Zeb1 Represses Mitf and Regulates Pigment Synthesis, Cell Proliferation, and Epithelial Morphology

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PURPOSE. Epithelial-mesenchymal transition (EMT) is important in fibrotic responses, formation of cancer stem cells, and acquisition of a metastatic phenotype. Zeb1 represses epithelial specification genes to enforce epithelial-mesenchymal phenotypic boundaries during development, and it is one of several E-box-binding repressors whose overexpression triggers EMT. The purpose of this study was to investigate the potential role for Zeb1 in EMT leading to the dedifferentiation of retinal pigment epithelial (RPE) cells.

METHODS. Real-time PCR was used to examine mRNA expression during RPE dedifferentiation in primary cultures of RPE cells from Zeb1(+/-) mice and after knockdown of Zeb1 by lentivirus shRNA. Chromatin immunoprecipitation was used to detect Zeb1 at gene promoters in vivo.

RESULTS. Zeb1 is overexpressed during RPE dedifferentiation. Heterozygous mutation or shRNA knockdown to prevent this overexpression eliminates the onset of proliferation, loss of epithelial morphology, and pigment, which characterizes RPE dedifferentiation. Zeb1 binds to the Mitf A promoter in vivo, and Zeb1 mutation or shRNA knockdown derepresses the gene. The authors link Zeb1 expression to cell-cell contact and demonstrate that forcing dedifferentiated RPE cells to adopt cell-cell only contacts via sphere formation reverses the overexpression of Zeb1 and reprograms RPE cells back to a pigmented phenotype.

CONCLUSIONS. Overexpression of the EMT transcription factor Zeb1 has an important role in RPE dedifferentiation via its regulation of Mitf. Expression of Zeb1 and, in turn, RPE dedifferentiation, is linked to cell-cell contact, and these contacts can be used to diminish Zeb1 expression and reprogram de-differentiated RPE cells. (Invest Ophthalmol Vis Sci. 2009;50: 5080–5088) DOI:10.1167/iovs.08-2911

Epithelial-mesenchymal transition (EMT) early in embryogenesis is responsible for delamination of neural crest cells from the neural tube and defining the ectodermal-mesodermal boundary required for gastrulation.1–3 However, EMT is also central to pathologic fibrotic responses. In addition, it is a hallmark of carcinomas, in which the loss of cell-cell contacts, tight junctions, and polarity and the onset of extracellular matrix degradation contribute to a proliferative, motile, fibroblastic phenotype that facilitates metastasis.2–4 Furthermore, recent studies demonstrate that EMT is responsible for inducing the CD44high/CD24low expression pattern associated with the formation of epithelial cancer stem cells.5

The phenotypic changes in EMT result from repression of epithelial specification genes by a set of related transcriptional repressors, including Snail (Snai) and zinc finger E-box binding homeodomain (Zeb) family members (Zeb1, also known as TCF8, δ-EFI, ZFHX1A, and ZHep and Zeb2, also known as Smad-interacting protein 1; reviewed in 6). These EMT transcription factors bind overlapping sets of E-box promoter elements to repress epithelial specification genes such as E-cadherin. They become overexpressed in cancer and in fibrotic responses,7–12 and overexpression of any one of them appears sufficient to initiate EMT.5 In Zeb1 mutants, mesenchymal progenitors in the craniofacial region and skeleton and neural progenitors in the CNS ectopically express epithelial markers including E-cadherin and cytokeratin, and they show proliferative defects.13 Accordingly, late-stage mutant embryos have severe craniofacial defects, skeletal curvatures, and shortened limbs and digits, and a subset of the embryos has a severe neural phenotype, with failure of neural tube closure at both cranial and caudal ends leading to exencephaly.14 Although Zeb1 heterozygous mice are viable, they show defective smooth muscle cell (mesenchymal) differentiation in response to vascular injury, leading to increased neo-intima formation.15 No defect in smooth muscle formation was evident in heterozygous mice before vascular injury, implying that this decrease in Zeb1 dosage is crucial only in response to injury. However, it has been demonstrated recently that heterozygous mutation of Zeb1 is responsible for posterior polymorphous corneal dystrophy (in both humans and mutant mice), which is characterized by a pathologic epithelial transition of the corneal endothelium, leading to corneal dysfunction.16–18 Thus, in some tissues, decreasing the level of Zeb1 by heterozygous mutation appears sufficient to drive an epithelial-like transition in the absence of injury.

