Characterization of a Transient TCF/LEF-Responsive Progenitor Population in the Embryonic Mouse Retina

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PURPOSE. High mobility group (HMG) transcription factors of the T-cell-specific transcription factor/lymphoid enhancer binding factor (TCF/LEF) family are a class of intrinsic regulators that are dynamically expressed in the embryonic mouse retina. Activation of TCF/LEFs is a hallmark of the Wnt/β-catenin pathway; however, the requirement for Wnt/β-catenin and noncanonical Wnt signaling during mammalian retinal development remains unclear. The goal of the study was to characterize more fully a TCF/LEF-responsive retinal progenitor population in the mouse embryo and to correlate this with Wnt/β-catenin signaling.

METHODS. TCF/LEF activation was analyzed in the TOPgal (TCF optimal promoter) reporter mouse at embryonic ages and compared to Axin2 mRNA expression, an endogenous readout of Wnt/β-catenin signaling. Reporter expression was also examined in embryos with a retina-specific deletion of the β-catenin gene (Ctbm1), using Six3-Cre transgenic mice. Finally, the extent to which TOPgal cells coexpress cell cycle proteins, basic helix-loop-helix (bHLH) transcription factors, and other retinal cell markers was tested by double immunohistochemistry.

RESULTS. TOPgal reporter activation occurred transiently in a subpopulation of embryonic retinal progenitor cells. Axin2 was not expressed in the central retina, and TOPgal reporter expression persisted in the absence of β-catenin. Although a proportion of TOPgal-labeled cells were proliferative, most coexpressed the cyclin-dependent kinase inhibitor p27/Kip1.

CONCLUSIONS. TOPgal cells give rise to the four earliest cell types: ganglion, amacrine, horizontal, and photoreceptor. TCF/LEF activation in the central retina does not correlate with Wnt/β-catenin signaling, pointing to an alternate role for this transcription factor family during retinal development.

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The neural retina develops from ventral forebrain neuroepithelium, and when mature, comprises seven major types of neurons and glia. These retinal cell types are generated in an evolutionary conserved order with ganglion cells formed first, then cones, horizontal cells, amacrine cells, and rods, with bipolar cells and Müller glia generated last.1–5 Retinal progenitors are generally multipotent; however, over time they exhibit both lineage and competence restrictions such that fewer and fewer distinct cell types arise as development proceeds.6–11 The competence of retinal progenitors is regulated by both extracellular and intrinsic factors.11–14 The intrinsic factors have so far fallen into two main protein classes: basic helix-loop-helix (bHLH) or homeobox transcription factors. In particular, the bHLH factors regulate the development of distinct retinal cell types, as they do throughout the vertebrate nervous system.11,14,15 For example, one bHLH factor, Math5, is required for ganglion cell formation,16–20 while another, NeuroD, is necessary for amacrine, S cone and rod photoreceptor cell genesis.21–25 Of note, different bHLH factors are further segregated by their expression at distinct stages of the mitotic cell cycle, with Ngn2 and Mash1 expressed by many S phase retinal progenitors,24 and Math5 present in newly postmitotic, transitional cells.25–27 Because the expression patterns and functions of these intrinsic factors are insufficient to explain how all retinal cell types develop, their integration with other pathways is essential to understanding retinal neurogenesis at the molecular level.

