Genes to Cells

Derivation of human differential photoreceptor cells from adult human dermal fibroblasts by defined combinations of *CRX, RAX, OTX2* and *NEUROD*

Yuko Seko[1,2]*, Noriyuki Azuma[3], Toshiyuki Ishii[4], Yukari Komuta[1], Kiyoko Miyamoto[1], Yoshitaka Miyagawa[2], Makoto Kaneda[4] and Akihiro Umezawa[2]

[1]*Visual Functions Section, Department of Rehabilitation for Sensory Functions, Research Institute, National Rehabilitation Center for Persons with Disabilities, Tokorozawa, Saitama 359-8555, Japan*
[2]*Department of Reproductive Biology, Center for Regenerative Medicine, National Institute for Child Health and Development, Okura, Setagaya, Tokyo, 157-8535, Japan*
[3]*Department of Ophthalmology, National Center for Child Health and Development, Setagaya, Tokyo 157-8535, Japan*
[4]*Department of Physiology, Nippon Medical School, Sendagi, Bunkyo, Tokyo 113-8602, Japan*

Redirecting differentiation of somatic cells by over-expression of transcription factors is a promising approach for regenerative medicine, elucidation of pathogenesis and development of new therapies. We have previously defined a transcription factor combination, that is, *CRX, RAX* and *NEUROD*, that can generate photosensitive photoreceptor cells from human iris cells. Here, we show that human dermal fibroblasts are differentiated to photoreceptor cells by the same transcription factor combination as human iris cells. Transduction of a combination of the *CRX*, *RAX* and *NEUROD* genes up-regulated expression of the photoreceptor-specific genes, recoverin, blue opsin and PDE6C, in all three strains of human dermal fibroblasts that were tested. Additional *OTX2* gene transduction increased up-regulation of the photoreceptor-specific genes blue opsin, recoverin, S-antigen, CNGB3 and PDE6C. Global gene expression data by microarray analysis further showed that photoreceptor-related functional genes were significantly increased in induced photoreceptor cells. Functional analysis, that is, patch-clamp recordings, clearly revealed that induced photoreceptor cells from fibroblasts responded to light. Both the *NRL* gene and the *NR2E3* gene were endogenously up-regulated in induced photoreceptor cells, implying that exogenous *CRX*, *RAX, OTX2* and *NEUROD*, but not *NRL*, are sufficient to generate rod photoreceptor cells.

Introduction

Redirecting differentiation of somatic cells by over-expression of transcription factors is a promising approach for regenerative medicine, elucidation of pathogenesis and development of new therapies. The process is called 'direct reprogramming' or 'direct conversion' and has been shown in β cells, cardiomyocytes, neurons, platelets and photoreceptors. A specific combination of three transcription factors (Ngn3, Pdx1 and MafA) reprogram differentiated pancreatic exocrine cells in adult mice into cells that closely resemble beta cells (Zhou *et al.* 2008) and a combination of three factors (Gata4, Tbx5 and Baf60c) induce noncardiac mesoderm to differentiate directly into contractile cardiomyocytes (Takeuchi & Bruneau 2009). We recently employed the strategy of 'direct reprogramming' to generate retinal photoreceptor cells from human somatic cells, defining a combination of transcription factors, *CRX, RAX* and *NEUROD*, that induce light responsive photoreceptor cells (Seko *et al.* 2012). In that study, we induced 'iris cells' into photoreceptor cells. During vertebrate eye development, the inner layer of the optic cup differentiates into the

Communicated by: Takashi Tada
**Correspondence*: seko-yuko@rehab.go.jp

DOI: 10.1111/gtc.12127

neural retina and iris-pigmented epithelium (IPE). Therefore, the common developmental origin of the iris and the retina may make photoreceptor-induction from iris cells easier than from other types of somatic cells.

The induced pluripotent stem cells (iPS) developed by Takahashi and Yamanaka were the first model for 'direct reprogramming', in which mouse adult fibroblasts were reprogrammed by transduction of four transcription factor genes, Oct3/4, Sox2, c-Myc and Klf4 (Takahashi & Yamanaka 2006). Additionally, functional neurons were generated from mouse fibroblasts by a combination of three factors (Ascl1, Brn2 and Myt1 l) (Vierbuchen *et al.* 2010), and functional platelets were generated from mouse and human fibroblasts by a combination of three factors (p45NF-E2, MafG and MafK) (Ono *et al.* 2012). Because human dermal fibroblasts are less specialized than iris cells, we tested whether human dermal fibroblasts could be converted into photoreceptors by the same defined combination of genes used successfully for human iris cells, *CRX, RAX* and *NEUROD*, to generalize and establish our technology for 'generating photoreceptors'.