We examined EMT in retinal pigment epithelial (RPE) cells as a clinically relevant model of this fibrotic transition. The retinal pigment epithelium forms a monolayer adjacent to photoreceptors in the retina, and these cells are essential for photoreceptor viability. RPE cells are normal in cell-cell contact and thus nonproliferative in vivo. However, on retinal detachment, RPE cells can be dislodged into the vitreous, where they bind the detached retina, initiate proliferation, and undergo EMT, and lose their pigment synthesis program. The resultant fibroblastic cells bound to the retina contract the retina, preventing its reattachment. This condition is known as proliferative vitreoretinopathy, and it is the major complication in retinal detachment surgery.19–21 It appears that the initiation of proliferation and inhibition of the pigment synthesis program in RPE cells is triggered by loss of cell-cell contact. If RPE cells are destroyed focally by laser treatment or by surgical debridement, cells on either side of the area of RPE loss initiate proliferation to fill in the gap.22 However, these proliferating...
cells are unable to initiate pigment synthesis and acquire a normal RPE phenotype, and they remain unpigmented after the gap is filled. Similarly, though sheets of RPE cells retain epithelial morphology and remain nonproliferative and pigmented in primary culture, dissociated cells in primary culture initiate proliferation and undergo EMT, losing their pigment synthesis program. Here, we link Zeb1 to initiation of proliferation, loss of pigment, and transition to fibroblastic morphology during RPE dedifferentiation.

**Materials and Methods**

**Primary Cell Culture**

Eyes were removed from C57BL/6 mice at postnatal day (P) 1 and were washed with Ca/Mg-free HBSS. The anterior segment and lens were removed, and RPE sheets were then dissected away from the posterior segment and digested with 0.25% trypsin for 10 minutes at 37°C before plating in DMEM with 10% fetal bovine serum at 5% CO₂. Cells were passaged 1:2 using trypsin/EDTA once they became confluent. All animals were handled according to the regulations of the Institutional Animal Care and Use Committee, and all procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Sphere Formation and Feeder Layer Preparation**

For sphere formation, cells were scraped from tissue culture plates and transferred to nonadherent plates. For reattachment, spheres were transferred to tissue culture plates or Petri dishes containing feeder layer cells. Feeder layers were created by irradiating the SNL fibroblast cell line 6 with 60 Gy to arrest cell proliferation and subsequently plating the irradiated cells on tissue culture or Petri dishes. Feeder layers remained viable and nonproliferative for at least 1 month in culture.

**RNA Extraction and Real-Time PCR**

Total cellular RNA was extracted (RNaseasy Mini Kit; Qiagen Sciences, Germantown, MD). cDNA was synthesized with a reverse transcriptase kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). SYBR Green real-time PCR was performed (Mx3000P Real-Time PCR System; Stratagen, Cedar Creek, TX) as described previously. The sequence and annealing temperature of PCR primers is shown in Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/50/11/5080/DC1. Three independent samples were analyzed for each condition or cell type, and each sample was compared in at least three independent RT-PCR amplifications.

**Chromatin Immunoprecipitation Assays**

Chromatin immunoprecipitation (ChIP) assays were based on the Up- State protocol (http://www.upstate.com/misc/protocol) using formaldehyde to cross-link genomic DNA. Polyclonal antiserum for Zeb1 and histone 3 were used for immunoprecipitation. Equal amounts of anti-IgG or preimmune serum were used as controls. ChIP PCR reactions were similar to those described for real-time PCR using primer sets (Supplementary Table S1) to amplify Mitf A, D, and H promoters and the GAPDH control promoter.