We and others observed activity of the HMG transcription factors TCF/LEF in retinal progenitors in the mouse embryo, consistent with endogenous TCF/LEF mRNA expression.27–29 TCF/LEFs (TCF1/TCF7, LEF1, TCF3/TCF7l1, TCF4/TCF7l2) are best known for mediating Wnt (wingless-type MMTV integration site family) signaling through interaction with the coactivator β-catenin.30–32 On Wnt binding to the Frizzled receptor, stabilized β-catenin translocates to the nucleus where it interacts with TCF/LEFs, to activate the transcription of target genes. Previous studies in Xenopus suggested that Wnt/β-catenin signaling is critical for early retinal development, where it controls neural competence of retinal progenitor cells by regulating Sox2 function.33 However, in the mammalian eye, the role of Wnt/β-catenin signaling in retinal progenitors is less clear. Some aspects of TCF/LEF activity in the developing retina directly correlate with Wnt/β-catenin signaling, for example, during ciliary body and iris formation.34–38 However, conditional deletion of β-catenin in the central embryonic retina resulted only in abnormal lamination, with no obvious defects in retinal progenitor proliferation or cell fate specification.39,40 In addition, direct modulation of LEF function in either chick or mouse retinal experiments did not affect progenitor proliferation or differentiation.41,42 Therefore, Wnt/β-catenin signaling appears largely dispensable during embryonic retinal neurogenesis.

In the present study, we characterized TCF/LEF-responsive retinal cells in greater depth during mouse embryonic development. Transgenic constructs with multimerized TCF/LEF binding sites upstream of a minimal promoter that drive expression of a reporter are commonly used as a read-out of
activated TCF/LEF-mediated transcription. At least four different mouse TCF/LEF reporter lines have been described.41-45 We analyzed the TOPgal reporter generated by DasGupta and Fuchs44 and demonstrate TCF/LEF activity within retinal progenitors that differentiate as ganglion cells, cone photoreceptors, amacrine or horizontal cells. Surprisingly, this reporter is active in the absence of β-catenin, suggesting that TCF/LEF transcription factors work independent of the Wnt/β-catenin pathway during retinal neurogenesis.

**Material and Methods**

**Animals**

TOPgal mice44 were crossed with Ctnnb1 mice containing an allele of β-catenin with exons 2 to 6 of the gene flanked by loxp sites (termed floxed β-catenin in this article).46 A separate stock of mice carrying the Six3-Cre transgene (Tg(Six3-cre)69Frty) was crossed with a β-catenin<del>/catenin</del> allele.46,47 For our experiments, Six3-Cre; β-catenin<del>/catenin</del> mice were crossed to those homozygous for both the TOPgal transgene and floxed β-catenin (β-catenin<sup>fl/del</sup>). In the resulting embryonic litters, Six3-Cre;TOPgal;β-catenin<sup>fl/del</sup> embryos (termed β-catenin mutant embryos) were compared to control littermates containing one wild-type allele of β-catenin or no Six3-Cre transgene. Genotyping for floxed β-catenin, β-catenin<del>/catenin</del>, and the Six3-Cre transgene was performed as described.46,47 TOPgal genotyping by PCR used the primers: 5’ cgataaagcgg 3’ (forward); 5’ cgataaagcgg 3’ (reverse) and 35 cycles with an annealing temperature of 62°C within a standard protocol. The day of the observed plug was designated embryonic day 0.5. Animal experiments were performed according to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Utah and Children’s Hospital Research Foundation Institutional Animal Care and Use Committees.

**Detection of X-Gal Activity**

Tissue was fixed with 4% PFA for 10 to 20 minutes, depending on the age. Cryostat sections (14–16 µm) were incubated with X-gal substrate for 8 to 15 hours, postfixed and mounted (Fluoromount G; Southern Biotech, Birmingham, AL). The pattern of β-galactosidase activity was confirmed by immunodetection of β-galactosidase protein expression (described later). Images were taken with a microscope (BX51; Olympus, Lake Success, NY) equipped with Nomarski optics and processed with image-analysis software (Photoshop CS3; Adobe System, San Jose, CA).

