Ectopic *Mitf* in the Embryonic Chick Retina by Co-transfection of β -Catenin and *Otx2*

Peter D. Westenskow,^{1,2} Jon B. McKean,¹ Fumi Kubo,³ Shinichi Nakagawa,³ and Sabine Fuhrmann^{1,2}

PURPOSE. Development of the retinal pigment epithelium (RPE) is controlled by intrinsic and extrinsic regulators including orthodenticle homeobox 2 (*Otx2*) and the Wnt/ β -catenin pathway, respectively. *Otx2* and β -catenin are necessary for the expression of the RPE key regulator microphthalmia-associated transcription factor (*Mitf*); however, neither factor is sufficient to promote *Mitf* expression in vivo. The study was conducted to determine whether *Otx2* and β -catenin act in a combinatorial manner and tested whether co-expression in the presumptive chick retina induces ectopic *Mitf* expression.

METHODS. The sufficiency of Wnt/ β -catenin activation and/or *Otx2* expression to induce RPE-specific gene expression was examined in chick optic vesicle explant cultures or in the presumptive neural retina using in ovo-electroporation. Luciferase assays were used to examine the transactivation potentials of *Otx2* and β -catenin on the *Mitf*-D enhancer and autoregulation of the *Mitf*-D and *Otx2*T0 enhancers.

RESULTS. In optic vesicles explant cultures, RPE-specific gene expression was activated by lithium chloride, a Wnt/ β -catenin agonist. However, in vivo, *Mitf* was induced only in the presumptive retina if both β -catenin and *Otx2* are co-expressed. Furthermore, both *Mitf* and *Otx2* can autoregulate their own enhancers in vitro.

CONCLUSIONS. The present study provides evidence that β -catenin and *Otx2* are sufficient, at least in part, to convert retinal progenitor cells into presumptive RPE cells expressing *Mitf*. *Otx2* may act as a competence factor that allows RPE specification in concert with additional RPE-promoting factors such as β -catenin. (*Invest Ophthalmol Vis Sci.* 2010;51:5328–5335) DOI:10.1167/iovs.09-5015

Mutations in RPE-specific genes and dysfunction of the RPE can lead to ocular diseases such as retinitis pigmentosa and age-related macular degeneration (AMD), the leading cause of blindness in industrialized countries. Encouraging studies demonstrate that RPE cells can be derived from human embry-

onic stem cells (hESCs) and can restore basic visual function when transplanted into dystrophic rat retinas.^{1–4} Generating and expanding RPE-like cells from stem cells, however, is difficult because of low yield and long generation times. Furthermore, isolated RPE cultures are inherently unstable, and cellular potency, function, transcriptomes, and morphologies fluctuate after only a few passages.^{5–7} Thus, elucidating the mechanisms underlying development and maintenance of the RPE may provide important clues for the identification of factors for generating stable homogenous cultures.

The RPE and neural retina originate from forebrain-derived neuroepithelium that invaginates to form the optic cup, the outer layer of which becomes RPE and the inner layer the neural retina. At early embryonic stages, bipotential eye progenitor cells receive divergent signals based on their positions in embryologic space. These signals regulate cell fate decisions that must be continually re-enforced through the actions of intrinsic and extrinsic signaling factors to prevent a change in cell fate. Few disparate RPE-promoting factors have been identified; however, in most cases the exact mechanisms for regulating RPE-specific gene expression are not well understood.^{8–18}

The two key transcription factors *Mitf* and *Otx2* are essential for regulating RPE specification and differentiation. *Mitf* isoforms activate melanogenic and RPE terminal differentiation genes, and *Mitf* gene inactivation in the mouse causes RPE cells to dedifferentiate, hyperproliferate, and upregulate neural retinal markers in a process termed RPE-to-retina transdifferentiation.^{19–24} We and others recently reported that *Mitf* may be regulated by Wnt/ β -catenin signaling in the RPE.^{10,17} RPE-specific inactivation of β -catenin induces pronounced pigment deficits and RPE-to-retina transdifferentiation. Furthermore, β -catenin binds *Mitf* enhancers in vivo and can transactivate these in vitro.^{10,17} (For a review of the Wnt/ β -catenin pathway, see Ref. 25.)