**Lentivirus shRNA**

The shRNA oligomers used for Zeb1 and Zeb2 silencing have been described previously. Controls were performed for these sequences previously. Furthermore, the Zeb2 sequence differs in only 5 of the 19 nucleotides; thus, it serves as a control for the Zeb1 sequence. We first cloned the shRNA into a CMV-GFP lentiviral vector, where its expression was driven by the mouse U6 promoter. Briefly, the shRNA construct was generated by synthesizing an 83-mer oligonucleotide containing: (i) a 19-nucleotide sense strand and a 19-nucleotide antisense strand, separated by a nine-nucleotide loop (TTCAAGAGA); (ii) a stretch of five adenines as a template for the Pol III promoter termination signal; (iii) 21 nucleotides complementary to the 5’ end of the Pol III U6 promoter; and (iv) a 5’ end containing a unique XbaI restriction site. The long oligonucleotide was used, together with a sp6 oligonucleotide (5‘-ATTTAGGTGACACTATAGAAT-3‘), to PCR-amplify a fragment containing the entire U6 promoter plus shRNA sequences; the resultant product was digested with XbaI and SpeI ligated into the XbaI and SpeI sites of the lentivirus vector, and the insert was sequenced to ensure that no errors occurred during the PCR or cloning steps. The detailed procedure is published elsewhere. Lentiviral particles were produced by a four-plasmid transfection system as described. Briefly, 293T cells were transfected with the lentiviral vector and packaging plasmids, and the supernatants, containing recombinant pseudoviruses, were collected and filtered. These pseudoviruses expressing shRNAs targeting Zeb1 were transduced into these lentiviral particles expressing shRNAs targeting Zeb1. A transduction efficiency of nearly 100% was achieved based on GFP-positive cells.

**Cell Images**

Photographs of cells were taken with an inverted microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany) and a digital camera (AxioCam MRC5; Carl Zeiss). Images were saved as jpg files and were directly imported into a presentation program (PowerPoint; Microsoft, Redmond, WA) for assembly into figures.

**Results**

**Dedifferentiation of RPE Cells**

Figure 1A (left) shows dissociated RPE cells at day 1 in primary culture. The cells are initially pigmented, and they exhibit epithelial morphology. After three passages, they lost pigment, but many of them retained epithelial morphology (Fig. 1A, middle). By passage 6, all the cells lost epithelial morphology and became fibroblastic in appearance (Fig. 1A, right). Thus, loss of pigment occurs initially in the cultured cells, followed by transition to fibroblastic morphology.

**Downregulation of mRNA for Mitf and Its Target Genes in Dedifferentiated RPE Cells**

Next, we used real-time PCR to compare mRNA expression in RPE tissue and dedifferentiated RPE cells in culture at passage 3. Notably, there was downregulation of Mitf mRNA in the dedifferentiated cells that was also associated with downregulation of important Mitf target genes, such as RPE65, and components of the pigment synthesis pathway, Tyr and Tyrp1 (Fig. 2A). Mitf is crucial for the differentiation of both melanocytes and retinal pigment epithelium, where it links differentiation to cell proliferation.

In retinal pigment epithelium, Mitf cooperates with Otx2 to activate transcription, and, as with Mitf, we found that Otx2 mRNA was also downregulated in the dedifferentiated RPE cells (Fig. 2A). Additionally, there was induction of PAI1 mRNA, a classic mesenchymal gene induced during EMT, which regulates extracellular matrix degradation and thus cell motility (Fig. 2B). We did not detect expression of E-cadherin mRNA (Cdh1) in the RPE cells, as occurs in other epithelia (Fig. 2B). We also did not detect expression of mRNA for the neuronal marker β-III tubulin, suggesting that the RPE cells are not undergoing transdifferentiation to neurons under these conditions in culture (Fig. 2B).