**In Situ Hybridization**

Wholemount in situ hybridization on separate eyes was performed as previously described43,53. At E10.5, the TOPgal reporter was also activated in the dorsal optic vesicle, consistent with the activity of other TCF/LEF reporters, we also observed transient expression in the ciliary body, iris, and RPE between birth and postnatal day 30 (not shown; Fuhrmann S, Westenskow P, unpublished observations, 2008).60,29

**Results**

**TOPgal Reporter Activity in the Embryonic Mouse Retina**

In this study, we used TOPgal transgenic mice to define which retinal cells have TCF/LEF activity during embryonic development. This transgene contains three TCF/LEF consensus-binding sites and the c-fos minimal promoter driving β-galactosidase expression.43,56 At embryonic day (E)9.5, β-galactosidase expression was first observed in the dorsal optic vesicle, consistent with other TCF/LEF reporters (not shown and Refs. 29, 43, 53). At E10.5, the TOPgal reporter was also activated in the dorsal retinal pigmented epithelium (RPE) of the cup, dorsal optic stalk and pericellular mesenchyme, whereas no activation is observed in the retina (Fig. 1A). At E11.5, scattered cells in the neural retina initiate reporter expression, and the number of these cells is obviously increased by E13.5 (Figs. 1B, 1C). Starting at E14, the spatial localization of the TOPgal-expressing (TOPgal+) population changes; as these cells accumulate near the ventricular/proliferative zone of the retina, adjacent to the RPE where M-phase progenitors and photoreceptor precursors reside (Fig. 1D, at E15.5). At E16.5, the number of TOPgal+ cells decreases substantially and is barely detectable by E17.5 (Figs. 1E, 1F). No TOPgal+ cells were present in the postnatal adult central retina (not shown). These results indicate that activation of the TCF/LEF reporter occurs transiently in a subpopulation of embryonic retinal cells. Consistent with the activity of other TCF/LEF reporters, we also observed transient expression in the ciliary body, iris, and RPE between birth and postnatal day 30 (not shown; Fuhrmann S, Westenskow P, unpublished observations, 2008).60,29

**Cell Counting**

Right and left eyes of three TOPgal transgenic animals were analyzed using two coronal, nonadjacent, 10- to 12-µm sections of the central retina. Images were taken using epifluorescence (BX51; Olympus) and confocal (FV1000; Olympus) microscopes and subsequently processed using ImageJ (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/ij/image); developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) and commercial software (Photoshop CS3; Adobe Systems) or images were taken using an epifluorescent microscope (Axioplan2; Carl Zeiss Meditec, Inc., Dublin, CA) equipped with deconvolution software (AxioVision; Carl Zeiss Meditec, Inc.) and processed using commercial software (Photoshop 7; Adobe Systems). For each marker, the percentage of double-positive cells per total number of β-galactosidase-positive cells was determined in each retinal section, from a minimum of three independent TOPgal embryos per age point.
TOPgal Activity in the Central Mouse Retina and Relation to Wnt/β-Catenin Signaling

We observed TOPgal reporter activity in the developing retina that is substantially different from TCF/LEF activity reported by other laboratories using different transgenic lines. To determine how well our TOPgal activity reports Wnt/β-catenin signaling activity, we examined the mRNA expression of the scaffold protein Axin2, which is a universal readout and antagonist for Wnt/β-catenin signaling. Axin2 mRNA is detectable at E13.5 in the RPE, periciliary mesenchyme and presumptive ciliary body and iris, but, neither the antisense (Fig. 2A) nor sense (Fig. 2B) probes showed any expression in the central retina (Figs. 2A–F).40 In β-catenin mutant embryos, we observed variable β-catenin deletion in large patches in the central retina, along with lamination defects consistent with other studies (Figs. 3H, 3K).40,54 Surprisingly, we also observed the persistence of TOPgal+ cells in regions lacking β-catenin expression (Figs. 3D–I). This TCF/LEF activity suggests that TCF/LEF may act independently of Wnt/β-catenin signaling during embryonic retinal development.

