Onecut1 Is Essential for Horizontal Cell Genesis and Retinal Integrity

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Horizontal cells are interneurons that synapse with photoreceptors in the outer retina. Their genesis during development is subject to regulation by transcription factors in a hierarchical manner. Previously, we showed that Onecut 1 (*Oc1*), an atypical homeodomain transcription factor, is expressed in developing horizontal cells (HCs) and retinal ganglion cells (RGCs) in the mouse retina. Herein, by knocking out *Oc1* specifically in the developing retina, we show that the majority (~80%) of HCs fail to form during early retinal development, implying that *Oc1* is essential for HC genesis. However, no other retinal cell types, including RGCs, were affected in the *Oc1* knock-out. Analysis of the genetic relationship between *Oc1* and other transcription factor genes required for HC development revealed that *Oc1* functions downstream of *FoxN4*, in parallel with *Ptf1a*, but upstream of *Lim1* and *Prox1*. By *in utero* electroporation, we found that *Oc1* and *Ptf1a* together are not only essential, but also sufficient for determination of HC fate. In addition, the synaptic connections in the outer plexiform layer are defective in *Oc1*-null mice, and photoreceptors undergo age-dependent degeneration, indicating that HCs are not only an integral part of the retinal circuitry, but also are essential for the survival of photoreceptors. In sum, these results demonstrate that *Oc1* is a critical determinant of HC fate, and reveal that HCs are essential for photoreceptor viability, retinal integrity, and normal visual function.

Introduction

In the vertebrate retina, there are six types of neurons (rod and cone photoreceptors, horizontal cells (HCs), bipolar cells, amacrine cells, and retinal ganglion cells (RGCs)), and one glial cell type (Müller cells). Each of these cell types plays a distinct role in the process of vision (Wässle and Boycott, 1991; Masland, 2001).

During development, all of the retinal cell types originate from a common pool of multipotent progenitor cells (Cepko et al., 1996). Both intrinsic and extrinsic pathways are involved in the process, and gene regulation is a major intrinsic mechanism controlling the formation of individual retinal cell types (Agathocleous and Harris, 2009; Xiang, 2013). However, a comprehensive understanding of the underlying gene regulatory pathways is still lacking. Many key regulators remain to be identified, and their functions to be characterized. Here we report the results of our investigation into the roles of a novel regulator, Onecut1 (*Oc1*; also known as *Hnf6*), in retinal development, and its function in the formation of HCs.

HCs are second-order neurons that process visual information laterally within the outer plexiform layer (OPL; Thoreson and Mangel, 2012). They receive input from rod and cone photoreceptors (Cervetto and MacNichol, 1972), and provide inhibitory input to photoreceptors and bipolar cells (Wu, 1992). HCs form during the first wave of retinal neurogenesis, which starts at embryonic day 11 (E11) and peaks at E14.5 in the mouse (Young, 1985; Cepko et al., 1996). Several transcription factors, including *FoxN4*, *Prox1*, *Ptf1a*, *Lim1*, *Sall3*, *AP-2α*, and *AP-2β*, have been reported to play roles in HC development (Dyer et al., 2003; Li et al., 2004; Fujitani et al., 2006; Nakhai et al., 2007; Pochè et al., 2007; de Melo et al., 2011; Bassett et al., 2012). Nevertheless, it

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remains unclear how and when fated HCs arise from the retinal progenitor pool and what factors determine the HC fate.

Oc1 belongs to the onecut transcription factor family, members of which have been shown to regulate development of the liver, pancreas, and the immune system (Lemaigre et al., 1996; Samadani and Costa, 1996; Landry et al., 1997; Jacquemin et al., 2003; Margagliotti et al., 2007; Furuno et al., 2008), as well as the CNS (Francius and Clotman, 2010; Espana and Clotman, 2012a, b; Roy et al., 2012; Wu et al., 2012). All three mouse *Onecut* factors (*Oc1*, *Oc2*, and *Oc3*) are also expressed in the developing retina, but only *Oc1* and *Oc2* are expressed at high levels (Wu et al., 2012). To investigate the role of *Oc1* in retinal development, we specifically inactivated *Oc1* in the developing mouse retina by Cre-mediated recombination. Our results indicate that *Oc1* is essential for HC development and determines HC fate in collaboration with *Ptf1a*. Further, we show that when the number of HCs is severely reduced, the synaptic connections between photoreceptors and bipolar cells in the OPL are abnormal, and that photoreceptors degenerate as the mice age, suggesting a previously unrecognized role of HCs in maintaining synaptic structure, photoreceptor viability, and retinal integrity.

Materials and Methods

Animals. The floxed *Oc1* allele (*Oc1^{fllox}*) and the *Six3-cre* transgenic line have been described previously (Furuta et al., 2000; Zhang et al., 2009). The *Ptf1a^{-/-}* (*Ptf1a^{cre}*) mice (Fujitani et al., 2006) were obtained from MMRRC-UNC (Stock number: 000435-UNC). All lines were maintained in C57BL/6 $\times$ 129 mixed background. All procedures using mice conform to the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of Roswell Park Cancer Institute and University at Buffalo.

Immunofluorescence labeling. Immunofluorescence labeling of frozen retinal sections followed our previously published procedure (Wu et al., 2012). Whole-mount immunostaining of the retina followed a protocol used by Kay et al., (2011). In cases where the primary antibody was from mouse, the sections were first blocked by donkey anti-mouse IgG Fab fragment (50 μ g/ml Jackson ImmunoResearch, 715-007-003) to reduce the background from endogenous mouse IgG. The sources and dilutions of antibodies against Pou4f2, HA tag, *Onecut 1* (*Hnf6*), *Onecut 2*, calbindin, *Isl1*, *Lim1* (mouse), *Chx10*, *Pax6*, *syntaxin 1*, *NF160*, protein kinase C α (*PKC α*), *FoxN4*, and rhodopsin were as reported by us in previous publications (Li et al., 2004; Mu et al., 2005, 2008; Fu et al., 2006, 2009; Wu et al., 2012). Other antibodies used in these studies include: rabbit anti-*Lim1* (1:100; Millipore, AB3200), mouse anti-*Prox1* (1:200; Millipore, MAB5654), rabbit anti-*Sox9* (1:500; Millipore, AB5535), rabbit anti-*Pgp9.5* (1:500; Millipore, AB1761), rabbit anti-cone arrestin (*CAR*) (1:500; Millipore, AB15282), guinea pig anti-doublecortin (*Dcx*) (1:500; Millipore, AB2253), guinea pig anti-*Ptf1a* (1:400; Dr. Jane Johnson, Utah Southwestern Medical Center), rabbit anti-*Ptf1a* (1:800; Dr. Helena Edlund, Umeå University), mouse anti-Bassoon (1:100; Enzo Life Sciences, ADI-VAM-PS003-D), and rabbit anti-GFP (1:150; Abcam, AB290). Alexa Fluor 488-conjugated and Alexa Fluor 546-conjugated secondary antibodies against IgGs of various species (Invitrogen) were used at 1:800 dilution. When necessary, the nuclei were counterstained with propidium iodide. Images were collected using a Leica TCS SP2 confocal microscope. To collect whole-mount images, image stacks of equal thickness in the z-plane were collected for both wild-type and *Oc1*-null retinas.

Cell counting. Cell counting was performed as described previously (Mu et al., 2005; Wu et al., 2012). Briefly, retinal sections from desired developmental stages were stained with cell type-specific antibodies as described above, counterstained with propidium iodide, and total cells and marker-positive cells were counted on arbitrary retinal section lengths in the central retinal regions. For each cell type, three to four sections from at least two individual animals were counted. To count cells in the whole-mount retinas, cells from corresponding regions of

wild-type and *Oc1*-null retinas were counted. Statistical significance was assessed by unpaired Student's *t* test, assuming equal variance with a two-tailed distribution; the threshold for statistical significance was set at $p < 0.05$.