Conversely, β -catenin is not sufficient to influence RPE fate. Gain-of-function experiments demonstrated that Wnt/ β -catenin acts to maintain an undifferentiated state of progenitor cells in the peripheral retina by repressing proneural gene expression and to promote peripheral fate by upregulating ciliary body marker expression.^{26–30} We hypothesize that the retinal environment is not permissive to allow *Mitf* induction in retinal progenitors and that additional factors besides β -catenin are necessary. In the present study, we tested the role of the candidate factor *Otx2* to induce ectopic *Mitf* expression in the presumptive chick retina in combination with β -catenin.

MATERIALS AND METHODS

Culture Experiments

Optic vesicles from chick embryos (White Leghorn) at Hamburger and Hamilton stages (HH) 11 to 13 were dissected, and extraocular tissues

From the ¹Department of Ophthalmology and Visual Sciences, Moran Eye Center, and the ²Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, Utah; and the ³RIKEN Advanced Science Institute, Wako, Saitama, Japan.

Supported by Grant EY014954 and Core Grant EY014800 from the National Institutes of Health/National Eye Institute (SF) and by an unrestricted grant from Research to Prevent Blindness, Inc., to the Department of Ophthalmology, University of Utah.

Submitted for publication December 3, 2009; revised March 26, 2010; accepted April 15, 2010.

Disclosure: P.D. Westenskow, None; J.B. McKean, None; F. Kubo, None; S. Nakagawa, None; S. Fuhrmann, None

Corresponding author: Sabine Fuhrmann, University of Utah Health Sciences Center, Dept. of Ophthalmology and Visual Sciences, John A. Moran Eye Center, Rm S3180, 65 Mario Capecchi Drive, Salt Lake City, UT 84132; sabine.fuhrmann@hsc.utah.edu.

were removed as previously described and cultured for 2 days in culture medium with 2.5 mM lithium chloride (LiCl).^{9,31}

Plasmid Construction

Xenopus constitutively active β -catenin (β catEGFP, CA- β -catenin), chick Otx2HA, chick DNLEf1, mouse MitfD:luc, and mouse Otx2T0:luc constructs have been described.^{17,27,32–34} Mouse MitfD:EGFP and Otx2T0:EGFP were generated through digestion with *KpnI* and *BglII* or *BamHI* enzymes, respectively, and ligated into pEGFP-1 (Clontech, Mountain View, CA). Otx2K50QHA, DN-Mitf-D (LIER sequence in the basic domain mutated to LIAR), and Otx2T0MBS:luc were generated by site-directed mutagenesis; the putative bicoid site conforming to TFMATRIX M00140 was mutated in the enhancer region (–964; CTGCAGCCCTG mutated to AAATCTCCCTG) to generate Otx2T0MBS:luc.^{35–37} The activity of β catEGFP was confirmed in vivo and in vitro by using TOPdGFP and TOPFlash reporters, respectively (data not shown). Mouse MitfD was synthesized by generating a synthon of the Mitf-D coding region of exon B1b (~250 bp) with long oligonucleotides designed using GeneDesign β 2.0 software (sequences available on request), which were fused with Mitf exons 2 to 9 by PCR.^{38,39}

Luciferase Assays

Luciferase assays were performed as previously described.¹⁷ Dose-response experiments were performed by transfecting HEK293T cells with 250 ng of total DNA including 1 ng renilla luciferase, 50 ng MitfD:luc (firefly luciferase), a range of 0 to 100 ng CA- β -catenin and/or Otx2HA, and pCMS-EGFP (empty vector). Data are presented as the mean $\pm$ SEM of results in four separate experiments (Excel; Microsoft, Redmond, WA, and Prism; GraphPad, San Diego, CA).