Chx10 is required to repress Mitf in forming retinal cells. Therefore, we asked whether the induction of Chx10 might be responsible for repressing Mitf in dedifferentiated RPE cells. However, we detected only a low level of Chx10 mRNA in RPE tissue, and this level did not increase in RPE cells in culture (Fig. 2B, solid bar). As a positive control, we did detect abundant Chx10 mRNA in the newborn mouse.

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**Zeb1 and Pigment Cell Differentiation**

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retina (Fig. 2B, open bar). Sox10 and Pax3 cooperate to induce Mitf expression in melanocytes31,32; however, we did not detect Sox10 or Pax3 mRNA in RPE tissue or cultured cells (Fig. 2B). Therefore, we conclude that neither induction of Chx10 nor loss of Sox10 or Pax3 is responsible for downregulation of Mitf mRNA in dedifferentiating RPE cells.

Overexpression of Zeb1 and Snai1 mRNA in Dedifferentiating RPE Cells

Because dedifferentiation of RPE cells has hallmarks of an EMT, we hypothesized that EMT transcription factors such as Zeb and Snai might be induced during the process. Real-time PCR was used to compare the level of mRNA expression for Zeb and Snai family members in RPE tissue and dedifferentiated RPE cells in culture. Both Zeb1 and Snai1 mRNAs were over-expressed in dedifferentiated RPE cells (Fig. 2B). However, mRNA for Zeb2 was not induced, and Snai2 mRNA was only modestly induced.

RPE Cells from Zeb1 Heterozygous Mice Do Not Undergo Dedifferentiation or Ectopic Proliferation

Heterozygous mutation of Zeb1 led to an approximately five-fold reduction in mRNA (Fig. 2C; >50% reduction with heterozygous mutation suggests that Zeb1 may be autoregulatory).
Thus, Zeb1 overexpression in dedifferentiated wild-type RPE cells (Fig. 2B) is largely offset by heterozygous mutation (Fig. 2C). We noted that the Zeb1 heterozygous RPE cells were highly pigmented at day 1 in culture (Fig. 1A, left), and, in contrast to cells from wild-type littermates, the mutant cells did not lose pigment, nor did they transition to fibroblastic morphology (Fig. 1B). Thus, heterozygous mutation of Zeb1 appears to prevent dedifferentiation of the RPE cells. As a control, the Zeb1 heterozygous mutation did not lead to a significant change in expression of other related EMT factors, such as Snai1, Snai2, or Zeb2 (Fig. 2C).

A key event in RPE dedifferentiation is the onset of proliferation in the normally quiescent cells.19–22 This ectopic proliferation along with EMT contributes to the failure in surgical retinal reattachment in patients with proliferative vitreoretinopathy. Wild-type RPE cells immediately began to proliferate in culture with a doubling time at passage 1 of 28 hours (Supplementary Fig. S1; all Supplementary Figures are online at http://www.iovs.org/cgi/content/full/50/11/5080/DC1). In addition, this proliferative capacity did not diminish by passage eight (doubling time, 24 ± 4 hours), when the cells had acquired fibroblastic morphology (as seen in Fig. 1A, right). By contrast, the number of Zeb1 mutant RPE cells remained stable for at least 1 month in culture. The same cells were counted each day, and there was no evidence of Zeb1 mutant cell loss during this time in culture. Thus, in addition to dedifferentiation, mutation of Zeb1 prevents ectopic proliferation of RPE cells in culture.

**Zeb1 Mutation Prevents Repression of Mitf and Mitf Target Genes in Cultured RPE Cells**

We compared expression of mRNAs for Mitf and its target genes in wild-type and Zeb1 heterozygous RPE cells in culture. Mitf and its target gene mRNAs were not downregulated in the mutant RPE cells, even after 1 month in culture (Fig. 2D). In fact, the level of Mitf mRNA in the heterozygous cells was higher than in normal RPE tissue (Figs. 2A, D; note that in these figures, both normal RPE tissue and Zeb1 heterozygous RPE cells are compared with the same RPE cells at passage 3). Thus, heterozygous mutation of Zeb1 is sufficient to prevent downregulation of Mitf and Mitf target genes in RPE cells, even after prolonged culture.

