Table 2. Primer sequences for qRT-PCR.

Gene name	Forward	Reverse
Recoverin	5′ – TTCAAGGAGTACGTCATCGCC – 3′	5′ – GATGGTCCCGTTACCGTCC – 3′
S-arrestin	5′ – GGACAAATCGGTGACCATCTAC – 3′	5′ – ACAGGAGGATACACCTGGACC – 3′
Phosphodiesterase 6B	5′ – ACGTGTGGTCTGTGCTGATG – 3′	5′ – CTTGCCGTGGAGGATGTAGTC – 3′
Rhodopsin	5′ – CACCTCTCTGCATGGATACTTCG – 3′	5′ – ATGGGCTTACACACCACCAC – 3′
Blue opsin	5′ – TAGCAGGTCTGGTTACAGGATG – 3′	5′ – GAGACGCCAATACCAATGGTC – 3′
Green opsin	5′ – CATCCGCAGGACAGCTATGAG – 3′	5′ – GTAAGCACAGTGGGTTCGTTTCCC – 3′
Phosphodiesterase 6C	5′ – AGGCTTCATCACACCAGCTAC – 3′	5′ – TGAAACTGTCGCTCAACATCTG – 3′
Cone channel A3	5′ – GGACTCTTTTCCTGATCGTTTCC – 3′	5′ – GCTGGTGTTAGTGTTGCATTTG – 3′
Cone channel B3	5′ – CTCCTGTGGCTCTTGCTTGTC – 3′	5′ – GCGGTTTGATATGGGAAGACGA – 3′
Arrestin3	5′ – GCACAAGCTAGGGGACAATG – 3′	5′ – CCAGCCGCACATAGTCTCTC – 3′
G3PDH	5′ – GCTCAGACACCATGGGGAAGGT – 3′	5′ – GTGGTGCAGGAGGCATTGCTGA – 3′

doi:10.1371/journal.pone.0035611.t002

0.2% ethanol as a vehicle, approximately 2 h prior to the electrical recording. The cells were kept at 37°C in the dark and were transferred to a recording chamber filled with Leibovitz's L-15 medium (Gibco) and mounted on the microscope stage (BX51WI; Olympus, Tokyo, Japan) under dim red light. Individual cells were visualized under an infrared light monitoring system. Electrical recordings were made in the whole-cell patch-clamp configuration. Patch pipettes were pulled from borosilicate glass (Hilgenberg GmbH, Marsfeld, Germany) using a two-stage electrode puller (PP-83; Narishige, Tokyo, Japan). The composition of the intra-pipette solution was (in mM) KCl, 135; $CaCl_2$, 0.5; HEPES, 5; EGTA, 5; ATP-2Na, 5; GTP-3Na, 1; and pH was adjusted to 7.3 with KOH. The resistance of patch pipettes was 12–15 MΩ when filled with an intra-pipette solution. An Ag-AgCl pellet submerged in a NaCl well and connected to a recording chamber via a 150 mM NaCl agar-bridge was used as a reference electrode. The membrane current was recorded with a patch-clamp amplifier (Axopatch-200B; Axon Instruments, Foster City, CA, USA), low-pass filtered with a cutoff frequency of 500 Hz, and digitized at 1 kHz through a DigiData 1322A Interface using pCLAMP software (version 8.0, Axon Instruments).

To assess whether a recorded cell had any response to light or not, we used the following criteria:

$$I_{photo} = I_{stim} - I_{base}$$

Where I_{base} was an average of holding current for 1 s just before light stimulation and I_{stim} was an average of holding current for 1 s just before the cessation of light stimulation. When I_{photo} was larger than the two times of standard deviation of I_{base}, I_{photo} was judged as a real response to light stimulation.

