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Activin Signals through SMAD2/3 to Increase Photoreceptor Precursor Yield during Embryonic Stem Cell Differentiation

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SUMMARY

In vitro differentiation of mouse embryonic stem cells (ESCs) into retinal fates can be used to study the roles of exogenous factors acting through multiple signaling pathways during retina development. Application of activin A during a specific time frame that corresponds to early embryonic retinogenesis caused increased generation of CRX^+ photoreceptor precursors and decreased $PAX6^+$ retinal progenitor cells (RPCs). Following activin A treatment, SMAD2/3 was activated in RPCs and bound to promoter regions of key RPC and photoreceptor genes. The effect of activin on CRX expression was repressed by pharmacological inhibition of SMAD2/3 phosphorylation. Activin signaling through SMAD2/3 in RPCs regulates expression of transcription factors involved in cell type determination and promotes photoreceptor lineage specification. Our findings can contribute to the production of photoreceptors for cell replacement therapy.

INTRODUCTION

The retina is a specialization of the forebrain developing from cells in the anterior neuroectoderm. These form the eye fields and eventually the optic vesicles, which subsequently invaginate to become the optic cups. The neural retina layer of the optic cup is composed of multipotential retinal progenitor cells (RPCs), which differentiate into the seven intrinsic cell types of the mature neural retina. Pluripotent stem cells such as embryonic stem cells (ESCs) can recapitulate parts of this development and efficiently form optic cup, RPCs, and cells of the inner neural retina, highlighting the potential use of ESC differentiation for model retina development in a controlled environment and as a source of transplantable cells (Eiraku and Sasai, 2012; Hirami et al., 2009; Ikeda et al., 2005; Lamba et al., 2006; Meyer et al., 2009; Osakada et al., 2009; Singh et al., 2015; Zhong et al., 2014). However, the yield of photoreceptor precursors from pluripotent stem cell differentiation, without the use of fluorescent transgene markers to select specific cell populations, varies between studies and is generally low compared with the percentage of photoreceptors in retinas *in vivo* (Jeon et al., 1998; Lamba et al., 2006; Singh et al., 2015; West et al., 2012).

Exogenous signals regulate neural stem cell fate decisions and mimicking morphogen gradients, such as those formed by bone morphogenetic proteins (BMPs), BMP antagonist Noggin, Wnts, Wnt/β-catenin signaling inhibitor Dickkopf-1 (Dkk1), basic fibroblast growth factor (bFGF), and insulin-like growth factor 1 (IGF1), during development can guide pluripotent stem cell differentiation into anterior neural fate and promote eye and retinal identity *in vitro* (Hirami et al., 2009; Ikeda et al., 2005; Lamba et al., 2006; Meyer et al., 2009; Osakada et al., 2009; Singh et al., 2015). Similarly, factors such as ciliary neurotrophic factor (CNTF), IGF1, and retinoic acid (Kelley et al., 1994; Pinzon-Guzman et al., 2011; Zhang et al., 2004) can alter photoreceptor development in retina explants and primary cells from perinatal retina. Therefore, it should be possible to improve the yield of photoreceptor precursors from pluripotent stem cells by elucidating the molecular signals that channel the differentiation of RPCs into photoreceptors.

Activin A is an important member of the Activin family of exogenous factors, which belong to the transforming growth factor β (TGF-β) superfamily of morphogens. Activin A, a dimer of inhibin βA (INHBA) subunits, and its receptors are expressed in developing neural retina, retinal pigment epithelium (RPE), and surrounding ocular tissue (Belecky-Adams et al., 1999; Davis et al., 2000; Feijen et al., 1994). Activin binds to its type 2A receptor (ACVR2A) and causes recruitment and phosphorylation of receptor type 1B (ACVR1B) (Pauklin and Vallier, 2015). ACVR1B phosphorylates SMAD2 and SMAD3, which then associate with SMAD4 to form a complex that translocates to the nucleus and recognizes SMAD binding elements (SBEs) containing the AGAC consensus sequence in regions surrounding the transcription start site (TSS) of target genes (Hill, 2016; Jonk et al., 1998; Kim et al., 2011). The specificity and directionality of gene regulation by SMADs depends on the presence of additional DNA binding cofactors. One of the best characterized cofactors that interacts with SMAD complex in multiple cell types and organ systems is Forkhead box protein H1 (FOXH1) (Yoon et al., 2011; Zhou et al., 1998). SMAD-FOXH1 binding has many effects, including upregulation of genes involved in retinoic acid signaling during anterior neuroectoderm development and forebrain patterning (Silvestri et al., 2008).

