

## Article

# Direct Conversion of Mouse Fibroblasts into Photoreceptor-like Cells

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## Highlights

### What are the main findings?

- The study identified a minimal combination of just three transcription factors—*Crx*, *Nrl*, and *Otx2* (CNO)—that is sufficient to directly convert MEFs into photoreceptor-like cells.
- The induced photoreceptor-like cells expressed a comprehensive panel of photoreceptor-specific markers. Notably, upon transplantation into adult mouse retinas, these induced photoreceptor-like cells migrated and integrated into the outer nuclear layer and continued to express photoreceptor markers in vivo.

### What are the implications of the main findings?

- This work explores a feasible method to generate photoreceptor-like cells from an abundant and easily accessible somatic cell source (fibroblasts). The direct conversion approach bypasses the pluripotent stem cell stage, making the process faster and eliminating the associated risk of teratoma formation, thereby presenting a potentially safer and more practical strategy for future cell replacement therapies for blinding diseases like AMD and RP.
- The finding that the CNO combination is the core driver of reprogramming provides preliminary experimental evidence for the synergistic role of these factors in defining photoreceptor identity and cell-based therapies for the photoreceptor repair.



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## Abstract

The purpose of our study is to explore the potential of a transcription factor-based strategy for directly converting mouse fibroblasts into photoreceptor-like cells. The mouse cDNAs of *Ascl*, *Crx*, *Ngn1*, *Nrl*, and *Otx2* were cloned into a modified commercial adenoviral vector. Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos, and mouse postnatal fibroblasts (MPFs) were isolated from three-day-old mice. A pool of adenoviruses containing five genes was prepared to infect MEFs or MPFs once daily for two days. The MEFs or MPFs were incubated in a specific medium supplemented with forskolin and were changed every two days. After 7 or 14 days, the photoreceptor-like cells were assayed via immunofluorescence or polymerase chain reaction with reverse transcription (RT-PCR). The photoreceptor-like cells were then transplanted into adult C57BL/6 mouse retinas and were assessed by immunofluorescence 14 days following transplantation. Screening from a

pool of five candidate genes, we reported that a combination of only three factors—*Crx*, *Nrl*, and *Otx2*—was sufficient to convert mouse embryonic and postnatal fibroblasts into photoreceptor-like cells. The induced photoreceptor-like cells expressed photoreceptor-specific proteins such as Recoverin, Rhodopsin, and Opsin and integrated into the outer nuclear layer of the retina following transplantation. This exploratory study provides preliminary evidence that fibroblasts can be directly converted into photoreceptor-like cells, suggesting a cellular model and potential source for future transplantation strategies aimed at retinal repair.

**Keywords:** direct conversion; photoreceptor; fibroblasts reprogramming; AAV infection

## 1. Introduction

The photoreceptors are terminally differentiated and specialized neural retinal cells, localized in the outer layer of the retina. These cells are essential for vision and responsible for capturing and converting light into electrical signals through a process of phototransduction. However, the fragility of photoreceptor outer segment membrane [1,2], their residence at the back of the retina and entire dependence on the underlying retinal pigment epithelium (RPE) and the choroidal capillaries for support and maintenance [3,4], and their demand for prodigious amounts of energy [5] make photoreceptor cells particularly vulnerable to damage and degeneration. Photoreceptor cell death is a vital cause of a variety of retinal disorders such as retinal detachment, retinitis pigmentosa (RP), and age-related macular degeneration (AMD) [6–8]. The loss of photoreceptors usually leads to severe visual damage and, eventually, blindness. Unlike teleost fish, such as zebrafish, which possess a prominent capacity for retinal regeneration and vision restoration after damage [9–11], mammalian retinas are similar to the central nervous system and lack this regenerative ability [12,13]. This significant difference between species underscores the difficulty of treating retinal diseases in humans. Novel therapeutic strategies for photoreceptor repair are urgently needed.

One of the most prominent and effective therapeutic strategies is the direct conversion method, which has drawn our attention.

Accumulated evidence indicates that fully differentiated cells can be directly stimulated to reprogram into other cell types by the overexpression of specific transcription factors. Several papers have also shown that fibroblasts can be directly converted into different cell types, such as functional neurons [14–18], cardiomyocytes [19] and hepatocyte-like cells [20]. Mahato et al. reported that a set of five small molecules could transform the fibroblasts into rod photoreceptor-like cells [21]. While pharmacologic modulation offers a valuable approach, we reasoned that a transcription factor-based strategy could provide a complementary and mechanistically distinct pathway. This rationale is grounded in the well-defined transcriptional hierarchy governing retinal development. Photoreceptor fate specification and terminal differentiation are directly orchestrated by a core set of transcription factors [22–24]. We therefore hypothesized that forcibly reactivating this endogenous transcriptional program would be a logical and feasible strategy to achieve a direct and faithful recapitulation of this cell fate conversion in vitro. Building upon this hypothesis, we aimed to design a transcription factor-driven conversion strategy that not only tests a fundamental biological principle but also holds significant translational potential. First, this approach directly tests the sufficiency of key developmental regulators to instigate cross-lineage fate conversion, offering a model for mechanistic dissection of photoreceptor programming. Second, compared to small molecule cocktails which have pleiotropic ef-

fects, a defined set of transcription factors provides a precise genetic toolkit, enhancing the specificity and reproducibility of the conversion process. Third, and most importantly, a transcription factor-based strategy inherently aligns with gene delivery platforms, thereby facilitating a more direct path towards future therapeutic development.

Retinal maturation occurs in a specific order and is regulated by transcription factors in the basic helix–loop–helix (bHLH) and the nuclear hormone receptor families. The transcription factors act in a combinatorial manner to define the cell fates of retinal progenitor cells. Recent studies report that the cone-rod homeobox (*Crx*), a member of the *Otx* homeodomain gene family, is expressed exclusively and abundantly in photoreceptor cells in the neural retina and is essential for the terminal differentiation and maintenance of the photoreceptors [25,26]. *Otx2*, another key member of the *Otx* homeobox gene family during photoreceptor development, works as a direct upstream regulator of *Crx*. *Otx2* is a crucial regulatory transcription factor for steering retinal progenitor cells into developing into photoreceptors, which is expressed in most of the forebrain and midbrain neuroepithelium, including the retina [27,28]. The neural retina leucine zipper (*Nrl*) is a basic motif-leucine zipper transcription factor of the *Maf* subfamily that is preferentially expressed in rod photoreceptors and regulates the rhodopsin expression synergistically with *Crx* [29,30]. Among the numerous proneural transcription factors expressed in response to injury, *Ascl1* is one of the rapidly upregulated genes in retinal Müller glia cells after retina damage in fish and is essential for retinal regeneration [31]. Neurogenin1 (*Ngn1*) is a member of the neurogenin subfamily of bHLH genes and participates in governing the differentiation of progenitor cells into photoreceptors [32,33]. Starting from this pool of five candidate genes (*Crx*, *Otx2*, *Nrl*, *Ascl1*, and *Ngn1*), we sought to identify a minimal transcription factor combination capable of directly installing this photoreceptor transcriptional program in fibroblasts, thereby testing their combined sufficiency for cross-lineage fate conversion.

