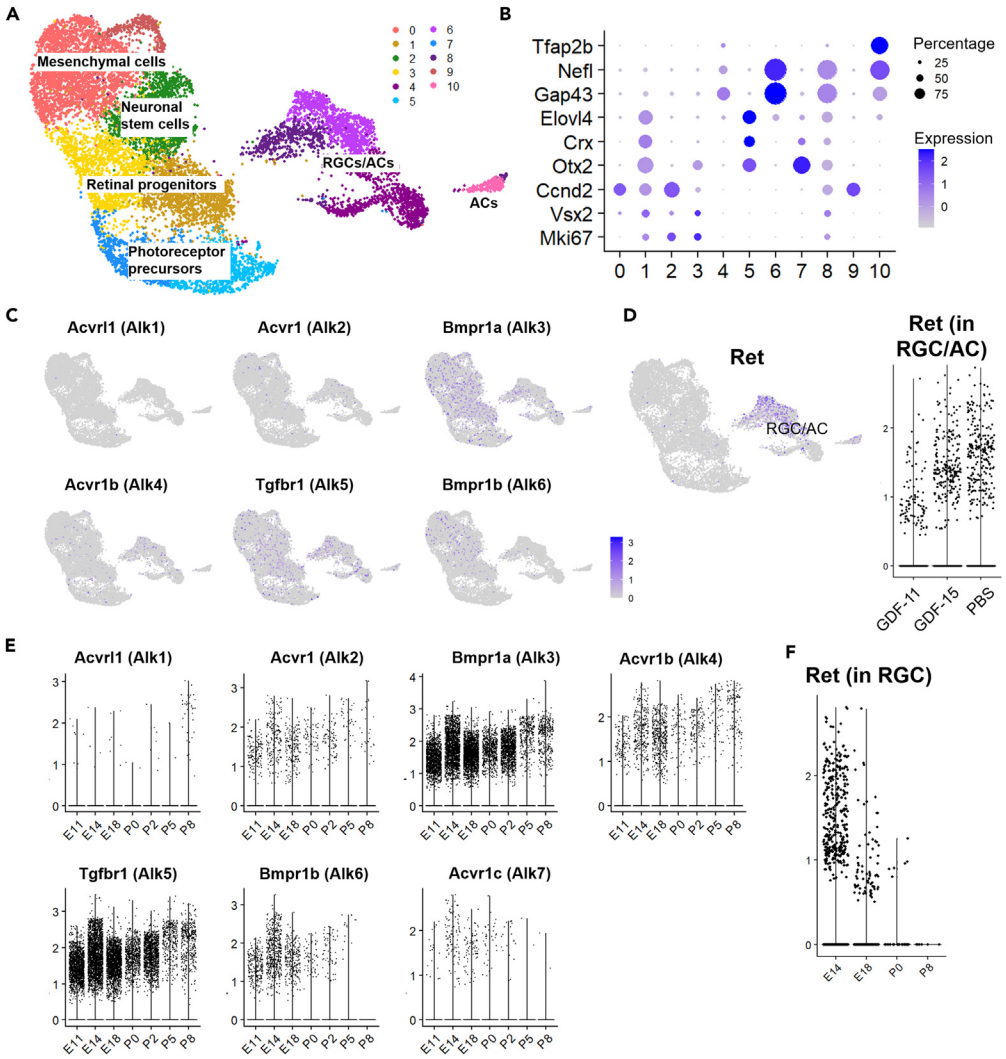

Figure 1. GDF receptors were present expressed in various cell types in both *ex vivo* culture and the developing retina *in vivo*

(A and B) UMAP plot in A shows 11 clusters that can be labeled by cell type using typical markers in (B). (B) Expression of retinal cell type-specific genes in each cluster.

(C) GDF-11/15 receptors, *Alk1-6*, were expressed in retinal progenitors (left clusters) and RGCs/ACs (right clusters) (*Alk7* was not detected).

(D) The GDF-15 co-receptor *Ret* was specifically expressed in the RGC/AC population and suppressed by exogenous GDF-11.

(E) *Alk1-7* expression remains relatively consistent throughout retinal development.

(F) Similar to its expression in retinal cultures, *Ret* was expressed in RGCs *in vivo*, declining after E14.

Single-cell RNA sequencing (scRNA-seq) is a powerful tool for studying evolutionarily conservation and species-specific mechanisms of retinal development.[32] Recent studies have successfully utilized scRNA-seq to classify as many as 46 RGC subtypes in the mouse,[33] 16 in the foveal retina and 18 types of RGCs in the peripheral retina of non-human primates[34] and 12 types in the human fovea and peripheral retina.[35] To understand how GDFs alter the differentiation of retinal cell types during retinal development, we treated embryonic 14 (E14) day mouse retinal cell cultures with GDF-11 and GDF-15 for five days and analyzed their transcriptional profiles. We find important differences between GDF-11 and GDF-15 regulation of proliferation and differentiation states early versus late in retinal development modeled *in vitro*. These data provide novel insights into GDF signaling and retinal cell development.

