Analysis of Transcriptional Regulatory Pathways of Photoreceptor Genes by Expression Profiling of the Otx2-Deficient Retina

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Abstract

In the vertebrate retina, the Otx2 transcription factor plays a crucial role in the cell fate determination of both rod and cone photoreceptors. We previously reported that Otx2 conditional knockout (CKO) mice exhibited a total absence of rods and cones in the retina due to their cell fate conversion to amacrine-like cells. In order to investigate the entire transcriptome of the Otx2 CKO retina, we compared expression profile of Otx2 CKO and wild-type retinas at P1 and P12 using microarray. We observed that expression of 101- and 1049-probe sets significantly decreased in the Otx2 CKO retina at P1 and P12, respectively, whereas, expression of 3- and 4149-probe sets increased at P1 and P12, respectively. We found that expression of genes encoding transcription factors involved in photoreceptor development, including Crx, Nrl, Nr2e3, Esrrb, and NeuroD, was markedly down-regulated in the Otx2 CKO at both P1 and P12. Furthermore, we identified three human retinal disease loci mapped in close proximity to certain down-regulated genes in the Otx2 CKO retina including Cdc126, Tnfsf13b, and Pitpnm1, suggesting that these genes are possibly responsible for these diseases. These transcriptomic data sets of the Otx2 CKO retina provide a resource on developing rods and cones to further understand the molecular mechanisms underlying photoreceptor development, function and disease.

Introduction

During mammalian retinogenesis, five major types of neurons arise from multipotent progenitor cells, which are common precursors for all retinal neurons and glia [1,2,3]. We previously demonstrated that Otx2, an Otx-like homeobox gene, is essential for the cell fate determination of retinal photoreceptor cells [4]. Otx2 conditional knockout (CKO) mice showed a cell fate switch from retinal photoreceptor precursor cells to amacrine-like cells. On the other hand, several transcription factors including Crx, Nrl, and Nr2e3 are essential for terminal differentiation of photoreceptors. Crx encodes an Otx-like homeodomain transcription factor essential for terminal differentiation of both rods and cones by regulating genes encoding phototransduction, photoreceptor metabolism and outer segment formation [5,6]. Crx knockout (KO) mice develop aberrant photoreceptors that lack both rod and cone photoreceptors [7]. In humans, mutations in CRX are associated with retinal degeneration diseases such as cone-rod dystrophy-2, retinitis pigmentosa (RP), and Leber congenital amaurosis (LCA) [6,8,9,10]. Nrl (neural retina leucine zipper gene) is a transcription factor of the leucine zipper family expressed predominantly in rods but not in cones [11]. Mice lacking the Nrl gene do not develop rods but produce an increased number of short wavelength-sensitive cones (S-cones) [12]. Nrl promotes rod development by directly activating rod-specific genes while simultaneously suppressing the S-cone related genes through the activation of transcriptional repressor Nuclear receptor subfamily 2 group E member 3 (Nr2e3) [12]. Mutations in human Nr2e3 cause enhanced S-cone syndrome [13]. The rd7 mouse has a genetic defect in the Nr2e3 gene and exhibits an increased number of cones [14]. Mice lacking retinoid-related orphan nuclear receptor β (Rorb) were shown to lose rods but overproduce primitive S-cones, similar to Nrl KO mice [15]. In addition, several other nuclear receptors are involved in both photoreceptor development and transcriptional regulation of photoreceptor-specific genes. During the terminal differentiation of cone

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photoreceptors, thyroid hormone receptor β2 (Trb2) is critical for M-opsin induction [16], whereas retinoid X receptor γ (Rxrγ) is essential for suppressing S-opsin in cone photoreceptors [17]. Retinoic acid receptor-related orphan receptor α (Rora) directly controls expression of cone opsins and arrestin3 [18]. In rod photoreceptor cells, estrogen-related receptor β (Errb) regulates expression of rod-specific genes and controls rod photoreceptor survival [19]. Recently, Pias3, an E3 SUMO ligase which is selectively expressed in developing photoreceptors, was shown to SUMOylate Nr2e3 and promote the differentiation of rod-photoreceptors [20]. In addition, Pias3 regulates expression of cone opsins by modulating Rxrg, Rora, and Trb1 [21].