**Lentivirus shRNA Knockdown of Zeb1 Prevents Ectopic Proliferation, Loss of Pigment, and Repression of Mitf mRNA in RPE Cells**

shRNA knockdown of Zeb1 has been described previously.15 We created a lentivirus for knockdown of Zeb1 expression based on this previously described construct. RPE cells were transfected with the lentivirus, and infection efficiency was assessed by GFP expression. Nearly 100% of the cells were infected, and Zeb1 mRNA and protein expression was effectively silenced (Fig. 3A). As with heterozygous mutation of Zeb1, lentivirus infection prevented proliferation of the RPE cells, and the cells retained pigment after 4 weeks in culture,
whereas uninfected cells lost pigment and transitioned to a fibroblastic morphology (Figs. 3B, C). Accordingly, Mitf mRNA expression was maintained at a high level with Zeb1 knockdown (Fig. 3D). These results provide additional evidence that Zeb1 expression is linked to repression of Mitf, proliferation of RPE cells, and their EMT in culture. As a control for these experiments, the closely related Zeb2 family member was also knocked down by lentivirus shRNA. This led to a loss of Zeb2 mRNA and protein expression; however, in contrast to knockdown of Zeb1, this knockdown of Zeb2 did not block proliferation (Supplementary Fig. S2).

Zeb1 Binds to the Mitf A Promoter In Vivo

Mitf can be transcribed from multiple promoters, including M, which is melanocyte-specific, H, which is used in the heart, A and D, which are more widely used in cells, including retinal pigment epithelium (32 and references therein). Real-time PCR primers were designed to specifically amplify transcripts arising from the unique first exon transcribed from these promoters (Fig. 4A; Supplementary Table S1). RPE tissue at P1 was examined. Transcripts for the A, H, and D promoters were detected, and the relative abundance was A > H > D (Fig. 4B). A low level of Mitf A promoter transcripts was evident in wild-type RPE cells at passage 3, but the level of these transcripts was high in Zeb1 heterozygous RPE cells (even after 1 month in culture [Fig. 4C], as occurred with total Mitf mRNA [Fig. 2D]). These results suggest that transcripts arising from the Mitf A promoter are repressed by Zeb1 overexpression in the wild-type cells. We did not detect transcripts from the H, D, or M promoters in either the wild-type or mutant RPE cells in culture (Fig. 4C), suggesting that under these conditions, the A promoter is primarily used to transcribe Mitf.

We wondered whether Zeb1-dependent repression of the Mitf A promoter in dedifferentiated RPE cells might be a direct effect of Zeb1 binding. Inspection of promoter sequences revealed potential Zeb1 E-box binding sites in the A promoter, but not the H or D promoter (results not shown). To determine whether Zeb1 was bound to these promoters in vivo, primers were designed to amplify a region upstream of each promoter, whether Zeb1 was bound to these promoters in vivo, primers were designed to amplify a region upstream of each promoter. Chromatin was cross-linked to bound proteins and sheared to an average size of 1 kb, allowing detection of binding at least 1 kb upstream and downstream of the primer sites. This chromatin was then precipitated with anti-Zeb1 antibody or, as a negative control, with preimmune sera (IgG). As a positive control, chromatin was precipitated with an antibody to histone H3, which is ubiquitously present. Real-time PCR was used to quantify the precipitated promoter fragments bound to Zeb1, and results were normalized to input DNA before immunoprecipitation. We found that Zeb1 was bound to the Mitf A promoter but not to the H or D promoter (Fig. 4D). Each of the promoters was bound by the positive control, histone H3. As an additional negative control, we did not detect Zeb1 binding to the Gapdh promoter (histone H3 was also evident at this promoter; Fig. 4D).