## 2. Materials and Methods

### 2.1. Animal Experiment Procedures

Young adult C57BL/6J mice were purchased from Vital River Company and kept in standard housing conditions. All animal studies were conducted in accordance with experimental protocols and approved by the Animal Care and Use Committees at the Peking University First Hospital.

### 2.2. Fibroblast Isolation and Culture

MEFs were isolated from E13.5 C57BL/6 mouse embryos under a dissection microscope (Leica Microsystems, Wetzlar, Germany) as previously described [14]. The head, vertebral column, dorsal root ganglia, and visceral organs were removed; and the remaining tissue was manually dissected into small pieces and incubated in 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) for 10–15 min to create a single-cell suspension. The dissociated cells were cultured in MEF media (high-glucose Dulbecco's Modified Eagle Medium, Invitrogen), supplemented with 10 percent fetal bovine serum (FBS, Biochrom, Berlin, Germany), 0.1 mM nonessential amino acids, and two mM of Glutamax (Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured at 37 °C with 5 percent CO<sub>2</sub> and incubated for two to three days until confluent; cells were passaged in a 1:4 split before being frozen. MEFs were used between passages two and four, and MPFs were obtained from three-day-old mice. Only forelimbs and forelegs were taken; the tissue was rinsed in 75% ethanol, washed with phosphate-buffered saline (PBS), dissociated, and digested in 0.25% trypsin. MPFs were cultured in MEF media.

### 2.3. Adenovirus Production and Infection

The mouse cDNAs of *Ascl*, *Crx*, *Ngn1*, *Nrl*, and *Otx2* were first cloned in the pEntr 3C vector (Invitrogen) and then in the pAD vector (Invitrogen) via a homologous L/R recombination. The complete vector maps for all constructs used in this study are provided in Supplementary Figures S1–S5. We then transduced the viral constructs into a 293A cell line and obtained high-titer ( $10^8$  IU/mL) viral particles by two rounds of amplification according to the manufacturer's instructions. MEFs or MPFs were infected twice with adenovirus for eight hours per day at multiplicities of infection (MOI, number of viral particles per cell) of 30 or 20. Twenty-four hours post-infection, half of the culture medium was changed into a neural medium (1 g/L glucose DMEM/F-12/neural basal 2:2:1, 1\*B-27, 10 or 20 ng/mL BDNF); 10 or 20  $\mu$ mol/L of forskolin was added each day for two successive days. Then, half of the medium was changed every two days until the cells were ready for immunostaining and RT-PCR experiments.

### 2.4. Conversion Efficiency

We counted the conversion efficiency using the photoreceptor purity as the percentage of Recoverin<sup>+</sup> cells relative to the total final population. The efficiency was calculated by dividing the number of photoreceptor-like cells by the number of total cells in each visual field. For each experimental condition, the entire reprogramming procedure was performed in three independent biological replicates. These replicates were conducted independently, involving separate cell preparations and experimental runs performed on different days. Within each replicate, at least three randomly selected wells were assessed. In each well, cells were counted across three randomly chosen, non-overlapping visual fields, and the average percentage from these three fields was calculated as the well-specific conversion efficiency. To obtain a single data point representing each independent biological replicate, the well-specific conversion efficiencies from the three randomly selected wells were averaged. Thus, the final data set for each condition consisted of three values ( $n = 3$ ), each derived from an independent biological replicate. The data presented are the mean  $\pm$  standard error of the mean (SEM) of the three independent replicate values. Statistical comparisons were performed using these replicate means.

### 2.5. Immunofluorescence

The photoreceptor-like cells were washed with phosphate-buffered saline (PBS) and then fixed with 4 percent paraformaldehyde for 20 min at room temperature. After washing twice with PBS, cells were blocked in a solution of PBS containing 5 percent BSA and 0.1 percent TritonX-100 for 30 min at room temperature. Primary antibodies were diluted in an antibody dilution solution (PBS with 1 percent BSA and 0.1 percent Triton X-100) in ratios from 1:100 to 1:1000, and secondary antibodies were diluted in a 1:1000 ratio in an antibody dilute solution. Photoreceptor-like cells were incubated in primary antibodies overnight at 4 °C, and secondary antibodies were incubated for 60 min at room temperature. The following primary antibodies were used: rabbit anti-Recoverin<sup>+</sup> (1:1000, AB5585, Millipore, Burlington, MA, USA), mouse anti-Rhodopsin (1:500, MAB5356, Millipore), rabbit anti-Opsin (1:200, AB5405, Millipore) and mouse anti-Tuj1 (1:2000; Millipore; MAB1637).

### 2.6. RT-PCR and Semi-Quantitative Analysis

Photoreceptor-like cells and wild-type photoreceptor cells were cultured in a neuronal medium. MEFs were cultured in DMEM with a 10 percent FBS medium. All cells were washed with a serum-free medium before collection. RNA was isolated using Trizol (Invitrogen), following the manufacturer's instructions. For each sample, 600 ng of total RNA was reverse-transcribed into cDNA using a standard reverse transcription

kit. PCR amplification was performed using gene-specific primers (sequences listed below).  $\beta$ -actin was amplified in parallel as an internal reference control for normalization. The PCR products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide, and visualized under UV light using a gel documentation system. For semi-quantitative analysis, the band intensity of each target gene was measured using ImageJ 1.54p (National Institutes of Health, USA) and normalized to that of  $\beta$ -actin. The relative expression level of the target gene was calculated as the ratio of the normalized band intensity of the experimental sample to that of the control sample. For gel quantification, values were derived from three independent gels, each representing one biological experiment. Primer Sequences: *Crx*: forward 5'-GTCCCATCACTCAAGTGCCCC-3', reverse 5'-CTTGAACCAGACCTGGACCC-3'; *Otx2*: forward 5'-GCAGTCAATGGGCTGAGTCT-3', reverse 5'-CACCTGGATTCTGGCAAGT-3'; *Nrl*: forward 5'-TTCACCCACCTTCAGTGAGC-3', reverse: 5'-GTCCGAAAATCTCTCGGGCA-3'; *Recoverin*: forward 5'-GACGGCAATGGGACCATCA-3', reverse 5'-CCCGCTTTTCTGGGGTGTTT-3'; *Rhodopsin*: forward 5'-GCCCCAATTTTATGTGCCCTT-3', reverse 5'-GTGACGTAGAGCGTGAGGAA-3'; *Fiz1*: forward 5'-TGCCCTAAGGGATTCCGAGA-3', reverse 5'-TGCAACATACTGAGCAGGGG-3'; *Sag*: forward 5'-GCCTGCGGGAAGACCAATA-3', reverse 5'-TTCACAAGCTCAGGGTCCAC-3'.