RESULTS

GDF-11 and GDF-15 alter gene expression and retinal differentiation in E14 retinal cultures

To understand target gene regulation induced by GDFs, we cultured mouse embyonic14 (E14) retinal progenitors for 5 days in the presence of PBS (control), GDF-11, or GDF-15. Three groups of data were integrated for computational analysis, and the data were visualized by Uniform Manifold Approximation and Projection (UMAP) dimensional reduction, revealing 11 distinct clusters (Figure 1A), which were further identified

Table 1. A list of gene markers used in the present analyses

Gene (Also known as)	Retinal cell type	Developmental stage/function	Reference
Mki67	Proliferating cells	Retinal progenitors that are still in the cell cycle	Clark et al.[45]
Ccnd2	Proliferating cells		Clark et al.[45]
Vsx2 (Chx10)	Retinal progenitors	Retinal progenitors	Clark et al.[45]
Atoh7 (Math5)	Transitional population	Transitional stage developing RGC or photoreceptor precursors	Sridhar et al.[39]
Fabp7	Photoreceptor precursosrs	Transitional stage developing photoreceptor precursors	Sridhar et al.[39]
Otx2	Photoreceptor precursosrs	Photoreceptor precursosrs	Clark et al.[45]
Crx	Photoreceptor precursosrs	Photoreceptor precursosrs	Clark et al.[45]
Nrl	Photoreceptors	Photoreceptors gaining rod-fate	Sridhar et al.[39]
Elovl4	Photoreceptors	Photoreceptors gaining rod/cone-fate	Cowan et al.[49]
Gap43	RGCs	Developing or regenerating RGC	Sridhar et al.[39]
Nefl	RGCs	Axon skeleton marker	Sridhar et al.[39]
Pou4f1 (Brn3a)	RGCs	Early-stage RGC	Sridhar et al.[39]
Pou4f2 (Brn3b)	RGCs	Early-stage RGC	Sridhar et al.[39]
Rbpms	RGCs	More mature RGC	Sridhar et al.[39]
Isl1	RGCs	More mature RGC	Luo et al.[50]
Ebf1	RGCs	More mature RGC	Wu et al.[12]
Tfap2b	ACs	Postmitotic ACs	Clark et al.,[45] Bassett et al.[51]
Dlx1	ACs or RGCs	Postmitotic ACs or late-born RGCs	Clark et al.,[45] de Melo et al.[52]

by specific retinal cell-type markers (listed in Table 1) including neuronal stem cells (cluster 2), retinal progenitors (clusters 1 and 3), RGCs/amacrine cells (ACs, clusters 4, 6, and 8), photoreceptor precursors (photoreceptor precursor clusters 5 and 7), mesenchymal cells (cluster 0), and amacrine cells (cluster 10) (Figure 1B).

To further investigate the ligand-receptor interactions, we evaluated the expression level of several well-known GDF receptors.[36] As shown in Figure 1C, genes of the Alk1-6 receptors were seen in retinal progenitors and RGCs/ACs (Alk7 was not detected). A previous study showed that GDF-15 promoted neurite length by binding to one of the glial-derived neurotrophic factor receptors, *GFRAL*, and its co-receptor *RET*.[22] However, *Gfral* was not detected in our dataset, which was probably limited by sequencing depth. Interestingly, the co-receptor *Ret* was found specifically in the RGC/AC population (Figure 1D). *Ret* is thought to regulate RGC type specification by interacting with *Pou4f1*(*Brn3a*).[37] In our cultures, *Ret* expression was slightly lower in the presence of exogenous GDF-11, which may correlate with the slight decrease in RGC number in this group.

Although in the present study only one time point in retinal progenitor-to-RGC differentiation was studied by RNA-seq, we also evaluated how GDF receptors' expression changes through retinal development. Analyzing a published database including mouse retinas in different developmental stages, we found that *Alk1-7* didn't change significantly throughout development (Figure 1E). These data were consistent with our finding that *Alk3* and *Alk5* were most highly expressed (Figure 1C). A previous study showed that Ret was stained positive in the newly forming RGCs at E14.5, and later in ACs and horizontal cells.[38] This result is also consistent with our finding in Figure 1F that the expression level of *Ret* peaks at E14, slightly earlier than the time that protein was detected.

Together these results demonstrate that GDF receptors were stably expressed in both *ex vivo* retinal cell culture and *in vivo* developing mouse retinas, indicating that the effects of GDF-11/-15 on retinal development may signal through one or more of these receptors. This is consistent with our previously finding effects directly on retinal progenitors in culture[20] and explaining observed effects of GDFs on retinal differentiations *in vivo*.[20,21] Next, we asked how GDF-15/-11 affected retinal progenitor development. GDF-11 treatment significantly increased photoreceptor precursor numbers, but in the analysis neither GDF treatment significantly altered RGCs/ACs differentiation (Figures 2A and 2B). Increased expression of proliferative genes in progenitors and neuronal stem cells compared to RGCs/ACs and photoreceptor precursors were seen in all conditions, as expected, but interestingly, the expression of anti-proliferative genes increased in RGCs/ACs and photoreceptor precursors only in response to GDF-11 (Figure 2C).

GDF-11 promotes photoreceptor development and maturation

To further elucidate the effect of GDF-11 on photoreceptor precursor differentiation (clusters 5 and 7 in Figures 1A, 3A, and 3D), this population was analyzed further. As described in human retinas previously,[39] photoreceptor development goes through two transitional stages characterized by the expression of *ATOH7* and *OTX2*. We found that *Atoh7* and *Otx2* were specifically expressed in cluster 7 (Figure 3B). In cluster 7, GDF-11 slightly increased but GDF-15 slightly decreased the number of these transitional cells (Figure 3A). Interestingly, *Otx2*