In Vivo Electroporation

All experiments with animal models were performed in strict compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fertilized White Leghorn eggs were incubated at 38.5°C in a humidified incubator. Optic vesicles (HH10-11, embryonic day [E]1.5) were usually injected with 2.5 μ g/ μ L DNA mixed with phenol red (0.1%), and five square 6-V current, 50.6-ms duration pulses (950-ms intervals), using 0.5-mm diameter, gold-plated wire electrodes (anode positioned lateral to the vesicles on the right side), were delivered with a pulse generator (ECM 830; BTX, Hawthorne, NY). We observed that the Otx2HA and β catEGFP expression constructs are active at E2.5, and ectopic Mitf is induced at E3. However, Otx2HA is undetectable and β catEGFP fluorescence is weak at E4.5. By E5.5 EGFP can be detected only with a GFP antibody. This expression may be transient because of transgene instability and dilution through repeated cell divisions.⁴⁰ Thus, for our co-expression analysis, embryos were harvested at E3 or E3.5 stages, to detect expression of Mitf, Otx2HA, and β catEGFP simultaneously. Electroporation procedures in mouse have been described.⁴¹ Briefly, CD-1 newborn pups (P0) were anesthetized on ice and injected with 0.5 μ L containing MitfD:EGFP or Otx2T0:EGFP and CMV:DsRed (1.25 μ g/ μ L DNA each) mixed with Fast Green (0.1%) into the subretinal space. Five square pulses (80-V current, 50.6-ms duration, 950-ms intervals) were delivered with the anode (electrode model 520, 7-mm diameter; BTX) positioned lateral to the left or right eye to target the RPE or retina, respectively.

Immunohistochemistry

Tissue processing and immunohistochemistry have been recently described.^{17,42} The following primary antibodies were used: mouse Mitf (1:400; Exalpha, Shirley, MA), rabbit Otx2 (1:1500; Chemicon, Billerica, MA), mouse MMP115 (MC/1 1:200–500; provided by Makato Mochii), goat Sox2 (1:750; Santa Cruz Biotechnology, Santa Cruz, CA), rat HA (1:400; Sigma-Aldrich, St. Louis, MO), rabbit GFP (1:500; Torrey Pines Biolabs, East Orange, NJ), and mouse NF-M (RMO270 1:1000; Zymed, San Francisco, CA). Alexa 488, 568, and 647-conjugated secondary antibodies (1:1000; Invitrogen-Molecular Probes, Carlsbad, CA)

and TRITC-conjugated donkey-anti-goat secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA) were used. We regarded MMP115 expression as positive when it was detectable in at least one tenth of the explants in a given cryostat section. Images were taken with a confocal microscope (model FV1000; Olympus, Tokyo, Japan) and processed (ImageJ, NIH; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html/> and Photoshop CS2; Adobe Systems, San Jose, CA).

Cell Counts

Ectopic Mitf-expressing cells in the neural retina that co-localized with DAPI, GFP(β catEGFP), and HA (Otx2HA) were counted in confocal images taken from E3.5 transfected eyes ($n = 5$) from eight cryosections (12 μ m) and at least two separate slides ($n = 5$). Only well-transfected eyes (strong GFP and HA signals in at least one third of the retina) were used in the analysis. Mitf-expressing cells in close proximity to the ciliary body or that appeared to have been scraped into the retina from the RPE or ciliary body, possibly during cryosectioning, were not counted. The total number of ectopic Mitf-expressing cells was recorded as a percentage of the total number of GFP- and HA-transfected cells. Statistical analyses were performed with ANOVA.

RESULTS

Role of the Wnt/ β -Catenin Pathway in Promoting RPE Differentiation in the Chick

To identify factors that promote RPE differentiation, we established an explant culture assay by using chick optic vesicles from E1.5 embryos, and we showed that removal of the extraocular mesenchyme prevents induction or maintenance of RPE-specific gene expression.⁹ Among the factors tested, we observed that indirect activation of the Wnt/ β -catenin pathway had a significant effect on RPE differentiation in a restricted domain of the explant. Lithium chloride (LiCl) stabilizes cytoplasmic β -catenin by inhibiting GSK-3 β , thereby activating the Wnt/ β -catenin pathway.⁴³ Addition of LiCl to the culture media greatly enhanced expression of the Mitf target gene melanosomal matrix protein MMP115 (93%; Figs. 1B, 1C; $n = 15$).⁴⁴ LiCl is also a noncompetitive inhibitor of inositol monophosphatase (IMPase), a component of the phosphatidylinositol pathway.⁴⁵ Treatment of explants with the specific IMPase inhibitor L690300 did not promote MMP115 expression (data not shown; $n = 9$), strongly suggesting that LiCl acts through inhibition of GSK-3 β and stabilization of β -catenin. Therefore, in explant cultures of chick optic vesicles, activation of Wnt/ β -catenin signaling is sufficient to upregulate RPE-specific gene expression, at least partially. One likely explanation is that the explants include presumptive RPE that is competent to upregulate RPE-specific genes. This explanation is consistent with previous studies that suggest a direct role for Wnt/ β -catenin signaling in regulating expression of Mitf isoforms in the embryonic RPE in mouse.^{10,17} In our explant cultures, the upregulation of MMP115 expression may be partial because the neural retina is already specified at the time of dissection⁹ and may have lost the ability to change to the RPE fate. In agreement with this, misexpression studies in mouse demonstrate that ectopic Wnt pathway activation is not sufficient to induce significant amounts of RPE-like tissue in the specified retina in vivo.^{30,46}