In this study, we also investigated an effect of additional transcription factor, *OTX2,* on transdifferentiation of somatic cells into retinal cells. Otx2 is essential for the cell fate determination of retinal photoreceptor cells (Nishida *et al.* 2003), and conditional disruption of the Otx2 gene decreases photoreceptor-associated genes (Omori *et al.* 2011).

Here, we show that the same combination of genes used for human iris cells, that is, *CRX, RAX and NEUROD*, generate human photoreceptor cells from human dermal fibroblasts, and that additional *OTX2* gene transduction further amplifies the expression of retina-specific genes. Our data therefore indicate that human dermal fibroblasts are a superior cell source for reprogramming into photoreceptor cells.

Results

Human dermal fibroblasts are induced into a rod- or cone-specific phenotype by defined transcription factors

We selected seven genes, *POU1F1*, *SOX2*, *PAX6*, *RAX*, *CRX*, *OTX2* and *NEUROD*, as candidate factors that may contribute to induce photoreceptor-specific phenotypes in human dermal fibroblasts, on the basis that such factors play a role in the development of photoreceptors. *CRX, RAX* and *NEUROD* are essential factors that induce photoreceptor cells from human iris cells (Seko *et al.* 2012) and *POU1f1,* Sox2 and Otx2 bind to the Rx promoter (Martinez-de Luna *et al.* 2010). Human dermal fibroblasts were infected with these genes and were examined for inducible expression of photoreceptor-specific genes. RT-PCR results showed that transduction of *CRX*, *RAX* and *NEUROD* (CRN) genes up-regulated the expression of the photoreceptor-specific genes recoverin, blue opsin and PDE6C, in all strains of fibroblasts tested (Fig. 1, panel A, B, C). Additionally, CRN-infected fibroblasts became positive for rhodopsin and blue opsin by immunohistochemistry (Fig. 1D). These results suggest that photoreceptor-specific phenotypes are induced by the same combination of transcription factors in human dermal fibroblasts as in human iris cells. However, it appeared that the combination of *CRX*, *RAX*, *NEUROD* and *OTX2* (CRNO) up-regulated the photoreceptor-specific blue opsin gene more strongly than the combination of CRN.

Additional *OTX2* gene transduction increases up-regulation levels of photoreceptor-specific genes

Expression levels of opsin- and phototransduction-related genes in induced- and noninduced fibroblasts were quantitated. Expression levels of S-antigen and recoverin, which are specifically expressed in rod photoreceptors, were much higher in CRNO-infected cells than in CRN-infected cells (S-antigen, $P < 0.01$, recoverin, $P < 0.05$; Welch's *t*-test, Fig. 2). In contrast, expression levels of rhodopsin, blue opsin, green opsin, recoverin, S-antigen, CNGB3 and PDE6C were not increased by additional *PAX6* gene infection (CRNP vs. CRN, in Fig. 2).

OTX2 is not an essential factor but an amplifier for induction of photoreceptor cells from human dermal fibroblasts

To investigate whether *OTX2* could be used as an alternative to the essential three genes, that is, *CRX, RAX* and *NEUROD*, we tested the effect of withdrawal of each individual factor from the four genes, that is, *CRX, RAX, NEUROD* and *OTX2*, on expression levels of the opsin- and phototransduction-related genes in induced photoreceptor cells (Fig. 3). Removal of either *CRX, RAX* or *NEUROD* resulted in a marked decrease in blue opsin, S-antigen, PDE6C