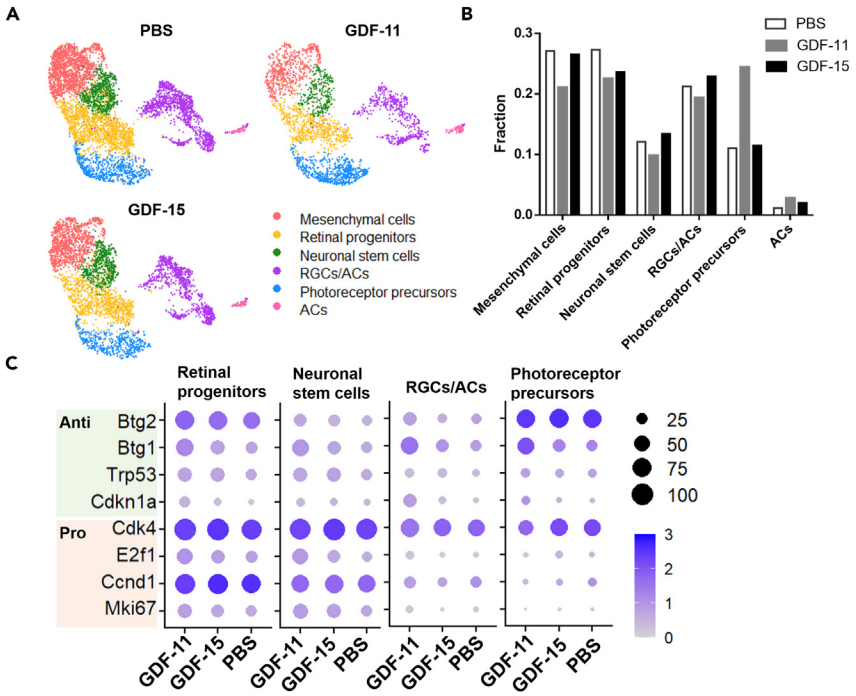

Figure 2. GDF-11 and GDF-15 alter gene expression and neuronal differentiation in embryonic retinal cultures

(A) Retinal cell type differentiation changes in response to exogenous treatment with control vehicle PBS or GDF-11 or GDF-15.

(B) Fractions of different retinal progeny cell types by treatment.

(C) Anti- and pro-proliferative gene expression in different retinal progeny cell types shows increased expression of proliferative genes in progenitors and neuronal stem cells compared to RGCs/ACs and photoreceptor precursors in all conditions, and increased expression of anti-proliferative genes in RGCs/ACs and photoreceptor precursors in response to GDF-11.

expression level was consistent in the three groups while *Atoh7* was suppressed by GDF-11 (Figure 3B), which was consistent with our previous findings.[20] Moreover, even in the whole mouse retina, *Atoh7*-positive cells were significantly decreased by GDF-11 (less than 5%, compared to 17% in PBS) (Figure 3C).

In contrast, GDF-11 significantly increased the cell fraction in cluster 5 (Figure 3D). This cluster co-expressed several well-known photoreceptor precursor markers, including *Crx* and *Nrl*. We found that the expression level of *Crx* was relatively consistent across three groups. However, *Nrl* was significantly upregulated in the GDF-11 group (Figure 3E). We next realigned cells in clusters 5 and 7 by pseudotime (Figure 3F). Cells that still expressed the proliferation marker, *Mki67*, were assigned as the youngest (pseudotime zero). Colored by clusters, the pseudotime UMAP demonstrated a clear distinction between cluster 7 (the less mature cells) and cluster 5 (more developed cells) shown in the middle panel on the first row of Figure 3F. Most of the cells from the GDF-11 group were distributed at the later pseudotime, which suggested that GDF-11 promotes photoreceptor development (the right panel on the first row of Figure 3F). At the earliest pseudotime, cells expressed *Atoh7*; in the mid-stage, *Fabp7*, *Crx*, and *Otx2* were highly expressed, consistent with the progression described previously in human fetal scRNA-seq data, in which the transition cells that eventually developed into photoreceptors and bipolar cells were characterized by high levels of *FABP7*, *OTX2*, and *DLL3*.[39] Moreover, cells expressing *Nrl* distributed in the latest pseudotime, confirming their further maturation, and were mainly contributed from the GDF-11 group. Thus, taken together, these data suggest that GDF-11 promotes photoreceptor development and maturation.

GDF-11 and GDF-15 play different roles in RGCs/ACs development

To further delineate the effect of GDF-11 and GDF-15 on RGC and AC development, the RGCs/ACs cluster (clusters 4, 6, and 8 in Figure 1A) were subset and re-clustered. Six clusters were defined (Figure 4A). Clusters 0, 1, 3, and 4 showed typical RGC marker expression, including *Pou4f1*, *Rbpms*, *Isl1*, *Nefl*, and *Gap43*. ACs were identified by *Tfap2b* in cluster 5 (Figure 4B). Interestingly, cluster 2 was characterized by *Dlx1* expression but did not co-express either RGC marker *Pou4f1*, or AC marker *Tfap2b* (Figure 4C). To further analyze the transcriptional differences between RGC-like clusters, we focused on cluster 0 versus clusters 1, 3, and 4, which were mapped at a greater distance. Genes that drove clustering were shown on a heatmap (Figure 4D). Typical RGC marker genes were widely expressed in all clusters, but the levels were significantly lower in cluster 0. In cluster 0, RGCs retained residual proliferative gene expression, including cell cycle genes *Ccnd1* and *Id2/3*, chromosomal accessibility and DNA replication genes *H2afv* and *Hmgb2*, and cell adhesion and migration genes *Mdk*, *Sparc*, *Serpinh1*, *Mest*, *Metrn*, and *Vim*. GDF-15 significantly decreased cell fraction in cluster 0 and slightly increased cell fractions in clusters 1 and 4, whereas