During the terminal differentiation of photoreceptors, the photoreceptor axon terminal develops a highly specialized synapse, the ribbon synapse, which connects photoreceptor axonal terminals with bipolar and horizontal dendritic terminals in the outer plexiform layer (OPL) of the retina [22]. The functional ribbon synapse structure is organized by the precise assembly of presynaptic components including CtxBP2, bassoon, pikachurin, CaBP4 and Cacna1f [23-24,25,26]. Photoreceptor cells develop the photosensitive outer segments which contain molecules involved in phototransduction, such as opsins and transducins, and outer segment morphogenesis factors, such as Rom1 and Peripherin2 [27]. Outer segments are formed from the primary cilia [28]. In humans disruption of photoreceptor ciliary function causes retinal diseases including retinitis pigmentosa, Bardet-Biedl syndrome (BBS) and Nephronophthisis (NPHP) [27,29,30]. Mutations in genes encoding ciliary components including Rpgrip1p, Rp1p, and Mak cause photoreceptor degeneration and retinal dysfunction in mice [31,32,33].

To our knowledge, the Otx2 CKO mouse is the only mutant which shows defects of both rods and cones in the retina from early developmental stages. In this study, we investigated the transcriptional profile of both developing rods and cones by taking advantage of the Otx2 CKO retina.

Results
Identification of differentially expressed genes in the Otx2 CKO retina
In order to clarify the molecular role of Otx2 in transcriptional regulation during development, we investigated the expression profile of the Otx2 CKO retina compared with that of the control retina with the genotype Otx2^{+/-} /Cre-cre using microarrays at two time points, postnatal day 1 (P1) and P12. From middle late embryonic stages cones are generated with almost all of the cones formed by P0 [34]. Rods begin to form in embryonic stages whereas, photoreceptor maturation, including ribbon synapse and outer segment formation, occurs in P0 to P14 [35,36]. By P12, all photoreceptor ciliary and ribbon synaptic components including Rpgrip1, Rp1p, Ctbp2, and Pikachurin, and Cacna1f showed drastically decreased expression both in microarray and Q-PCR analysis (Fig. 3).

In contrast, the expression profile of strongly up-regulated genes was grouped into two clusters (Fig. 2B, Table 2). Most of members in the first cluster (15 genes) were up-regulated in the P12 control retina compared to that of P1. This cluster includes transcription factors Dlx1, Dlx2, Sox11 and Myc11. These genes are possibly involved in several retinal development events such as proliferation of progenitor cells, maturation of early photoreceptor precursors, and cell fate determination of progenitor cells. Consistent with this result, more probes categorized in “cell cycle” were observed in the up-regulated groups (8% at P1 and 4% at P12) compared to those in the down-regulated groups (2% at both P1 and P12) (Fig. 1). Most of members in the second cluster (22 genes) were up-regulated in the P12 control retina compared to that of P1. This group includes Car3, Spp1, Cst7 and Cot12a1. We suppose that these genes encode components associated with the increase of amacrine-like cells in the Otx2 CKO retina.

To determine whether up- or down-regulated genes in the Otx2 CKO retina are indeed enriched for sets of genes expressed selectively in individual retinal cell subtypes, we performed a statistical analysis. We compared our microarray data sets with previously reported cellular expression patterns of each retinal gene obtained from published in situ hybridization data [37]. Forty-five of 84 probes corresponding to photoreceptor-specific genes previously identified by in situ hybridization analysis were found in the group down-regulated in the Otx2 CKO retina (1049 probes; signal log ratio ≤-1.0, signal intensity ≥50). Twenty-one of 70 probes corresponding to amacrine-specific genes previously identified by in situ hybridization analysis were found in the group up-regulated in the Otx2 CKO retina (4149 probes; signal log ratio ≥+1.0, signal intensity ≥50). This data shows that photoreceptor-specific genes are strongly enriched in the group down-regulated in P12 Otx2 CKO (P<0.01), whereas, the amacrine-specific genes are enriched in the group up-regulated in the P12 Otx2 CKO retina (P<0.01).
Furthermore, we compared our microarray data with previously reported cellular expression patterns of each retinal gene obtained from published results of in situ hybridization [37,38], immunohistochemistry [39,40], and cell type–specific GFP expression in the retina using BAC transgenic mice from the GENSAT project [41] (Table 1 and 2). In addition, we searched cone- and rod-specific genes based on the microarray data from Nrl KO mice [42] (signal log ratio ≤−2.0 for “down” or ≥+2.0 for “up”). Most of the down-regulated genes in P12 Otx2 CKO were known cone, rod or pan-photoreceptor genes (Table 1), whereas, several genes up-regulated in the Otx2 CKO retina were known amacrine- or INL-expressed genes. Expression patterns of many genes in the latter group were previously unidentified in the retina (Table 2).