## 2.7. Transplantation of Photoreceptor-like Cells In Vivo and Tissue Dissection

The induced photoreceptor-like cells were labeled with GFP by lentivirus infection and concentrated to  $\sim 10^6$  cells/ $\mu$ L. Two  $\mu$ L per site were transplanted into the subretinal space of eight-to-ten-week-old C57BL/6 mice (anesthetized with 70 mg/kg of pentobarbital sodium). We performed three independent transplantation experiments, with a total of  $n = 6$  animals in the experimental group and  $n = 3$  animals in the control group. Each experiment included 2 experimental and 1 control mouse. For each animal, only one eye was randomly selected for cell transplantation, while the contralateral eye remained untreated. Two weeks post-transplantation, the eyes of the mice were harvested and fixed with 4 percent paraformaldehyde overnight, followed by dehydration in PBS containing 30 percent sucrose for 24 h at 4 °C. Eyeballs were then embedded in an optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan, <https://www.sakura-finetek.com/> (accessed on 1 February 2026)) and frozen at  $-20$  °C. The eyeballs were cryosectioned at ten  $\mu$ m using a cryostat microtome (Leica CM1950) and mounted onto slides. Slide-mounted sections were kept at  $-80$  °C until needed. Three representative sections per eye were quantified via whole-slide scanning, and the values from these sections were averaged to obtain a single value per animal. Statistical comparisons were performed at the animal level (experimental group:  $n = 6$ ; control group:  $n = 3$ ).

## 2.8. Definition of Endpoints and Consideration of Multiplicity

To address the potential for inflated Type I error due to multiple comparisons across related outcome measures, endpoints were categorized as follows: The in vitro conversion efficiency, quantified as the percentage of Recoverin-positive cells, was pre-specified as the primary measure of successful reprogramming. The expression levels of a panel of photoreceptor-specific genes (*Rhodopsin*, *Opsin*, *Fiz1* and *Sag*) were assessed as secondary, phenotypic validation markers to provide a more comprehensive characterization of the converted cells.

The primary in vivo transplantation endpoint was the number of integrated, Recoverin-positive transplanted photoreceptor-like cells in the host retina. Additional histological assessments served as complementary descriptive outcomes.

Statistical inferences are primarily drawn from the pre-specified primary endpoints. Findings from secondary endpoints are interpreted as supportive within this context.

## 2.9. Statistical Analysis

Statistical analysis was evaluated by the GraphPad Prism 10 software. To minimize observer bias, all quantitative image analyses were performed under blinded conditions. The blinding procedure was as follows: all identifiers revealing the experimental batch, group assignment (e.g., control vs. treatment), or staining condition were removed from raw image files. Raw image files were anonymized by removing all condition labels, then randomized and assigned arbitrary codes prior to analysis. Two independent researchers performed the measurements, and their respective values for each sample were averaged to obtain the final quantitative result. To assess inter-rater reliability, the intraclass correlation coefficient (ICC) was calculated using a two-way mixed-effects model for absolute agreement, based on independent counts of Recoverin<sup>+</sup> cells from ACNN1O-introduced cells. The analysis yielded an ICC of 0.981 (95% CI: 0.917–0.996), indicating excellent agreement between observers.

Given the exploratory nature of this work and the limited sample size, the analysis was intentionally designed to emphasize data description and effect size estimation over underpowered statistical hypothesis testing. The primary analytical approach was to calculate effect sizes (mean differences) for a pre-specified, limited set of pairwise comparisons of biological interest, along with their 95% confidence intervals (95% CI). These intervals, calculated using standard methods (Welch's formula for unpaired comparisons, which does not assume equal variances), provide a direct measure of the estimated effect's magnitude and its associated uncertainty. All individual data points are presented graphically to ensure full transparency.

In *in vitro* conversion efficiency and RT-PCR/semi-quantitative analyses, the experimental unit for statistical comparison was the independent biological replicate ( $n = 3$ ). The estimated mean difference and its 95% CI for the key comparisons of interest are reported. In *in vivo* transplantation studies, the experimental unit was the individual animal. Data from three sections per eye were averaged to yield a single value per animal. The mean difference and its 95% CI are reported for the comparisons between the two groups. The independent biological replicate was the sole unit of analysis. For *in vitro* studies, this was defined as a complete, independent experiment conducted on a separate day with distinct cell preparations ( $n = 3$ ). The hierarchical data aggregation was performed solely for the purpose of obtaining a single, representative value for each independent biological replicate. No statistical tests, calculations of variance, or measures of uncertainty were ever performed on, or derived from, data at the level of technical replicates (wells or visual fields).

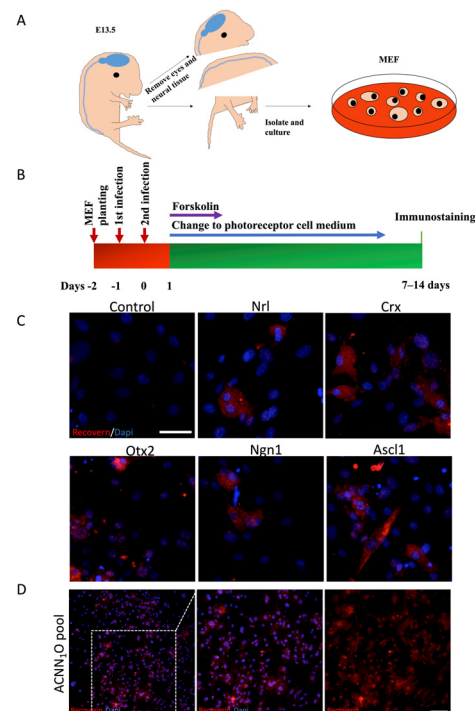
Pre-defined exclusion criteria were applied blinded to group identity. A sample was excluded only for objective technical failures independent of outcome: microbial contamination; extensive non-specific cell death (>90%); or irreparable imaging artifacts. Two researchers independently flagged such cases, with exclusions requiring consensus. No samples from the experimental groups met these criteria; therefore, no data were excluded. The decision to exclude a sample was made during quality control, prior to its data entering the final analysis dataset. No data points were excluded post hoc as statistical outliers. All planned data points were obtained, with no missing outcomes. The analyses were therefore conducted using the complete dataset.