Histology. Tissue processing and hematoxylin and eosin (H&E) staining of mouse retinal sections were performed as described previously (Mu et al., 2008). Briefly, eyes from wild-type and mutant mice were enucleated, fixed in buffered mixed aldehydes (3% paraformaldehyde and 2% glutaraldehyde, in PBS, pH 7.4), and embedded in paraffin. The tissues were then sectioned (7 μ m in thickness), dewaxed, and stained with H&E sequentially. Images were collected using a Nikon Eclipse 80i microscope using a SPOT RT3 digital camera (Diagnostic Instruments).

In utero electroporation. DNA constructs expressing *Oc1* or *Ptf1a* under the CAG promoter were made by replacing the GFP coding sequence with *Oc1* or *Ptf1a* full-length cDNA in the previously reported pCAG-GFP plasmid (Addgene; Plasmid 11150) (Matsuda and Cepko, 2004). *In utero* electroporation was performed following a previously published procedure (Petros et al., 2009). Briefly, pregnant female mice were anesthetized at E13.5 using vaporized isoflurane (2–5%) mixed with oxygen. A 2 cm vertical incision was made on the midline of the abdomen to expose the uterine horns, and 0.5 μ l of DNA solution (2.5 μ g/ μ l of each DNA construct, 0.5 μ g/ μ l pCAG-GFP, and 0.025% Fast Green Dye) was injected through the uterine wall and amniotic sac into an embryonic retina with a fine-tipped micropipette. After injection, wet electroporation paddles were placed on the sides of the embryo head, and five 40 V, 50 ms square pulses were delivered by an electroporation device (ECM 830; Harvard Apparatus). The embryonic chain was then placed back into the abdominal cavity, the peritoneum and the skin were then closed, and the embryos were allowed to develop further to E17.5, and analyzed by immunofluorescence.

Transmission electron microscopy. Mouse eyes were fixed with buffered mixed aldehydes, osmicated, serially dehydrated, and embedded in plastic resin in preparation for transmission electron microscopy (TEM) analysis, as described previously (Ding et al., 2004; Stricker et al., 2005). Thin sections (600–800 Å) were obtained with an ultramicrotome (Reichert–Jung Ultracut E Microtome; American Instruments) using a diamond knife, collected onto copper 75/300 mesh grids (Electron Microscopy Sciences), and stained with 2% (w/v) uranyl acetate and Reynolds' lead citrate. Sections were viewed using a JEOL 100CX electron microscope (JEOL) at an accelerating voltage of 60 keV, and digital images were collected and stored on a computer for subsequent viewing and analysis.

Electroretinogram recording. Electroretinogram (ERG) recording was performed as previously reported (Umino et al., 2012). In brief, dark-adapted mice were placed in a light-proof cage and anesthetized with 60 mg/kg pentobarbital (Nembutal; Lundbeck). Pupils were dilated with a couple of drops of 1% tropicamide, corneas were kept moist with 0.3% glycerine/1.0% propylene glycol, and body temperature was maintained at 37°C with a heating pad. ERGs were recorded using the Espion E2 system and a ColorDome Ganzfeld stimulator (Diagnosys) in response to brief LED flashes (520 nm). The scotopic (dark-adapted) b-wave amplitude was measured from the a-wave trough to the peak of the corneal-positive b-wave. For light-adapted ERG, retinas were exposed to a steady adapting background (520 nm) of 10 cd/m². The number of photoisomerizations/rod/s produced by the background was estimated as described previously (Umino et al., 2012).

Results

Retina-specific deletion of *Oc1*

To delete *Oc1* specifically in the developing retina, we crossed the *Oc1^{fllox}* line with the *Six3-Cre* transgenic mice. The *Oc1^{fllox}* allele has two *loxP* sites flanking the first exon, which encodes the N-terminal part of the protein including the “cut” domain, which is the main DNA binding domain. Cre-mediated recombination leads to the deletion of this exon and results in a null allele (Zhang et al., 2009). The *Six3-Cre* transgene is specifically expressed in the neural retina, starting from E9, a time when retinal neurogenesis has yet to begin and *Oc1* expression in the retina has not yet

Figure 1. *Oc1* is efficiently deleted in the retina by *Six3-Cre* and its deletion leads to defects in the OPL. **A–D**, Immunofluorescence labeling using anti-*Oc1* antibodies on *Oc1*^{flox/flox} and *Oc1*^{flox/flox}; *Six3-Cre* (*Oc1*-null) retinal sections from E12.5 and E14.5. *Oc1* shows identical expression patterns in *Oc1*^{flox/flox} retinas to those previously reported in wild-types at E12.5 (**A**) and E14.5 (**C**). In *Oc1*^{flox/flox}; *Six3-Cre* retinal sections, almost no *Oc1* could be detected at both E12.5 and E14.5. **E, F**, H&E staining of *Oc1*^{flox/flox} and *Oc1*^{flox/flox}; *Six3-Cre* retinal sections from P16 mice. Compared with the wild-type control retina, the OPL of the *Oc1*-null retina is greatly reduced. All other cell layers are comparable to those in the control retina. Scale bars: (in **A**), **A–D**, 150 μ m; (in **E**), **E, F**, 75 μ m.

initiated (Cepko et al., 1996; Furuta et al., 2000; Wu et al., 2012). To examine the efficiency of deletion of *Oc1* by *Six3-cre*, we examined *Oc1* expression by immunofluorescence on *Oc1*^{flox/flox} and *Oc1*^{flox/flox}; *Six3-cre* retinal sections at E12.5 and E14.5 (Fig. 1*A–D*). Whereas *Oc1* was readily detectable in control *Oc1*^{flox/flox} retinas as previously reported for wild-type retinas (Wu et al., 2012), there was essentially no signal in *Oc1*^{flox/flox}; *Six3-cre* retinas at either E12.5 or 14.5, indicating that *Oc1* was efficiently deleted in the developing retina at an early stage. We refer to *Oc1*^{flox/flox}; *Six3-cre* as *Oc1*-null (*Oc1*^{−/−}) hereafter. Adult *Oc1*-null mice showed no gross health issues and were fertile. Since *Oc1*^{flox/flox} mice exhibit the wild-type phenotype, both *Oc1*^{flox/flox} and true wild-type littermates were used as controls and will be referred to as wild-type (*Oc1*^{+/+}) hereafter. To first assess the consequences of *Oc1* deletion, we examined postnatal day 16 (P16) retinas histologically (Fig. 1*E,F*). Retinas from *Oc1*-null mice exhibited normal cell layer lamination; the outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) were of similar thickness to the corresponding layers of control retinas, and showed no overt morphological abnormalities. However, the OPL, which is composed of photoreceptor terminals and processes from HCs and bipolar cells, was markedly reduced and even absent in some locations (Fig. 1*F*). Since HCs are the only *Oc1*-expressing cell type that extends processes into the OPL (Wässle and Boycott, 1991; Masland, 2001; Wu et al., 2012), it was likely that changes in the OPL of the *Oc1*-null retina reflected effects on HCs.

Effect of *Oc1* deletion on retinal cell differentiation

Next, we performed immunofluorescence labeling using cell type-specific markers to examine whether the different retinal cell types formed normally at P16. These markers included rhodopsin (rods; Fig. 2*A,B*), CAR (cones; Fig. 2*C,D*), Chx10, and PKC α (bipolar cells; Fig. 2*E,F*; data not shown), Pax6 (amacrine cells and RGCs; Fig. 2*G,H*), syntaxin I (amacrine cells; Fig. 2*I,J*), Sox9 and vimentin (Müller cells; Fig. 2*K–N*), Pou4f2 (RGCs; Fig. 2*O,P*), and calbindin (HCs and amacrine cells; see Fig. 4*A,B*). We observed no obvious changes in the locations and numbers of rods, cones, bipolar cells, Müller cells, amacrine cells, or RGCs in *Oc1*-null retinas compared with wild-type retinas (Fig. 2*A–P*). All these cell types had normal morphology, and cell counting confirmed that their individual proportions to the total cell number were not impacted by deletion of *Oc1* (Fig. 2*Q*). However, consistent with the observed thinning of the OPL in *Oc1*-null retinas (Fig. 1*F*), the number of HCs detected by calbindin immunostaining was significantly reduced (Figs. 2, 4*B*; see details below).