TOPgal Expression in Proliferating and Postmitotic Retinal Progenitors

To define TOPgal+ retinal cells more fully, we next examined the cell cycle status of these cells by performing antibody double-labeling for β-galactosidase and cell cycle markers (Fig. 4; Table 1). To determine whether TCF/LEF activity is confined to a particular stage of the cell cycle, we quantified the proportion of TOPgal+ cells that coexpress proliferating cell nuclear antigen (PCNA), Ki67, BrdU, phosphohistone H3, and the cyclin-dependent kinase inhibitor p27/Kip1. The markers PCNA and Ki67 are expressed in all phases of the cell cycle. Our analysis shows that 53% (E13.5) and 56% (E15.5) of the TOPgal+ population coexpressed PCNA (Figs. 4A–C; Table 1). Likewise, we observed 69% (E13.5) and 65% (E15.5) coexpress-

expression was observed in the central retina (white arrowhead). (B) Axin2 sense control showing no specific labeling of ocular tissues. (C) By comparison, simultaneous in situ hybridization for Math5 mRNA showed strong expression in the central retina (white arrowhead), but not in the RPE (black arrowhead) or peripheral retina (arrow).
Next we found that there was essentially no overlap of TOPgal expression with BrdU (Table 1) and only 7% of TOPgal+ cells coexpressed the M phase marker phosphohistone H3 (Figs. 4G–I; Table 1). However, more than 76% of TOPgal+ cells coexpressed the CKI p27/Kip1 at E13.5 and E15.5 (Figs. 4J–L; Table 1). These observations indicate that TCF/LEF-responsive cells are largely nonproliferative, with the greatest degree of overlap occurring with p27/Kip1 expression. This finding suggests that TCF/LEF is most active when retinal progenitors are transitioning out of the cell cycle to differentiate.

### TOPgal Reporter Expression in Early Retinal Cell Types

Next, we wanted to correlate TOPgal+ cells with markers of neuronal specification and differentiation. First, we compared β-galactosidase expression with that of Math5 and NeuroD, two bHLH factors known to be expressed by several retinal cells at E13.5 and E15.5. However, only rare TOPgal+ cells were found to coexpress either Math5 or NeuroD in TOPgal retinal sections (not shown). Next we tested the bHLH factor Neurogenin2 (Ngn2), whose lineage contributes to all seven retinal cell types, but for which an obvious role in cell type specification has not yet been determined. We found that at E13.5, but not at E15.5, a subset of TOPgal cells was also Ngn2+. The double-positive cohort represented 26% of the TOPgal+ population (Figs. 5A–C; Table 1). Then, expression of the bHLH factor Olig2 was tested for the extent to which it overlaps with the TOPgal-expressing cells. In the mouse retina, Olig2 initiates expression at E13 in undifferentiated progenitors and is present postnatally in ganglion, amacrine, and horizontal cells; bipolar neurons; and Müller glia. Of interest, we found substantial coexpression of β-galactosidase and Olig2 at both E13.5 and E15.5, 64% and 57%, respectively (Figs. 5D–L; Table 1).
Finally, no overlap of the TOPgal reporter was seen with the bHLH factor Hes1 (Figs. 5G–I), which is expressed in proliferating progenitors and acts as a transcriptional repressor to regulate the timing of neuronal differentiation. Thus, when combined with the high number of TOPgal-p27Kip1 coexpressing cells, our data suggest that TCF/LEF activity may act within retinal progenitors as they exit the cell cycle.

### TCF/LEF Activity in Neurons or Neuronal Precursors Generated during the Embryonic Period

Our observations suggest that TOPgal+ cells largely represent the postmitotic transitional cells of separate neuronal lineages. Therefore, we predicted that TOPgal reporter expression would be present in nascent ganglion cells, photoreceptors, amacrine cells, and horizontal cells. The paired-type ho-