Supporting Information

Figure S1 Southern blot analysis. A. Genomic DNA was isolated using the DNeasy kit (Qiagen). Genomic DNA (500 ng) was digested with BamHI restriction enzyme, separated via 0.8% agarose gel electrophoresis, and transferred to Hybond-N membranes (GE Healthcare). The membrane was then fixed under UV irradiation. The full-length RB gene probe was labeled by the AlkPhos Direct Labelling Reagent (GE Healthcare) and hybridized to the blot and detected using CDP-Star detection reagent (GE Healthcare). Lane 1: iris-derived cells (EY1420), lane 2: iris-derived cells (EY1406), lane 3: iris-derived cells (EY1408), lane 4: menstrual blood-derived cells (control), lane 5: endometrium-derived cells (control). B. Ethidium bromide stain of the BamHI-digested genomic DNA after electrophoresis.
(DOC)

Figure S2 RT-PCR analysis for genes of MAP2, rhodopsin, blue opsin, green/red opsin and G3PDH in iris-derived cells after gene transduction of several transcription factors. As negative controls, data of iris tissue and cultured iris-derived cells without gene transduction (w/o) are shown. We selected six genes, SIX3, PAX6, RX, CRX, NRL, and NEUROD, as candidate factors that may contribute to induce photoreceptor-specific phenotypes in iris cells. SIX3, PAX6, RX, CRX, NRL, and NEUROD are indicated as S, P, R, C, NR and ND, respectively. Left panel: Transduction of each single gene of SIX3, PAX6, RX, CRX, NRL, or NEUROD. Right panel: Transduction of all six genes and 5 genes. To determine which of the six candidates are critical, we examined the effect of withdrawal of individual factors from the pool of the candidate genes on expression of the opsin genes. As a result, individual withdrawal of NEUROD resulted in loss of expression of rhodopsin and withdrawal of CRX resulted in loss of blue opsin.
(DOC)

Figure S3 Immunocytochemistry using antibodies to rhodopsin (left panels), blue opsin (middle panels) and green/red opsin (right panels) on the cultured iris cells without gene infection (upper panels) and frozen sections of human retina (lower panels). Human iris cells without gene infection (upper panels) and the macular area of human retina (lower panels) served negative and positive controls, respectively, for Fig. 2C. The primary antibodies used were as follows: rhodopsin (goat polyclonal, I-17, Santa Cruz), blue opsin (goat polyclonal, P-13, Santa Cruz), and green/red opsin (goat polyclonal, C-19, Santa Cruz). The secondary antibody used was rabbit polyclonal to goat IgG conjugated with FITC. Nuclei were stained with DAPI. The photoreceptor layer in the retina is positive for rhodopsin, blue opsin and green/red opsin (lower panels from left to right).
(DOC)

Figure S4 Immunocytochemistry using antibodies to blue opsin (red) and rhodopsin (green). Double-stained immunocytochemistry was performed, by using 2 primary antibodies (mouse monoclonal Ab to rhodopsin and rabbit polyclonal Ab to blue opsin) and 2 secondary antibodies (goat

anti-mouse polyclonal FITC-labeled Ab and goat anti-rabbit polyclonal rhodamine-labeled Ab). Nuclei were stained with DAPI (blue). Experiments were performed at two weeks after infection. Scale bar represents 50 μm in the rightmost panel.
(DOC)

Figure S5 Electron-microscopic observation. Cells were initially fixed in PBS containing 2.5% glutaraldehyde for 24 h, and were embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and were viewed under a JEM-1200EX transmission electron microscope (JEOL, Ltd.). After transduction of the RX, CRX, and NEUROD genes into human cultured iris cells, a cilia-associated structure, i.e. centriole (arrow head) surrounded by mitochondria (arrows), was detected.
(DOC)