Expression profiles of Crx, Nrl, and N2e3-null retinas were reported previously [42,43,44,45]. We compared datasets of the Otx2 CKO retina with those of the Crx, Nrl, and N2e3-null retinas (Fig. 4, Text S1). We found that the expression of 84 probes was strongly decreased in both the Otx2 CKO and Crx KO retinas (signal log ratio ≤−2.0, Fig. 4A). This group includes both rod and cone photoreceptor genes such as Rhodopsin (Rho) and S-opsin (Opn1sw). The expression of 48 probes was markedly decreased in both the Otx2 CKO and Nrl KO retinas (signal log ratio ≤−2.0, Fig. 4B). These genes include rod-specific genes such as Pde6b and Rho. We identified 18 probes that were down-regulated in the Otx2 CKO retina but up-regulated in the Nrl KO retina, including cone photoreceptor genes such as Opal1sw, cone arrestin (Arr3) and Pde6c (Fig. 4B). Five of six genes down-regulated in the Otx2 CKO retina but up-regulated in the N2e3-null retina overlapped with the probes down-regulated in the Otx2 CKO and up-regulated in the Nrl KO retina (Fig. 4C).

Otx2 regulates expression of transcription factors involved in retinal development

To investigate the transcriptional network of Otx2-regulated genes, we first focused on the expression of transcription factors involved in retinal development. We previously showed that Crx expression was absent in the Otx2 CKO retina at E18.5, however, the expression of other transcription factors involved in photoreceptor development was not determined [4]. We selected the microarray data sets of 28 transcription factors known to be involved in retinal development (Table 3), and found that the expression of several transcription factors genes (Crx, Nrl, N2e3, Esrb, Isl1, Blimp1, Pax6 and NeuroD) was strongly reduced at P12 (signal log ratio ≤−2.1), whereas the expression level of Pax6 was increased consistent with the previous result by immunohistochemical analysis [4,46](Table 3). In addition, we found that the expression of Crx, Nrl and N2e3 was strongly reduced at P1 as well.
Table 1. Genes down-regulated in the Otx2 CKO retina (signal log ratio $\leq -5.0$).

| Probe ID   | Gene symbol | P1 WT | P12 WT | P1 CKO | P12 CKO | SLR  | Cell Type | Nrl KO |
|------------|-------------|-------|--------|--------|--------|------|-----------|--------|
| 1430817_at | Samd7       | 12.4  | 221.6  | 1.9    | 2.0    | -6.8 | Photoreceptor |        |
| 1431010_a_at | Rdh12     | 2.2   | 1411.2 | 0.4    | 19.9   | -6.2 | Photoreceptor |        |
| 1457855_at | Rbp3        | 8.4   | 190.0  | 1.2    | 2.7    | -6.1 | Photoreceptor |        |
| 1429133_at | Nxn12       | 48.6  | 1742.8 | 2.4    | 31.3   | -5.8 | Photoreceptor |        |
| 1458506_at | Gm626/Frmpd2 | 10.8  | 962.8  | 1.7    | 19.1   | -5.7 | Photoreceptor |        |
| 1451785_at | Rgrip1      | 27.6  | 653.9  | 3.8    | 13.0   | -5.6 | Photoreceptor |        |
| 1440605_at | Fscn2       | 0.3   | 128.2  | 1.0    | 2.6    | -5.6 | Rod down     |        |
| 1425757_a_at | Impg1     | 1.6   | 266.2  | 1.5    | 5.4    | -5.6 | Photoreceptor |        |
| 1419740_at | Pde6b       | 1.6   | 4001.8 | 1.2    | 83.8   | -5.6 | Rod down     |        |
| 1425306_at | BC027072/C2orf71 | 26.6  | 1241.9 | 2.2    | 26.1   | -5.6 | Photoreceptor |        |
| 1423631_at | Nr2e3       | 95.0  | 2540.0 | 7.2    | 83.8   | -5.6 | Rod down     |        |
| 1428986_at | Slc17a7     | 3.0   | 1384.0 | 2.6    | 32.0   | -5.4 | Photoreceptor |        |

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To validate these microarray results, we carried out quantitative real-time RT-PCR analysis (Q-PCR) for these genes. The Q-PCR results clearly reflect the changes observed in the microarray analysis for all of the genes we tested (Fig. 5).

To compare expression of the transcription factor genes examined with those in the Crx KO retina, we analyzed their expression level in the P12 Crx KO retina by Q-PCR. In contrast to the drastic decrease of expression in the Otx2 CKO retina, the expression of most of the transcription factors examined showed only a minor change in the Crx KO retina at P12 compared to that of the control retina from wild-type 129/SvEv. The only notable exception was Esrrb which was strongly down-regulated in the Crx KO (Fig. 5) [43]. Similar to these transcription factors, we found that the expression changes in genes encoding ciliary and ribbon synaptic components in the Crx KO retina were milder than those in the Otx2 CKO retina (Fig. 3).