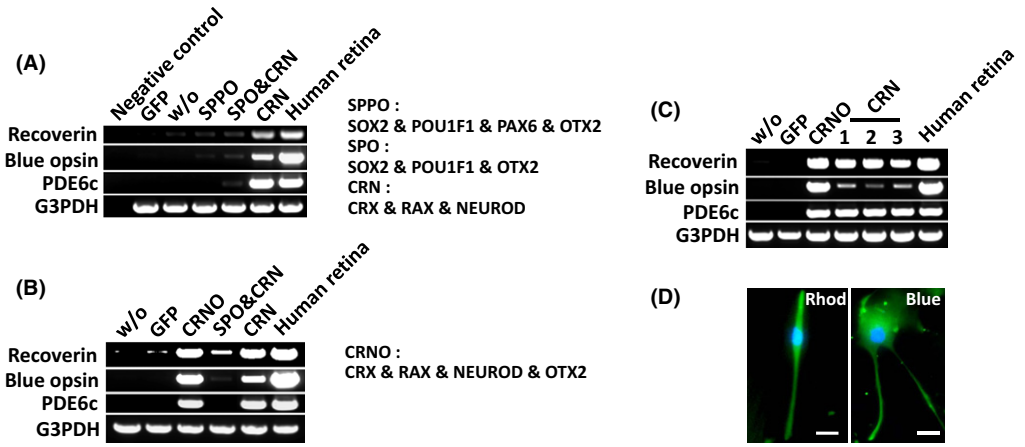

Figure 1 Induction of retina-specific genes in human dermal fibroblasts by the retroviral infection of genes for defined transcription factors. (A) RT-PCR analysis for photoreceptor-specific genes in cultured human dermal fibroblasts (NHDF) obtained from Lonza after gene transfer of several kinds of transcription factors. Recoverin, blue opsin and PDE6c genes were up-regulated by CRN transduction. 'Negative control': amplified water as a negative control. 'GFP': cultured fibroblasts after retroviral gene transfer of the GFP gene as another negative control. 'w/o': cultured fibroblasts without gene transfer as the other negative control. 'SPPO': *SOX2, POU1F1, PAX6* and *OTX2*. 'SPO': *SOX2, POU1F1* and *OTX2*. 'CRN': *CRX, RAX* and *NEUROD*. 'Human retina': human retinal tissue as a positive control. The amount of cDNA as a template was a half in the positive control. (B) RT-PCR analysis for photoreceptor-specific genes in cultured human dermal fibroblasts (NHDF) obtained from Promo Cell after gene transfer of several transcription factors. Recoverin, blue opsin and PDE6c genes were up-regulated by CRN or CRNO transduction. 'w/o': cultured fibroblasts without gene transfer as a negative control. 'GFP': cultured fibroblasts after retroviral gene transfer of the GFP gene as another negative control. 'CRNO': *CRX, RAX, NEUROD* and *OTX2*. (C) RT-PCR analysis for photoreceptor-specific genes in cultured human dermal fibroblasts (HDF-a) obtained from ScienCell after gene transfer of several transcription factors. Recoverin, blue opsin and PDE6C genes were up-regulated by CRN or CRNO transduction. Expression levels of blue opsin were increased by additional *OTX2* gene transduction. 'w/o': cultured fibroblasts without gene transfer as a negative control. 'GFP': cultured fibroblasts after retroviral gene transfer of the GFP gene as another negative control. 'CRNO': *CRX, RAX, NEUROD* and *OTX2*. '1', '2' and '3' mean independently cultured, transfected and harvested cells by the same combination of CRN genes. (D) Immunocytochemistry using antibodies to rhodopsin and blue opsin (green). Nuclei were stained with DAPI (blue). Experiments were carried out at 2 weeks after infection. The cells in the left panel and the right panel are CRN-infected Fib#2 and Fib#1, respectively. Scale bars represent 10 μm.

and CNGB3 levels; withdrawal of *RAX* resulted in a marked decrease in expression of blue opsin, and withdrawal of *NEUROD* resulted in a striking decrease in expression of PDE6C. Alternatively, withdrawal of *OTX2* alone did not affect the up-regulation of any of the tested photoreceptor-specific genes. These results indicate that *OTX2* is not an essential factor but an amplifier for induction of photoreceptor cells from human dermal fibroblasts, suggesting that additional *OTX2* plays a role in improving the balance and stability of photoreceptor-related gene expression in induced photoreceptor cells. Removal of either *CRX, RAX* or *NEUROD* resulted in a marked decrease in blue opsin, S-antigen, PDE6C and CNGB3 levels, suggesting that each transcription factor plays a role for specific molecular functions along with a role as a constituent of a combination for transdifferentiation to photoreceptor cells.