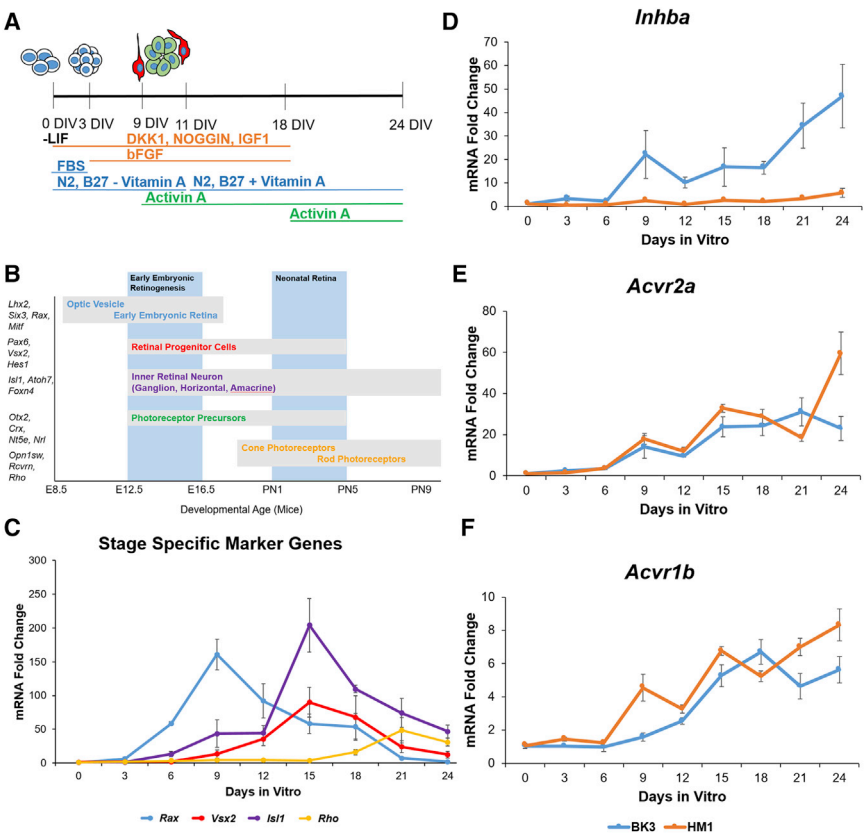


Figure 1. Expression of Eye and Retinal Marker Genes, Activin A, and Activin Receptors during ESC Retinal Differentiation

(A) Schematic representation of the *in vitro* protocol used to induce retinal differentiation from mouse ESCs and the time points during which activin A is introduced to culture medium.

(B) Schematic of expression patterns of selected developmental stage- and cell type-specific marker genes over the time course of retina development. Summarized from qRT-PCR analysis of expression in mouse retinas and previously published *in vivo* transcript expression patterns (see Supplemental Experimental Procedures). y axis, gene name; x axis, developmental age in mouse.

(C) qRT-PCR measuring expression of *Rax*, *Vsx2*, *Isl1*, and *Rho* over the course of BK3 differentiation.

(D–F) qRT-PCR of *Inhba* (D), *Acvr2a* (E), and *Acvr1b* (F) expression throughout differentiation.

n ≥ 3 independent experiments per cell line per time point; error bars denote SEM.

Activin promotes eye field formation in ESCs (Bertacchi et al., 2015; Lupo et al., 2013) and generation of mature photoreceptors in primary rodent retina cultures (Davis et al., 2000), but also prevents differentiation of pluripotent stem cells by regulating the expression of key stem cell genes such as *Oct4* and *Nanog* (Beattie et al., 2005; Sun et al., 2014; Vallier et al., 2009; Xu et al., 2008) and needs to be inhibited in ESCs and blastula stage embryos to allow initial differentiation (Wong et al., 2015; Zhou et al., 2015). Thus, while activin is involved in the earliest and last steps of retinal development, there is currently no information about its role in intermediate steps.

In the present study, we directed the differentiation of mouse ESCs into a retinal lineage to test the hypothesis that activin A can promote photoreceptor precursor generation from RPCs. Treating ESCs at a time corresponding to early embryonic retinogenesis decreased the expression of RPC marker genes and elevated and maintained the expression of genes associated with photoreceptor precursors in a dose-dependent manner. Activin A caused these changes in gene expression and photoreceptor precursor yield by activation and direct binding of SMAD2/3 to regulatory regions of key retinal genes in RPCs.

RESULTS

Expression of Activin A and Activin Receptors Increase during ESC Retinal Differentiation

To determine whether activin signaling can affect retinal differentiation at specific times, we differentiated BK3 mouse ESCs using a modified stepwise protocol (La Torre et al., 2012) (Figure 1A). Time points during *in vitro* differentiation were aligned to well-established *in vivo* developmental stages by comparing expression patterns of selected eye field transcription factors (EFTFs), marker genes of RPCs, and specific neural retinal cell types (Figure 1B). *In vitro* dates comparable with early embryonic retinogenesis were defined by high expression of the EFTF *Rax*, increasing expression the RPC marker *Vsx2*, and the emergence of the ganglion cell marker *Isl1* at 9 days *in vitro* (DIV). A stage corresponding to neonatal retina was characterized by decreasing expression of *Vsx2* and increasing expression of the photoreceptor marker *Rho* after 18 DIV (Figure 1C).