To further determine whether regulation of RPE-specific gene expression by Wnt/ β -catenin signaling occurs similarly in chick and mouse, we performed loss-of-function experiments during early eye development in the chick embryo. We conducted this experiment using electroporation, by which gene expression can be precisely targeted to specific regions or single cells in the optic vesicle, such as the retina. To inactivate Wnt/ β -catenin signaling in the presumptive RPE, we forced the

FIGURE 1. Effects of Wnt/ β -catenin pathway modulation on RPE differentiation in chick optic vesicle explants and in the embryonic chick eye. (A–C) Chick optic vesicles without extraocular tissues were cultured for 2 days under different conditions and examined for MMP115 expression. (A) MMP115 was not detected in most control explants (Con). (B) MMP115 was induced when culture medium was supplemented with 2.5 mM lithium chloride (LiCl), a Wnt/ β -catenin pathway agonist. (C) Quantification of explants expressing MMP115. (D–F) Inactivating Wnt/ β -catenin signaling in embryonic chick RPE by using DNLe1 induced transdifferentiation of RPE into neural tissue, possibly retina. Separate DNLe1 and EGFP constructs were co-electroporated into presumptive RPE in chick embryos at E1.5 and examined 1 day later. (D, E) EGFP marked the transfected regions; (D, box) magnified in (E, F). (F) In DNLe1/EGFP-transfected RPE (arrowheads), MMP115 (red) was downregulated, whereas the neuronal marker neurofilament-M (NF-M; green) was upregulated.

FIGURE 2. Misexpression of β -catenin or Otx2 is not sufficient to induce Mitf in the presumptive chick retina. (A–F) A bicistronic expression vector containing CA- β -catenin and EGFP (β catEGFP) was electroporated into E1.5 chick optic vesicles that were examined 2 days later. (A, D; same image) EGFP detected with GFP antibody marks transfected regions. (B, E) Mitf and Otx2 proteins were normally expressed in the RPE (brackets), but no ectopic expression was observed in the neural retina. (C, F) Merged files. (G–I) Electroporation of Otx2 (Otx2HA) into presumptive chick retina at E1.5 followed by examination for gene expression at E3.5. (G) Ectopic Otx2 expression, detected by HA immunolabeling, was observed in several cells of the retina. (H) Mitf was not detected in the transfected cells. (I) Merged image.

FIGURE 3. Otx2 and β -catenin activated the Mitf-D enhancer in an additive manner. Dose-response curves were generated by transfecting HEK293T cells with 50 ng MitfD:luc and increasing nanogram amounts of constitutively active β -catenin (CA- β -catenin), Otx2, or both constructs. Luciferase values were plotted as the ratio of induction to basal levels (fold induction). Values for CA- β -catenin (blue diamonds) and Otx2 (red squares) were summed and plotted (orange crosses) to represent additive predicted values. Measured values from CA- β -catenin/Otx2 co-transfection are also plotted (green triangles), and trend-lines were fit to predicted (dashed line; $R^2 = 0.9581$) and measured mean values (solid line; $R^2 = 0.9118$). Slopes were calculated (predicted, 0.115 ± 0.011 ; measured, 0.114 ± 0.016) along with intercept values (predicted, 2.00 ± 0.610 ; measured, 1.64 ± 0.896). ANCOVA tests of the slope and elevation values clearly show that differences between predicted and measured mean values are not statistically significant ($P = 0.949$ and $P = 0.470$, respectively).