### Table 2. Genes up-regulated in the Otx2 CKO retina (signal log ratio $\leq +3.0$).

| Probe Set ID | Gene Symbol | P1 WT | P12 WT | P1 CKO | P12 CKO | P12/P1 | P12WT/CKO | Cell type |
|--------------|-------------|-------|--------|--------|---------|--------|-----------|-----------|
| 1449470_at   | Dlx1        | 26.4  | 0.7    | 66.0   | 273.4   | -5.2   | 8.6       | GCL(d)    |
| 1424010_at   | Mfap4       | 23.7  | 1.5    | 10.5   | 102.5   | -4.0   | 6.1       |           |
| 1450960_at   | Ifgbl1      | 13.6  | 10.9   | 26.3   | 445.7   | -0.3   | 5.3       |           |
| 1431225_at   | Lmcd1       | 1.5   | 3.0    | 1.4    | 108.6   | 1.0    | 5.2       |           |
| 1450680_at   | Ragl1       | 6.3   | 6.0    | 3.0    | 180.6   | -0.1   | 4.9       |           |
| 1429945_at   | Klh35       | 17.4  | 16.8   | 33.4   | 392.0   | 0.0    | 4.5       |           |
| 1419202_at   | Cst7        | 2.0   | 4.5    | 1.3    | 101.0   | 1.2    | 4.5       |           |
| 1434411_at   | Col12a1     | 0.2   | 10.3   | 0.8    | 220.8   | 5.5    | 4.4       |           |
| 1426004_at   | Rnasel      | 103.1 | 11.9   | 90.2   | 234.4   | -3.1   | 4.3       | PR+INL(B) |
| 1416846_a_at | Pdzm3       | 106.1 | 55.7   | 103.4  | 878.1   | -0.9   | 4.0       |           |
| 1439426_x_at | Lyz1        | 50.6  | 11.9   | 18.5   | 182.9   | -2.1   | 3.9       |           |
| 1453125_at   | Sox11       | 344.6 | 60.7   | 255.6  | 845.5   | -2.5   | 3.8       | PR+INL(B) |
| 1438245_at   | AK034813    | 77.5  | 96.7   | 68.2   | 1295.1  | 0.3    | 3.7       |           |
| 1418139_at   | Dcx         | 116.4 | 59.5   | 106.0  | 797.1   | -1.0   | 3.7       | HC(W)     |
| 1448877_at   | Dlk2        | 17.7  | 9.0    | 36.5   | 119.8   | -1.0   | 3.7       | INL+GCL(d)|
| 1449343_at   | Car3        | 11.9  | 53.5   | 11.3   | 700.0   | 2.2    | 3.7       | AM(G)     |
| 1449254_at   | Spp1        | 5.3   | 35.8   | 2.0    | 412.2   | 2.7    | 3.5       |           |
| 1428184_at   | 3110035E14Rik| 66.3 | 21.5   | 45.4   | 237.6   | -1.6   | 3.5       |           |
| 1460009_at   | Ier5        | 22.6  | 43.7   | 32.4   | 442.5   | 1.0    | 3.3       | AM+PR+GCL(G)|
| 1436364_x_at | Nfix        | 87.8  | 376.3  | 82.3   | 3784.4  | 2.1    | 3.3       | BP+GCL(G) |
| 1442214_at   | AK141047    | 59.0  | 63.3   | 44.6   | 635.9   | 0.1    | 3.3       |           |
| 1437156_at   | Necab1      | 4.0   | 26.5   | 3.6    | 264.5   | 2.7    | 3.3       |           |
| 1456261_at   | Sh3kb1      | 5.4   | 11.4   | 4.8    | 109.2   | 1.1    | 3.3       |           |
| 1448891_at   | Fcrls       | 3.9   | 12.9   | 6.0    | 122.4   | 1.7    | 3.2       |           |
| 1434777_at   | Mycl1       | 108.0 | 28.2   | 100.9  | 264.9   | -1.9   | 3.2       |           |
| 1435165_at   | Cntn2       | 9.0   | 20.5   | 25.9   | 186.8   | 1.2    | 3.2       |           |
| 1416034_at   | Cd24a       | 580.5 | 176.8  | 592.6  | 1602.4  | -1.7   | 3.2       | MG(B)     |
| 1449130_at   | Cd1d1       | 150.3 | 40.8   | 116.0  | 367.1   | -1.9   | 3.2       |           |
| 1447020_at   | AK042819    | 2.8   | 11.6   | 2.5    | 103.1   | 2.1    | 3.1       |           |
| 1438072_at   | AK042460    | 63.3  | 77.1   | 45.6   | 662.9   | 0.3    | 3.1       |           |
| 1433236_at   | 3300001A09Rik| 26.3 | 13.0   | 6.7    | 110.5   | -1.0   | 3.1       |           |
| 1457261_at   | A930025H08Rik| 4.5  | 12.0   | 4.9    | 100.6   | 1.4    | 3.1       |           |
| 1439808_at   | A130900K04Rik| 19.1 | 24.6   | 26.9   | 204.9   | 0.4    | 3.1       |           |
| 1448288_at   | Nfib        | 33.4  | 41.4   | 24.3   | 333.9   | 0.3    | 3.0       |           |
| 1448194_a_at | H19         | 191.6 | 47.4   | 188.1  | 379.1   | -2.0   | 3.0       |           |
| 1457227_at   | A1843755    | 8.9   | 26.3   | 7.2    | 207.1   | 1.6    | 3.0       |           |