We then followed the expression of the activin A monomer, *Inhba*, and the activin receptors *Acvr2a* and *Acvr1b* over the *in vitro* time course in BK3 and another mouse ESC line, HM1. Notably, while absolute levels of *Inhba*

transcript were comparable between differentiating cell lines prior to 6 DIV, expression increased substantially over time in BK3 whereas expression in HM1 remained at the same level (Figure 1D). Similar levels of *Acvr2a* and *Acvr1b* were detected in the two cell lines and showed progressive increases with time (Figures 1E and 1F). These results suggest that activin signaling may operate at time points comparable with early embryonic retinogenesis and that some variations in endogenous expression of its pathway components exist between cell lines. To maintain consistency in the interpretation of the effects of activin signaling, we performed the majority of our subsequent experiments using BK3 ESCs.

Stage-Specific Activin A Treatment Decreases Expression of RPC Genes while Increasing Expression of Photoreceptor Precursor Genes

To test the concentration dependence of activin signaling on differentiating ESC-derived retinal cultures, we treated cells with 0, 10, 50, or 100 ng/mL activin A at 9 DIV, which corresponds to early embryonic retina, and cells were analyzed after 15 days (at 24 DIV) for expression of cell type-specific genes. In particular, expression of *Pax6* and *Vsx2* were used as representative RPC markers. *Otx2* and *Crx*, two photoreceptor-specific transcription factors (TFs) whose expression precedes, and regulates the expression of mature photoreceptor genes involved in phototransduction, were used as representative photoreceptor precursor markers. Treatment with 10 ng/mL activin A resulted in reduced expression of *Vsx2* but caused no changes in *Pax6*, *Otx2*, or *Crx*. However, 50 ng/mL resulted in significant decreases in *Pax6* and *Vsx2* expression and increases in *Otx2* and *Crx* expression. Similarly, 100 ng/mL reduced *Pax6* (0.6-fold) and *Vsx2* (0.4-fold), and elevated *Otx2* (5.0-fold) and *Crx* (82.1-fold) (Figure 2A). Treatment with 100 ng/mL activin A resulted in no changes in expression of recoverin or rod or cone opsins (Figure 2B), which shows that the changes in *Otx2* and *Crx* represent an increase in photoreceptor precursors but not in mature photoreceptors. When cells were treated at a later stage (18 DIV, equivalent to neonatal retina), 100 ng/mL activin A treatment showed no change in expression of *Pax6*, *Vsx2*, *Otx2*, or *Crx* (Figure 2A), so subsequent experiments were conducted with activin A application at 9 DIV unless otherwise noted. These data suggest that the effects of activin A on gene expression are dependent on both developmental stage and dose.

We next examined the effect of activin signaling on a wider array of marker genes. Activin A treatment at 50 ng/mL and 100 ng/mL did not cause significant change in marker genes of other germ layers, surface ectoderm, or telencephalon (Figure 2C). Expression of EFTFs was increased only following 100 ng/mL treatment. Treatment with 100 ng/mL resulted in higher expression of bipolar marker *Grm6*, and application of either 50 ng/mL or 100 ng/mL activin A caused variable or no change in markers associated with RPE and inner retinal cells, suggesting that activin signaling during this time frame preferentially affects late-born retinal neuronal cell types (Figure 2C). Further investigation into bipolar cell marker genes revealed that neither dose altered expression of TFs involved in bipolar cell lineage determination (Figure 2C). Similarly, markers of Müller glia were unchanged or decreased following treatment. These data demonstrate that activin signaling reduces the expression of select RPC genes and elevates the expression of photoreceptor precursor genes during an *in vitro* period that corresponds to early embryonic retinogenesis.

Because there were no statistically significant differences between treating BK3 cells with 50 ng/mL or 100 ng/mL activin A for the majority of RPC and photoreceptor precursor markers studied (Figures 2A and 2C) and to limit potential effects outside the scope of photoreceptor specification from RPCs resulting from high treatment doses, subsequent experiments were conducted using 50 ng/mL of activin A unless otherwise noted.

The initial changes in expression of *Pax6* and *Vsx2* were detectable 6 days after treatment began and preceded the first statistically significant changes in *Otx2* and *Crx*, which appeared 9 days after treatment (Figure 2D). This suggests that activin causes RPC genes to decrease before it upregulates expression of photoreceptor precursor markers.

To determine whether the effects of activin A were specific to retinal lineages, we tested its actions on non-retinal lineages including ESCs differentiated into midbrain neurons, primary embryonic neurons, and Neuro2a cells. When ESCs were differentiated to generate midbrain dopaminergic neurons, we found a >75-fold increase in neuronal and dopaminergic gene expression at 20 DIV, which was reduced following activin A treatment (Figures S1A and S1B). Conversely, the effects of activin A on *Rax*, *Pax6*, *Vsx2*, *Otx2*, and *Crx* were similar to those seen in ESC retinal differentiation (Figure S1B). This suggests that activin exerts differing effects on retinal versus dopaminergic lineage.

Interestingly, while the effects of activin on *Rax* and *Otx2* were recapitulated in embryonic brain-derived primary cultures, it caused no changes in RPC genes and decreased *Crx* (Figure S1C). Neuro2a and primary cerebellar cells did not show any significant changes in response to treatment (Figures S1D and S1E). These data indicate that activin signaling alters RPC and photoreceptor gene expression specifically in cells with the potential to differentiate into retinal lineage or committed to anterior neural fate.