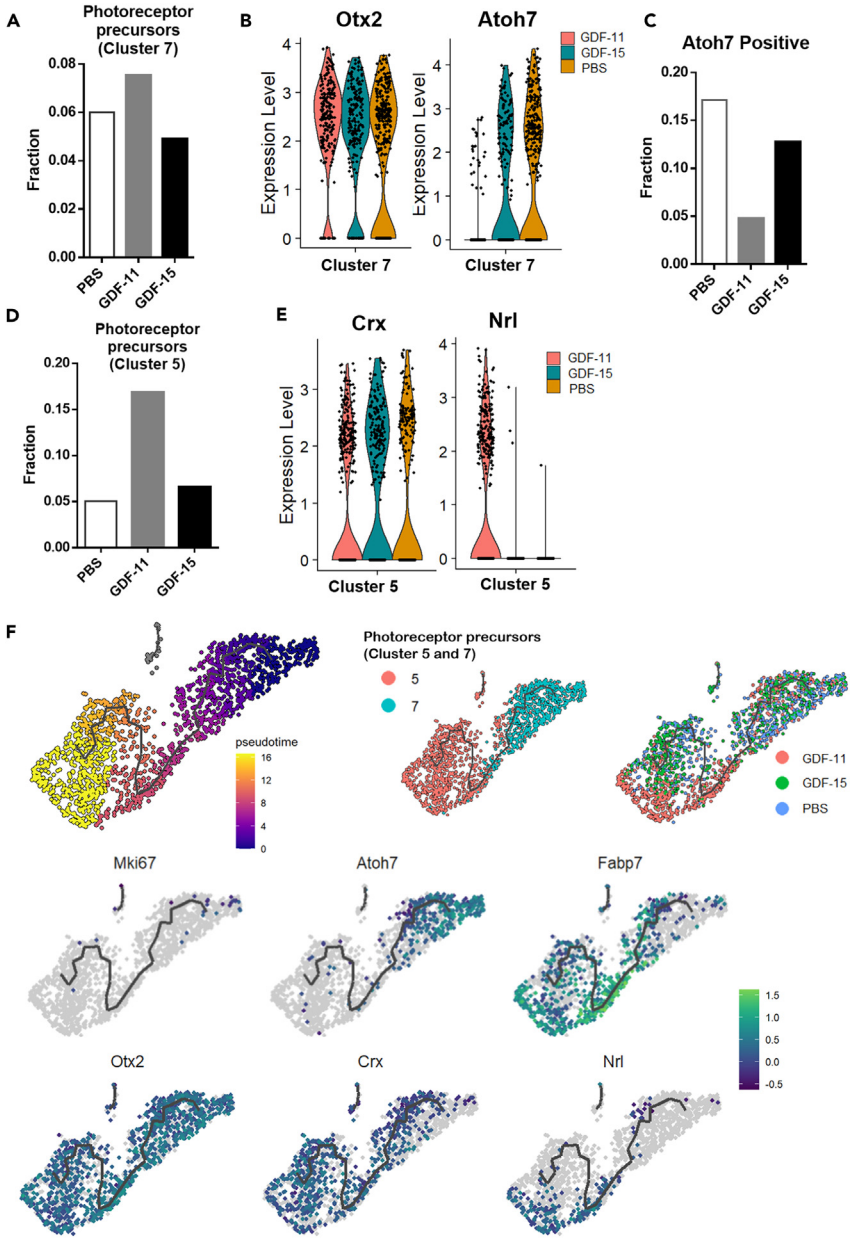

Figure 3. GDF-11 promotes photoreceptor development and maturation

(A) GDF-11 increased cell fraction in photoreceptor precursors cluster 7.

(B) *Otx2* was consistent in the three groups, while *Atoh7* was suppressed by GDF-11.

(C) In the whole retinal dataset, *Atoh7* expression was suppressed by GDF-11.

(D) GDF-11 significantly increased cell fraction in photoreceptor precursors cluster 5.

(E) *Crx* expression was consistent across the three groups, whereas GDF-11 treatment dramatically induced *Nrl* expression.

(F) Trajectory analysis of clusters 5 and 7 showed that cells were more mature in the GDF-11-induced cells (cluster 5). The top left panel was colored by pseudotime and the black line demonstrated the structure of the trajectory. The right two plots on the top row showed distributions of cells from different clusters or samples.

GDF-11 increased cell fraction in the amacrine cell cluster (cluster 5) (Figures 4E and 4F). In addition, we also found that cluster 2, which was missing markers typical of either RGCs or amacrine cells, was upregulated by GDF-15 and downregulated by GDF-11. Although GDF-11 did not significantly affect the cell fraction segregating into cluster 0, it drove RGCs to increase anti-proliferative *p21* expression compared to PBS- or GDF-15-treated cultures (Figure 4G), suggesting that GDF-11 downregulates cell proliferation in this immature or newly differentiating RGC cluster in differentiating retinal progenitors in culture.