Since *Oc1* is also highly expressed in nascent RGCs and their precursors (Wu et al., 2012), we examined the expression of six RGC markers at E14.5, a time of peak RGC production, to ascertain whether or not RGCs were affected by deletion of *Oc1* (Fig. 3). These markers included Pou4f2 (Fig. 3*A,B*), Isl1 (Fig. 3*C,D*), *Oc2* (Fig. 3*E,F*), NF160 (Fig. 3*G,H*), *Dcx* (Fig. 3*I,J*), and Pgp9.5 (Fig. 3*K,L*). With the exception of *Oc2*, these markers showed no change in their expression patterns and levels in *Oc1*-null retinas compared with wild-type controls. In the case of *Oc2*, whereas its expression pattern did not change, there was a marked increase in its expression level in the GCL (Fig. 3*E,F*), suggesting that *Oc2* may compensate and function redundantly with *Oc1*. These observations indicate that deletion of *Oc1* did not affect the development and maintenance of RGCs. Therefore before P16, when retinal cell generation is essentially complete (Young, 1985; Cepko et al., 1996), only HCs were affected in the *Oc1*-null retinas.

Oc1 is required for HC development

Calbindin is expressed by HCs, which are located in the INL adjacent to the OPL, and by subsets of amacrine cells and RGCs found in the inner INL and GCL, respectively (Fig. 4*A*; Pochet et al., 1989). Deletion of *Oc1* affected HCs, but not amacrine cells at P16 (Fig. 4*B*). The number of HCs in *Oc1*-null retinas was reduced by 75% ($\pm 10.2\%$, $n = 3$) compared with wild-type retinas (Fig. 2*Q*). To further investigate HC deficiency in the *Oc1*-null retina, we examined the expression of several other HC-specific markers, including *Oc2*, Lim1, Pgp9.5, and NF160 (Fig. 4*C–J*). Consistent with the reduction in calbindin-positive HCs (Fig. 4*B*), the numbers of HCs positive for these other markers were similarly reduced in the *Oc1*-null retina (Fig. 4*D,F,H,J*). In addition to HCs, NF160 is expressed in RGCs (Fig. 4*G*), while Pgp9.5 is expressed in amacrine cells (Fig. 4*I*), but the levels of expression of these genes in non-HCs did not change in *Oc1*-null retinas (Fig. 4*H,J*). These data confirmed that *Oc1* deletion selectively affected HCs and resulted in the loss of the majority of HCs. To further examine the residual HCs in *Oc1*-null retinas, we double immunolabeled for *Oc1* and either calbindin or *Oc2* (Fig. 4*A–D*). As we reported previously, *Oc1* and *Oc2* are expressed in all HCs of wild-type retinas, and they completely overlap with calbindin in these cells (Fig. 4*A,C*; Wu et al., 2012). In *Oc1*-null retinas, the vast majority (>95%) of residual HCs were negative for *Oc1* (Fig. 4*B,D*), but all were positive for *Oc2* (Fig. 4*D*; data not shown). These results suggest that *Oc2*, instead of undeleted *Oc1* due to inefficient Cre activity, was responsible for the residual HCs. To further confirm these findings and to assess the

distribution and morphology of the remaining HCs, we performed whole-mount staining of HCs with anti-Lim1 and anti-calbindin antibodies (Fig. 4*K–P*). The same degree of HC loss ($80.5 \pm 0.3\%$, $n = 4$) was observed in the *Oc1*-null retinas (Fig. 4*L,N*) compared with the wild-type retinas (Fig. 4*K,M*). In wild-type retinas, HCs are distributed in a non-random mosaic pattern (Fig. 4*K,M*; Cook and Chalupa, 2000). In the *Oc1*-null retinas, this mosaic distribution pattern was maintained for the remaining HCs, although the distances between neighboring HCs became larger due to reduction of HC numbers (Fig. 4*L,N*). In addition, immunostaining of calbindin revealed that the HC processes were longer and thicker in the *Oc1*-null retinas than those in the wild-type, but the overall density of HC processes was markedly reduced (Fig. 4*M–P*), indicating that the remaining HCs attempted to fill in the increased space between them with their processes, but unsuccessfully.

The loss of HCs in the mature *Oc1*-null retina could be due to either initial failure of HC birth or defective differentiation/maintenance after their birth. To distinguish between these two possibilities, we examined HC formation at E14.5 by immunofluorescence staining for Lim1 and Prox1 (Dyer et al., 2003; Pochè et al., 2007). These markers are expressed in newly born HCs, which migrate basally to the boundary between the neuroblast layer (NBL) and the GCL in wild-type retinas by E14.5 (Fig. 5*A,C,E,G*). Prox1 is also expressed in retinal progenitor cells (Fig. 5*E,G*; Dyer et al., 2003) at E14.5, but its expression in newly formed HCs is much higher and colocalizes with *Oc1* (Fig. 5*E,G*). In E14.5 *Oc1*-null retinas, the number of Lim1⁺ HC precursors was reduced by 85% ($\pm 3.9\%$, $n = 3$) compared with wild-type controls (Fig. 5*A–D*). The numbers of HC precursors strongly expressing Prox1 showed a similar reduction (Fig. 5*E–H*). Thus, the loss of HCs in E14.5 *Oc1*-null retinas was comparable to that in mature retinas. The deficiency of HC formation continued at E17.5 (Fig. 5*I–P*). At this stage, HCs normally are scattered through the NBL while migrating apically to their final destination, and more HC marker genes begin to be expressed (Fig. 5*I,K,M,O*). Examination of some of these markers, including Lim1, *Oc2*, Prox1, and NF160 in *Oc1*-null retinas, showed that the numbers of HCs remained at $\sim 15\%$ of those in wild-type retinas (Fig. 5*I–P*). The few remaining HCs migrated normally and all expressed *Oc2* (Fig. 5*L*), but very few expressed *Oc1* (Fig. 5*N,P*), further supporting the idea that *Oc2* was responsible for the formation of these residual HCs. These data indicated that HC de-

Figure 2. Deletion of *Oc1* does not affect formation of photoreceptors, bipolar cell, amacrine, ganglion, or Müller cells. Immunofluorescence labeling for cell type-specific markers was used to examine the formation of six retinal cell types in wild-type (*Oc1*^{+/+}) (*A, C, E, G, I, K, M, O*) and *Oc1*-null (*Oc1*^{-/-}) (*B, D, F, H, J, L, N, P*) retinal sections from P16 mice. *A, B*, Rhodopsin staining for rod photoreceptors. *C, D*, CAR staining for cone photoreceptors. *E, F*, Chx10 staining for bipolar cells. *G, H*, Pax6 staining for amacrine cells and retinal ganglion cells. *I, J*, Syntaxin I staining for amacrine cells. *K, L*, Sox9 staining for Müller cells. *M, N*, Vimentin staining for Müller cells. *O, P*, Pou4f2 staining for ganglion cells. *A–P*, Green is the cell-type marker and red is propidium iodide (nuclear) staining. Scale bar: (in *A*) *A–P*, 75 μ m. *Q*, The proportion of the total retinal cell population accounted for by each cell type was calculated and statistical significance was determined using Student's *t* test ($p < 0.05$ criterion level for significance). There were no significant differences in the numbers of ganglion cells (GC), bipolar cells (BC), cone photoreceptors (CP), rod photoreceptors (RP), Müller cells (MC), or amacrine cells (AC) between wild-type and *Oc1*-null retinas. However, there were significantly fewer HCs (Fig. 4) in the *Oc1*-null retinas compared with wild-type retinas ($p < 0.001$).

ciency in *Oc1*-null retina occurred at very early stages of development, with a failure of HC differentiation. To confirm this, we performed terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay to detect apoptotic cells in wild-type and *Oc1*-null retinas at E14.5, E17.5, and P0, which span the time window for HC formation, and observed no change in apoptosis in *Oc1*-null retinas compared with wild-type controls (Fig. 5*Q–V*). Thus increased apo-

Figure 3. RGC development is not affected in *Oc1*^{−/−} retina. Six RGC markers were examined in wild-type (*Oc1*^{+/+}) (**A, C, E, G, I, K**) and *Oc1*-null (*Oc1*^{−/−}) (**B, D, F, H, J, L**) retinal sections from E14.5 mouse embryos. The markers include Pou4f2 (**A, B**), Isl1 (**C, D**), Oc2 (**E, F**), NF160 (**G, H**), Dcx (**I, J**), and Pgp9.5 (**K, L**). Except for Oc2, all other markers show no changes in either the levels or patterns of expression in *Oc1*-null retinas compared with wild-type controls. The expression pattern of Oc2 does not change in *Oc1*-null retinas, but its level is increased (**F**). Scale bar, (in **A–L**), 150 μ m.

ptosis was not the cause of HC loss in *Oc1*-null retinas. Together these data strongly suggest that in *Oc1*-null retinas, the majority of HCs failed to form during development.