expression of dominant-negative Lef1 (DNLeF1), which binds DNA but is unresponsive to β -catenin.³³ In DNLeF1-transfected RPE cells, the expression of MMP115 is downregulated, whereas neuron-specific neurofilament expression is ectopically induced (Figs. 1E, 1F) suggesting that RPE-to-retina trans-differentiation may be occurring. Next, we asked whether Wnt/ β -catenin signaling is sufficient to induce ectopic RPE differentiation in the presumptive chick retina. Constitutively active β -catenin (β catEGFP) was electroporated into the distal chick optic vesicle at E1.5. Immunohistochemical analyses revealed no ectopic Mitf or Otx2 expression 2 to 4 days later (Figs. 2B, 2E; $n = 7$), indicating that, just as in the mouse, activation of Wnt/ β -catenin signaling is not sufficient to promote RPE fates in the presumptive retina. Together, these results show that the role of Wnt/ β -catenin signaling during RPE development is conserved between mouse and chick.

Effect of Otx2 Misexpression on Mitf Expression

We hypothesized that since a single RPE-promoting factor such as Wnt pathway activation is not sufficient to induce ectopic expression of RPE genes in the presumptive retina tissue, additional factors are required. A good candidate is Otx2: It is expressed before the RPE is specified in the optic vesicle, it is transiently downregulated in the presumptive retina during optic vesicle patterning, loss of function results in the loss of Mitf expression, and it can cooperate with Mitf to activate some pigment genes such as tyrosinase.^{21,36} Thus, we asked whether misexpression of Otx2 could promote Mitf expression in the embryonic chick retina. In the developing retina, Otx2 is normally essential for photoreceptor and bipolar cell development.^{47–49} At E3.5, a very small number of scattered cells express endogenous Otx2 in the chick retina and we confirmed that they do not express Mitf (Supplementary Fig. S1; all Supplementary Figures are available at <http://www.iovs.org/cgi/content/full/51/10/5328/DC1>). For misexpression of Otx2, we used an HA-tagged expression construct (Supplemen-

tary Fig. F2).³² Ectopic expression of Otx2 did not induce Mitf expression in the presumptive chick retina (Figs. 2G, 2H; $n = 7$). Thus, it appears that Otx2 alone is not sufficient or, alternatively, that factors are present that create a nonpermissive environment for Otx2 to transactivate RPE-specific genes in the retina.

Mitf Activation by Co-expression of Otx2 and β -Catenin

We hypothesized, therefore, that a combination of both β -catenin and Otx2 would induce Mitf. To examine how co-expression of both factors can activate the Mitf-D enhancer in vitro, we co-transfected HEK293T cells with increasing amounts of both expression constructs. The response curves show an additive effect of β -catenin and Otx2 on Mitf enhancer activation, suggesting that, at least in vitro, both constructs exert a graded response and do not appear to interfere with each other (Fig. 3). To determine whether the combination of β -catenin and Otx2 can induce Mitf expression in vivo, β -catenin and Otx2 expression constructs were transfected into the distal chick optic vesicle at E1.5. After 2 days, 14% of cells co-

FIGURE 4. Misexpression of β -catenin and Otx2 induced Mitf expression. (A–D) E3.5 chicken retinas transfected with Otx2 (Otx2HA) and β -catenin (β catEGFP) were stained with HA (A; red), GFP (B; green), and Mitf antibodies (D; red) and counterstained with DAPI (blue). (C) Merged image (yellow nuclei): coexpression of β -catenin and Otx2. (A–D) arrows: Co-transfected cells with strong HA signals; arrowheads: weakly HA-transfected cells. (D) Co-transfected cells showed induction of ectopic Mitf expression in the retina. (E) Ectopically Mitf-expressing cells were counted and categorized according to colocalization with EGFP (β catEGFP) or HA (Otx2HA). Expression vectors containing only EGFP and HA were used as the control. ANOVA revealed statistical significance between the experimental and the control groups ($P < 0.0001$). (F) The percentage of cells with ectopic Mitf expression was higher in co-transfected cells with strong HA staining.