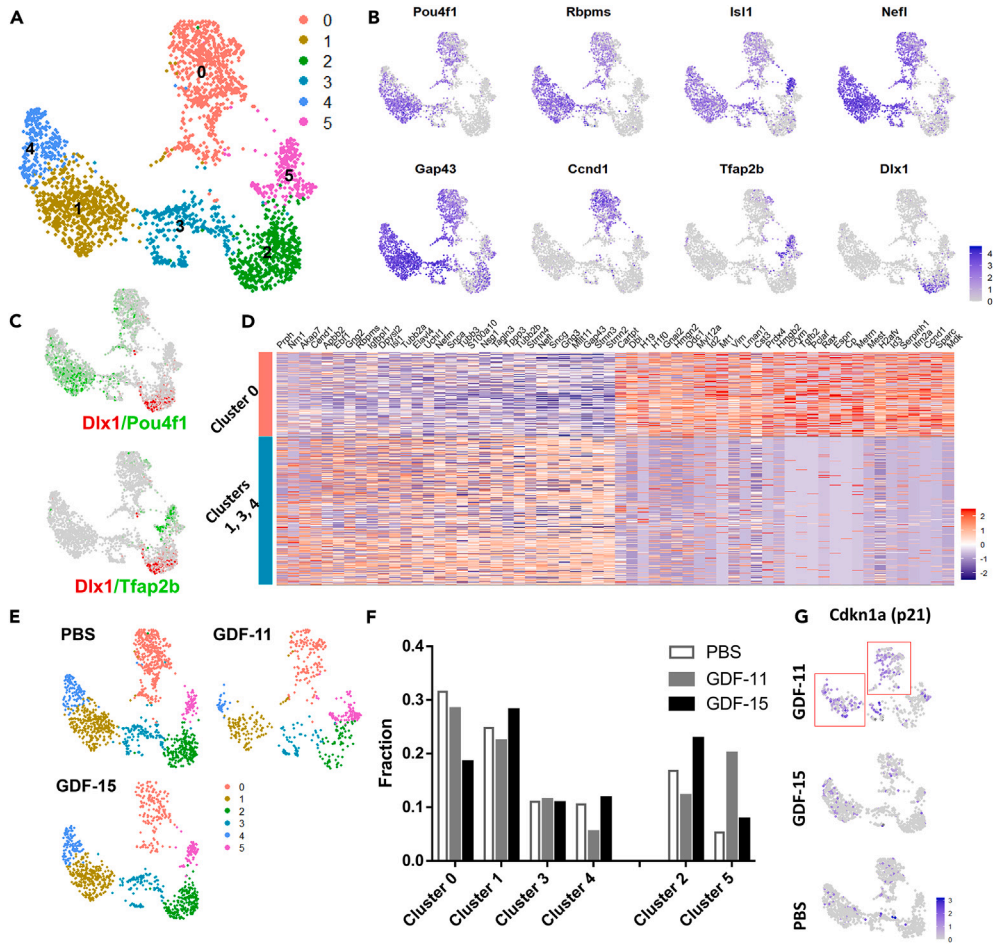

Figure 4. Role of GDFs in RGC and amacrine cell gene expression

(A) Re-clustering of RGCs/ACs (clusters 4, 6, and 8 in Figure 1A) generated 6 clusters.

(B) Expression patterns of RGC marker genes, including *Gap43*, *Nefl*, *Pou4f1*, *Isl1*, and *Rbpms*, and amacrine marker gene, *Tfap2b*.Cluster 0 was characterized by additional expression of *Ccnd1*, and cluster 2 highly expressed *Dlx1*.

(C) *Dlx1* was not co-expressed with either RGC marker *Pou4f1*, or amacrine cell marker *Tfap2b*.

(D) Heatmap of genes differentiating RGC cluster 0 from RGC clusters 1, 3, and 4.

(E and F) GDF-15 significantly decreased cell fraction in cluster 0, and slightly increased cell fractions in clusters 1 and 4, whereas GDF-11 dramatically increased cell fraction in cluster 5.

(G) GDF-11-treated retinal progenitors increased anti-proliferative gene *Cdkn1a* (also known as *p21*) compared to PBS- or GDF-15-treated cultures.

Trajectory analysis of whole retinal culture differentiation confirms that GDF-15 promotes RGC maturation

To further understand the effect of GDF-11 and GDF-15 in regulating retinal development and maturation, we analyzed the data from Figure 1 by trajectory construction using Monocle3. Two main populations were found on the UMAP plot of the trajectory analysis, with proliferating markers (*Mki67*and *Ccnd1*) expressed in a significant subset of both populations (Figure 5A). The right clusters were identified as RGCs/ACs based on their marker genes, including *Atoh7*, *Pou4f1/2*, *Ebf1*, *Dlx1*, and *Tfap2b* (Figure 5B). We specifically focused on RGCs/ACs and pseudotime zero was assigned based on the expression of *Mki67* and *Ccnd1* (Figure 5C). Assigning the cluster distribution from Figure 3 on the trajectory analysis showed cluster 0 distributed at the earliest, least mature stage, and clusters 1, 3, and 4 were at a later, more mature RGCs (Figure 5D). Moreover, the AC cluster (cluster 5) was seen at the end of the trajectory, after the *Dlx*-positive cluster 2. Since cluster 2 showed a similar trend as clusters 1, 3, and 4 in response to GDFs, and was identified as a late stage in the trajectory analysis, it may represent transitional cells between RGCs and amacrine cells. These data validate that each algorithm approach yields similar results. Compared to PBS, GDF-15 treatment significantly increased and GDF-11 treatment slightly decreased the ratio of later RGCs (clusters 1, 3, and 4) compared to earlier RGCs (cluster 0) (Figures 5E and 5F), suggesting that GDF-15 may promote RGC differentiation and maturation into "late" developmental stages (Figure 5F).