Oc1 is downstream of FoxN4, but acts in parallel with Ptf1a in HC genesis

Our finding that *Oc1* was required for HC genesis, and that expression of Prox1 and Lim1 were dependent on *Oc1*, suggested that *Oc1* functions upstream of Prox1 and Lim1 during HC development. To further decipher the underlying gene regulatory cascade, we examined the relationship of *Oc1* to FoxN4 and Ptf1a, two transcription factors that are also essential for the HC lineage (Li et al., 2004; Fujitani et al., 2006; Nakhai et al., 2007).

We focused our analysis on E14.5, a stage when most HCs are generated (Young, 1985; Pochè et al., 2007). At E14.5, FoxN4 is expressed in a large subset, but not all, of retinal progenitor cells (Fig. 6*A, C*) and is critical for the formation of HCs and most amacrine cells (Li et al., 2004). Ptf1a is also expressed in the NBL at this stage, but there are far fewer Ptf1a⁺ cells than FoxN4⁺ cells (Fig. 6*E, G, I, K*; Fujitani et al., 2006; Nakhai et al., 2007). *Oc1* is expressed in both the NBL and GCL at E14.5 (Fig. 6*C, G, K, O*). Cells expressing *Oc1* in the GCL are RGCs since they also express Pou4f2 and Isl1, two RGC markers (Wu et al., 2012), but the identity of *Oc1*-expressing cells in the NBL at E14.5 has been uncertain, since they generally do not express markers for RGCs or their precursors (Wu et al., 2012). We observed that only a small subset of FoxN4⁺ cells expressed *Oc1*, while many *Oc1*⁺ cells in the NBL were FoxN4[−] (Fig. 6*A, C*). FoxN4 expression

Figure 4. The great majority of HCs are missing in *Oc1*-null retina at P16. **A, B**, Coimmunolabeling for *Oc1* (red) and calbindin (green). In wild-type retina (**A**), cells expressing both calbindin and *Oc1* (arrows) are HCs, and those expressing only calbindin in the INL and GCL are amacrine cells and RGCs. In *Oc1*-null retinas, very few HCs remain and are *Oc1*-negative (**B**, arrows). Amacrine cells and RGCs are not affected (**B**). **C, D**, Colabeling for *Oc1* (red) and *Oc2* (green). Both are expressed in all HCs in the wild-type retina (**C**). In *Oc1*-null retina, only very few HCs remain, as indicated by *Oc2* staining (**D**). Although occasional *Oc1*/*Oc2* double-positive HCs are observed (yellow arrow in **D**), the vast majority of remaining HCs are negative for *Oc1*, but positive for *Oc2* (white arrows in **D**). **E, F**, Immunofluorescence labeling for Lim1, which is expressed only in HCs in wild-type retina (**E**). Lim1⁺ cells (green) were greatly reduced in *Oc1*-null retinas (**F**, arrows). **G, H**, Immunolabeling (green) for NF160, which is expressed in HC processes in the OPL (**G**) and RGCs. The numbers of HC processes are reduced in the *Oc1*-null retina (**H**), but there is no change in RGCs. **I, J**, Staining for Pgp9.5, which is expressed in HCs (**I**, arrows) and a subset of amacrine cells in wild-type retinas (**I**). In *Oc1*-null retinas, Pgp9.5 expression in HCs (**J**, arrows), but not in amacrine cells, was greatly reduced. **K–P**, Whole-mount staining of HCs by anti-Lim1 (**K, L**) and anti-calbindin (**M–P**). Images were obtained from equivalent regions of wild-type and *Oc1*-null retinas. **O, P**, High-magnification images from anti-calbindin staining to show the processes of HCs. Scale bars: (in **A–J**), 25 μ m; (in **K–N**), 12.5 μ m; (in **O, P**), 2.125 μ m.

also showed limited overlap with *Ptf1a* localization; only a subset of *FoxN4*⁺ cells also expressed *Ptf1a*, while many *Ptf1a*⁺ cells expressed little or no *FoxN4* (data not shown). Coimmunolabeling for *Oc1* and *Ptf1a* revealed that these two factors were only partially overlapping in their expression, and that many cells in the NBL were positive for *Ptf1a* or *Oc1* alone (Fig. 6*E, G, I, K*).

We then examined how deletion of the gene encoding one transcription factor affected the expression of the others. In *Oc1*-null retinas, there was no significant change in the expression of either *FoxN4* or *Ptf1a* (Fig. 6*B, D, F, H*). In *FoxN4*-null retinas, as reported previously (Fujitani et al., 2006), *Ptf1a* expression was completely abolished (Fig. 6*J, L*). A marked decrease of *Oc1*⁺ cells in the NBL, but not in the GCL, also was observed in *FoxN4*-null retinas (Fig. 6*I–L*). The dependence of these *Oc1*⁺ cells on *FoxN4* in the NBL indicates that they were likely HC precursors. These results suggest that *Oc1*, like *Ptf1a*, functions downstream of *FoxN4* in the HC lineage. Consistent with *FoxN4* being upstream of *Ptf1a*, *FoxN4* expression does not change in *Ptf1a*-null retinas (Fujitani et al., 2006). Interestingly, we also did not observe any changes in *Oc1* expression in the NBL of *Ptf1a*-null retinas (Fig. 6*M–P*). Similar to *Oc1*, *Ptf1a* functions before HC fate determination, since expression of *Lim1*, an early HC marker, is already diminished at E14.5 in the *Ptf1a*-null retina (Fig. 6*M–P*). These results indicate that, during HC genesis, *FoxN4* functions upstream in a large set of retinal progenitor cells, perhaps as a competence factor. *Oc1* and *Ptf1a* operate downstream of *FoxN4*. The observation that many *Oc1*⁺ or *Ptf1a*⁺ cells were *FoxN4*⁺ also suggests that *FoxN4* expression was extinguished once *Oc1* or *Ptf1a* was expressed. Although both *Oc1* and *Ptf1a* are essential for HC formation, they are expressed in two distinct, but overlapping, sets of cells in the NBL and largely do not regulate each other. Since *Oc1* and *Ptf1a* are coexpressed in only a small subset of cells, it is possible that one transcription factor can regulate the other in those cells, but this might not be detected readily due to the small size of this cell population. To examine this possibility, we ectopically expressed either *Oc1* or *Ptf1a* by *in utero* electroporation, using GFP to track cells that took up the DNA constructs, and then analyzed if one factor could induce the other (Fig. 6*Q–T*). When *Oc1* was ectopically expressed, although GFP⁺*Ptf1*⁺ cells could be detected occasionally, no increase of these double-positive cells was observed compared with controls expressing GFP alone (Fig. 6*Q, R*). Similarly, when *Ptf1a* was ectopically expressed, there was no induction of *Oc1* in the GFP⁺ cells com-