PR, photoreceptor cell; AM, amacrine cell; BP, bipolar cell; MG, Müller glia; GCL, cells in ganglion cell layer; HC, horizontal cell; INL, cells in inner nuclear layer. d, de Melo et al., 2003; W, Wakabayashi et al., 2008; B, Blackshaw et al., 2004; G, GENSAT database.
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Identification of retinal disease candidate genes

Human homolog mutations of many genes with photoreceptor-associated expression have been shown to be associated with retinal diseases including RP [27]. Since many of the Otx2 CKO
down-regulated genes at P12 were photoreceptor-associated genes (Table 1), we supposed that mutations of human homologs of these down-regulated genes may be responsible for retinal degeneration diseases. To identify retinal disease candidate genes, we determined the chromosomal loci of human homologs of these down-regulated genes and compared these loci with the mapped loci of various hereditary retinal diseases (RetNet, the Retinal Information Network, http://www.sph.uth.tmc.edu/RetNet/). We found that three human retinal disease loci were mapped in close proximity to the markedly down-regulated genes in the Otx2 CKO retina. Human CCDC126 is located on 7p15.3 where dominant cystoid macular dystrophy has been mapped [47]. Human PITPNM1 is located on 11q13 where autosomal dominant neovascular inflammatory vitreoretinopathy was reported [48].

Central areolar choroidal dystrophy was confined to a critical region, an interval of approximately 2.4 Mb (5 cM) flanked by...
The main goal of this study is to obtain information and gain insights into the transcriptional network in photoreceptor development regulated by Otx2. Thus, we examined the retinal gene expression profiles of the Otx2 CKO retina at P1 and P12, and identified significant changes in the expression of genes encoding transcription factors, components of the cilium and photoreceptor development.

**Otx2 regulates the transcriptional network in photoreceptor development**

The main goal of this study is to obtain information and gain insights into the transcriptional network in photoreceptor development regulated by Otx2. Thus, we examined the retinal gene expression profiles of the Otx2 CKO retina at P1 and P12, and identified significant changes in the expression of genes encoding transcription factors, components of the cilium and photoreceptor development.