Induction of p21CDKN1a in Zeb1 Mutant RPE Cells

In addition to its role in the transactivation of genes important for RPE specification, Mitf inhibits cell proliferation because of its transactivation of cyclin-dependent kinase (cdk) inhibitors p21CDKN1a and p16INK4a.27, 28 These inhibitors block cdk2 and cdk4/6 activity, respectively.24, 25 Each of these cdkjs can drive proliferation, at least in part, through hyperphosphorylation and inactivation of the cell cycle inhibitory retinoblastoma
protein (Rb). Because Mitf mRNA does not diminish in the Zeb1 mutant RPE cells in culture, we hypothesized that p21CDKN1a and p16INK4a mRNAs would be elevated in the mutant RPE cells, correlating with their lack of proliferation. Therefore, we used real-time PCR to compare the level of p21CDKN1a and p16INK4a mRNA in wild-type and Zeb1 mutant RPE cells in culture. We did not detect p16INK4a mRNA in either the wild-type or Zeb1 mutant RPE cells, nor did we detect mRNA for p15INK4b, which also inhibits cdk4/6 (Fig. 2D). However, we did detect an increased level of p21CDKN1a mRNA in the mutant cells (Fig. 2D). This level of p21CDKN1a mRNA induction was similar to that seen in response to p53 activation during senescence of mouse embryo fibroblasts.35

Sphere Formation Reprograms Dedifferentiated RPE Cells Back to a Differentiated Phenotype

We noticed that, on primary culture, RPE cells formed clusters of highly pigmented cells on tissue culture plates (Fig. 5A, left; Supplementary Fig. S3). Cells in the central regions of these clusters retained pigment and epithelial morphology and were nonproliferative, whereas at the periphery of these clusters, a monolayer of proliferating cells appeared; these proliferating cells migrated away from the original cluster, lost pigment, and acquired fibroblastic morphology (Fig. 5A, middle and right; Supplementary Fig. S2). With time, the central cluster became progressively smaller, finally giving way to dedifferentiated cells that could be maintained in culture for more than 10 passages. These observations suggested that dedifferentiation may be initiated by the loss of cell-cell contacts because cells at the periphery migrated away from neighboring cells. Indeed, the importance of confluence in maintaining RPE differentiation in culture has been suggested previously.36

We then wondered whether dedifferentiated RPE cells in monolayer culture could be reprogrammed to a differentiated phenotype by reestablishing cell-cell contacts. To test this possibility, we forced dedifferentiated cells to establish cell-cell only contacts through sphere formation. Dedifferentiated RPE cells (Fig. 5B, left) at passage 8 in monolayer culture were scraped from culture dishes and placed in suspension in nonadherent dishes. The cells immediately formed spheres, which remained viable in culture for weeks (Fig. 5B, middle). The efficiency of sphere formation was high, with most cells forming spheres. If cells were trypsinized and placed in suspension, they did not efficiently form spheres, and the single cells rapidly (within hours) underwent apoptosis (known as anoikis when epithelial cells in suspension undergo apoptosis9; results not shown). We noticed that the cells became pigmented within 1 day after sphere formation (Fig. 5B, middle), demonstrating that pigment synthesis was restored.

Real-time PCR was used to compare the expression of mRNAs in dedifferentiated RPE cells at passage 8 in monolayer culture with cells from the same culture but that underwent 2 additional days of sphere formation. Sphere formation led to reexpression of Mitf mRNA and upregulation of mRNAs for Mitf target genes, including RPE65 and genes involved in pigment synthesis (Fig. 6A). Additionally,
Consistent with this notion, real-time PCR analysis revealed a diminished level of mRNAs for Mitf and Otx2 (compare Fig. 6A).

**Forcing Reprogrammed RPE Cells from Spheres to Maintain Association with the Feeder Layer Prevents Dedifferentiation**

We wondered whether reprogrammed RPE cells derived from spheres would maintain a differentiated phenotype if they were forced to remain adherent to feeder layer cells (e.g., if they were prevented from migrating onto the surrounding tissue culture dish). We noticed that though the SNL cell line used to derive feeder layer cells could adhere to Petri dishes, cells from RPE spheres could not (they would attach only to tissue culture dishes). Therefore, we allowed RPE spheres to adhere to feeder layers plated on Petri dishes. Cells from the spheres migrated onto the feeder layer, but they did not subsequently migrate onto the Petri dish. Under these conditions in which cell-cell contact with the feeder layer was forcibly maintained, the cells from RPE spheres remained viable and pigmented as a nonproliferative monolayer for more than 1 month in culture (Fig. 5D, right).