Figure 5. Loss of HCs in *Oc1*-null retinas occurs at early developmental stages. **A–H**, Immunolabeling for *Lim1* and *Prox1* at E14.5. *Lim1* and *Prox1* are expressed in newly generated HCs located at the boundary between the GCL and the NBL (**A, C, E, G**). *Prox1* also is expressed in the NBL, but its expression in the newly generated HCs is at a much higher level and overlaps with that of *Oc1* (red in **E** and **G**). In the *Oc1*-null retina, the numbers of *Lim1*⁺ (**B, D**) and *Prox1*⁺ (**F, H**) HCs (arrow in **D** and **H**) are greatly reduced. **I–P**, Immunolabeling for four HC markers at E17.5. These markers show HCs migrating to their final destination scattered throughout the NBL in the wild-type retina (**I–O**). In the *Oc1*-null retina (**J–P**), the numbers of labeled HCs are greatly reduced, compared with wild-type controls, but these cells still migrate normally. Note that *Oc2* and *NF160* are also expressed in the GCL in wild-type retinas (**K, O**), and that their expression in RGCs does not change in the *Oc1*-null retina (**L, P**). In addition, all HCs at E17.5 express *Oc1* in wild-type retina (**M, O**), but few are *Oc1*⁺ in *Oc1*-null retinas (**N, P**). **Q–V**, TUNEL assay for wild-type and *Oc1*-null retinas at E14.5, E17.5, and P0. No obvious differences were observed between wild-type and mutant retinas at any of the three stages. Scale bars: (in **A, B, E, F, Q, R**, 150 μ m; (in **C, D, G, H**, 25 μ m; (in **I, J, L, P, S–V**, 25 μ m).

pared with the controls (Fig. 6*S, T*). These results further support the conclusion that *Oc1* and *Ptf1a* are independent of one other in expression.

***Oc1* and *Ptf1a* codetermine the HC fate**

Since *Ptf1a* is essential for both HC and amacrine cell formation (Fujitani et al., 2006; Nakhai et al., 2007), yet *Oc1* is required only for HC fate, it is likely that only progenitor cells expressing both

Figure 6. Relationship of *Oc1* to other transcription factor genes involved in HC genesis. **A–D**, FoxN4 expression pattern overlaps partially with that of *Oc1* in the NBL of E14.5 wild-type retinas, but its expression does not change in *Oc1*-null retinas. Arrows (**C**) indicate some FoxN4/*Oc1* double-positive cells. **E–H**, The expression pattern of *Ptf1a* only partially overlaps with that of *Oc1* in the NBL and its expression is independent of *Oc1*. Some *Ptf1a*/*Oc1* double-positive cells are indicated by arrows (**G**). **I–L**, Both *Oc1* and *Ptf1a* require FoxN4 for expression in the NBL. All *Ptf1a*⁺ cells and most *Oc1*⁺ cells are lost in the NBL of *FoxN4*-null retinas (**J**). **M–P**, Newly formed HCs coexpress Lim1 and *Ptf1a* in wild-type retinas (arrows, **O**), but HCs fail to form in *Ptf1a*-null retinas, as indicated by the absence of Lim1⁺ cells (**P**). Expression of *Oc1* in the NBL of *Ptf1a*-null retinas does not change in **P**. **Q–T**, Ectopic expression by *in utero* electroporation of *Oc1* and *Ptf1a* did not induce the expression of each other. There was no increase in the number of GFP⁺*Ptf1a*⁺ cells when *Oc1* and GFP were expressed (**R**) compared with when only GFP was expressed (**Q**). Similarly, when *Ptf1a* was expressed (**T**), there was no increase in GFP⁺*Oc1*⁺ cells compared with the control (**S**). Dotted lines demarcate the NBL (top) from the GCL (bottom) in **C, D, G, H, K, L, O, P**. Arrows in **C, G, O**, and **Q–T** indicate double-positive cells. Scale bars: (in **A, B, E, F, I, J, M, N**, 150 μ m; (in **C, D, G, H, K, L, O–T**, 25 μ m).

Ptf1a and *Oc1* adopt the HC fate (i.e., *Ptf1a* and *Oc1* may cooperate to specify HC fate). To test this hypothesis, we performed *in utero* electroporation of plasmids expressing *Oc1* and *Ptf1a* into wild-type retinas. A plasmid-expressing GFP was coelectroporated in all experiments to track cells that had taken up the DNA. Since both *Ptf1a*⁺ and *Oc1*⁺ cells are sparse in the NBL and are postmitotic, most cells transfected by electroporation should have only expressed the ectopic genes, but not the endogenous *Oc1* or *Ptf1a*. If our hypothesis is correct, cells expressing only ectopic *Oc1* or *Ptf1a* should not adopt the HC fate efficiently, whereas cells transfected with both *Oc1* and *Ptf1a* should become HCs. Retinal electroporation was performed on E13.5 embryos, a time when HC formation starts to peak, and the fate of electroporated retinal cells was examined at E17.5 by coimmunofluorescence labeling for GFP and Lim1 or Prox1 (Fig. 7). In retinas that

were electroporated with the GFP plasmid alone, only a very small subset ($4.7 \pm 1.6\%$, $n = 4$) of all GFP⁺ cells were also Lim1⁺ (Fig. 7A,B). The Lim1⁺ cells (either GFP⁺ or GFP[−]) were HCs that formed as a process of normal development. In retinas electroporated with *Oc1* and GFP or *Ptf1a* and GFP constructs, significantly more GFP⁺Lim1⁺ cells were observed in both cases (Fig. 7E,F,I,J). In retinas electroporated with *Ptf1a* and GFP, 13.3% ($\pm 0.6\%$; $n = 4$, $p < 0.001$) of electroporated cells (GFP⁺) were also Lim1⁺ (Fig. 7E,F). In retinas electroporated with *Oc1* and GFP, 29.1% ($\pm 4.4\%$; $n = 4$, $p < 0.001$) of GFP⁺ cells were Lim1⁺ (Fig. 7I,J). Critically, when *Oc1* and *Ptf1a* were electroporated together, most GFP⁺ cells ($70.8 \pm 2.2\%$; $n = 4$, $p < 0.001$) were also Lim1⁺ (Fig. 7M,N), indicating these cells adopted the HC fate. Similar results were observed with Prox1 (Fig. 7C,D,G,H,K,L,O,P). In the control (GFP alone) retinas, $3.6 \pm 2.9\%$ ($n = 4$) of GFP⁺ cells were Prox1⁺ (Fig. 7C,D). In retinas expressing *Ptf1a* and GFP, $2.9 \pm 0.4\%$ ($n = 4$) of GFP⁺ cells were Prox1⁺ (Fig. 7G,H). In retinas expressing *Oc1* and GFP, $6.3 \pm 1.5\%$ ($n = 4$) of GFP⁺ cells were Prox1⁺ (Fig. 7K,L). In retinas expressing both *Ptf1a* and *Oc1*, $62.4 \pm 6.8\%$ ($n = 4$) of GFP⁺ cells were Prox1⁺ (Fig. 7O,P). We were unable to further track these cells and examine whether they further developed into mature HCs, since the GFP signals were lost at later stages. This could be due to either the loss of the expression constructs from the cells or loss of the cells themselves since they were ectopically produced. Nevertheless, we conclude that either *Ptf1a* or *Oc1* is very inefficient when acting alone to promote HC fate. When both *Ptf1a* and *Oc1* were present, however, most cells adopted the HC fate. Therefore, these two factors appear to be not only necessary but also sufficient to direct retinal progenitor cells to adopt the HC fate.

Loss of HCs leads to defects in the synaptic connections in the OPL

The OPL is comprised of photoreceptor terminals, and the processes of HCs and bipolar cells. Photoreceptors form synapses with HCs and bipolar cells in a stereotypical fashion (Olney, 1968; Blanks et al., 1974), but the developmental mechanisms that regulate formation of the complex synaptic interactions remain unclear. Because the first synapses to develop in the OPL are those between photoreceptors and HCs (Olney, 1968; Blanks et al., 1974), an obvious question is what role HCs play in the formation of synapses between photoreceptors and bipolar cells. The deficiency of HCs in *Oc1*-null retinas allowed us to examine this question directly by TEM.

