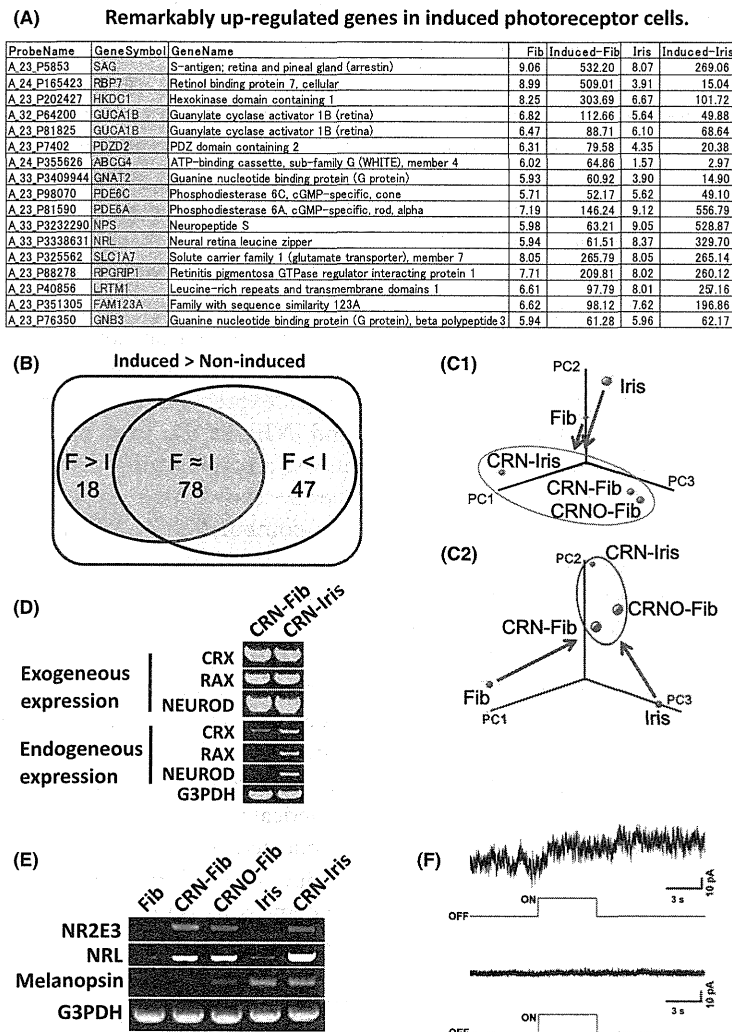

(A) **Remarkably up-regulated genes in induced photoreceptor cells.**

| ProbeName | GeneSymbol | GeneName | Fib | Induced-Fib | Iris | Induced-Iris |
|---|---|---|---|---|---|---|
| A_23_P5853 | SAG | S-antigen; retina and pineal gland (arrestin) | 9.06 | 532.20 | 8.07 | 269.06 |
| A_24_P165423 | RBP7 | Retinol binding protein 7, cellular | 8.99 | 509.01 | 3.91 | 15.04 |
| A_23_P202427 | HKDC1 | Hexokinase domain containing 1 | 8.25 | 303.69 | 6.67 | 101.72 |
| A_32_P64200 | GUCA1B | Guanylate cyclase activator 1B (retina) | 6.82 | 112.66 | 5.64 | 49.88 |
| A_23_P81825 | GUCA1B | Guanylate cyclase activator 1B (retina) | 6.47 | 88.71 | 6.10 | 68.64 |
| A_23_P7402 | PDZD2 | PDZ domain containing 2 | 6.31 | 79.58 | 4.35 | 20.38 |
| A_24_P355626 | ABCG4 | ATP-binding cassette, sub-family G (WHITE), member 4 | 6.02 | 64.86 | 1.57 | 2.97 |
| A_33_P3409944 | GNAT2 | Guanine nucleotide binding protein (G protein) | 5.93 | 60.92 | 3.90 | 14.90 |
| A_23_P98070 | PDE6C | Phosphodiesterase 6C, cGMP-specific, cone | 5.71 | 52.17 | 5.62 | 49.10 |
| A_23_P81590 | PDE6A | Phosphodiesterase 6A, cGMP-specific, rod, alpha | 7.19 | 146.24 | 9.12 | 556.79 |
| A_33_P3232290 | NPS | Neuropeptide S | 5.98 | 63.21 | 9.05 | 528.87 |
| A_33_P3338631 | NRL | Neural retina leucine zipper | 5.94 | 61.51 | 8.37 | 329.70 |
| A_23_P325562 | SLC1A7 | Solute carrier family 1 (glutamate transporter), member 7 | 8.05 | 265.79 | 8.05 | 265.14 |
| A_23_P88278 | RPGRIP1 | Retinitis pigmentosa GTPase regulator interacting protein 1 | 7.71 | 209.81 | 8.02 | 260.12 |
| A_23_P40856 | LRTM1 | Leucine-rich repeats and transmembrane domains 1 | 6.61 | 97.79 | 8.01 | 257.16 |
| A_23_P351305 | FAM123A | Family with sequence similarity 123A | 6.62 | 98.12 | 7.62 | 196.86 |
| A_23_P76350 | GNB3 | Guanine nucleotide binding protein (G protein), beta polypeptide 3 | 5.94 | 61.28 | 5.96 | 62.17 |