**Table 3. Expression of transcription factors involved in retinal development in the Otx2 CKO retina.**

| Probe ID         | Gene symbol | P1 WT  | P1 CKO | SLR  | P12 WT  | P12 CKO | SLR  |
|------------------|-------------|--------|--------|------|---------|---------|------|
| 1418705_at       | Cx            | 253.7  | 15.6   | -4.0 | 2932.7  | 65.3    | -5.5 |
| 1423631_at       | Nr2e3        | 95.0   | 7.2    | -3.7 | 2540.0  | 57.4    | -5.5 |
| 1450946_at       | Nrl          | 293.9  | 18.6   | -4.0 | 6073.3  | 191.9   | -5.0 |
| 1436926_at       | Esrb         | 5.1    | 3.3    | -0.6 | 6320.0  | 24.3    | -4.7 |
| 1450723_at       | Isl1         | 196.5  | 219.1  | 0.2  | 1542.2  | 243.0   | -2.7 |
| 1420425_at       | Prdm1/Blimp1 | 202.4  | 23.0   | -3.1 | 188.5   | 34.4    | -2.5 |
| 1426413_at       | NeuroD       | 296.1  | 96.7   | 3.2  | 396.7   | 93.9    | 0.6  |
| 145115_at        | Plas3        | 39.1   | 24.7   | -1.3 | 860.0   | 199.7   | -2.1 |
| 1419628_at       | Chxl0        | 423.7  | 411.8  | 0.0  | 2082.0  | 927.8   | -1.2 |
| 1424034_at       | Rora         | 49.2   | 33.8   | -0.5 | 411.0   | 291.4   | -0.5 |
| 1418558_at       | Rax          | 168.4  | 117.3  | 0.3  | 309.0   | 222.1   | -0.5 |
| 1418782_at       | Rxrg         | 24.7   | 17.3   | -0.5 | 41.1    | 32.7    | -0.3 |
| 1422202_at       | Thrb         | 4.8    | 2.6    | -0.9 | 40.9    | 34.0    | -0.3 |
| 1450796_at       | Math5        | 32.8   | 21.4   | 0.6  | 32.6    | 3.1     | 0.0  |
| 1455799_at       | Rorb         | 465.0  | 387.2  | -0.3 | 747.8   | 765.4   | 0.0  |
| 1418102_at       | Hes1         | 111.4  | 94.5   | 0.6  | 90.1    | 140.0   | 0.0  |
| 1437588_at       | Bm3b/Pou4F2  | 96.1   | 123.3  | 0.4  | 155.9   | 284.4   | 0.9  |
| 1427253_at       | Six3         | 190.5  | 181.7  | -0.1 | 299.7   | 568.2   | 0.9  |
| 1421336_at       | Prox1        | 18.6   | 22.8   | 0.3  | 199.1   | 414.2   | 1.1  |
| 1419408_at       | Six6         | 237.1  | 238.1  | 0.0  | 335.8   | 774.0   | 1.2  |
| 1418633_at       | Notch        | 63.7   | 61.7   | 0.0  | 57.4    | 141.2   | 1.3  |
| 1418054_at       | Math3        | 165.5  | 47.0   | -1.8 | 504.4   | 1277.6  | 1.3  |
| 1419271_at       | Pax6         | 723.9  | 723.6  | 0.0  | 1042.4  | 3083.1  | 1.6  |
| 1423146_at       | Hes5         | 145.0  | 155.6  | 0.1  | 46.7    | 157.4   | 1.8  |
| 1422839_at       | Neurog2      | 216.2  | 277.0  | 0.4  | 48.4    | 296.6   | 2.6  |
| 1437086_at       | Mash1        | 392.6  | 266.4  | 0.6  | 15.9    | 119.4   | 2.9  |
| 1448877_at       | Dlx2         | 17.7   | 36.5   | 1.0  | 9.0     | 119.8   | 3.7  |
| 1449470_at       | Dlx1         | 26.4   | 66.0   | 1.3  | 0.7     | 273.4   | 8.6  |

Figure 3. Expression of genes encoding photoreceptor ciliary and ribbon synaptic components in the Otx2 CKO and Crx KO retina. Expression levels of genes encoding photoreceptor ciliary and ribbon synaptic components in the control, Otx2 CKO and Crx KO retinas at P12 were analyzed by Q-PCR. Expression levels of selected genes were normalized to the expression levels of a housekeeping gene, Gapdh. Primer sequences for PCR were shown in Table S1. The mean of the value of control at P12 was set as 1.0. Error bars show the SD (n = 3). *, P<0.03.

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Figure 4. Comparison gene expression profiles with the Otx2 CKO retina. (A-C) Microarray analysis datasets from Crx (A), Nrl (B), Nr2e3 (C) KO retinas were compared with that from the Otx2 CKO retina at P12. We identified 367 down-regulated probes in the Otx2 CKO retina (signal log ratio ≤ -2.0, signal intensity ≥ 74). We used datasets from previous microarray analysis of the Crx, Nrl and Nr2e3-null retinas at P21 with the following results: 154 probes down-regulated in the Crx KO retina (signal log ratio ≤ -2.0, signal intensity ≥ 352), 63 probes up-regulated in the Crx KO retina (signal log ratio ≥ +2.0, signal intensity ≥ 345), 95 probes down-regulated in the Nrl KO retina (signal log ratio ≤ -2.0, signal intensity ≥ 354), 186 probes up-regulated in the Nrl KO retina (signal log ratio ≥ +2.0, signal intensity ≥ 347), 9 probes down-regulated in the Nr2e3-null (d7) retina (signal log ratio ≤ -2.0, signal intensity ≥ 354), 37 probes up-regulated in the Nr2e3-null retina (signal log ratio ≥ +2.0, signal intensity ≥ 358). The numbers of probes in each category were indicated.

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Figure 5. Comparison gene expression profiles with the Otx2 CKO retina and identified significant changes in the expression of genes encoding transcription factors, components of the cilium and photoreceptor development.
Figure 5. *Otx2* regulates expression of transcription factors involved in retinal development. Expression levels of selected transcription factor genes in the control (at P1 and P12), Otx2 CKO (at P1 and P12) and Crx KO (at P12) retinas were analyzed by Q-PCR. Expression levels of selected genes were normalized to the expression levels of a housekeeping gene, *Gapdh*. The mean of the value of each control at P12 was set as 1.0. Error bars show the SD (n = 3). *, P < 0.03.