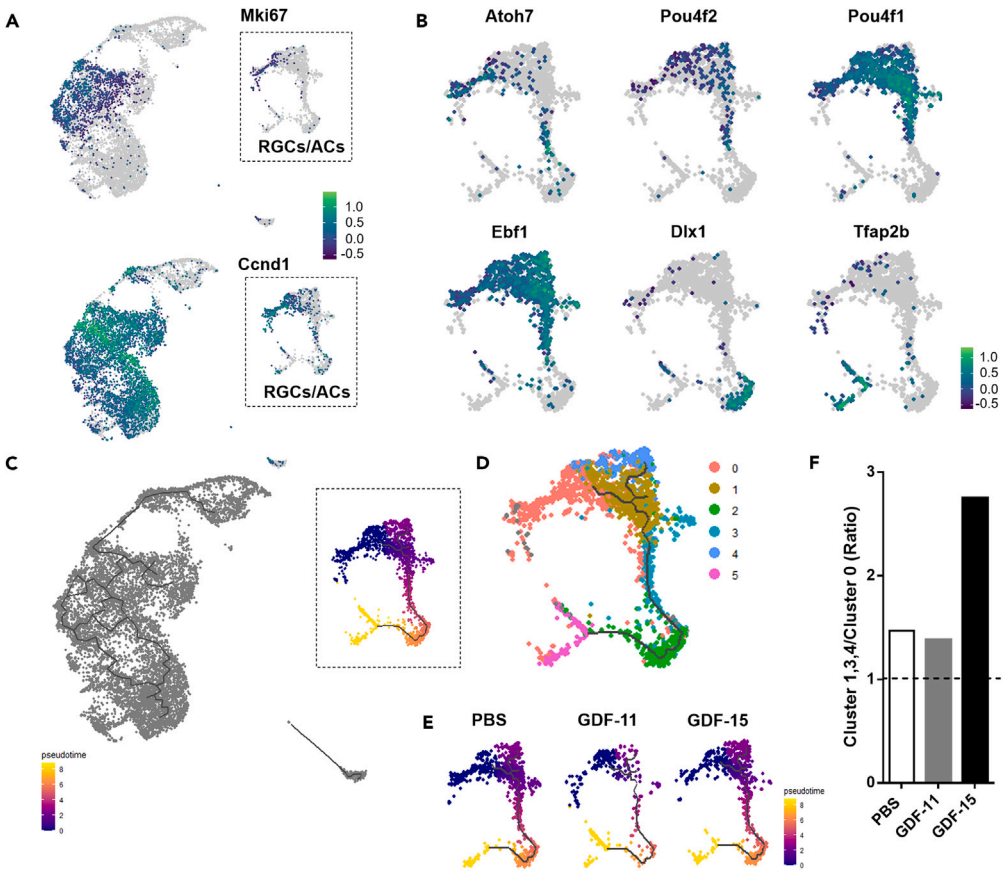

Figure 5. Trajectory analysis of whole retinal culture differentiation confirms that GDF-15 promotes RGC maturation

(A) Two populations were generated in the UMAP plot of trajectory analysis. Proliferative markers Mki67 and Ccnd1 were expressed in both populations.

(B) The right population consisted of RGCs, ACs and their precursors, based on the expression of typical marker genes.

(C) Pseudotime plot focusing on RGCs/ACs confirms directionality suggested by expression of proliferative genes in (A) and precursor markers in (B).

(D) Assigning the cluster distribution from Figure 3 onto the distribution mapped through trajectory analysis validates that each approach yields similar results, with cluster 0 a more immature/early RGC, clusters 1, 3, and 4 a more mature RGC, cluster 5 at the end ACs, and the less well-defined Dlx-positive cluster 2 in an intermediate zone.

(E) Pseudotime plot split by treatment groups.

(F) The ratio of later RGCs (clusters 1, 3, and 4) to earlier RGCs (cluster 0) shows an increase in maturation with GDF-15 and a slight decrease with GDF-11.

DISCUSSION

This study marks a significant milestone in the exploration of two GDF-family ligands that we previously found play opposing roles in retinal development.[20] Here, we extend these previous functional data by exploring gene expression as a marker of differentiation in mouse embryonic retinal progenitor culture, and find exogenous GDF-15 promotes RGC development whereas GDF-11 promotes photoreceptor maturation. A number of our new findings extend prior observations. For example, GDF-15 barely affected Atoh7 expression in retinal progenitors in culture, consistent with our previous study, in which GDF-15's effect on promoting RGC differentiation may not be via Atoh7 but rather depended on Sox4 expression.[20] Together, these new scRNA-seq data help to molecularly confirm the previous cellular phenotypes and vice versa.

Although we and others previously showed that GDFs regulate RGC number in the developing retina, the effects of GDFs on RGC specification that may occur before or after final mitosis remains unknown. In the present study, we first observed that nearly every cell in cluster 0 (Figure 4B) that expressed typical RGC markers also expressed residual proliferation-associated gene expression, which is consistent with a previous study showing that the two key RGC-fate regulators, *Brn3b* and *Isl1* were expressed before cell cycle exit in the early developmental stages (<E14).[40] Interestingly, in their study, Brn3b did not co-express with the G1/early-S phase marker *cycD1*(*Ccnd1*) at the protein level, while in our study, several RGC markers co-expressed with *Ccnd1* at the RNA level, which further indicates that RGC cell fate commitment can happen early before final mitosis or a residual expression of cell cycle genes can be detected after final mitosis and differentiation.