In the wild-type retina, rod and cone synaptic terminals stratify in the outer half of the OPL, with the cone terminals residing more proximally (vitread) than most rod terminals. The inner half of the OPL is occupied by the processes of HCs and bipolar cells (Fig. 8A; Olney, 1968; Blanks et al., 1974). We first performed TEM on P30 retinas. In the *Ocl*-null retinas, the rod and cone terminals did not exhibit normal stratification and were disorganized (Fig. 8B–D). Furthermore, photoreceptor terminals in the *Ocl*-null retina often penetrated deep into the inner portion of the OPL, sometimes extending all the way to the border with the INL (Fig. 8C). Rod cell bodies and cells from the INL also could be found penetrating into the OPL (Fig. 8B–D). In wild-type retinas, rods, bipolar cells, and HCs formed the classic “triad” ribbon synaptic complexes (Fig. 8E). However, in *Ocl*-null retinas, typical triad ribbon synapses were rarely seen, although they possibly could exist at very low frequency (Fig. 8F–I). However, synaptic ribbons were found in *Ocl*-null rod terminals, but much more rarely compared with the wild-type, and they typically were not anchored to the plasma membrane, i.e., they appeared to be “free floating” (Fig. 8G,H). Despite this, invaginations of postsynaptic processes sometimes were observed in rod terminals of the *Ocl*-null retina, although they did not show the classic triad organization (Fig. 8F). Wild-type cone terminals normally show multiple triadic ribbon synapses organized around short synaptic ribbons, in addition to flat contacts located on the base of the terminal that represent contacts with OFF-cone bipolar cell processes (Fig. 8J). In contrast, in *Ocl*-null cone terminals, no synaptic ribbons were seen, but invaginations of postsynaptic processes were present and flat contacts between cone terminals and OFF-cone bipolar cell dendrites also were observed (Fig. 8K,L). In addition, extensive tubulovesicular cisternae were observed in both rod and cone terminals (Fig. 8F,I,M). To rule out the possibility that the observed defects were secondary, we also performed TEM at P16, a stage immediately after completion of synapse formation in the OPL. At P16, HC plexus in the developing OPL was already severely reduced in the *Ocl*-null retina as shown by anti-NF160 staining (Fig. 4H). Consistently, TEM revealed that the OPL of P16 *Ocl*-null retina showed defects comparable to those observed at P30, including less frequent and aberrantly positioned ribbons; absence of clear triadic organization to the postsynaptic complexes; and contact of at least some postsynaptic cells with photoreceptor terminals (data not shown).

These results suggest that, in the reduction of HC processes, synaptic ribbons did not anchor properly to the plasma membrane, leading to the loss of the stereotypical triadic organization of postsynaptic processes associated with ribbon synapses of both rods and cones. In contrast, flat contacts between cone terminals and OFF-cone bipolar cells still formed. The appearance of the large cisternae in the rod and cone terminals of the *Ocl*-null retina may reflect a functional change in membrane trafficking in these terminals.

Figure 7. *Oc1* and *Ptf1a* are sufficient to specify HC fate. Retinal sections from embryos electroporated with plasmid constructs expressing GFP, *Oc1*, and *Ptf1a* were double immunolabeled with anti-GFP (green) and anti-Lim1 or anti-Prox1 (red). **A–D**, In retinas electroporated with the construct for GFP alone, few GFP⁺ cells express Lim1 or Prox1. **E–H**, When constructs for GFP and *Ptf1a* were coelectroporated into the retina, some GFP⁺ cells are also Lim1⁺, but few were Prox1⁺. **I–L**, Coelectroporation of constructs for *Oc1* and GFP also resulted in only a few GFP⁺Lim1⁺ or GFP⁺Prox1⁺ cells. **M–P**, Electroporation of constructs for *Oc1*, *Ptf1a*, and GFP resulted in most GFP⁺ cells also being Lim1⁺ and Prox1⁺. Arrows point to double-positive cells. Scale bar, (in **A**) **A–P**, 25 μ m.

To extend our TEM observations, we immunolabeled synaptic ribbons with anti-Bassoon (Fig. 8N,Q,T,W) and flat contacts with peanut agglutinin (PNA; Fig. 8O,P,R,S). Consistent with the TEM data, many fewer synaptic ribbons were observed in the OPL of *Ocl*-null retinas (Fig. 8Q,W), and these ribbons tended to be shorter than those in wild-type retina. In addition, some regions in the OPL were devoid of synaptic ribbons (Fig. 8Q,W). Staining with PNA indicated that flat contacts between cones and OFF-cone bipolar cells were present in the OPL of *Ocl*-null retinas, but fewer in number, compared with wild-type controls (Fig. 8Q,R).

In addition, we performed immunolabeling with antibodies to PKC α to visualize rod bipolar cell dendrites in the OPL (Fig. 8U–Y). In the wild-type retina, rod bipolar cell dendrites contacted rod terminals and were confined within the OPL, as expected (Fig. 8U,V). In the *Ocl*-null retina, contacts between rod bipolar cell dendrites and photoreceptor terminals were present, but infrequently (Fig. 8X,Y). Furthermore, rod bipolar cell dendrites often showed abnormal sprouting and invasion of the ONL, consistent with disruption of the normal synaptic connections of the OPL (Fig. 8X,Y). These results indicated that although loss of HCs in the *Ocl*-null retinas severely disrupted photoreceptor terminals and their synaptic connections in the OPL, some contacts between photoreceptors and bipolar cells persisted.

HCs are required for retinal integrity and photoreceptor survival

To further evaluate how loss of HCs affected the function of *Ocl*-null retinas, we performed flash ERG recordings on 5-month-old mice

Figure 8. The deficiency of HCs disrupts synaptic ultrastructure and organization of the OPL in *Oc1*-null retinas. **A–D**, Low-magnification (5000×) TEM images of the OPL from P30 wild-type (WT; **A**) and *Oc1*-null mutant mice (MT; **B–D**). The boundaries of the OPL are indicated by dotted lines. **A**, In the WT retina, the OPL is of uniform thickness and is well laminated, with the synaptic terminals of cones and rods localized to the outer (sclerad) portion and processes from secondary neurons localized to the inner (vitread) portion. **B–D**, In the MT retinas, this laminar structure of the OPL is disrupted, with aberrant localization of processes from photoreceptors and bipolar cells. The thickness of the OPL in the MT retina varies from region to region, resulting in irregular boundaries. In addition, intruding bipolar cell (**B**, **C**) and photoreceptor cell bodies (**D**) can be found in the OPL. **E–I**, High-magnification images (30,000×) of rod terminals. **E**, WT rod terminals show normal synaptic ultrastructure with a large synaptic ribbon (arrowhead) in the presynaptic terminal and a postsynaptic “triad” comprised of two HC processes (H) flanking the ribbon laterally and a rod bipolar dendrite (B) occupying the central position. **F**, Rod terminals in the MT retina contain numerous synaptic vesicles but typically do not show synaptic ribbons. However, invaginations containing postsynaptic processes (arrowheads) are present. Arrows point to tubulovesicular cisternae. **F–I**, In rod terminals from MT retinas, “free-floating,” amorphous ribbon material (arrowhead in **G**), abnormal synaptic ribbons devoid of associated synaptic vesicles (arrowhead in **H**), and tubulovesicular cisternae (**I**, arrows) can be found. **J**, WT cone terminals show invaginations of postsynaptic processes forming multiple synaptic complexes organized around short synaptic ribbons (arrowheads). Flat contacts (FC) are present along the base of the cone terminal. **K**, **L**, Cone terminals in the MT retina. No synaptic ribbons are present, although invaginations of postsynaptic processes (arrowheads, in **K**) and FC (**L**) are present, but are sometimes ectopically positioned. **M**, Extensive tubulovesicular cisternae (arrows) in cone terminals in the MT retina. **N–S**, Colabeling using anti-Bassoon and PNA to visualize synaptic ribbons (red) and FCs (green), respectively, in the OPL of P16 WT and MT retinas. In the OPL of the MT retina, the number of synaptic ribbons is markedly reduced, compared with controls; there are also fewer FCs (arrowheads). **T–Y**, Colabeling of synaptic ribbons and rod bipolar cells using anti-Bassoon and anti-PKCα, respectively. In the WT retina, processes of rod bipolar cells contact rod terminals but do not extend beyond the OPL into ONL. In the MT retina, rod bipolar cell dendrites still make contacts with rod terminals, but in some areas project ectopically into the ONL. Scale bars: (in **A**) **A–D**, 2 μm; **E–I**, **L**, **M**, 500 nm; **J**, **K**, 2 μm; (in **N**) **N–Y**, 10 μm.