Figure S6 Quantitative analysis of photoreceptor-specific/associated genes expression (blue opsin, s-antigen and recoverin). Individual RNA expression levels were normalized by respective G3PDH expression levels. Vertical axis is relative expression levels of each gene in the siCRX & siNEUROD-transfected cells versus negative control siRNA-transfected cells (%, the mean of relative expression levels of two experiments). **siRNA transfection.** The cells at 7 days after transduction of the CRX, RX, and NEUROD genes in 6 well plates were transfected with siRNA using Lipofectamine RNAi-MAX Reagent (Invitrogen) according to the protocols recommended by the manufacturer. The cells were harvested 48 h after transfection and analyzed by quantitative RT-PCR. Stealth RNAiTM siRNA Duplex Oligoribonucleotides (siCRX and siNEUROD1, Invitrogen) were used as siRNAs to the CRX and NEUROD genes, and StealthTM RNAi Negative Control Duplexes (Invitrogen) were used as control siRNA.
(DOC)

Table S1 Primer sequences for exogenous/endogenous expression of transcription factors.
(DOC)

Table S2 Opsin expression by combination of transcription factors.
(DOC)

Acknowledgments

We are grateful for Dr. M. Yamada for discussion and critical reading of the manuscript, the National Eye Institute, National Institutes of Health for gift of 11-*cis* retinal, Y. Shichida, Y. Fukada, and D. Kojima for scientific comments on 11- *cis* retinal handling, M. Toyoda, Y. Fukawatase, Y. Takahashi, K. Miyado, H. Akutsu, M. Terai, C.H. Cui, T. Yokoi, M. Yamada, H. Makino, and Y. Nishina for the support throughout the work, and K. Saito and Y. Suehiro for the secretarial work.

Author Contributions

Conceived and designed the experiments: YS NA HO AU. Performed the experiments: YS YN RK. Analyzed the data: YS MK KN. Contributed reagents/materials/analysis tools: YM. Wrote the paper: YS AU.