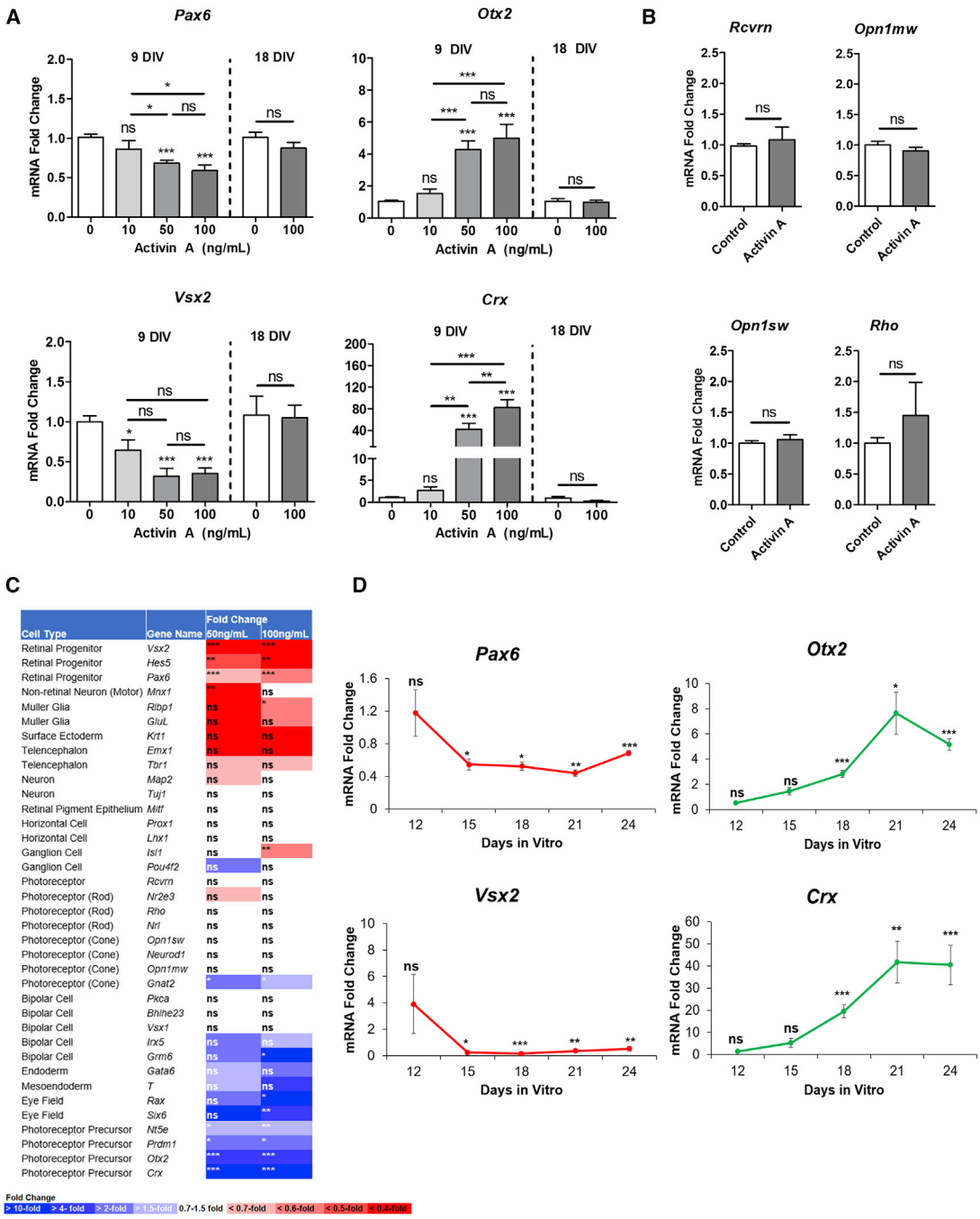


Figure 2. Changes in Expression of Retinal Genes following Activin A Treatment

(A) qRT-PCR of samples treated at 9 DIV (n ≥ 6 independent experiments, one-way ANOVA with Newman-Keuls post hoc test) or 18 DIV (n = 3 independent experiments, Student's t test) and collected at 24 DIV.

(B) qRT-PCR of samples treated with 100 ng/mL activin A beginning at 9 DIV and collected at 24 DIV; n = 3 independent experiments, Student's t test.

(C) qRT-PCR analysis of eye field and retina marker gene expression in cell treated with 50 or 100 ng/mL activin A at 9 DIV and collected at 24 DIV compared with cells treated in control medium. n ≥ 3 independent experiments, Student's t test.

(D) qRT-PCR of samples treated with 50 ng/mL activin A beginning at 9 DIV and collected every 3 days. n = 3 independent experiments, one-way ANOVA with Newman-Keuls post hoc test.

*p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant; error bars denote SEM. See also Figure S1.