TABLE 1. Percentage of β-Galactosidase–Positive Cells Coexpressing the Listed Markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>E13.5</th>
<th>E15.5</th>
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<tbody>
<tr>
<td>PCNA</td>
<td>53.4 ± 6.66</td>
<td>55.83 ± 1.26</td>
</tr>
<tr>
<td>Ki67</td>
<td>69.3 ± 3.6</td>
<td>62.9 ± 3.3</td>
</tr>
<tr>
<td>BrdU</td>
<td>10.5 ± 1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>PHH3</td>
<td>6.97 ± 1.74</td>
<td>7.32 ± 1.94</td>
</tr>
<tr>
<td>P27Kip1</td>
<td>75.7 ± 2.3</td>
<td>78.8 ± 13</td>
</tr>
<tr>
<td>Ngn2</td>
<td>26.1 ± 4.98</td>
<td>No overlap</td>
</tr>
<tr>
<td>Olig2</td>
<td>63.0 ± 9.73</td>
<td>66.7 ± 6.5</td>
</tr>
<tr>
<td>Otx2</td>
<td>58.77 ± 2.51</td>
<td>67.53 ± 6.44</td>
</tr>
<tr>
<td>Ptf1a</td>
<td>19.63 ± 5.46</td>
<td>25.26 ± 1.10</td>
</tr>
<tr>
<td>Brn3b (E12.5)</td>
<td>30.3 ± 3.05</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

Data are expressed as percentage ± SD.
FIGURE 5. Overlap of TOPgal reporter expression with transcription factors expressed in retinal progenitor cells at E13.5. Transverse sections were double-labeled for β-gal (A, D, G; green) and transcription factors (B, E, H; red). (C, F, I) Merged images. Retinal progenitor cells with TCF/LEF activation coexpressed Ngn2 (A–C) and Olig2 (D–F), but not Hes1 (G–I), suggesting that TOPgal+ cells may start to differentiate soon. Arrowheads: double-labeled cells; arrows: single-labeled TOPgal+ cells. Scale bar, 50 μm.

FIGURE 6. TCF/LEF is active in embryonic cone photoreceptors and ganglion, amacrine, and horizontal cells. Transverse sections of E15.5 TOPgal embryonic retina colabeled for β-gal (A, D, G; green) and different cell-type-specific proteins (B, E, H, red). (C, F, I) Merged images. Substantial overlap was observed with Otx2 (A–C), RXRγ (D–F), and Ptf1a (G–I). Arrowheads: double-labeled cells in the outer retina; arrows: double-labeled cells in the presumptive ganglion cell layer. Scale bar, 50 μm.
meobox transcription factor Otx2 controls maturation of photoreceptor and bipolar cells in the rodent retina.63-66 Otx2 is coexpressed in many TOPgal+ cells: 50% at E13.5 and 67% at E15.5 (Figs. 6A–C; Table 1). To determine independently that TCF/LEF activity is present in photoreceptor precursors, we looked in the outer retina for coexpression of β-galactosidase with RXR α, a nuclear hormone receptor essential for fine-tuning the differentiation of cone subpopulations (Figs. 6D–F, arrowheads).67-69 In the embryonic retina, RXR α also labels ganglion cells, and we observed β-galactosidase: RXR α double-positive inner retinal cells as well (Figs. 6D–F, arrows). To quantify the percentage of TOPgal+ ganglion cells, we compared β-galactosidase and Brn3b coexpression70,71 and found 30% of E12.5 TOPgal+ cells also express Brn3b (Table 1, data not shown). Finally, to examine early amacrine and horizontal neurons, we compared β-galactosidase expression to that of Ptf1a, a bHLH factor expressed specifically by amacrine and horizontal72,73. Between 20% (E13.5) and 23% (E15.5) of the TOPgal+ cells coexpress Ptf1a indicating that the TOPgal+ cells also contribute to amacrine and horizontal cell fates (Figs. 6G–I; Table 1).

**Discussion**

We defined more fully the transient TCF/LEF-responsive population of retinal progenitors that contribute to the four earliest retinal cell types: ganglion, amacrine, and horizontal cells and cone photoreceptors. We conclude that TCF/LEF reporter activity does not require Wnt/β-catenin signaling, because Axin2 is not expressed in the central retina, and TOPgal expression persists in the absence of functional β-catenin. Our results point to a possible regulatory role for TCF/LEFs during early mammalian retinal neurogenesis, which correlates with the cell cycle exit of these early-generated cell types.