**Data Presentation and Reporting:** All quantitative findings are presented with individual biological replicate data points and the mean  $\pm$  SEM. The primary statistical results are effect sizes (mean differences) accompanied by 95% CI.

### 3. Results

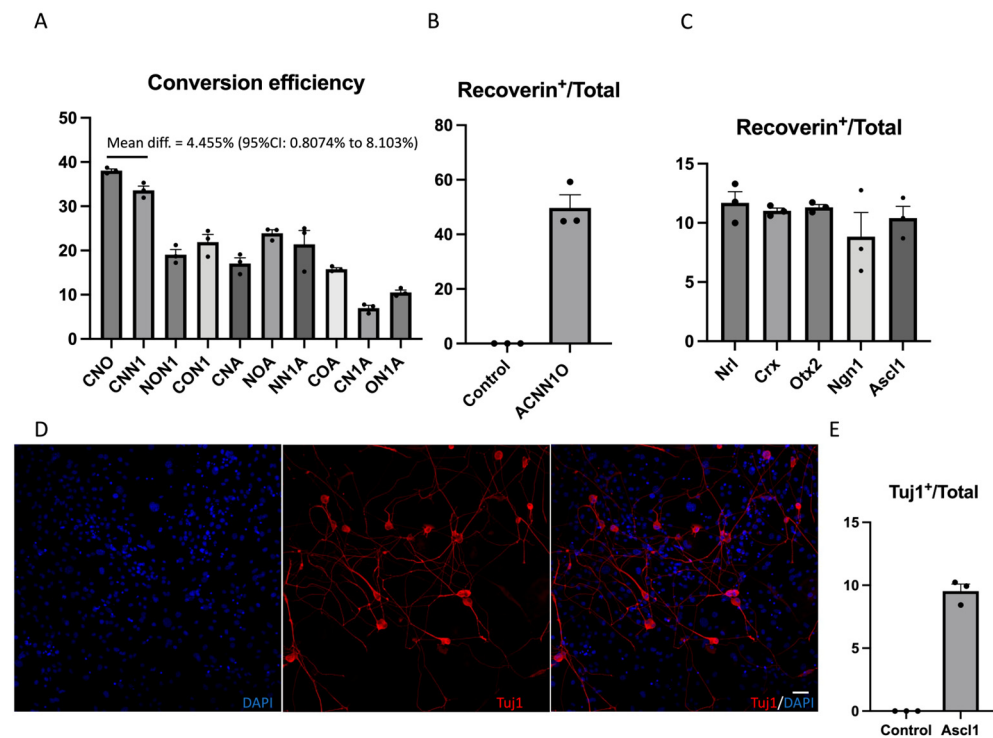
#### 3.1. Screening for Photoreceptor-Fate-Inducing Factors

Five genes expressed in the neural retina and known to contribute to the development of photoreceptor cells were cloned: *Ascl1*, *Crx*, *Nrl*, *Ngn1*, and *Otx2* (abbreviated as A, C, N, N1, and O, respectively). A pool of adenoviruses including all five genes was constructed to infect the MEFs taken from the E13.5 mice. We carefully excluded the neural tissue and eyes from the MEF preparation according to the previous description [14] (Figure 1A). We were unable to detect any photoreceptor cells in the MEFs (Supplementary Figure S6).



**Figure 1.** Screen for photoreceptor-fate-inducing factors. (A) MEF preparation from E13.5 mice. Tissues with neurogenic potential (head, vertebral column, dorsal root ganglia, and internal organs) are removed as indicated. The remaining tissue is then processed for primary MEF culture. (B) Diagram depicting the transdifferentiation procedures of MEFs into photoreceptor-like cells by AAV delivery. The horizontal bar indicates the experimental timeline from Day -2 to Day 7–14. (C) Recoverin<sup>+</sup> cells induced by *Ascl1*, *Crx*, *Nrl*, *Ngn1*, and *Otx2* individually after seven days of infection. (D) Recoverin<sup>+</sup> cells induced by ACNN10 pool after seven days of infection. Shown are a low-magnification field (left) and higher-magnification insets (middle and right) of the region outlined in the left panel. The middle inset is a merged image of DAPI (blue, nuclei) and Recoverin (red) staining; the right inset shows the Recoverin signal alone. Dapi (blue) shows nucleus. (Scale bar: 50  $\mu$ m).

We also investigated the individual potential of these five genes to reprogram MEFs into photoreceptor cells. MEFs were infected once daily for two consecutive days with adenoviruses containing each of the five genes, according to transdifferentiation procedures (Figure 1B). Although the conversion efficiencies were very low, seven days after infection, we detected recoverin-positive cells in cultures individually infected by *Ascl1*, *Crx*, *Nrl*, *Ngn1*, or *Otx2* (Figures 1C and 2C). The dynamic progression of cell transdifferentiation is provided in Supplementary Figure S7. Furthermore, to potentially increase the conversion efficiency, all five genes (ACNN10 pool) were combined to infect E13.5 MEFs using the same method. We performed the recoverin staining seven days after infection to identify photoreceptor cells. The recoverin-positive cell ratio notably increased in the ACNN<sub>1</sub>O pool (Figures 1D and 2B).



**Figure 2.** Defining the most efficient combination for photoreceptor-like cell induction. (A) Quantitative analysis of reprogramming efficiency for different transcription factor combinations. Conversion efficiency was calculated as the percentage of Recoverin-positive cells. Key comparisons are quantified as follows: CNO vs. CNN1: Mean diff. = 4.455%, 95% CI: 0.8074% to 8.103%. (B) Recoverin<sup>+</sup> cell ratios of ACNN10 combination. (C) Recoverin<sup>+</sup> cell ratios of each individual transcription factors. (D) Tuj1 staining seven days after infection by AAV carrying *Ascl1*. (Scale bar: 50  $\mu$ m.) (E) The quantification analysis of Tuj1<sup>+</sup> cells. Data are presented as mean  $\pm$  SEM of  $n = 3$  independent biological replicates. Each replicate value was derived from the average of 3 randomly selected wells per replicate, with each well's efficiency calculated from the average of 3 randomly chosen, non-overlapping visual fields. Individual data points ( $n = 3$ ) are shown.