**Figure 5** Comparison of gene expression profiles of up-regulated genes in induced photoreceptor cells from dermal fibroblasts and from iris cells. (A) Remarkably up-regulated genes in induced photoreceptor cells ([CRNO-Fib]/[Fib] > 9.0). (B) Microarray analysis data sets from up-regulated genes in induced photoreceptor cells from dermal fibroblasts and from iris cells. F > I: Signal ratio of F/signal ratio of I ≥ 2.0. F < I: Signal ratio of I/signal ratio of F ≥ 2.0. The numbers of probes in each category are indicated. (C) Three-dimensional representation of PCA of gene expression levels (C-1: PCA based on the expression of all genes. C-2: PCA based on the expression of retina-related genes). It was shown that CRN-Fib, CRNO-Fib and CRN-Iris were grouped into the same group (shown in circle), suggesting that genes expression patterns in the CRN-Fib, CRNO-Fib and CRN-Iris were similar based on component 2 (PC2) and were apart from parent cells (Fib and Iris). (D) RT-PCR analysis of the exogenous and endogenous genes in induced retinal cells. Expression of the *CRX, RAX* and *NEUROD* and genes in the transgene-induced cells was analyzed by RT-PCR, using the exogenous and endogenous gene-specific primers (Seko *et al.* 2012). Equal amounts of RNAs were examined as determined by normalization by expression of the G3PDH gene. The levels of endogenous genes expression of *CRX, RAX* and *NEUROD* were clearly higher in CRN-Iris than in CRN-Fib. (E) RT-PCR analysis of genes expression of the transcription factor, *NRL* and *NR2E3*, and melanopsin. Expression of *NRL* and *NR2E3* was clearly up-regulated in the transgene-induced cells. The combination of CRN may be sufficient to up-regulate those transcription factors genes. As for melanopsin, expression was detected in CRNO-fib, but not in Fib or CRN-Fib. By microarray analysis, any expression of melanopsin was not detected (uploaded to GEO accession #GPL16699 at http://www.ncbi.nlm.nih.gov/geo/index.cgi). (F) Responses to light in infected cells and noninfected cells. Responses to light in infected cells (upper panel) and noninfected cells (lower panel). In a CRNO-infected cell (Fib #2), there was a large outward current when cell was exposed to light (upper panel). However, no detectable outward current was evoked when light stimulation was given to a noninfected cell (lower panel). A timing and duration of light stimulation is shown under the current trace. Holding potential was 0 mV.