Photoreceptor-related functional genes are clearly up-regulated in induced photoreceptor cells from human dermal fibroblasts

To clarify the specific gene expression profile in induced photoreceptor cells, we compared the expression profiles of 50 599 probes in the induced photoreceptor cells (CRN-infected fibroblasts (CRN -Fib), CRNO-infected fibroblasts (CRNO-Fib) and parental cells [fibroblast (Fib)] by microarray analysis (uploaded to GEO accession #GPL16699 at http://www.ncbi.nlm.nih.gov/geo/index.cgi). We first extracted the intersection of the two groups of genes, that is, up-regulated genes by CRN-infection ([CRN-Fib] vs. [Fib]) and those by CRNO-infection ([CRNO-Fib] vs. [Fib]) (signal ratio ≥ +1.5 for 'up'). According to gene ontology (GO) term annotation, the differentially expressed genes (4124 probes), which were

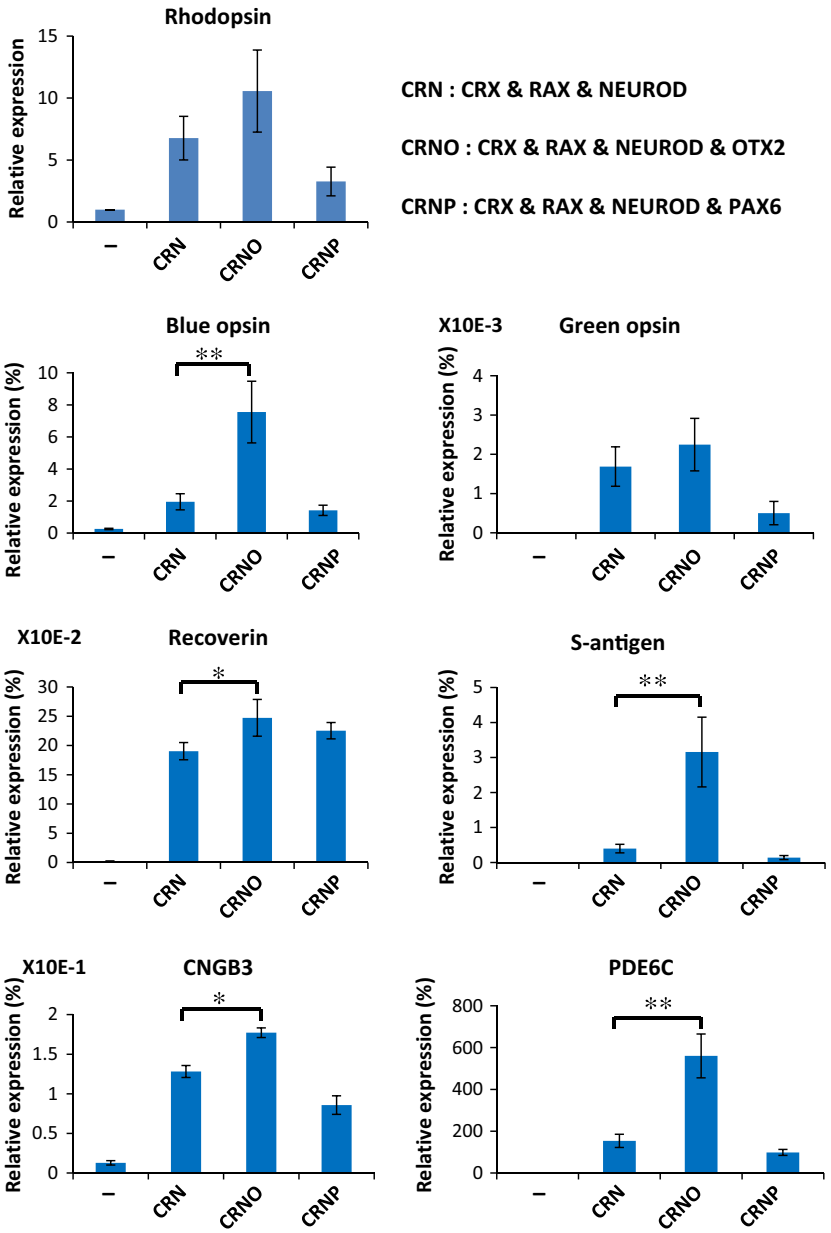

Figure 2 Effect of additional *OTX2* gene infection. Quantitative RT-PCR results for expression levels of rod- or cone-specific genes in induced photoreceptor cells from human dermal fibroblasts by the defined transcription factors. Quantitative expression levels of rhodopsin, blue opsin, green opsin, recoverin, S-antigen, CNGB3 and PDE6c genes were investigated. The data of green opsin, recoverin and CNGB3 were the results in experiments using Fib#2, and the data of rhodopsin, blue opsin and S-antigen were the results in experiments using Fib#3. The vertical axis indicates expression levels of each gene (%) in the indicated cells, relative to human retinal tissues. For rhodopsin, expression in cultured fibroblasts is regarded as 1.0. Results of statistical analyses for comparison of expression levels between CRNO-infected cells and CRN-infected cells are shown [$\star P < 0.05$ and $\star\star P < 0.01$ (Welch's *t*-test)]. '–': cultured fibroblasts without gene transfer as a negative control. 'CRN': *CRX, RAX* and *NEUROD*. 'CRNO': *CRX, RAX, NEUROD* and *OTX2*. 'CRNP': *CRX, RAX, NEUROD* and *PAX6*.