under dark-adapted conditions (Fig. 9A). The corneal-negative a-wave of the ERG provides information about phototransduction (Pugh et al., 1998) and precedes the corneal-positive b-wave that originates from bipolar cell activity in the inner retina (Robson et al., 2004). The a- and b-wave amplitudes recorded from *Oc1*-null retinas were much smaller than those recorded from control retinas, but

the kinetics of the responses, as inferred by the time to peak of the b-wave, was not affected. However, the *Oc1*-null retinas did not exhibit the robust oscillatory potentials, suggesting that the function of the inner retina also was impaired (Wachtmeister, 1998). The intensity-response functions (Fig. 9B,C) show that the a- and b-waves in *Oc1*-null retina were uniformly attenuated (by threefold)

Figure 9. *Oc1*-null mice exhibit abnormal ERGs and undergo retinal degeneration. **A–C**, Dark-adapted ERG. **A**, Average ERGs recorded from *Oc1*-null (MT) and littermate control (WT) retinas at 5 months of age in response to brief (4 ms) flashes. Flash intensities are indicated to the left of each trace. **B**, **C**, Intensity-response functions of b- and a-waves, respectively, recorded from *Oc1*-null mice (MT, red) and their wild-type (WT) control siblings (black) at 5 months of age. Gray dashed lines indicate the response of WT mice divided by a factor of 3. Error bars indicate SEM, $n = 4$ in each group. **D**, **E**, Light-adapted ERGs. **D**, Average ERGs recorded from *Oc1*-null (MT) and control retinas (WT) in response to brief (4 ms) flashes superimposed on a steady background of 10 cd/m². Intensities of the flashes are indicated in the left column. **E**, Intensity-response functions of b-waves recorded from *Oc1*-null mice (MT) and their wild-type (WT) siblings at 5 months of age. Gray dashed lines indicate the response of WT mice divided by a factor of 3. Bars indicate SEM, $n = 4$ in each group. **F–K**, Histological analysis of H&E stained wild-type (**F**, **H**, **J**) and *Oc1*-null (**G**, **I**, **K**) retinal sections from mice at 3 months (**F**, **G**), 5 months (**H**, **I**), and 8 months (**J**, **K**) of age shows progressive loss of photoreceptors in the *Oc1*-null retina. See text for detailed description. Scale bar, (in **F**) **F–K**, 25 μ m.

over much of the intensity range, indicating that the loss of HCs nonselectively impairs retinal function over the entire intensity range. The increase of the a-wave at -2 log sc cd s/m² probably resulted from the relative reduction of the b-wave and unmasking of the a-wave.

HCs provide lateral inhibitory input to photoreceptors as well as bipolar cells and are thought to contribute to light adaptation (Werblin and Dowling, 1969; Wu, 1992; Thoreson and Mangel, 2012). To determine whether light adaptation was impaired in *Oc1*-null retinas we recorded flash ERG responses in the presence of steady back-

ground lights of 10 cd/m². These light levels produce ~8000 photoisomerizations/rod/s (Umino et al., 2012), which is sufficient to saturate rods (Nakatani et al., 1991) and induce light adaptation in cones (Dunn et al., 2007). We found that background illumination sped up the ERG response, reduced the amplitude of the b-wave, and limited the range of both wild-type and *Oc1*-null retinas to flash intensities $> -1 \log \text{cd s/m}^2$ (Fig. 9D). However, much as in dark-adapted conditions, the b-waves of *Oc1*-null retinas were ~3-fold smaller than those of control retinas over the entire intensity range (Fig. 9D,E). These results suggest that the cone-driven light responses in *Oc1*-null retinas are attenuated, although their light adaptive mechanisms appeared to remain intact.

Since we did not observe overt structural abnormalities in either the ONL or INL of *Oc1*-null retinas at P16, the impaired ERGs at 5 months of age prompted us to examine whether *Oc1*-null retinas underwent degeneration as the mice aged. At 3 months of age, the OPL was similarly defective as at P16. However, there were no obvious defects in the other layers of the *Oc1*-null retinas (Fig. 9F,G). In contrast, at 5 months, the OPL in *Oc1*-null retinas had virtually disappeared, while the thickness of the other retinal layers remained comparable to those in wild-type retinas. Some early signs of degeneration were noticeable in the ONL at this time: the ONL was slightly thinner and the nuclei in the ONL appeared less tightly packed than those in wild-type retinas (Fig. 9H,I). By 8 months of age, degeneration of photoreceptors was obvious in *Oc1*-null retinas, with the thickness of the ONL being only approximately one-third that of wild-type retinas (Fig. 9J,K). The degeneration of the ONL in *Oc1*-null retinas likely contributed to the reduction of the ERG a-waves under dark-adapted conditions. Nevertheless, the thickness of the INL did not change significantly, suggesting that the observed reduction in the ERG b-waves was due to diminished photoreceptor input, rather than bipolar cell loss. The observed age-dependent degeneration and loss of photoreceptors must be secondary to the loss of HCs in the *Oc1*-null retina, since photoreceptors do not express *Oc1* themselves and formed normally. Thus, in addition to processing visual signals, HCs are also essential for the postnatal survival of photoreceptors.

Discussion

Roles of *Oc1* in HC development and its potential redundancy with *Oc2*

We previously showed that *Oc1* is expressed in developing HCs (Wu et al., 2012), but it was not clear from that study the stage at which *Oc1* is involved in HC development. Nevertheless, at E14.5, *Oc1*⁺ cells, which are mostly postmitotic and typically do not coexpress factors that regulate the RGC lineage, exist in the NBL and their identities heretofore were not known (Wu et al., 2012). Our finding that deletion of *Oc1* results in a loss of ~80% of HCs and that this loss takes place early during HC development suggests that *Oc1* is required for the initial genesis of HCs. The lack of increased apoptosis in the *Oc1*-null retinas supports this idea. Further, we found that the *Oc1*⁺ cells located in the NBL are dependent on *FoxN4*. This observation, in combination with the deficient HC development in both *FoxN4*-null and *Oc1*-null retinas, indicates that these *Oc1*⁺ cells in the E14.5 NBL are HC precursors and arise from *FoxN4*⁺ progenitor cells. Upon expressing *Oc1*, they become committed to the HC fate and migrate inward to the boundary of the NBL and GCL, where they undergo further differentiation and begin expressing mature HC markers, including *Lim1* and *Prox1*. *Oc2* exhibits very similar expression patterns to those of *Oc1* in the developing retina (Wu et al., 2012). Since *Oc1* and *Oc2* are highly similar in their DNA-binding domains and bind to the same consensus sequence (Iyaguchi et al.,

Figure 10. Summary diagram, illustrating key transcriptional regulators that regulate HC genesis. **A**, Model for the genesis of HCs. 1, Expression of *FoxN4* (blue) in a large subset, but not all, of retinal progenitor cells confers competence toward the HC fate. For simplicity, *FoxN4*[−] cells and the GCL are not depicted. 2, From HC-competent cells emerge two populations of cells: one expressing *Ptf1a* (red) and the other expressing *Oc1* (green). Only a small subset of cells coexpress both of these factors (yellow cells). 3, Only those cells that express both *Ptf1a* and *Oc1* (yellow) adopt the HC fate. 4, Fate-determined HC precursors migrate basally and begin to express *Lim1*, *Prox1*, and *Sall3* (pink). 5, These cells then migrate apically and begin to express other HC markers. 6, Nascent migrating HCs reach their final destination and differentiate into mature HCs. **B**, Schematic illustration of the gene regulatory cascade underlying HC formation. *FoxN4* is the most upstream factor, *Ptf1a* and *Oc1* are downstream of *FoxN4* and in parallel to each other, and *Lim1* and *Prox1* are downstream of *Oc1* and *Ptf1a*. *Ptf1a* directs cells toward an amacrine cell fate in the absence of *Oc1*, and inhibits the ganglion cell fate by repressing *Math5*.