**Zeb1 Expression Is Regulated during RPE Dedifferentiation and Reprogramming**

We compared the levels of Zeb1 mRNA in RPE tissue, dedifferentiated cells in monolayer culture, dedifferentiated cells induced to form spheres, and monolayer cells derived from the spheres that had migrated onto culture dishes, lost pigmentation, and begun proliferation (cells resembling those in Fig. 5D, middle). We found that sphere formation repressed Zeb1 mRNA and that its level was reinduced as cells from the spheres reformed a monolayer on tissue culture plates and again lost pigment (Fig. 6C). Thus, the level of Zeb1 mRNA is inversely correlated with RPE differentiation. The feeder layer cells that had migrated back onto a tissue culture plate for 1 week (TC plate; see Fig. 5D, middle). (B) Real-time PCR showing the effects of sphere formation on expression of cdk inhibitor mRNAs. n.d., not detected. (C) Real-time PCR comparing the effects of RPE cell culture, sphere formation, and migration of cells from spheres back onto a tissue culture dish (sphere-TC plate, as in B) on Zeb1 mRNA levels. Fold differences in mRNA levels are shown by real-time PCR. (D) Real-time PCR for the Neo gene in SNL cells (filled bars) and a mixture of RPE cells plated on SNL cells (open bars). Results were normalized to the level of ACTB mRNA in the two samples.

Cells from RPE Spheres Can Reform Monolayers and Again Undergo Dedifferentiation

We wondered whether reprogrammed RPE cells derived from spheres would migrate from the spheres to reform a monolayer; however, the spheres did not attach efficiently to tissue culture dishes. Therefore, we plated them on a feeder layer of embryonic fibroblasts that had been irradiated to block their proliferation (as is used for culture of embryonic stem cells; Fig. 5B, right). The spheres attached efficiently to the feeder layer (Fig. 5C, left), and cells began migrating from the spheres onto the feeder layer (Fig. 5C, middle and right). The RPE cells initially migrating from the spheres were round and pigmented; however, as they attached to the feeder layer, the cells took on an epithelial morphology, and they maintained pigmentation (Fig. 5C, middle and right; Fig. 5D, left). After 1 week in culture, cells began to migrate from the feeder layer onto the surrounding culture dish, and, as this occurred, they became proliferative and lost pigment (Fig. 5D, middle). At this point, the cells resembled cells in monolayer culture at passage 3 (Fig. 1A, middle). Subsequently, the cells transitioned to a fibroblastic morphology (appearing like cells in Fig. 1A, right; results not shown). These results suggested that the cells from the spheres were again undergoing dedifferentiation as they migrated onto the tissue culture dish. Consistent with this notion, real-time PCR analysis revealed a diminished level of mRNAs for Mitf and Otx2 (compare Fig. 6A).
normalized to β-actin (ACTB) mRNA, and they indicate that the SNL cells contributed only a small portion of the mRNA to the mixture (Fig. 6D).