FIGURE 5. The DNA-binding domain of Otx2 was essential for ectopic Mitf expression in combination with β -catenin. Chick retinas transfected with β catEGFP and with a K50Q Otx2 mutation construct, Otx2K50QHA (resulting in loss of DNA binding), were labeled with HA (A; red), GFP (B; green), and Mitf (D; red) antibodies and counterstained with DAPI (blue). No ectopic Mitf expression was detectable in co-transfected cells in the presumptive chick retina at E3.5 (arrows). (C) Merged image; arrows point to cells co-expressing β -catenin and Otx2.

transfected for β -catenin and Otx2 upregulated ectopic Mitf expression, compared with $<2\%$ in controls transfected with empty vectors (Figs. 4A–E, $n = 5$ embryos). The percentage of Mitf-expressing cells with either β -catenin or Otx2 alone was similar to that in the control groups (Fig. 4E). Of interest, closer observations revealed that, in particularly well-transfected cells (Fig. 4A, arrows; identified by strong HA signals), ectopic Mitf-expressing cells were observed in higher proportions (35% , $n = 5$; Fig. 4F), suggesting that the expression level of Otx2 is critical for inducing Mitf in retinal progenitor cells. In addition, we observed that the DNA binding ability of Otx2 is necessary, as shown by co-transfecting optic vesicles with β -catenin and a mutated form of Otx2 (Otx2K50QHA), which lacks a functional DNA binding domain (Fig. 5, $n = 7$).^{36,50,51} These results demonstrated that Otx2 and β -catenin act cell autonomously and probably directly, to promote ectopic Mitf expression in the embryonic chick retina.

To further determine whether cell fate is affected by β -catenin and Otx2 co-transfection, we examined whether the neural progenitor marker Sox2 is downregulated in ectopic Mitf-positive cells in the retina. Sox2 regulates proliferation and terminal differentiation of retinal progenitors in vertebrates and is downregulated in the chick RPE before cells with characteristic RPE morphology and pigmentation are observed.^{12,52–54} Therefore, in the controls, Mitf and Sox2 expression was normally restricted to the RPE and retina, respectively (Figs. 6A–C). Co-transfection of β -catenin and Otx2 resulted in a decrease in Sox2 expression in a subpopulation of transfected retinal cells with ectopic Mitf expression (Figs. 6G–I; $n = 7$). This finding is consistent with our hypothesis that ectopic expression of β -catenin and Otx2 may induce a change in cell fate from retinal progenitors to presumptive RPE cells by upregulating Mitf expression.

Autoregulation of Mitf and Otx2

Continuous RPE differentiation is ensured by maintaining Mitf and Otx2 expression. Mitf and Otx2 did not appear to directly cross-regulate each other, as shown in our study and by Martinez-Morales et al.³⁶ We asked, therefore, whether Mitf-D and Otx2 can autoregulate their own promoters. The melanocytic Mitf isoform (Mitf-M) is capable of autoregulation, in contrast to Mitf-A.^{36,55} However, it is not known whether Mitf-D can regulate its own enhancer, which contains 12 putative E-box sequences based on the consensus sequence (CANNTG).^{55,56} We generated a mouse Mitf-D expression construct (MitfD) and tested it using electroporation in chicken optic vesicles. Misexpression of MitfD induced ectopic pigmentation and Mitf expression in the presumptive chick retina (Supplementary Fig. S3; $n = 5$). Furthermore, we confirmed that the Mitf-D reporter construct is specifically activated in the native RPE when electroporated into eyes of newborn mice, but not in the postnatal retina (Supplementary Figs. S4A–F). In HEK293T cells, significant induction of the *Mitf-D* enhancer was observed when Mitf-D was co-transfected ($P = 0.002$; Fig. 7A). Conversely, dominant-negative Mitf-D barely activated the *Mitf-D* enhancer, thereby demonstrating that the interaction is specific (Fig. 7A).

Otx2 has been demonstrated to induce *Otx2T0* enhancer activity in luciferase assays³⁶; however, it is not known whether this is mediated through putative bicoid binding sites in the enhancer. We cloned the mouse *Otx2T0* enhancer and confirmed RPE-specific activation by using electroporation of perinatal mouse eyes ($n = 7$; Supplementary Figs. S4G–I). Of interest, the *Otx2T0* enhancer is not activated in the mouse retina at P2, suggesting that Otx2 expression in retinal progenitors is regulated differently ($n = 5$; Supplementary Figs. S4J–K). We identified a putative bicoid binding site in the *Otx2T0* enhancer that is conserved in humans at position -964 (TCTA-ATCCCTA). Although Otx2 significantly activates the wild-type *Otx2T0* enhancer, luciferase expression is not induced when this site is mutated (Fig. 7B; Otx2T0MBS:luc),³⁷ suggesting that activation is direct and depends specifically on Otx2 binding to the putative bicoid site. These results support the idea that once Mitf and Otx2 are induced, they may act in a positive feedback loop by autoregulating their promoters and maintaining their own expression.