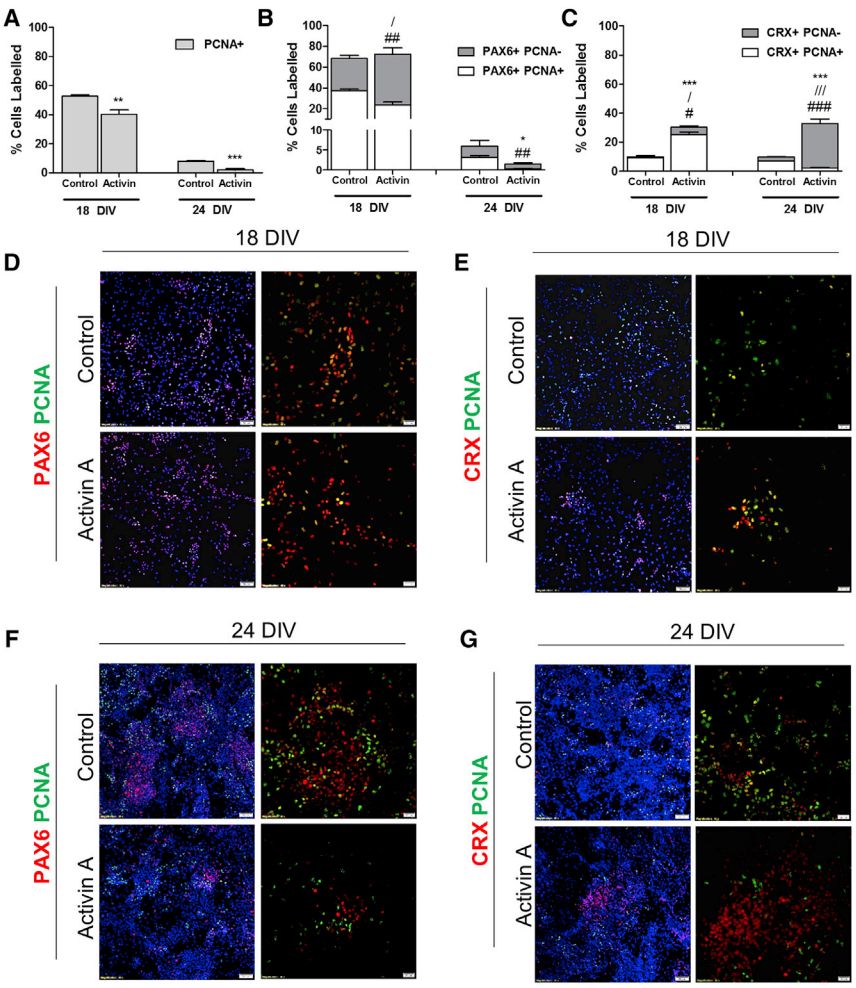


Figure 3. Effect of Activin A on Proportions of Proliferating RPCs and Postmitotic Inner Retinal Neurons and Photoreceptor Precursors

(A–C) Cells were treated beginning at 9 DIV, dissociated at 15 DIV, and fixed at either 18 DIV or 24 DIV for immunofluorescent detection of PCNA (A), PAX6 (B), or CRX (C). Quantification: number of primary antibody-labeled nuclei/number of Hoechst-labeled nuclei, n = 3 independent experiments, Student's t test. $^{*,\#,/}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,\#\#\#,///}p < 0.001$ (*, total number of PAX6$^+$ or CRX$^+$; #, double-labeled cells; /, single-labeled cells); error bars denote SEM.

(D–G) Representative images of control and activin A-treated cells expressing PAX6 (red: D, F) or CRX (red: E, G) and PCNA (green) overlaid with nuclear counterstain (Hoechst, blue). Left column: scale bar, 50 μm; right column (higher magnification of left column region showing only red and green channels): scale bar, 20 μm.

(H) Summary of changes in proportions of cells labeled by primary antibodies following treatment at 9 DIV. Decrease (↓), increase (↑), or no change (–) in number of cells labeled. See also Figure S2 and Table S1.

H

Antibody	18 DIV	24 DIV
PCNA+	↓	↓
PAX6+/PCNA+	↓	↓
PAX6+/PCNA-	↑	–
CRX+/PCNA+	↑↑	↓
CRX+/PCNA-	↑	↑↑↑

Activin Signaling Shifts the Proportion of RPCs and Photoreceptor Precursors

To identify the cells exhibiting changes in transcript expression in response to activin A and to investigate whether these were due to altered levels of expression per cell or proportion of expressing cells, we quantified cell populations by immunofluorescence after 9 days (at 18 DIV) or 15 days (at 24 DIV) of treatment. To correlate expression with proliferating cells, we colabeled cells with proliferating cell nuclear antigen (PCNA) antibody.