2007), it is possible that *Oc1* and *Oc2* function redundantly by regulating the same target genes in the developing retina. They both are expressed in all HCs during development and in adulthood. However, *Oc2* is expressed in a much smaller cohort of HC precursors in the NBL during early development than is *Oc1* (Wu et al., 2012). Consistent with this, only ~20% of HCs still form in *Oc1*-null retinas and all of these remaining HCs express *Oc2*, suggesting that *Oc2* is also involved in HC development.

Both *Oc1* and *Oc2* also are expressed in developing RGCs (Wu et al., 2012). However, we did not observe any RGC defects in the *Oc1*-null retina either during development or in adulthood. This also may be due to redundancy with *Oc2*, as suggested by the upregulation of *Oc2* in *Oc1*-null retinas. Examining RGC development in the *Oc2*-null and *Oc1/Oc2*-double-null retinas in the future will help resolve this.

The gene regulatory cascade for HC development

The expression patterns and knock-out phenotypes of genes involved in HC development suggests a stepwise scenario of HC genesis (Fig. 10A). In this scenario, *FoxN4* is most upstream and is expressed in a large population of, but not all, proliferating progenitor cells (Fig. 10A, 1). The function of *FoxN4* is to render the progenitor cells competent for HC and amacrine cell fates. From these

FoxN4-expressing cells emerge two distinct, but overlapping, cell populations: those expressing *Ptf1a* and those expressing *Oc1* (Fig. 10A, 2). Although both *Ptf1a* and *Oc1* are essential for the HC fate, neither is sufficient individually. As indicated by *in utero* electroporation, most, if not all, cells expressing both factors will eventually adopt the HC fate (Fig. 10A, 2,3), i.e., *Oc1* and *Ptf1a* act together to determine the HC fate. Those precursor cells expressing *Ptf1a* alone become amacrine cells. In the *Oc1*-null retinas, precursor cells that normally adopt the HC fate may switch fate and become amacrine cells, since they now only express *Ptf1a*; however, this remains to be demonstrated unequivocally by further experiments. At present, it is not clear what fate(s) cells expressing only *Oc1* adopt and what happens to them when *Oc1* is inactivated. The HC fate-committed cells then migrate to the basal side of the retina at ~E14.5 (Fig. 10A, 4) and begin to express transcription factors such as *Lim1*, *Prox1*, *Sall3*, and *AP-2 α* and *β* , which regulate subsequent HC migration and differentiation (Dyer et al., 2003; Pochè et al., 2007; de Melo et al., 2011; Bassett et al., 2012). At later stages (e.g., E16.5), the immature HCs begin to migrate apically (Fig. 10A, 5), reach their final destination in the retina at ~P0 (Fig. 10A, 6), and differentiate into mature HCs, ultimately becoming part of visual circuitry. Clearly, HC formation is subject to regulation by a hierarchical pathway comprised of factors that include FoxN4, *Ptf1a*, *Oc1*, *Lim1*, *Prox1*, *Sall3*, and *AP-2 α* and *β* (Fig. 10B). Thus, our results identify *Oc1* as an essential player in this pathway, placing it downstream of FoxN4, in parallel with *Ptf1a*, but upstream of *Lim1*, *Prox1*, *Sall3*, and *AP-2 α* and *β* (Fig. 10B). It should be noted that *Ptf1a* inhibits RGC formation by repressing *Math5* (Fujitani et al., 2006; Lelièvre et al., 2011), and is also required for amacrine cell formation (Fujitani et al., 2006; Nakhai et al., 2007; Jusuf et al., 2011), indicating that this regulatory cascade also interacts with gene regulatory pathways for other retinal cell types (Fig. 10B). Currently it is not clear how *Oc1* and *Ptf1a* collaborate to specify the HC fate. Interestingly, both *Ptf1a* and *Oc1* are expressed in the multipotent pancreatic progenitor cells and they coregulate the expression of *Pdx1*, a critical transcription factor for pancreas development (Jacquemin et al., 2006). *Ptf1a* and *Oc1* may coregulate downstream genes through a shared mechanism in the two tissues.

HCs are essential for retinal integrity and function, and photoreceptor viability

During normal development, HC processes are the first to contact photoreceptors; this leads to the recruitment of nascent synaptic ribbons to the site of contact, after which a second HC process is recruited and the synaptic complex begins to invaginate, followed by the subsequent addition of ON-type bipolar cell dendrites into the central position of the synaptic “triad” (Blanks et al., 1974). In *Oc1*-null retinas, this process is severely disrupted, although in some cases bipolar cell processes can still contact photoreceptor terminals and invaginations can still form. However, similar to the *Basoon* knock-out retina (Dick et al., 2003), synaptic ribbons in the *Oc1*-null retina are rarely recruited to sites of contact with postsynaptic processes and formation of the stereotypical triadic synaptic complexes is impeded. The presence of these defects at p16, which coincides with the completion of photoreceptor synapse formation (Blanks et al., 1974) indicates that HCs are important for the establishment of normal synaptic connections in the OPL. The disruption of connectivity is entirely consistent with the abnormal ERG responses in the *Oc1*-null retina and indicates that the deficiency of HCs leads to severely compromised transmission of signals from photoreceptors to bipolar cells and the inner retina. Although it is clear that the synaptic organization of the OPL is disrupted in the *Oc1*-null retina,

several important issues remain to be resolved including the extent of contacts, if any, that form between photoreceptors and HCs, and whether HC deficiency disrupts the specificity of contact formation between photoreceptors and the various classes of bipolar cells. All these issues may be addressed by further analyzing the *Oc1*-null retinas, as well as the *Oc1/Oc2*-double-null retinas we have just created, which are expected to have more severe HC defects, at stages of synaptogenesis in the OPL during development. These future studies may reveal the exact roles HCs play during synaptogenesis in the OPL.

In addition, HCs are also essential for the integrity and maintenance of the photoreceptors in the adult retina. Because *Oc1*-null retinas are largely normal when the mice are young, deficiency of HC formation does not affect the formation of the rest of the retina on a large scale. However, severe retinal degeneration develops as the mice age. This is manifested mainly in the loss of photoreceptors, revealing a previously unrecognized but essential role for HCs in the survival of photoreceptors. Consequently, there were severe reductions in the a- and b-waves of the ERGs from these mice, although the change in the b-wave also likely arose from the severe compromise of synapse formation and the aberrant development of the presynaptic transmitter release machinery of the photoreceptor terminals due to reduction of HCs. Consistent with our findings, a recent study using a mouse model in which HCs were ablated by diphtheria toxin A revealed a similar degeneration of rod photoreceptors (Sonntag et al., 2012). The mechanism by which HCs promote and preserve photoreceptor viability is as yet unknown. It is possible HCs provide essential trophic factors for the survival of photoreceptors. Alternatively, electrical activity and/or feedback neurotransmission from HCs to photoreceptors may be required to maintain photoreceptor viability. Further investigations will be needed to identify the actual mechanisms involved. Once discovered, augmentation of these endogenous processes may provide a novel means of preventing photoreceptors from degenerating and dying in diseased retinas. Although retinal degeneration caused by HC deficiency has not been reported in humans, this model may have relevance to some types of hereditary retinal degenerations for which the underlying mechanisms are not yet understood and the genes involved have not been identified.

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