included in the intersection, were categorized into functional groups. Interestingly, when phototransduction-related genes were extracted, they accounted for up to 0.2% of the total (Fig. 4A; Table S1 in Supporting Information). In fact, signals of 16 probes were increased among the 30 phototransduction-related probes.

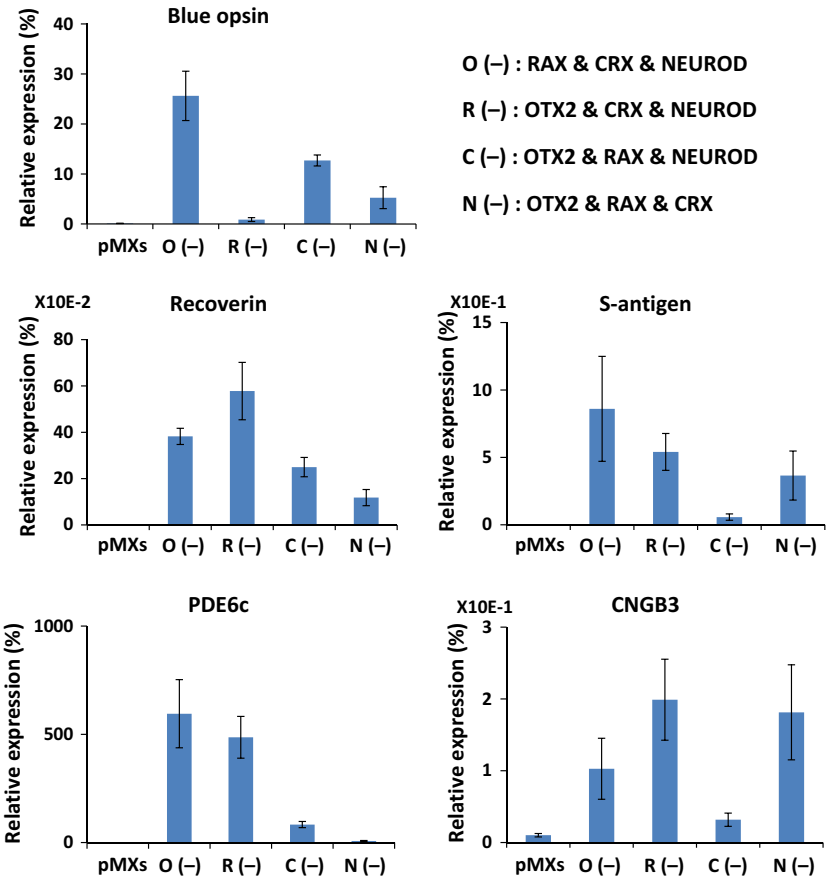

Figure 3 Effect of individual withdrawal of each gene from the combination of *CRX, RAX, NEUROD* and *OTX2*. Quantitative RT-PCR results for expression levels of rod- or cone-specific genes in induced photoreceptor cells from human dermal fibroblasts by the defined transcription factors. To determine which of the four genes, that is, *CRX, RAX, NEUROD* and *OTX2,* are critical, we examined the effect of withdrawal of individual factors from the pool of the candidate genes on expression of the opsin genes. In this experiment, Fib#3 was used. Quantitative expression levels of blue opsin, recoverin, S-antigen, CNGB3 and PDE6C genes were investigated. Vertical axis indicates expression levels of each gene (%) in the indicated cells, relative to human retinal tissues. Individual withdrawal of *RAX* resulted in a significant decrease in expression of blue opsin and withdrawal of *CRX* resulted in a significant decrease in S-antigen PDE6C and CNGB3. Individual withdrawal of *NEUROD* resulted in a significant decrease in PDE6C. However, withdrawal *OTX2* could up-regulate all of the retina-specific genes tested. 'O(-)': *CRX, RAX* and *NEUROD*. 'R(-)': *CRX, OTX2* and *NEUROD*. 'C(–)': *OTX2, RAX* and *NEUROD*. 'N(-)': *CRX, RAX* and *OTX2*. 'pMXs': cultured fibroblasts after retroviral gene transfer of the pMXs gene as a negative control.