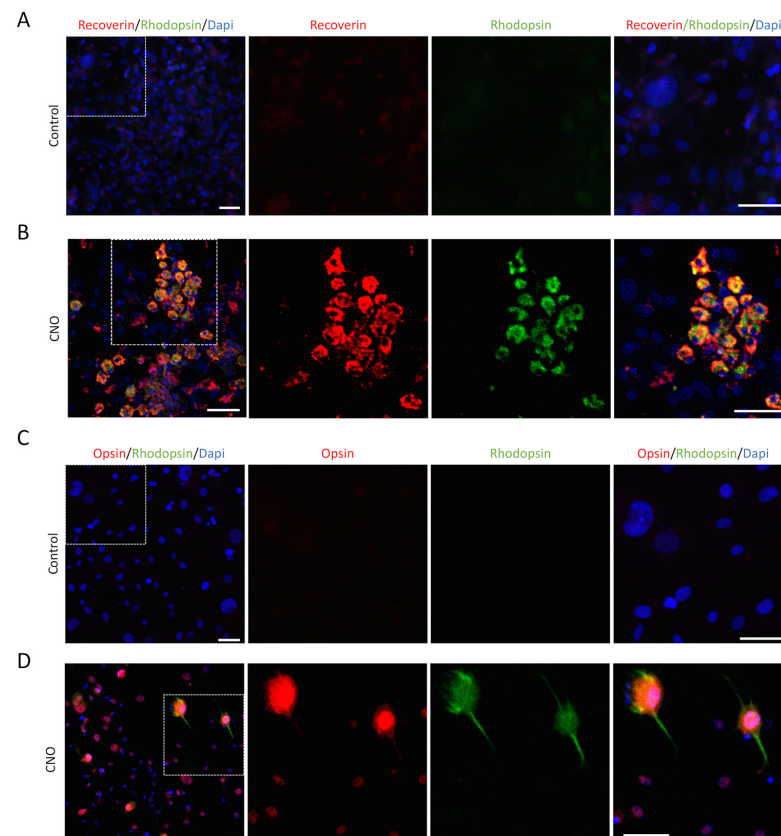
### 3.2. Exploratory Identification of a Candidate Factor Combination for Induction of Photoreceptor-like Cells

To narrow down the number of required transcription factors, we exploratively evaluated all possible three-gene combinations derived from the original five. The conversion efficiency for each of the ten combinations was assessed by counting recoverin-positive cells seven days post-infection. Given the exploratory nature and small sample size of this study, we present all data points and focus on describing the observed effect sizes for pre-specified comparisons of interest. The individual data points and mean conversion efficiency for all combinations are shown in Figure 2A.

We observed considerable variation in mean conversion efficiency across the different combinations. The CNO combination was numerically higher than other combinations. The CNO combination exhibited a higher mean conversion efficiency than CNN1 (mean diff. = 4.455%, 95% CI: 0.8074% to 8.103%) (Figure 2A). Therefore, we focused our further analysis on the CNO pool. Surprisingly, the combinations including *Ascl1* did not exhibit higher conversion ratios. We thus performed immunostaining for the other retinal neuron marker, Tuj1, in cultures infected by AAV carrying *Ascl1* at the seven days after infection. We found that most of the cells were Tuj1-positive, implying that these cells differentiated into neuron-like cells rather than photoreceptors (Figure 2D,E).

### 3.3. The Expression of Multiple Markers in Induced Photoreceptor-like Cells

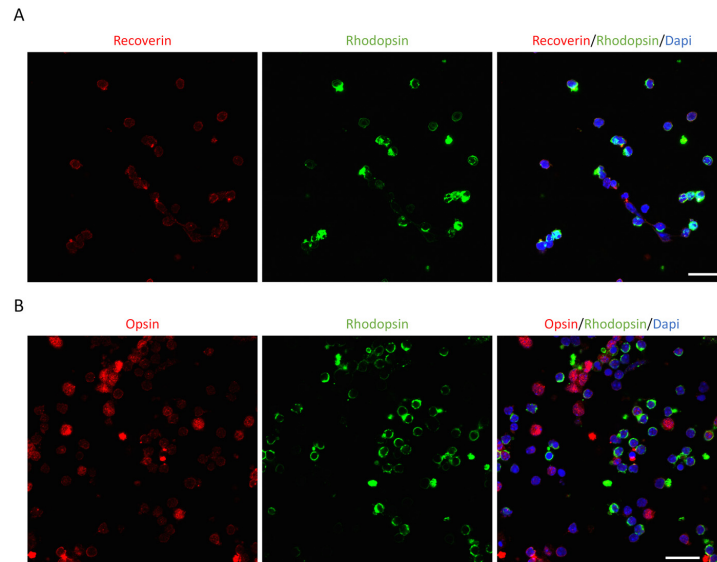
To obtain more photoreceptor-like cells, the MEFs were infected twice by CNO and cultured for 14 days. In addition to Recoverin, we also labeled the cells with other photoreceptor markers, Rhodopsin and Opsin, and performed double immunolabeling of Recoverin and Rhodopsin or Opsin and Rhodopsin in support of the conversion phenotype. MEFs infected by the empty AAV were used as controls (Figure 3A–D), and postnatal retinal cells from P3 mouse pups were used as positive controls. We observed that induced photoreceptor-like cells were similar in morphology and immunolabeling to those of positive control cells (Figure 4A,B).



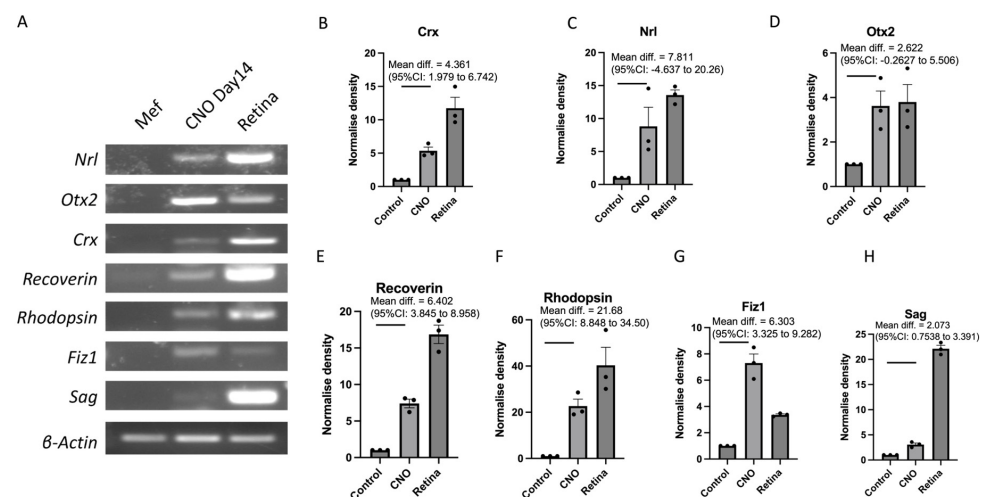
**Figure 3.** Induced photoreceptor-like cells from MEFs express multiple photoreceptor specific markers. (A) MEFs infected by empty AAV showed no positive signals of Recoverin (red) and Rhodopsin (green) staining. (B) Recoverin (red) and Rhodopsin (green) double staining indicate photoreceptor-like cells induced by CNO pool from MEFs. (C) MEFs infected by empty AAV showed no positive signals of Opsin (red) and Rhodopsin (green) staining. (D) Opsin (red) and Rhodopsin (green) double staining indicated photoreceptor-like cells induced by CNO pool from MEFs. Dapi (blue) shows nucleus. (Scale bar: 50  $\mu$ m.).