Unlike GDF-15, GDF-11 had been well-studied in retinal development. A previous study demonstrated that GDF-11-null embryos possessed 50% more RGCs, suggesting a role for GDF-11 in suppressing RGC fate specification, but not directly demonstrating a cell-autonomous function in retinal progenitors and arguing that GDF-11 did not directly regulate proliferation.[21] Our previous GDF-11 RNA *in situ*

hybridization data[20] and others' scRNA seq data[41] showed that GDF-11 is expressed in the retinal progenitor cells and other retinal cells during retinal development, which suggests that endogenous GDF-11 can also contribute to the cell-autonomous effects in a paracrine fashion. Our data also demonstrate GDF receptors expression in progenitor cells. Undertaking a broader exploration of which receptors may be most active e.g., by doing 7–8 receptor knockouts alone and in various combinations is beyond the scope of this paper, but will drive an interesting future work. Furthermore, we showed that GDF-11 directly inhibited progenitor-to-RGC differentiation and also human stem cell-to-RGC differentiation.[20] In the present study, in addition to observing that GDF-11 treatment slightly inhibits RGC generation (Figure 2B), we further found that GDF-11-treated retinal progenitors drive toward RGCs with an increased anti-proliferative gene expression (Figures 2C and 4G), reinforcing a model relying on early cell cycle exit as a mechanism for promoting differentiation. However, unlike previous data showing that GDF-11 was responsible for a large reduction in Brn3b+ RGCs in E13.5 retinal explants, we only observed a minimum decrease in RGC differentiation in culture. One possible explanation is that most RGCs are already specified by E14 when exposed to exogenous GDF-11. However, a recent study showed that deletion of *Atoh7* leads to increased apoptosis in RGCs via Bax signaling,[42] suggesting *Atoh7* which we found was suppressed by GDF-11 in our cultures plays an important role in regulating RGC number by inhibiting apoptosis, not studied here.

In these *in vitro* studies, GDF-11 also demonstrated a significant effect in promoting photoreceptor differentiation and maturation, although *in vivo* knockout of GDF-11 didn't appear specific to regulation of photoreceptor numbers in the developing mouse retina.[21] GDF-11 controls the duration of expression of various bHLH and homeobox genes to ultimately control the temporal window of progenitors' competence to generate various cell types.[43] For example, GDF-11 suppresses Atoh7 expression, allowing retinal progenitors to acquire competence to produce later-born cell types, including photoreceptors. Indeed, our data showed that GDF-11 increased the *Otx2*-positive photoreceptor precursor population, and Atoh7 expression was suppressed in these cells. Furthermore, our study indicated that GDF-11 advanced photoreceptor maturation. Such an effect was not only demonstrated by upregulation of the more mature marker *Nrl*, but also by increasing cell numbers in this cluster. In addition, GDF-11 also significantly increased amacrine cell marker gene expression (*Tfap2b*), suggesting an effect on traditionally earlier-born cell types—with the important caveat that some amacrine cells are born in later retinal development and this may reflect effects on specific late-born amacrine cell types. This is the first study to show that GDF-11 regulates the balance of photoreceptors, amacrine, and RGC differentiation in retinal progenitor cell cultures. However, the underlying mechanism remains unclear and will be an interesting future research direction.

Hence, comparing the present gene expression analyses to prior experimental work, we see some similarities and conserved elements, such as the suppressive effect of GDF-11 and the promoting effects of GDF-15 in RGC differentiation. But in other cases, the gene expression now suggests new hypotheses that didn't match prior *in vivo* or human cell data, such as GDF-11 treatment induces photoreceptors and amacrine cell gene expression *ex vivo*, but we did not observe such phenotypes *in vivo*.[20] The potential confounds in interpreting scRNAseq data versus *in vivo* experimental data include the simplified environment of *in vitro* vs. *in vivo* (where other molecules may compensate for the loss of GDF-11 *in vivo*), differences between humans and mice, and the far-from-perfect correlation between RNA and protein expression in cells. However, these new hypotheses derived from these scRNA-seq data may be interesting to drive future work, such as studying whether GDF-11 and GDF-15 compete for RGC fate, to further investigate the underlying mechanisms of GDFs in retinal development.

There are 46 RGC subtypes in the mouse retina[33] and little is known about what regulates RGC subtype fate determination. Although our study reveals the transcriptional profiles of RGC fate that are regulated by GDFs, we did not observe any differences in RGC subtypes mimicking those in the mature retina, possibly because it is still too early to identify RGC subtypes in earlier developmental stages.[44] Longer exposure to GDFs may allow for the study of such a question in future experiments, but in any case it is not clear from these data whether GDFs play a role in RGC subtype specification.

In sum, these data enhance our understanding of the mechanisms underlying the differentiation of RGCs and photoreceptors from retinal progenitors. Extending these data to human stem cells may offer insights into the optimization of protocols for the differentiation of these cell types. These new findings may be applied to human stem cell-to-RGC or stem cell-to-photoreceptor differentiation, which may one day be a source for retinal neuron transplants and cell replacement therapies for retinal and optic nerve degenerative diseases.

Limitations of the study

Limitations in these analyses included the small sample size, comprising with one sample from each treatment group for scRNA-seq, although our sequencing depth and quality were excellent. Furthermore, progenitor cell death over longer periods in culture limited our exploration into the effects of prolonged GDF treatment. While our study successfully explored the transcriptional profiles of GDF-treated retinal progenitors, it is essential to note that the regulatory mechanisms governing translational regulation and therefore proteomic profiles within these groups remain unknown, and would be an interesting area for future work.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