To clarify the difference in gene expression profiles between fibroblast-derived and iris-derived photoreceptor cells, we investigated the expression profiles of default cells (iris cells) and induced cells (CRN-infected iris cells). We carried out GO analysis based on the differentially expressed genes (2585 probes), which were included in the commonly up-regulated genes, that is, ([CRNO-Fib] vs. [Fib]) and ([CRN-Iris] vs. [Iris]) (signal ratio $\geq +1.5$ for 'up'). The phototransduction-related genes were extracted and accounted for up to 4.4% (Fig. 4B; Table S2 in Supporting Information). Although detection/perception, which includes detection of external stimulus, detection of abiotic stimulus and detection of light stimulus, accounted for up to 0.6% of the total in Fig. 4A, the detection/perception accounted for up to 21.1% in Fig. 4B.

A dermal fibroblast could be a cell source as well as an iris cell

We searched up-regulated genes both in the CRNO-infected fibroblasts and in CRN-infected iris cells (signal ratio ≥ 2.0 for 'up') and named as 'intersection

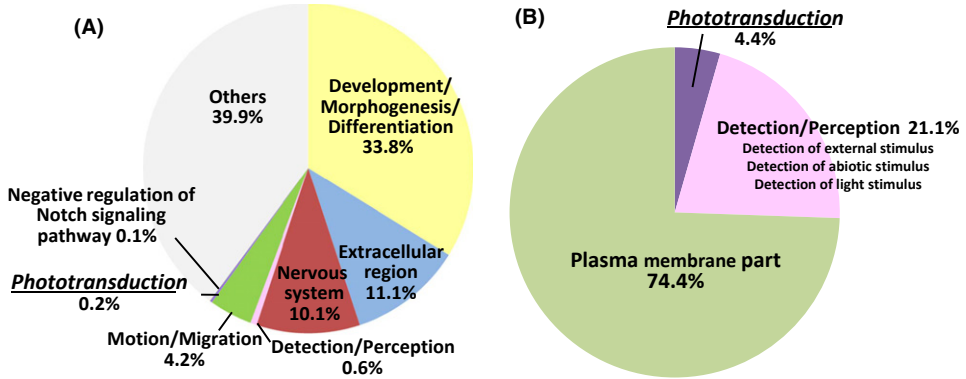

Figure 4 Categorization of the genes differentially expressed in induced photoreceptor cells from human dermal fibroblasts by the defined transcription factors. (A) Categorization of commonly up-regulated genes in induced photoreceptor cells from human dermal fibroblasts by genes transduction of CRN and CRNO. 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 (CRN-infected fibroblasts (CRN-Fib), CRNO-infected fibroblasts (CRNO-Fib) and parental cells [fibroblast (Fib)] by microarray analysis. We searched up-regulated genes in the induced photoreceptor cells by CRN- and CRNO-infection compared with parental cells (signal ratio ≥ +1.5 for 'up'), respectively. We then extracted the intersection of the two groups of genes, that is, up-regulated genes by CRN-infection and those by CRNO-infection. According to gene ontology (GO) term annotation, the genes differentially expressed in the induced photoreceptor cells by CRN- and CRNO-infection (4124 probes) were categorized into functional group, figuring out the relative importance or significance of the GO-term [corrected *P*-value < 0.01)]. After that, we carried out additional categorization into eight groups. Interestingly, phototransduction-related genes were extracted and account for up to 0.15% of the total. (B) Categorization of commonly up-regulated genes (2585 probes) in the CRNO-transfected dermal fibroblasts and CRN-transfected iris cells (signal ratio ≥ 1.5 for 'up'). According to gene ontology (GO) term annotation, the genes differentially expressed in the induced photoreceptor cells (2585 probes) were categorized into functional groups to figure out the relative importance or significance of the GO term (corrected *P*-value < 0.01).