Furthermore, we sought to explore that the induced photoreceptor-like cells shared more commonality with the photoreceptors from P3 mice retinas. We detected the expressions of *Recoverin*, *Rhodopsin*, *Fiz1* (Flt-3 Interacting Zinc-finger, *Fiz1*), and *Sag* (S-antigen) by RT-PCR in both the CNO-induced photoreceptor-like cells and retinal cells isolated from P3 mice; none of these four genes were expressed in negative control MEFs infected by empty AAV (Figure 5A,E–H). Semi-quantitative RT-PCR analysis confirmed the induction of a photoreceptor-like gene expression profile in CNO-reprogrammed cells. The analysis indicated the specific upregulation of *Crx* (Mean diff. = 4.361, 95% CI: 1.979 to 6.742), *Nrl* (Mean diff. = 7.811, 95% CI: −4.637 to 20.26), and *Otx2* (Mean diff. = 2.622, 95% CI: −0.2627 to 5.506) transcripts following AAV-CNO infection compared to control (Figure 5B–D). Compared to the control group, CNO-treated cells showed elevated mRNA

levels of key photoreceptor markers, including *Recoverin* (Mean diff. = 6.402, 95% CI: 3.845 to 8.958), *Rhodopsin* (Mean diff. = 21.68, 95% CI: 8.848 to 34.50), *Fiz1* (Mean diff. = 6.303, 95% CI: 3.325 to 9.282) and *Sag* (Mean diff. = 2.073, 95% CI: 0.7538 to 3.391). This result suggests that our induced photoreceptor-like cells were similar to the photoreceptors of P3 mouse pups (Figure 5A–H).

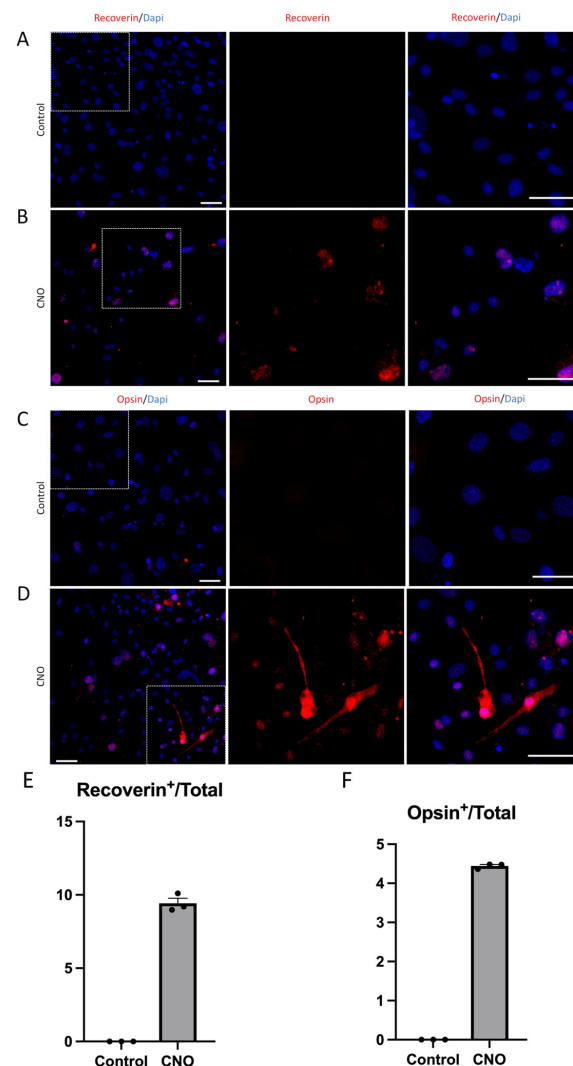


**Figure 4.** Postnatal retinal cells culture and staining. (A) Recoverin (red) and Rhodopsin (green) double staining show photoreceptor cells from P3 mice retinas, which were similar to photoreceptor-like cells induced by CNO pool at 14 dpi. (B) Opsin (red) and Rhodopsin (green) double staining showed photoreceptor cells from P3 mice retinas that were similar to photoreceptor-like cells induced by CNO pool at 14 dpi. Dapi (blue) shows nucleus. (Scale bar: 20  $\mu$ m.).



**Figure 5.** RT-PCR shows expressions of more photoreceptor markers. (A) Expressions of *Nrl*, *Otx2*, *Crx*, *Recoverin*, *Rhodopsin*, *Fiz1*, and *Sag* by RT-PCR in both photoreceptor-like cells induced by CNO pool and retinal cells isolated from P3 mice; however, MEFs infected by empty AAV showed no expressions of these four genes.  $\beta$ -Actin was used as a loading control for all experiments. (B–D) Quantification RT-PCR analysis of the three transcriptional factors. Control vs. CNO: *Crx*: Mean diff. = 4.361, 95% CI: 1.979 to 6.742; *Nrl*: Mean diff. = 7.811, 95% CI: -4.637 to 20.26; *Otx2*: Mean diff. = 2.622, 95% CI: -0.2627 to 5.506. (E–H) Quantification RT-PCR analysis of the indicated markers. Control vs. CNO: *Recoverin*: Mean diff. = 6.402, 95% CI: 3.845 to 8.958; *Rhodopsin*: Mean diff. = 21.68, 95% CI: 8.848 to 34.50; *Fiz1*: Mean diff. = 6.303, 95% CI: 3.325 to 9.282; and *Sag*: Mean diff. = 2.073, 95% CI: 0.7538 to 3.391. Data are presented as the mean  $\pm$  SEM from 3 independent biological experiments, each represented by a separate agarose gel. Individual data points ( $n = 3$ ) are shown.

To further verify the capacity of the CNO-induction of photoreceptor-like cells from fibroblasts, we isolated MPFs from three-day-old mice. Although the conversion efficiency using the MPFs was much lower than when using MEFs, we detected the expression of Recoverin and Opsin after being cultured for 14 days (Figure 6A–D).