Nine days of activin A treatment resulted in a significant reduction in PCNA$^+$ mitotically active progenitors (control 52.7%, activin A 40.2%) (Figure 3A). While there was no significant change in the total proportion of PAX6$^+$ cells,

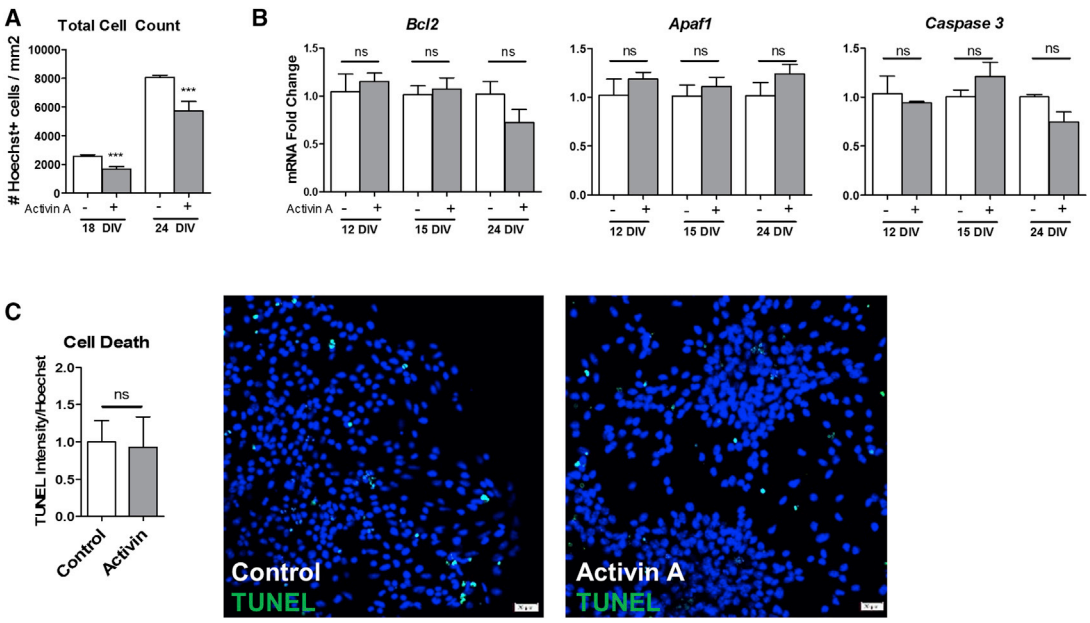


Figure 4. Effect of Activin A Treatment on Cell Death

(A) Number of cells (cells/mm^2) identified by Hoechst staining at 18 and 24 DIV. Cells were dissociated at 15 DIV to facilitate cell counting. n = 9 independent experiments, one-way ANOVA with Newman-Keuls post hoc test.

(B) qRT-PCR of apoptosis-associated genes; n = 3 independent experiments, Student's t test.

(C) Cells were treated at 9 DIV with 50 ng/mL activin A and fixed 6 days following treatment (15 DIV) for TUNEL (green) overlaid with nuclear counterstain (Hoechst, blue). Quantification: TUNEL fluorescence intensity/number of Hoechst-labeled nuclei, n = 6 independent experiments, Student's t test. Scale bar, 20 μm.

***p < 0.001; ns, not significant; error bars denote SEM.

there was a significant change in subpopulations from PAX6$^+$/PCNA$^+$, which represent putative RPCs (control 37.3%, activin A 27.4%), to PAX6$^+$/PCNA$^-$ (control 30.9%, activin A 41.0%) (Figures 3B and 3D). This was accompanied by an elevation in percentage of potential photoreceptor precursors labeled by CRX (control 9.8%, activin A 30.2%), which was primarily due to an increase in the CRX$^+$/PCNA$^+$ population (control 9.1%, activin A 25.1%) and a smaller increase in the CRX$^+$/PCNA$^-$ population (control 0.7%, activin A 5.1%) (Figures 3C and 3E).

After 15 days of treatment, there was a substantial decrease in the proportion of PCNA$^+$ mitotically active progenitors in the control, and this was further reduced by activin A (control 8.1%, activin A 2.2%) (Figure 3A). Similarly, there was a significant reduction in total PAX6$^+$ cells (control 6.0%, activin A 1.5%), which was accounted for by a decrease in PAX6$^+$/PCNA$^+$ (control 3.1%, activin A 0.3%) and no statistically significant change in percentage of single-labeled PAX6$^+$/PCNA$^-$ cells, which might represent post-mitotic cells of the inner retina (Figures 3B and 3F). The elevation in total CRX$^+$ cells induced by activin A was sustained at 15 days of treatment (control 9.1%, activin A 33.4%) but was marked by a large increase in the proportion of CRX$^+$/PCNA$^-$ cells from 5.1% after 9 days to 30.7% after 15 days of treatment (Figures 3C and 3G). OTX2 behaved similarly to CRX while quantification of RHO and OPN1SW fluorescence did not reveal any statistically significant changes (Figures S2A, S2B, and S2C).

These changes in proportion of cells reflect changes in the total number of cells labeled (Table S1). Taken together, activin A caused a reduction in proliferating PAX6$^+$/PCNA$^+$ RPCs and a transient rise in PAX6$^+$/PCNA$^-$ inner retinal neurons. This was followed by an increase in post-mitotic CRX$^+$/PCNA$^-$ photoreceptor precursors (Figure 3H).