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ribbon synapse. Our microarray analysis and Q-PCR validation of the Otx2 CKO retina revealed strong down-regulation of multiple transcription factors involved in photoreceptor development, including Crx, Nrl, Esrrb, NeuroD, Isl1, Blimp1, Pias3 and Nr2e3. These data support the hypothesis that Otx2 organizes a transcription factor network in photoreceptor development. A previous study showed that a considerable amount of Nrl expression remains in the Crx KO retina, and, similarly, Crx expression remains in the Nrl KO retina [43,44], suggesting that Otx2 regulates the expression of Nrl and Crx by parallel pathways. Otx2 directly regulates Crx expression through binding to cis-regulatory elements on the Crx promoter region [4]. Does Otx2 directly regulate other transcription factors involved in photoreceptor development? The 2.4 kb fragment in the 5′ region of the Nrl locus is essential for its expression in photoreceptors and contains Crx/Otx2 binding sites [43]. Since Otx2 and Crx can bind to the same DNA consensus sequence [5,50], it is possible that Otx2 binds to these cis-regulatory elements and directly regulates Nrl expression. In contrast, we found that Esrrb expression is almost abolished in both the Otx2 CKO and the Crx KO retinas, showing that Esrrb is likely to be regulated directly by Crx and indirectly by Otx2. Similarly, Nr2e3 expression in the Nrl KO was almost abolished [12], suggesting that Nr2e3 expression is directly regulated by Nrl and indirectly regulated by Otx2. We found that expression of Isl1, NeuroD, Blimp1, and Pias3 was markedly decreased in the P12 Otx2 CKO retina, however, considerable amounts of expression remained in the Nrl KO and Crx CKO retinas [43]. This result suggests that Isl1, NeuroD, Blimp1, and Pias3 are the direct targets of Otx2. We searched for the Otx2/Crx-binding consensus sequence “TAATC” within 5 kb of the 5′ upstream region of each gene, and found that each of the 5′ regions of Isl1, NeuroD and Pias3 genes contains clusters of the Otx2/Crx-binding sites (2.3 to 2.7 kb, 5 sites for Isl1; 4.2 to 4.9 kb, 4 sites for NeuroD; and 1.3 to 2.0 kb, 4 sites for Pias3). Otx2 may directly regulate expression of these transcription factors through these Otx2/Crx-binding sequence clusters. In contrast to the down-regulation of transcription factors involved in photoreceptor development, we observed a 3.0-fold increase of Pax6 expression in the P12 Otx2 CKO retina. This is probably due to the increase of the amacrine cell population, since amacrine cell markers including Glyt1 and Gad65 also increased in the Otx2 CKO at a similar level (3.0-fold increase for Glyt1, 3.5-fold increase for Gad65). The result from microarray analysis is consistent with that from immunohistochemical analysis of the Otx2 CKO retina [4].

We found that photoreceptor-specific genes are strongly enriched in the group of down-regulated probes in the P12 Otx2 CKO retina, whereas amacrine-specific genes are enriched in the group of up-regulated probes in the P12 Otx2 CKO retina. Although many of down-regulated genes in the P12 Otx2 CKO retina were known photoreceptor genes, the expression patterns of several genes, including BC027072/C2orf71, LOC100048701, A930003A15Rik, and 2610034M16Rik, have not been analyzed in the retina. These genes are strong candidates for photoreceptor-specific genes. Expression of 2610034M16Rik was decreased in the Nrl KO retina, suggesting that this gene is expressed in rod photoreceptors. In contrast to the high enrichment of photoreceptor-specific genes in the Otx2 down-regulated probes, amacrine genes were enriched in the group up-regulated in the Otx2 CKO retina. This group seems to contain not only amacrine associated genes but also various genes from cell types including Muller glia and cells in ganglion cell layer. This result may reflect the increase of aberrant “amacrine-like cells” in the Otx2 CKO retina.

We found that genes encoding synaptic components (e.g., CaBP4, Cacna1f and Pikaehum)+ and ciliary components (e.g., Rpgp1 and Rpl) were also significantly downregulated in the Otx2 CKO retina. We previously demonstrated that rhodopsin- and Crx-positive cells were absent in the Otx2 CKO retina [4], however, there is a possibility that the increased amacrine-like cells converted from photoreceptors in the Otx2 CKO retina still express photoreceptor-related molecules. Our findings in the present study excluded this possibility and support the idea that Otx2 executes a genetic program on photoreceptor cell fate determination and differentiation. Thus, the transcriptional profile data in this study will be a useful resource to identify genes involved in development and maintenance of both rods and cones.