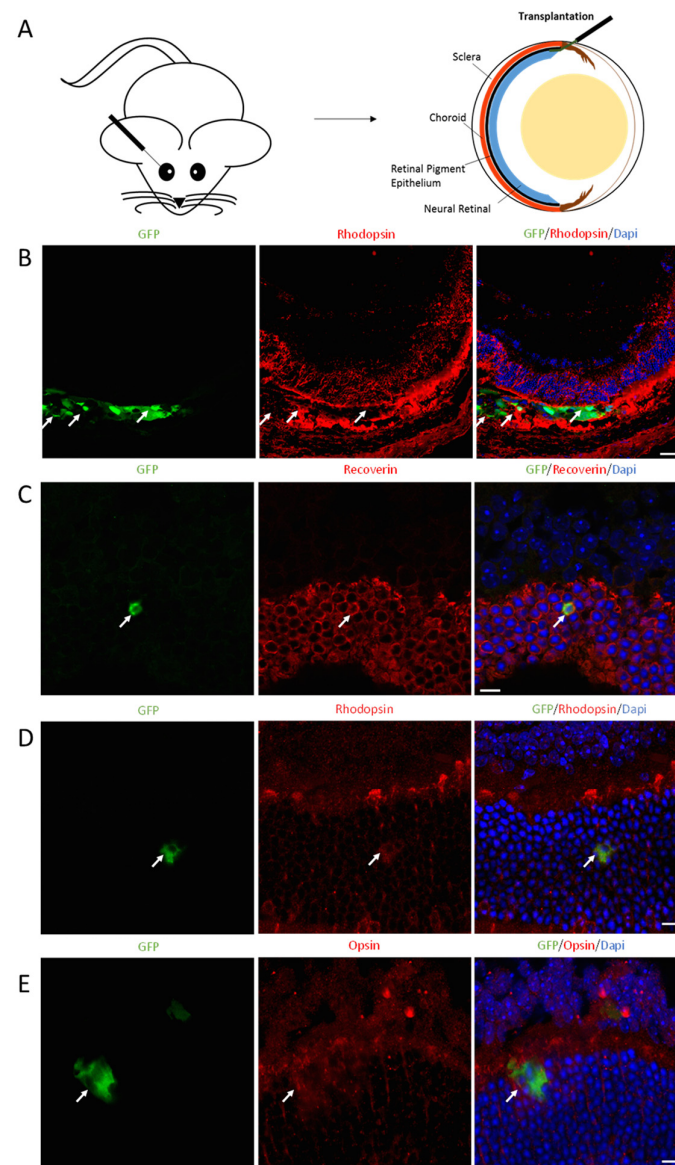


**Figure 6.** Induction of photoreceptor-like cells by CNO pool from MPFs. (A) MPFs infected by empty AAV show no positive signals of Recoverin (red) staining. (B) Recoverin (red) staining indicated photoreceptor-like cells induced by CNO pool from MPFs. (C) MPFs infected by empty AAV showed no positive signals of Opsin (red) staining. (D) Opsin (red) staining indicated photoreceptor-like cells induced by CNO pool from MPFs. Dapi (blue) shows nucleus. (Scale bar: 50  $\mu$ m.) (E) Quantification analysis of Recoverin. (F) Quantification analysis of Opsin. Data are presented as the mean  $\pm$  SEM of  $n = 3$  independent biological replicates. Each replicate value was derived from the average of 3 randomly selected wells per replicate, with each well's efficiency calculated from the average of 3 randomly chosen, non-overlapping visual fields. Individual data points ( $n = 3$ ) are shown.

### 3.4. Transplantation of Photoreceptor-like Cells

We next examined the transplantation potential of CNO-induced photoreceptor-like cells. We transplanted 200,000 CNO-iPs into the subretinal space of eight-to-ten-week-old adult C57BL/6J mice. To trace the transplanted cells in the retina, we infected MEFs with a lentivirus that stably expressed GFP before they were infected by AAV-CNO. Infected cells were transplanted into the subretinal space after three days of viral infection (Figure 7A,B). The MEFs only infected by the lentivirus expressing GFP were used as controls (Supplementary Figure S8A). The transplanted eyes were har-

vested two weeks after transplantation, and the eye sections were collected for analysis. We detected Recoverin-, Rhodopsin-, and Opsin-positive cells labeled with GFP in the outer nuclear layer (ONL) where the photoreceptors were located, suggesting that the CNO-induced photoreceptor-like cells were successfully migrated and integrated into the host retinas (Figure 7C–E). The analysis revealed the following mean difference for GFP<sup>+</sup>/Rhodopsin<sup>+</sup> cells (Mean diff. = 1.667, 95% CI: 0.5828 to 2.751); GFP<sup>+</sup>/Recoverin<sup>+</sup> cells (Mean diff. = 1.333, 95% CI: 0.2495 to 2.417); GFP<sup>+</sup>/Opsin<sup>+</sup> cells (Mean diff. = 0.667, 95% CI: −0.1902 to 1.524) (Supplementary Figure S8B–D).



**Figure 7.** Migration and integration of photoreceptor-like cells induced by CNO pool after transplantation. (A) Schematic diagram described subretinal transplantation of photoreceptor-like cells. (B) Representing the immediate/early post-transplantation period. At this stage, the injected cells resided within the transient subretinal space created by the injection procedure, which is located between the RPE and the neural retina. GFP<sup>+</sup> cells indicated the photoreceptor-like cells had been transplanted into subretinal spaces. Rhodopsin staining shows host photoreceptors. (Scale bar: 50  $\mu$ m.) (C–E) GFP shows photoreceptor-like cells collocated with red signals of Recoverin (C), Rhodopsin (D), and Opsin (E) in outer nuclear layer of host retinas two weeks post-transplantation. (Scale bar: 10  $\mu$ m.) Dapi (blue) shows nucleus. Data are derived from a total of  $n = 9$  mice (6 experimental, 3 control) across three independent transplantation experiments (2 experimental + 1 control mouse per experiment). For each animal, one injected eye was analyzed.

## 4. Discussion

Photoreceptor cell loss represents the final common pathological endpoint in a range of blinding retinal disorders, including AMD and RP [34]. While endogenous regenerative capacity in the mammalian retina is severely limited [12,13], significant advances have been made in generating photoreceptors both in vivo and in vitro. Most in vivo studies have focused on Müller glia cells and RPE [35] as the resource of photoreceptor regeneration. However, the number of differentiated photoreceptors from the currently endogenous regeneration is not sufficient for retinal repair, especially in the later stage of AMD and RP, in which there is a severe loss of photoreceptors. In vitro, studies have shown that photoreceptors can be generated from different tissues, such as the iris [36], ciliary tissues [37], and embryonic stem cells [38–40]. Some remarkable studies have demonstrated the potential of pluripotent stem cells (PSCs). These include their capacity to self-organize into laminated optic-cup structures in three-dimensional culture [41,42], their directed differentiation into functional rod and cone photoreceptors [39], and the successful transplantation of such derived cells to restore visual function in animal models [43]. In parallel, innovative direct reprogramming strategies, such as pharmacological conversion of fibroblasts using small molecules, have emerged [21]. Within this evolving landscape, our exploratory study provides preliminary evidence for a minimal transcriptional module—comprising *Crx*, *Nrl*, and *Otx2* (CNO)—that efficiently drives the direct conversion of fibroblasts into photoreceptor-like cells. The significance of this finding extends beyond methodological development, offering fundamental insights into fate determination and establishing a versatile platform for photoreceptor regenerative research.