Activin Signaling Does Not Cause Cell Death in Differentiating ESCs during Early Retinogenesis

While there was an overall increase in cell number between 18 and 24 DIV, at each time point, activin A treatment resulted in fewer cells (9 days treatment: 35.2% fewer cells; 15 days treatment: 29.2% fewer cells) (Figure 4A). Activin A also reduced the proportion of PAX6$^+$/PCNA$^+$ proliferating RPCs at both 9 and 15 days after treatment (Figure 3B). To understand whether the activin A induced reduction in both total number of cells and the number RPCs was due to exit of post-mitotic cells from the RPC pool or to elevated cell death, we measured markers of apoptosis.

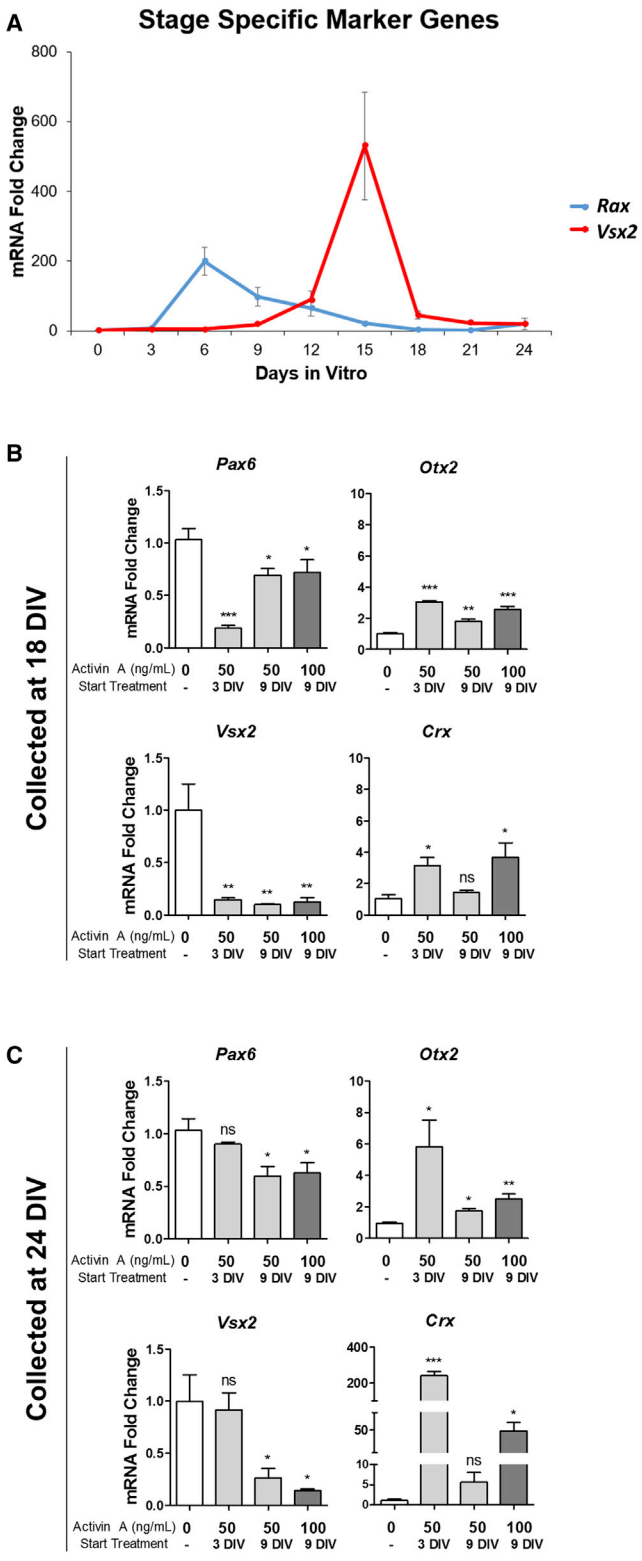


Figure 5. Effect of Activin A Is Conserved between ESC Lines

(A) Changes in expression of *Rax* and *Vsx2* mRNA over the time course of *in vitro* differentiation measured by qRT-PCR. n ≥ 3 independent experiments per time point.

(B) qRT-PCR analysis of *Pax6*, *Vsx2*, *Otx2*, and *Crx* in cells collected at 18 DIV; n = 6 independent experiments.

(C) qRT-PCR analysis of *Pax6*, *Vsx2*, *Otx2*, and *Crx* in cells collected at 24 DIV. n = 3 independent experiments, one-way ANOVA with Newman-Keuls post hoc test.