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*NEUROD* (Seko *et al.* 2012), that were used successfully for iris cells (Fig. 1). An additional gene added to the combination, *OTX2*, further increases expression levels of photoreceptor-specific genes (Fig. 2). Global gene expression data by microarray analysis further shows that photoreceptor-related functional genes are significantly increased in induced photoreceptor cells (Fig. 4). Our data suggest that *OTX2* plays a role as an amplifier of photoreceptor-related functions (Figs 2 and 3; Fig. S1 in Supporting Information). Functional analysis also revealed that induced photoreceptor cells from fibroblasts by *CRX*, *RAX*, *NEUROD* and *OTX2* are photoresponsive *in vitro* (Figs 5F; Fig. S2 in Supporting Information).

Dermal fibroblasts are of mesodermal origin and immunogenic, whereas iris-pigmented epithelial cells (IPE cells) are of neural ectoderm-origin and show immune tolerance. Iris cells studied here include not only IPE cells but also iris stromal cells, which are of neural crest origin. We have previously shown that iris cells, IPE cells and iris stromal cells are differentiated into photoreceptor cells in the same way (Seko *et al.* 2012). However, dermal fibroblasts are harvested easily and safely, and iris cells are obtained surgically. To find a more suitable cell source than the iris cells for reprogramming into photoreceptor cells, we compared signal ratios between CRNO-Fib and CRN-Iris by a microarray analysis. The results show that there is an increase in both the expression levels and the variety of up-regulated photoreceptor-specific genes in induced cells from iris when compared with dermal fibroblasts (Fig. 5B; Table S3 in Supporting Information). From the standpoint of regenerative medicine, iris cells may be more suitable than dermal fibroblasts based on their characteristics of immune tolerance and higher expression of retina-specific genes in differentiated cells. The difference in induced endogenous expression of transcription factors *CRX, RAX* and *NEUROD* between CRN-Fib and CRN-Iris as well as the difference in up-regulated photoreceptor-specific genes may suggest a difference in reprogramming potential between the human dermal fibroblasts and the human iris cells (Fig. 5C). It may be possible to improve dermal fibroblasts as a source by use of other transcription factors or manipulating the histone methylation signature (Bramswig *et al.* 2013). However, dermal fibroblasts have an important advantage in that these cells are obtained safely and easily from patients. Because the direct reprogramming method may be suitable to provide the small numbers of cells required for individualized drug screening and disease modeling, dermal fibroblasts may be useful for such purposes despite their limitations.

We have previously shown that the combination of *CRX* and *NEUROD*, but not *NRL*, is sufficient for rod-specific gene expression (Seko *et al.* 2012), but Mears *et al.* (2001) reported that *Nrl* is necessary for rod-photoreceptor development. The present study indicates that both the *NRL* gene and the *NR2E3* gene are endogenously up-regulated in induced photoreceptor cells (CRNO-Fib and CRN-Iris) by microarray analyses and RT-PCR (Fig. 5D; Table S3 in Supporting Information). Endogenous NRL expression by the three factors, *CRX, RAX* and *NEUROD*, may promote retinal differentiation in the absence of the exogenous *NRL* gene. This fact clearly shows that exogenous gene transduction of the combination, *CRX, RAX* and *NEUROD*, is sufficient but *NRL* is not essential to induce rod photoreceptor-specific gene expression.

Several retinal diseases, including retinitis pigmentosa (RP), age-related macular degeneration and cone dystrophy, lead to loss of vision, due to loss of photoreceptors and retinal pigment epithelium (RPE), especially, RP leads to visual impairment due to irreversible retinal degeneration, which is determined genetically in most cases. Gene therapy has been implicated for Leber's congenital amaurosis (Bainbridge *et al.* 2008). Another promising therapeutic strategy is to transplant functional photoreceptor cells and retinal pigment epithelial cells. Sheets of human fetal neural retina with retinal pigment epithelium (Radtke *et al.* 2004) and ES cell-derived photoreceptors (Osakada *et al.* 2008) have been implicated for use as sources for the photoreceptor cells. The technology for producing retinal sheets from ES cell/ iPSCs by self-organogenesis (Eiraku *et al.* 2011) is promising for retinal transplantation. Recently, Tanaka *et al.* (2013) reported that inducible expression of myogenic differentiation 1 (*MYOD1*) in immature human iPSCs drives cells along the myogenic lineage, with efficiencies reaching 70–90%. Although induction of human neural retina takes a long time (Nakano *et al.* 2012), there is a possibility that the induction period could be shortened by the aid of the defined factors that we determined. We have previously reported the defined combination of transcription factors, that is, *CRX*, *RAX* and *NEUROD,* induce light-responsive photoreceptor cells in humans using iris cells (Seko *et al.* 2012). We show here the function of the *OTX2* gene as an amplifier of retinal transdifferentiation of human dermal fibroblasts (Figs 2 and 3; Fig. S1 in Supporting Information). In