Previously, we showed that Otx2 is essential for bipolar cell development [31]. Consistent with this, we observed significant decreases in bipolar genes, including Bikhh4, Otx10, Calb3 and Pep2, in the Otx2 CKO retina.

The morphological features of rod and cone photoreceptor ribbon synaptic terminals are different; rod photoreceptors form small synaptic terminals with a single ribbon, whereas, cone photoreceptors form larger terminals containing several ribbons with a shorter active zone [24]. These structural differences might be derived from differences of synaptic components. Transcription...
factors involved in photoreceptor terminal differentiation such as Nrl and Nr2e3 may regulate the expression of rod- or cone-specific synaptic components and contribute to the formation of different structures between cone and rod photoreceptors. Compared with the previous microarray studies, we identified 48 down-regulated probes in both the Otx2 CKO and Nrl KO retinas, while at the same time, 18 probes were down-regulated in the Otx2 CKO but up-regulated in the Nrl KO retina. These data can be a useful resource for finding different mechanisms between cone and rod photoreceptor formation, including ribbon synapse structures.

Identification of retinal disease candidate genes from Otx2 downstream genes

The expression profiles of several transcription factors which were shown to be critical for photoreceptor terminal differentiation have been analyzed by microarray or SAGE analysis. The expression profile of the Crx KO retina was analyzed using both cDNA microarray and SAGE [38,43,52]. Analyses of the expression profiles of Nrl KO, Nr2e3 KO or Nrl & Nr2e3 double KO retinas were performed using microarrays [42,43,44,45]. Lack of Crx does not affect photoreceptor cell fate but does result in abnormal photoreceptor morphogenesis [7]. Lack of either Nrl or Nr2e3 causes photoreceptor subtype conversion from rod photoreceptor to S-cone photoreceptors [12,14]. Since deletion of Otx2 leads to a total loss of photoreceptors in the retina, by comparing expression profiles between the Otx2 CKO retina and control retina, we were able to identify photoreceptor-associated genes more clearly than by using other mutant retinas. Comparing the expression profiles between the Otx2 CKO and other mutant mice may provide novel insights into genetic transcriptional networks in photoreceptor development. Furthermore, we identified several retinal disease candidate genes among the genes down-regulated in the Otx2 CKO retina based on information from RetNet’s mapped retinal disease loci. Linking information from other databases to the set of Otx2 down-regulated genes presented in our study may give unexpected insights into photoreceptor biology. For example, proteome analysis of purified photoreceptor sensory cilium revealed that this complex contains 1,968 proteins [53]. By comparing our data and the data from other studies, a novel insight on the mechanisms of retinal disease might be obtained in the future.

Materials and Methods

Animals

The Otx2 CKO and Crx KO mice were generated in our previous studies [4,7]. All procedures conformed to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and these procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments and the Animal Research Committee of Osaka Bioscience Institute (approval ID 10-401). Mice were housed in a temperature-controlled room with a 12 h light/dark cycle. Fresh water and rodent diet were available at all times.

Microarray profiling

The P1 or P12 retinas of mice were dissected. Total RNA (5 μg) of the retina was isolated using TRIzol reagent (Invitrogen) and converted to cDNA using One-Cycle cDNA synthesis kit (Affymetrix) according to the manufacturer’s instruction. Biotin-labeled cRNA was prepared using IVT labeling kit and hybridized to GeneChip mouse genome 430 2.0 array (Affymetrix). Signal intensity was determined using GeneChip Operating Software 1.4. Microarray expression data are MIAME compliant and have been deposited in a MIAME compliant database (GEO accession number GSE21900).

Q-PCR

The P1 or P12 retinas of mice were dissected. Total RNA (1 μg) of the retina was isolated using TRIzol reagent (Invitrogen) and converted to cDNA using Superscript II RTase (Invitrogen). Real time qPCR was performed using Cyber Green ER qPCR Super MIX (Invitrogen) and Thermal Cycler Dice Real Time System single MRQ TIP80 (Takara) according to the manufacturer’s instruction. Quantification was performed by Thermal Cycler Dice Real Time System software Ver. 2.0 (Takara). To amplify the gene fragments, we used primers as listed in Table S1.

Supporting Information

Table S1 Primers for Q-PCR analysis.

Text S1 Probe IDs and gene symbols for each group in Figure 4.

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Author Contributions

Conceived and designed the experiments: T. Furukawa. Performed the experiments: YO KK SS YM TC AO TM. Analyzed the data: YO KK SS T. Wrote the paper: YO T. Furukawa. Supervised the experiments: T. Fujikado T. Furukawa.
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