DISCUSSION

We and others have shown that Wnt/ β -catenin signaling is necessary to maintain RPE cell fate in the optic cup in mouse; our results suggest that this mechanism is evolutionary conserved in chick. Furthermore, although a complex of β -catenin/TCF/LEF can directly bind to and transactivate the *Otx2* and RPE-specific *Mitf* enhancers,^{10,17} it is not sufficient to induce ectopic Mitf expression in the intact presumptive retina (Fig. 2), which is derived from the same embryonic origin as the RPE and is competent to differentiate into RPE at these early stages.^{57–59} The present study provided evidence that the combined activities of Otx2 and the Wnt pathway are sufficient, at least in part, to mediate respecification of chick retinal progenitor cells into presumptive RPE. We propose that Otx2 is a competence factor and that its expression must be maintained to ensure RPE specification and differentiation in the proximal optic vesicle and optic cup.

Otx2 may establish competence and regulate cell-fate decisions in the eye based on when it is expressed and in tissue-specific contexts. In the early optic vesicle, Otx2 is expressed in the presumptive RPE and in the presumptive retina. One possibility is that additional factors cooperate directly or indirectly with Otx2 to specifically promote the RPE fate. We have demonstrated in the chick that a signal from the adjacent extraocular mesenchyme promotes Mitf

FIGURE 6. Effect of β -catenin and Otx2 co-transfection on Sox2 expression in the chick retina. (A–F) In E3 chick eyes transfected with the control vectors EGFP and HA, Mitf was detected in the RPE (A), and Sox2 was detected in the nuclei of all retinal progenitor cells (B, C). DAPI (D) and Sox2 colocalized in the nucleus (E), and the transfected region is shown in (F). (G–L) In embryos co-transfected with Otx2HA and β catEGFP, ectopic Mitf expression was detected in the presumptive retina (G; arrowheads and arrows), often resulting in downregulation of Sox2 expression in retinal progenitors (H, I). Arrows: Mitf-positive cells with very weak or no detectable Sox2 expression; arrowheads label Mitf-positive cells co-expressing Sox2. DAPI (J) and DAPI/Sox2 merged images (K) confirm that Sox2 was downregulated in the nuclei of cells with ectopic Mitf expression (arrows). (K, inset) Magnification of the area indicated with an asterisk; arrow: a transfected, Sox2-negative cell adjacent to Sox2-positive cells. (L) GFP marks transfected cells.

expression, and we propose that this signal (possibly an activin-like factor) influences RPE cell fate.⁹ Otx2 is expressed at these stages; thus, factors downstream of the activin pathway (e.g., Smad2) may directly or indirectly interact with Otx2 to activate Mitf expression. In contrast to the presumptive RPE, in which Otx2 is continuously expressed, Otx2 expression is downregulated in the presumptive retina of the distal optic vesicle shortly before optic cup morphogenesis. Later, it is re-expressed in some retinal precursor cells to regulate photoreceptor and bipolar cell neurogenesis.^{48,60,61} We observed activation of the *Otx2T0* enhancer in the perinatal mouse RPE but not in the retina. Although we frequently obtained successful transfection in the outer retina, it is possible that the construct was not electroporated into photoreceptor or bipolar precursor cells that express Otx2. Alternatively, distinct Otx2 isoforms have been identified that may control different retina- and RPE-specific functions during ocular development.^{62–64} The *Otx2T0* enhancer may regulate the expression of an Otx2 isoform that only functions in the RPE. Tissue-specific ablation of distinct Otx2 isoforms, therefore, may provide more insight into how Otx2 exerts retina- and RPE-specific functions in the developing eye.