References

1. Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, et al. (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci U S A 86: 5434–5438.
2. Gurdon JB, Melton DA (2008) Nuclear reprogramming in cells. Science 322: 1811–1815.
3. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455: 627–632.
4. Takeuchi JK, Bruneau BG (2009) Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. Nature 459: 708–711.
5. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, et al. (2010) Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463: 1035–1041.
6. Bainbridge JW, Smith AJ, Barker SS, Robbie S, Henderson R, et al. (2008) Effect of gene therapy on visual function in Leber's congenital amaurosis. N Engl J Med 358: 2231–2239.
7. Radtke ND, Aramant RB, Seiler MJ, Petry HM, Pidwell D (2004) Vision change after sheet transplant of fetal retina with retinal pigment epithelium to a patient with retinitis pigmentosa. Arch Ophthalmol 122: 1159–1165.
8. Osakada F, Ikeda H, Mandai M, Wataya T, Watanabe K, et al. (2008) Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat Biotechnol 26: 215–224.
9. Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, et al. (2012) Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 379: 713–720.
10. Morrow EM, Furukawa T, Lee JE, Cepko CL (1999) NeuroD regulates multiple functions in the developing neural retina in rodent. Development 126: 23–36.
11. Ochocinska MJ, Hitchcock PF (2009) NeuroD regulates proliferation of photoreceptor progenitors in the retina of the zebrafish. Mech Dev 126: 128–141.
12. Liu H, Etter P, Hayes S, Jones I, Nelson B, et al. (2008) NeuroD1 regulates expression of thyroid hormone receptor 2 and cone opsins in the developing mouse retina. J Neurosci 28: 749–756.
13. Sidman R (1961) Histogenesis of the mouse retina studied with thymidine-H3. In: Smelser GK, ed. The structure of the eye; proceedings of the symposium held April 11–13, 1960, during the Seventh International Congress of Anatomists. New York: Academic Press. pp 487–506.
14. Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, et al. (2000) Retinal stem cells in the adult mammalian eye. Science 287: 2032–2036.
15. Fischer AJ, Reh TA (2001) Müller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci 4: 247–252.
16. Sun G, Asami M, Ohta H, Kosaka J, Kosaka M (2006) Retinal stem/progenitor properties of iris pigment epithelial cells. Dev Biol 289: 243–252.
17. Coles BL, Angenieux B, Inoue T, Del Rio-Tsonis K, Spence JR, et al. (2004) Facile isolation and the characterization of human retinal stem cells. Proc Natl Acad Sci U S A 101: 15772–15777.
18. Frøen RC, Johnsen EO, Petrovski G, Berenyi E, Facsko A, et al. (2011) Pigment epithelial cells isolated from human peripheral iridectomies have limited properties of retinal stem cells. Acta Ophthalmol 89: e635–644.
19. MacNeil A, Pearson RA, MacLaren RE, Smith AJ, Sowden JC, et al. (2007) Comparative analysis of progenitor cells isolated from the iris, pars plana, and ciliary body of the adult porcine eye. Stem Cells 25: 2430–2438.
20. Akagi T, Akita J, Haruta M, Suzuki T, Honda Y, et al. (2005) Iris-derived cells from adult rodents and primates adopt photoreceptor-specific phenotypes. Invest Ophthalmol Vis Sci 46: 3411–3419.
21. Inoue T, Coles BL, Dorval K, Bremner R, Bessho Y, et al. (2010) Maximizing functional photoreceptor differentiation from adult human retinal stem cells. Stem Cells 28: 489–500.
22. Foster RG (1998) Shedding light on the biological clock. Neuron 20: 829–832.
23. Berson DM, Dunn FA, Takao M (2002) Phototransduction by retinal ganglion cells that set the circadian clock. Science 295: 1070–1073.
24. Provencio I, Jiang G, De Grip WJ, Hayes WP, Rollag MD (1998) Melanopsin: An opsin in melanophores, brain, and eye. Proc Natl Acad Sci U S A 95: 340–345.
25. Hattar S, Liao HW, Takao M, Berson DM, Yau KW (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295: 1065–1070.
26. Hartwick AT, Bramley JR, Yu J, Stevens KT, Allen CN, et al. (2007) Light-evoked calcium responses of isolated melanopsin-expressing retinal ganglion cells. J Neurosci 27: 13468–13480.
27. Xue T, Do MT, Riccio A, Jiang Z, Hsieh J, et al. (2011) Melanopsin signalling in mammalian iris and retina. Nature 479: 67–73.
28. MacLaren RE, Pearson RA, MacNeil A, Douglas RH, Salt TE, et al. (2006) Retinal repair by transplantation of photoreceptor precursors. Nature 444: 203–207.
29. Lamba DA, Gust J, Reh TA (2009) Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 4: 73–79.
30. Lamba DA, McUsic A, Hirata RK, Wang PR, Russell D, et al. (2010) Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. PLoS One 5: e8763.
31. Manavathi B, Peng S, Rayala SK, Talukder AH, Wang MH, et al. (2007) Repression of Six3 by a corepressor regulates rhodopsin expression. Proc Natl Acad Sci U S A 104: 13128–13133.
32. Glaser T, Walton DS, Maas RL (1992) Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 gene. Nat Genet 2: 232–239.

33. Mathers PH, Grinberg A, Mahon KA, Jamrich M (1997) The Rx homeobox gene is essential for vertebrate eye development. Nature 387: 603–607.
34. Furukawa T, Morrow EM, Cepko CL (1997) Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91: 531–541.
35. Rehemtulla A, Warwar R, Kumar R, Ji X, Zack DJ, et al. (1996) The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc Natl Acad Sci U S A 93: 191–195.
36. Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, et al. (2001) Nrl is required for rod photoreceptor development. Nat Genet 29: 447–452.
37. Miyagawa Y, Okita H, Nakaijima H, Horiuchi Y, Sato B, et al. (2008) Inducible expression of chimeric EWS/ETS proteins confers Ewing's family tumor-like phenotypes to human mesenchymal progenitor cells. Mol Cell Biol 28: 2125–2137.
38. Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, et al. (2001) Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 68: 235–244.