K-C.C. performed cell culture, retina dissection, data collection and analysis. Z.L. performed scRNA-seq analysis. S.S. performed scRNA-seq sample preparation. B.T. performed scRNA-seq analysis. K-C.C., Z.L., and J.L.G. were involved in the study design, data analysis, and manuscript writing. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
GDF 11	R & D systems	1958-GD-010
GDF 15	R & D systems	89-44G-D025
B27	Gibco	17504-044
Transferrin	Sigma	T-1147
BSA	Sigma	A-4161
Putrescine	Sigma	P7505
Sodium selenite	Sigma	S5261
Progesterone	Sigma	P8783
Insulin	Sigma	I-6634
Epidermal growth factor	Peprotech	AF-100-15
Fibroblast growth factor	Peprotech	100-18B
Penicillin/streptomycin	Thermo Fisher Scientific	P4333
Accutase	Innovative Cell Technologies	AT-104
Critical commercial assays		
Chromium GEM-X Single Cell 3′ Kit	10X Genomics	1000686
Chromium GEM-X Single Cell 3′ Chip Kit	10X Genomics	1000690
Dual Index Kit TT Set A	10X Genomics	1000215
Library Construction Kit C	10X Genomics	1000694
Deposited data		
Raw and analyzed scRNA-seq data	This paper	GSE252861
scRNA-seq data of developing mouse retinas	Clark et al.[45]	GSE118614
Experimental models: Organisms/strains		
E14.5 mouse	Charles River Laboratories	C57BL/6
Software and algorithms		
CellRanger	10X Genomic	V3.0.0.
Seurat	Satija Lab	v4.0.2
Monocle 3	Trapnell Lab	v0.2.2.0
Prism 9	GraphPad	Prism 9.0.0

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kun-Che Chang, PhD (kcchang@pitt.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Single-cell RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

- This paper does not report the original code. All analyses were performed as recommended by Seurat (v4.0.2)[46] and Monocle 3 (v0.2.2.0)[47]
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal and cell culture

All animal research was conducted in compliance with ARVO statement for the use of animals in ophthalmic and vision research and was approved by the Institutional Animal Care and Use Committee at University of Pittsburgh and Stanford University. Mouse GDF-11 and GDF-15 were purchased from R & D systems. Since RGC formation peaks at E14 and is gradually decreased, we chose E14.5 retinal tissue for GDFs treatment for 5 days, reaching P0 for RGC measurement. Primary retinal tissues were isolated from E14.5 mouse (C57BL/6, Charles River Laboratories) eye following a previous study.[6] Timed-pregnant mice were euthanized and the retinas from E14.5 mouse pups were swiftly dissected. Retinal tissues were dissociated by Accutase (Stemcell Technology) and the dissociated cells were cultured in the 60 mm dishes with 70% confluency. Retinal cells were incubated in the retinal progenitor media prepared with DMEM/F12 medium containing glucose (0.6%, Sigma), B27 (1:50, Gibco), Sato supplement (1:100), insulin (5 μg mL^{-1}, Sigma), epidermal growth factor (20 ng mL^{-1}, Peprotech), fibroblast growth factor (20 ng mL^{-1}, Peprotech), penicillin/streptomycin (1%, Thermo Fisher Scientific). Each well was cultured with 500 μL of retinal progenitor medium in a humidified atmosphere incubator at 5% CO_2, 37°C. Since the dose of GDFs (50 ng mL^{-1}) was validated for RGC fate specification in the previous publications,[20,21] we then treated E14.5 retinal cell cultures with GDFs (50 ng mL^{-1}) for 5 days before scRNA sequencing. Medium and GDFs were replaced on day 3 of the 5-day incubation.

METHOD DETAILS

Cell dissociation for 10X genomics

Cells were dissociated using Accutase (Innovative Cell Technologies) for 15–20 min on day 5. Cell pellets were resuspended in PBS containing 0.04% bovine serum albumin and filtered through a 40μm cell strainer (Falcon, Cat#352340) to remove cell clumps. 10,000 cells were then input into the 10X library construction. GEM generation, reverse transcription, cDNA amplification, and library construction steps were performed according to manufacturer's instructions (Chromium Single Cell 3'v1/v2/v3 platform (10X Genomics, Pleasanton, CA). Libraries were sequenced on a NovaSeq 6000 sequencer (Illumina, San Diego, CA) with paired-end 150 bp reads.

scRNA-sequencing analysis

Data processing

We applied fastp with the default parameters to filter the adaptor sequence and remove the low-quality reads to achieve clean data. Then the feature-barcode matrices were obtained by aligning reads to the mm10 genome using CellRanger v3.0.0. Seurat analysis was performed in R using Seurat (v4.0.2),[46] ggplot2, dplyr. The data was normalized and passed quality control to remove dead cells. The three control and treatment datasets were integrated by identify 'anchors' across single-cell datasets[48]; the data was then scaled. Cell clustering was visualized by UMAP dimensional reduction and cell types were identified by different markers. In Figure 2, the two clusters of photoreceptors (clusters 5 and 7) were subset for further analysis. Similarly, in Figures 3 and 4, the RGC/AC cluster in Figure 1C was subset and re-clustered (as shown in Figure 3A). Gene markers used to identify retinal cell types were listed in Table 1. The datasets generated during this study are available on GEO: GSE252861. An additional dataset used for comparison can be found at GEO: GSE118614.[45] The E12, E16, and P14 samples were excluded due to their significantly lower sequencing depth. Materials are available upon reasonable request.

Pseudotime analyses

We applied the single-cell Trajectory analysis utilizing Monocle 3 (v0.2.2.0) with default parameters.[47] Two datasets, the whole retinas and the photoreceptors, were input and analyzed separately. We perform a completely "unsupervised" analysis for its machine learning approach. Batch effects were removed by aligning across samples.

QUANTIFICATION AND STATISTICAL ANALYSIS

The present study does not include statistical analysis or quantification. All the graphs were based on cell numbers in scRNA-seq data, in which definitions of center and dispersion and precision measures are not applicable.