Our *in vivo* electroporation experiments demonstrate that the DNA-binding domain of Otx2 is necessary for the induction of Mitf in combination with β -catenin (Fig. 5) and support a cell-autonomous activation, since Mitf is induced only in cells

FIGURE 7. Mitf-D and Otx2 autoregulate their own enhancers *in vitro*. (A) Mitf-D significantly induced MitfD:luc activity in HEK293T cells ($P = 0.002$), whereas minimal activity was induced by DN-Mitf-D containing a mutated basic DNA binding domain. (B) The Otx2T0:luc construct was significantly activated by Otx2 ($P = 0.0001$), whereas induction was greatly reduced by the K50Q Otx2 mutation (Otx2^{K50Q}), which results in loss of DNA binding, or by a mutation in a putative bicoid site in the reporter construct (Otx2T0MBS:luc). pCMS was used in both assays as filler DNA.

that co-express Otx2 (Fig. 4). In addition, several studies, including our own, imply that the amount of Otx2 protein could be critical for inducing *Mitf* expression. Ablation of one Otx2 allele results in severe RPE specification defects, including loss of *Mitf* expression in an Otx1 null background.²¹ Consistent with this, we observed a higher percentage of ectopic *Mitf*-positive cells when cells that exhibit weak expression of ectopic Otx2 are excluded from the analysis. We also showed with a transactivation assay that β -catenin and Otx2 activate *Mitf-D* in an additive manner (Fig. 3). Although our observations suggest that the combined activity of Otx2 and TCF/LEFs is necessary to transactivate the *Mitf-D* enhancer, the exact mechanism is not known. Thus, further experiments are necessary to determine exactly how Otx2 and TCF/LEF cooperate to promote activation of the *Mitf-D* enhancer.

In the present study, we observed a switch in retinal cells to RPE fate in up to 35% of cells co-transfected with Otx2 and activated β -catenin. The efficacy of both factors in inducing *Mitf* may be dependent on the expression levels of both genes, as mentioned. It is also likely that negatively acting factors such as receptor tyrosine kinase (RTK) signaling (e.g., activated by fibroblast growth factors [FGFs]) negatively regulate *Mitf* protein stability. FGFs are highly expressed in the presumptive retina, and activation of this pathway can result in phosphorylation and subsequent degradation of *Mitf* protein.⁶⁵ Furthermore, the paired-like homeodomain transcription factor *Vsx2* is robustly expressed in the retina at the optic vesicle stage and can directly suppress *Mitf* expression.¹⁹ Thus, *Vsx2* may act as a strong suppressor in preventing upregulation of ectopic *Mitf* expression in co-transfected cells in our electroporation experiments. Another explanation is the lack of RPE-promoting factors, for example an activin-like signal. In mouse and chick, recent reports suggest that activin signaling may support maintenance of the RPE status; *Smad2* phosphorylation is detectable in the differentiating RPE and inhibition of activin signaling facilitates transdifferentiation into retina.⁶⁶ In the developing chick retina, the activin antagonist follistatin is highly expressed⁶⁷; thus, activin signaling may be suppressed during early stages to ensure proper patterning of the retina in the optic cup.

Enhancement of Generation of RPE-like Cells from Stem Cells

The generation of RPE-like cells from stem cells is a strategy to obtain RPE cells for transplantation in blinding conditions caused by dysfunction and loss of RPE. Under certain culture conditions, cells with RPE-like characteristics can be spontaneously derived from induced pluripotent stem cell lines (iPSCs)^{68,69} or hESCs. However, the generation of pigmented foci is slow and rare, and the identification of RPE-promoting factors is critical for improving the yield of RPE-like cells. Recently, treatment of hESC with nicotinamide and activin A, originally identified in our experiments as an RPE-promoting factor,⁹ increased the formation of RPE-like cells dramatically.¹ iPSCs and hESCs express Otx2 endogenously, which we propose to be an important prerequisite for *Mitf* induction.^{1,4,68} We identified Wnt signaling as an additional RPE-promoting factor that could further improve the efficacy by which RPE cells are induced from stem cells.

Acknowledgments

The authors thank Andrew Loudon, Kayla Dyorich, and the Levine and Baehr laboratories for technical help; Chang Jiang Zou for providing the *Mitf-D* enhancer fragment; Harukazu Nakamura and David Kimelman for expression constructs; and Wolfgang Baehr, Rich Dorsky, Kathryn Moore, and Ed Levine for helpful comments.

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