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conclusion, *OTX2* and the three transcription factors, *CRX*, *RAX* and *NEUROD,* are promising as tools for effective retinal induction.

## Experimental procedures

### Cell culture

Three strains of cultured human dermal fibroblasts were used: one was obtained from Lonza (NHDF), another was from Promo Cell (NHDF) and the other was from ScienCell (HDF-a). These three kinds of fibroblasts were designated as Fib#1, Fib#2 and Fib#3, respectively. The cells were cultured in the recommended medium by the manufactures (FGM-2 Bullet kit, Fibroblast Growth Medium Kit, and Fibroblast Medium, respectively). Iris cells were obtained as previously reported (Seko *et al.* 2012) with the approval (approval number, #156) of the Ethics Committee of the National Institute for Child and Health Development (NCCHD), Tokyo. The ethics committees of the NCCHD and National Rehabilitation Center for Persons with Disabilities specifically approved this study. Signed informed consent was obtained from donors, and the surgical specimens were irreversibly de-identified. All experiments handling human cells and tissues were carried out in line with the Tenets of the Declaration of Helsinki. The iris cells were cultured in the growth medium [Dulbecco's modified Eagle's medium (DMEM)/Nutrient mixture F12 (1:1) supplemented with 10% fetal bovine serum, insulin–transferrin–selenium, and MEM-NEAA (GIBCO)].

### Preparation and infection of recombinant retrovirus

Full-length transcription factors, *SOX2* (Martinez-de Luna *et al.* 2010), *POU1F1* (Martinez-de Luna *et al.* 2010), *OTX2* (Nishida *et al.* 2003), *PAX6* (Glaser *et al.* 1992), *RAX* (Mathers *et al.* 1997), *CRX* (Furukawa *et al.* 1997) and *NEUROD* (Morrow *et al.* 1999), were amplified from cDNAs prepared from total RNA of adult human retina (Clontech, CA, USA) by PCR and cloned into the XmnI-EcoRV sites of pENTR11 (Invitrogen). Each vector contained one transcription factor, and a mixture of vectors was used.

Preparation and infection of recombinant retrovirus were carried out as previously reported (Seko *et al.* 2012). In brief, the resulting pENTR11-transcription factors were recombined with pMXs-DEST by use of LR recombination reaction as instructed by the manufacturer (Invitrogen). The retroviral DNAs were then transfected into 293FT cells, and 3 days later, the media were collected and concentrated. The human dermal fibroblasts and the iris cells were infected with this media containing retroviral vector particles. After the retroviral infection, the media were replaced with the DMEM/F12/B27 medium supplemented with 40 ng/ml bFGF, 20 ng/ml EGF, fibronectin and 1% FBS. The retrovirus-infected cells were cultured for up to 14 days. We transfected retroviral eGFP under the same condition to measure efficiency of infection. The frequency of eGFP-positive cells was 90–94% of all cells at 48 h after infection.

### Reverse transcriptase-PCR

Total RNA was isolated with an RNeasy Plus mini-kit® (Qiagen, Maryland, USA) or PicoPure™ RNA Isolation Kit (Arcturus Bioscience, CA, USA) according to the manufacturer's instruction. An aliquot of total RNA was reverse transcribed using an oligo (dT) primer. The design of PCR primer sets is shown in our previous paper (Seko *et al.* 2012).