**DISCUSSION**

Overexpression of Zeb1, which occurs as RPE cells undergo dedifferentiation in culture, leads to repression of Mitf A expression. Concomitant with this repression, there is downregulation of Mitf target genes, including genes involved in pigment synthesis, RPE65, and the cdk inhibitor p21CDKN1a. Thus, Zeb1-mediated repression of Mitf could conceivably account for both the loss of pigment gene expression and the ectopic proliferation seen in dedifferentiating RPE cells. However, Zeb1 overexpression also repressed expression of Otx2 (which is required along with Mitf for RPE formation in vivo), and Zeb1 is known to repress epithelial specification genes involved in cell-cell contact (E-cadherin), tight junction formation (the Pals1 family), and polarity (Crumbs3 and Hugl2) in nonpigmented epithelial cells. 5,8 Repression of these genes is thought to facilitate the characteristic transition from epithelial to fibroblastic morphology seen in EMT. Taken together, these results imply that Zeb1 overexpression may have effects beyond repression of Mitf. For example, mutation of Zeb1 leads to alterations in epithelial versus mesenchymal phenotypic balance and proliferation in nonpigmented cells in vivo, 13 and in mouse embryo fibroblasts (MEFs) Zeb1 mutation leads to induction of epithelial specification genes such as E-cadherin, to epithelial morphology in the fibroblasts, and to induction of cyclin-dependent kinase inhibitors and growth arrest. Mitf mRNA is not induced in the mutant MEfS, and the mutant cells do not become pigmented 15 (Supplementary Fig. S5). Thus, the role of Zeb1 in regulating proliferation and epithelial morphology appears separable from its repression of Mitf and its role in maintaining pigment. In addition, we suggest that its repression of Mitf and inhibition of RPE differentiation only become evident when it is overexpressed.

Interestingly, Zeb1 mutation inhibits cell proliferation in MEFS and at sites of developmental defects in nonpigmented cells in vivo. 13 These proliferative defects are associated with an induction of cdk inhibitors but not with a change in Mitf expression (Supplementary Fig. S5). Zeb1 can bind directly to the p21CDKN1a promoter, 13 and Mitf can also bind to this promoter in vivo; 27; thus, ectopic proliferation and repression of p21CDKN1a seen with Zeb1 overexpression in dedifferentiating RPE cells may be caused by both direct repression by Zeb1 and downregulation of the transcriptional activator Mitf. Although overexpression of a repressor and downregulation of a transactivator would provide for efficient repression of p21CDKN1a, coordinate regulation by Zeb1 and Mitf may prove even more complex. Both Zeb1 and Mitf are E-box-binding proteins, which bind to variations of the core sequence CANNTG. Mitf can bind to CATGTG, CACGTG, and CACCTG, whereas Zeb1 binds efficiently to CACCTG. 38 However, Zeb1 also binds to the CACGCTG sequence bound by Mitf. 38 Further, the E-box sites in the p21CDKN1a promoter contain the sequence CACGCTG, and both Mitf and Zeb1 have been shown to bind these sites in vivo. 15, 27 Thus, it is possible that overexpression of Zeb1, coupled with downregulation of Mitf, allows Zeb1 to compete effectively with Mitf for binding to common E-boxes, not only on the p21CDKN1a promoter but also on the promoters of other Mitf target genes. In this regard, it is of note that E-boxes in the Tyr promoter contain the same CACGCTG sequence. 37

We demonstrate here that Zeb1 expression is repressed by cell-cell contact, and its induction in RPE cells, which have lost cell-cell contact, triggers proliferation and loss of epithelial morphology and pigment synthesis. Cell contact inhibition is mediated by the cell cycle control pathway consisting of the Rb family and its E2F transcription factor–binding partners. Indeed, mutation or inhibition of the Rb family eliminates cell contact inhibition. 50 E2F binds directly to the Zeb1 gene promoter, and an Rb-E2F complex represses transcription of Zeb1. 50 Therefore, it is likely that Zeb1 is repressed on cell-cell contact by the Rb family-E2F complexes, which form to mediate the cell cycle arrest leading to contact inhibition. Consistent with this notion, Zeb1 expression in vivo in the developing mouse embryo is closely linked to cell proliferation. 13, 31

Our results link cell-cell contact to proliferation and maintenance of pigment synthesis in RPE cells. These processes in RPE cells are all regulated by Zeb1 expression, which in turn is controlled by cell-cell contact. Finally, this ectopic proliferation and the loss of pigment synthesis can be reversed by forcing dedifferentiated cells to adopt cell-cell only contacts through sphere formation.

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**References**