of Fib and Iris'. Then, we extracted retina-related genes from them according to Gene Ontology and a previous paper (Omori *et al.* 2011). We focused on remarkably up-regulated genes ([CRNO-Fib]/[Fib] > 9.0) and extracted them (Fig. 5A). We then compared signal ratios between [CRNO-Fib]/[Fib] (ΔFib) and [CRN-Iris]/[Iris] (ΔIris). The signal ratios of 18 probes were higher in [CRNO-Fib] (ΔFib/ΔIris ≥ 2.0); however, the signal ratios of 47 signals were higher in [CRN- Iris] (ΔIris/ΔFib ≥ 2.0). As for other 78 probes, the signal ratios were regarded not to be significantly different (Fig. 5B; Table S3 in Supporting Information). To analyze the gene expression data in an unsupervised manner, we carried out principal component analysis (PCA). The gene expression patterns in the CRN-Fib, CRNO-Fib and CRN-Iris were close based on component 2 (PC2) but were apart from the parent cells (Fib and Iris) (Fig. 5C). We investigated the difference in endogenous expression of *CRX, RAX* and *NEUROD* between CRN-Fib and CRN-iris by RT-PCR (Fig. 5D). The endogenous *CRX* genes started to be expressed in CRN-Fib, but the expression levels of *RAX* and *NEUROD* were higher in CRN-Iris than in CRN-Fib. Both the *NRL* gene and the *NR2E3* gene were endogenously up-regulated in the induced photoreceptor cells, that is, CRN-Fib, CRNO-Fib and CRN-Iris (Fig. 5E).

Induced photoreceptor cells from fibroblasts are photoresponsive *in vitro*

Light stimulation was applied to infected or non-infected human fibroblasts because CRN- or CRNO-infected cells showed the photoreceptor-like phenotypes by RT-PCR and global gene expression analyses. Among cells tested, significant light responses were detected in a portion of infected cells (Fig. 5F; Fig. S2 in Supporting Information). An infected cell presented a large outward current when exposed to light (Fig. 5F, upper panel). However, no detectable outward current was evoked when light stimulation was given to a noninfected cell (Fig. 5F, lower panel).

Discussion

This is the first report that human dermal fibroblasts can differentiate into photoreceptor cells by the same combination of transcription factors, *CRX*, *RAX* and

(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

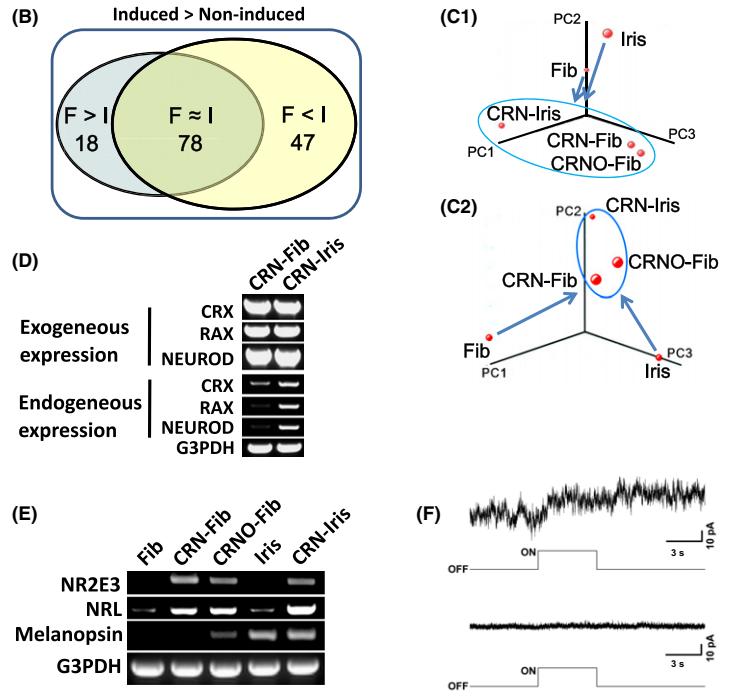

Figure 5 Comparison of gene expression profiles of up-regulated genes in induced photoreceptor cells from dermal fibroblasts and from iris cells. (A) Remarkably up-regulated genes in induced photoreceptor cells ([CRNO-Fib]/[Fib] > 9.0). (B) Microarray analysis data sets from up-regulated genes in induced 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.

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

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-

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).

Acknowledgements

We are grateful for the National Eye Institute, National Institutes of Health for gift of 11-*cis* retinal, S. Kato, K. Mori, S. Toyama and Y. Takahashi for the support throughout the work and for H. Miyauchi for the technical assistance. This research was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, by Ministry of Health, Labour and Welfare Sciences (MHLW) research grants, by a Research Grant on Health Science focusing on Drug Innovation from the Japan Health Science Foundation, by the program for the promotion of Fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency, by the Grant of National Center for Child Health and Development to AU, by the Nippon Medical School Grant-in-Aid for Medical Research to MK and by grants of National Rehabilitation Center for Persons with Disabilities.