### Quantitative RT-PCR

cDNA template was amplified (ABI7900HT Sequence Detection System) using the Platinum Quantitative PCR SuperMix-UDG with ROX (11743-100, Invitrogen). Fluorescence was monitored during every PCR cycle at the annealing step. The authenticity and size of the PCR products were confirmed using a melting curve analysis (using software provided by Applied Biosystems) and a gel analysis. A mRNA level was normalized using G3PDH as a housekeeping gene. The design of PCR primer sets is shown in our previous paper (Seko *et al.* 2012).

### Immunocytochemistry

Immunocytochemical analysis was carried out as previously described (Kohyama *et al.* 2001). As a methodological control, the primary antibody was omitted. The primary antibodies used were as follows: rhodopsin (goat polyclonal, I-17, Santa Cruz) and blue opsin (goat polyclonal, P-13, Santa Cruz).

### Global gene expression analysis

To clarify the specific gene expression profile in induced photoreceptor cells, we compared the expression levels of 50 599 probes in the induced photoreceptor cells and parental cells using the SurePrint G3 Human Gene Expression Microarray 8 × 60 K, ver.2.0 (Agilent) using total RNA extracted from those cells. To average experimental variations, extracted total RNA samples were pooled into one tube from three independent induction experiments of human dermal fibroblasts (Fib#2) and 12 independent induction experiments of human iris cells, respectively, and pooled samples were served to microarray analyses. To normalize the variations in staining intensity among chips, the 75th percentile of intensity distributions was aligned across arrays using GeneSpring software, version 12.5 (Agilent Technologies, Palo Alto). We then carried out GO analysis based on the normalized expression data of induced and noninduced cells. Commonly up-regulated genes in CRN- and CRNO-transfected fibroblasts (4124 probes) and those in CRNO-transfected fibroblasts and CRN-transfected iris cells (2585 probes) were extracted and were catego-

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rized into functional groups, respectively, to figure out the relative importance or significance of the gene ontology (GO) term (corrected *P*-value < 0.01). To analyze and compare the gene expression data of the induced cells and parent cells in an unsupervised manner, we used principal component analysis (PCA).

### Light stimulation and electrophysiological recordings

We followed the method in our previous paper (Seko *et al.* 2012). Briefly, a high pressure UV lamp (USH-102D, Ushio) was used as a light source. Diffuse, unpolarized blue light was generated through bandpass filters attached with the fluorescent emission system (BX-FLA, Olympus, Tokyo, Japan). Wavelength of light for stimulation was 460–490 nm. Duration and timing of light stimulation was controlled by an electrically controlled shutter attached to the UV lamp box. The trigger signals to the electrically controlled shutter were given by commercially available software (pClamp 9) through AD/DA. Light intensity used for stimulation was 390 $W/m^2$. To activate the phototransduction cascade, 11-*cis* retinal (a gift from the vision research community, the National Eye Institute, National Institutes of Health) was added to the culture medium of human fibroblasts to a concentration of 37.5 μM with 0.15% ethanol as a vehicle, approximately 45 min before the electrical recording. Electrical recordings were made in the whole-cell patch-clamp configuration. The composition of the intrapipette 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 intrapipette solution. 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 1 kHz and digitized at 2 kHz through a DigiData 1322A Interface using pCLAMP software (version 8.0, Axon Instruments). We recorded light responses from noninfected cells, CRN-infected cells and CRNO-infected cells. Recorded data were pooled for further analysis (for details, see Fig. S2 in Supporting Information).

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## Author contributions

YS carried out all of the experiments; MK, TI, YS, YK carried out electrophysiological analyses; YS, YM and KM prepared viral vectors; YS, NA, AU made experimental designs; and YS and AU wrote the manuscript.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

**Figure S1** Categorization of the genes differentially expressed in induced photoreceptor cells from human dermal fibroblasts (CRNO-Fib versus CRN-Fib).

**Figure S2** Method for analysis of light responses.

**Table S1** List of the enriched GO term (corrected *P*-value < 0.01) for Fig. 4A

**Table S2** Up-regulated retina-related genes both in the CRNO-infected fibroblasts and in CRN-infected iris-derived cells (signal ratio ≥ 2.0 for 'up')

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