TCF/LEFs encode different protein isoforms and, depending on the interaction with other cofactors, they can activate or repress target gene transcription.74-77 (for reviews, see Refs. 75–77) TCF/LEFs promote a variety of processes when activated by the Wnt/β-catenin pathway, such as embryonic patterning, regeneration, stem cell renewal or differentiation in both embryonic and adult tissues, and deregulated activity can lead to tumor formation (for reviews, see Refs. 78, 79). Substantial progress has been made in elucidating the interaction of β-catenin with TCF/LEFs, however, previous reports show that other coregulators can interact with TCF/LEFs to promote transcription in the absence of β-catenin. LEFI can transactivate the T-cell receptor-α enhancer in a complex with the coactivators ALY and AML-1, which does not require the β-catenin interaction domain of LEFI.80,81 Similarly, an LEFI version lacking the β-catenin binding domain activates transcription of the Xenopus homeobox gene twin by interacting with effectors of the TGFβ/Activin signals Smad2, -3, and -4.82 Our findings also suggest that not all TCF/LEF functions are dependent on Wnt/β-catenin signaling. In the developing retina, LEFI and TCF3 expression is present in the central retina between E12.5 and E14.5, whereas TCF1 and -4 show weak expression at E14.5.83 However, targeted inactivation of TCF1, LEFI, and TCF4 do not appear to cause eye defects, which may be due to functional redundancy.83-89 Mutations in both TCF1 and LEFI result in severe developmental defects and lethality around E10, which has so far prevented further analyses of retinal neurogenesis.90 Thus, loss-of-function studies in which multiple TCF/LEF genes are simultaneously deleted during retinal development are needed to elucidate the role of TCF/LEFs in this tissue.

Surprisingly, our results strongly suggest that Wnt/β-catenin signaling is not active in the embryonic retina in the mouse, despite TCF/LEF reporter activation. TOPgal activation in the embryonic mouse retina is not artifactual, since endogenous TCF/LEF and TOPgal reporter expression overlap and an independently generated reporter shows a very similar expression pattern in the embryonic mouse retina (TCF/LEF line).26,27 For example, this TCF/LEF reporter is expressed in apical embryonic retinal cells that express CRX, a transcription factor expressed in photoreceptor precursors, consistent with TOPgal+ Otx2 coexpression in our study. However, ectopic activation of Wnt/β-catenin suppresses CRX expression indicating that this pathway must be inactive in committed precursors, to ensure proper development of photoreceptors.37 The data in the present study agree with this notion. Why the previously characterized TCF/LEF reporter shows persistent activity in embryonic and adult ganglion and amacrine cells is unclear.91 Since we observed TOPgal activity in embryonic ganglion cells and putative amacrine precursors, it is possible that the activity levels and perdurance of these different reporters vary, since not all transgenic constructs are equivalent and undoubtedly reside in different insertion sites throughout the mouse genome.

To provide context for the TCF/LEF reporter activity during retinal development, we compared TOPgal expression to that of transcription factors promoting retinal cell fates, mitotic cell cycle and differentiation markers for the four main embryonic retinal cell types. Together, these data show that TCF/LEF reporter activity is not confined to a single retinal lineage. Among the bHLH factors examined, very little TOPgal expression was seen in Math5+ or NeurodD+ cells, but more extensive in early Ngn2+ cells. However, the highest coincidence occurred in Olig2+ retinal cells. Intriguingly, there is extensive TOPgal activity in both Olig2+ and Otx2+ cohorts, which are complementary. Because very few Math5-TOPgal double-positive cells were observed, yet a subset of Brn3b+ cells express TOPgal, the onset of TCF/LEF reporter activity is consistent with retinal progenitor progression toward differentiation. In support of this idea, we found that many more TOPgal+ cells express p27/Kip1 than any of the other cell cycle markers examined. Therefore, we propose that TCF/LEF activity assists in some aspect of retinal progenitor transition into a postmitotic precursor. The integrated expression of TCF/LEF proteins with bHLH and homeobox factors appears to be one means by which the complexity of retinal cell type specification may occur. Future studies will address the combinatorial functions of these pathways.

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