The CNO module defines a core transcriptional logic for photoreceptor fate specification. Our systematic exploratory screening, beginning with five candidate factors, identified the CNO combination as a core regulatory unit. The consistent numerical advantage of CNO over the majority of alternatives, combined with its ability to induce a more mature photoreceptor marker profile, suggests its designation as a core, synergistic transcriptional module sufficient for driving this cell fate conversion. Future studies with increased sample sizes may further refine the comparative efficacy ranking among the top candidates. This finding carries profound biological implication. During retinal development, a precise hierarchical network operates: *Otx2* acts as an upstream determinant specifying photoreceptor progenitors, *Crx* functions as a terminal selector gene activating the phototransduction cascade, *Nrl* works synergistically with *Crx* to regulate rhodopsin transcription, which is essential for photoreceptor genesis. The mutations of each of these three genes cause severe retinal degeneration. The mutations of *Crx* are associated with dominant cone-rod dystrophy, late-onset dominant RP, and dominant congenital Leber amaurosis [44]. The differentiating photoreceptor cells were converted to amacrine-like retinal neurons in *Otx2* deficiency [45]. Mutations of *Nrl* have resulted in the autosomal dominant RP [46,47]. Our results indicate that the forced co-expression of this endogenous regulatory triad is sufficient to reconstitute this developmental program in a distant somatic cell type—the fibroblast. This effectively overrides its native epigenetic state and installs a photoreceptor identity. This collectively suggests that photoreceptor fate can be initiated and stabilized by a remarkably concise set of master regulators. The power and generality of this module are further emphasized by its independent identification in a different cellular context, where the same CNO factors were shown to reprogram reactive Müller glia into photoreceptors in vivo [48]. Therefore, our work identifies CNO not merely as a reprogramming cocktail but as a fundamental and transferable transcriptional code for photoreceptor commitment.

This work provides a mechanistically direct and developmentally informed strategy complementary to existing paradigms. Current approaches to photoreceptor generation primarily involve multi-step differentiation from PSCs, a process that recapitulates retino-

genesis to yield cells capable of forming structured tissues [39,41,42] and restoring function upon transplantation [43]. Alternatively, small-molecule-based reprogramming offers a non-genetic route [21]. Our transcription factor-driven direct conversion strategy provides a distinct, complementary pathway with unique advantages. It bypasses the prolonged timeline and teratoma risk associated with PSC intermediates and offers a genetically precise, developmentally rationalized alternative to small molecule screens. The induced cells expressed not only early markers (Recoverin, Rhodopsin, Opsin) but also later functional components like *Fiz1* and *Sag* (*Arrestin1*) [49–52], suggesting activation of maturation pathways. Most importantly, the CNO induced photoreceptor-like cells demonstrated competency for in vivo integration into the host outer nuclear layer. By utilizing an abundant and accessible somatic cell source, this approach addresses practical challenges of scalability and autologous transplantation.

Beyond its utility as a cell source, the CNO system serves as a useful discovery platform for photoreceptor biology. The “minimal sufficiency” of CNO makes it an ideal, reductionist tool for dissecting the molecular mechanisms of photoreceptor differentiation and maturation. This system can be leveraged to build in vitro models of retinal disease using patient-derived fibroblasts, to screen for factors that enhance functional maturation, and to map the epigenetic landscape changes during fate conversion. Thus, the value of this work is dual: it delivers a practical method for generating photoreceptor-like cells and, more fundamentally, provides a defined genetic toolkit to probe the core regulatory principles governing photoreceptor identity and development.

We explicitly acknowledge the key limitations of this study, primary among them being the limited biological replication ( $n = 3$  for in vitro studies). This constrains the statistical power and definitiveness of our conclusions, necessitating the exploratory framing presented here. The assessment of multiple gene expression and transplantation outcomes further underscores the preliminary nature of these findings. Consequently, this work should be viewed as a foundational, hypothesis-generating step. Future studies with larger cohorts are essential to validate the robustness and efficiency of CNO-mediated conversion. In the next phase, we intend to perform functional validation—including electrophysiological confirmation of light responses in vitro and the restoration of visual function in animal models of photoreceptor degeneration.

## 5. Conclusions

This exploratory study provides preliminary evidence that a core transcriptional module is sufficient to enable photoreceptor fate acquisition in somatic cells. This finding establishes a basis for future investigations into retinal cell fate determination and offers a genetically tractable, testable hypothesis for regenerative approaches. Future work will focus on enhancing functional maturation, employing transient delivery systems for clinical translation, and harnessing this CNO platform to unravel the complexities of photoreceptor development and degeneration.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15040320/s1>, Figure S1: Complete vector map for the adenoviral construct expressing *Ascl1*; Figure S2: Complete vector map for the adenoviral construct expressing *Crx*; Figure S3: Complete vector map for the adenoviral construct expressing *Ngn1*; Figure S4: Complete vector map for the adenoviral construct expressing *Nrl*; Figure S5: Complete vector map for the adenoviral construct expressing *Otx2*; Figure S6: Control experiments confirm the absence of photoreceptor-like cells in starting MEF population; Figure S7: Morphological dynamics and marker induction in MEFs infected with individual photoreceptor transcription factors; Figure S8: Control transplantation of un-reprogrammed fibroblasts into the subretinal space and quantification cell counts of GFP-positive cells co-expressing retinal markers.

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## Abbreviations

The following abbreviations are used in this manuscript:

|      |                                  |
|------|----------------------------------|
| MEF  | Mouse embryonic fibroblast       |
| MPF  | Mouse postnatal fibroblast       |
| RP   | Retinitis pigmentosa             |
| AMD  | Age-related macular degeneration |
| bHLH | basic helix–loop–helix           |
| Crx  | cone-rod homeobox                |
| Nrl  | Neural retina leucine            |
| Ngn1 | Neurogenin1                      |
| PBS  | Phosphate-buffered saline        |
| RPE  | Retinal pigmented epithelium     |
| PSC  | Pluripotent stem cell            |

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