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.

References

Bainbridge, J.W., Smith, A.J., Barker, S.S. *et al.* (2008) Effect of gene therapy on visual function in Leber's congenital amaurosis. *N. Engl. J. Med.* **358**, 2231–2239.

Bramswig, N.C., Everett, L.J., Schug, J., Dorrell, C., Liu, C., Luo, Y., Streeter, P.R., Naji, A., Grompe, M. & Kaestner, K.H. (2013) Epigenomic plasticity enables human pancreatic alpha to beta cell reprogramming. *J. Clin. Invest.* **123**, 1275–1284.

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., Sekiguchi, K., Adachi, T. & Sasai, Y. (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* **472**, 51–56.

Furukawa, T., Morrow, E.M. & Cepko, C.L. (1997) Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* **91**, 531–541.

Glaser, T., Walton, D.S. & Maas, R.L. (1992) Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 gene. *Nat. Genet.* **2**, 232–239.

Kohyama, J., Abe, H., Shimazaki, T., Koizumi, A., Nakashima, K., Gojo, S., Taga, T., Okano, H., Hata, J. & Umezawa, A. (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.

Martinez-de Luna, R.I., Moose, H.E., Kelly, L.E., Nekkalapudi, S. & El-Hodiri, H.M. (2010) Regulation of retinal homeobox gene transcription by cooperative activity among cis-elements. *Gene* **467**, 13–24.

Mathers, P.H., Grinberg, A., Mahon, K.A. & Jamrich, M. (1997) The Rx homeobox gene is essential for vertebrate eye development. *Nature* **387**, 603–607.

Mears, A.J., Kondo, M., Swain, P.K., Takada, Y., Bush, R.A., Saunders, T.L., Sieving, P.A. & Swaroop, A. (2001) Nrl is required for rod photoreceptor development. *Nat. Genet.* **29**, 447–452.

Morrow, E.M., Furukawa, T., Lee, J.E. & Cepko, C.L. (1999) NeuroD regulates multiple functions in the developing neural retina in rodent. *Development* **126**, 23–36.

Nakano, T., Ando, S., Takata, N., Kawada, M., Muguruma, K., Sekiguchi, K., Saito, K., Yonemura, S., Eiraku, M. & Sasai, Y. (2012) Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell* **10**, 771–785.

Nishida, A., Furukawa, A., Koike, C., Tano, Y., Aizawa, S., Matsuo, I. & Furukawa, T. (2003) Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. *Nat. Neurosci.* **6**, 1255–1263.

Omori, Y., Katoh, K., Sato, S., Muranishi, Y., Chaya, T., Onishi, A., Minami, T., Fujikado, T. & Furukawa, T. (2011) Analysis of transcriptional regulatory pathways of photoreceptor genes by expression profiling of the Otx2-deficient retina. *PLoS ONE* **6**, e19685.

Ono, Y., Wang, Y., Suzuki, H., Okamoto, S., Ikeda, Y., Murata, M., Poncz, M. & Matsubara, Y. (2012) Induction of functional platelets from mouse and human fibroblasts by p45NF-E2/Maf. *Blood* **120**, 3812–3821.

Osakada, F., Ikeda, H., Mandai, M., Wataya, T., Watanabe, K., Yoshimura, N., Akaike, A., Sasai, Y. & Takahashi, M. (2008) Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. *Nat. Biotechnol.* **26**, 215–224.

Radtke, N.D., Aramant, R.B., Seiler, M.J., Petry, H.M. & 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.

Seko, Y., Azuma, N., Kaneda, M., Nakatani, K., Miyagawa, Y., Noshiro, Y., Kurokawa, R., Okano, H. & Umezawa, A. (2012) Derivation of human differential photoreceptor-like cells from the iris by defined combinations of CRX, RX and NEUROD. *PLoS ONE* **7**, e35611.

Takahashi, K. & Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676.

Takeuchi, J.K. & Bruneau, B.G. (2009) Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature* **459**, 708–711.

Tanaka, A., Woltjen, K., Miyake, K. *et al.* (2013) Efficient and reproducible myogenic differentiation from human iPS cells: prospects for modeling miyoshi myopathy *in vitro*. *PLoS ONE* **8**, e61540.

Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Sudhof, T.C. & Wernig, M. (2010) Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* **463**, 1035–1041.

Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J. & Melton, D.A. (2008) *In vivo* reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature* **455**, 627–632.

Received: 17 July 2013
Accepted: 26 November 2013

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')