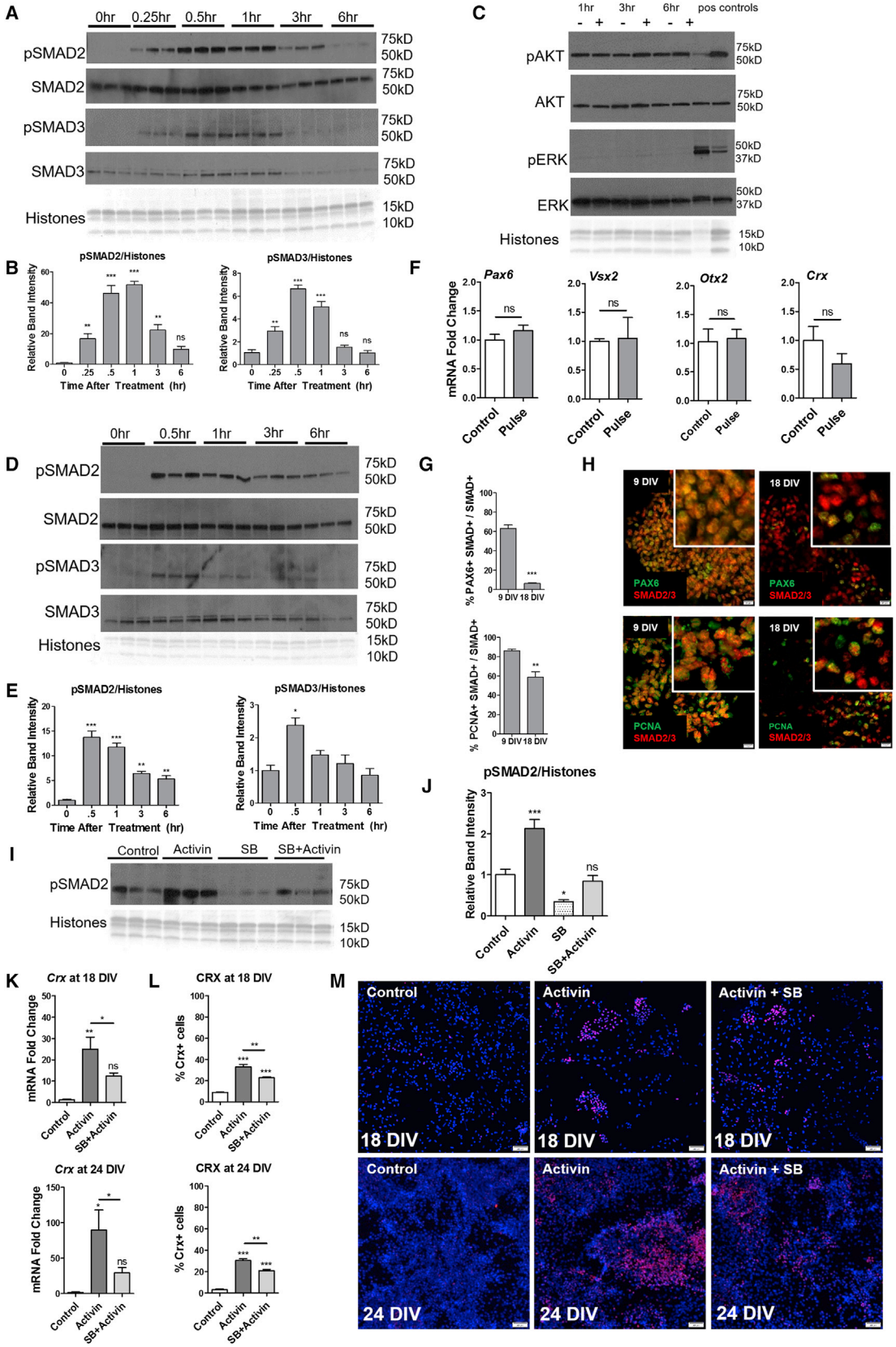
*p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant; error bars denote SEM.

There were no statistically significant changes in mRNA expression of *Bcl2*, *Apaf1*, or *Caspase 3* after 3, 6, and 15 days of treatment (Figure 4B). *Pax6* and *Vsx2* expression first declined after 6 days (Figure 2D), so we quantified TUNEL labeling at this time. No statistically significant difference in TUNEL labeling was found between control and treated cells (Figure 4C). The decrease in $PCNA^+$ cells following activin A treatment (Figure 3A) corresponded to a lower total number of cells after 9 or 15 days of treatment (Figure 4A) in the absence of obvious increase in apoptotic markers (Figures 4B and 4C), suggesting that activin A caused proliferating cells to exit the cell cycle without inducing apoptosis.

Activin A Effect on RPC and Photoreceptor Gene Expression Is Conserved in HM1 ESCs

We investigated whether any effects of activin A on the transition from RPCs to photoreceptor precursors were due to individual properties of the BK3 mouse ESC line. We performed the same retinal differentiation protocol on HM1 ESCs and followed expression of *Rax* and *Vsx2* over the time course of differentiation. HM1 progressed through the steps of retinal differentiation in the same sequence but several days sooner than BK3 ESCs (compare Figures 1C and 5A). HM1 cultures had lower endogenous levels of activin A after differentiation progressed past a stage similar to early embryonic retina (Figure 1D). Because of these observations, we hypothesized that HM1 cells would behave similarly to BK3 cells but would require either a higher concentration of activin A or earlier treatment start time.

We treated HM1 retinal differentiation cultures at 3 DIV with 50 ng/mL activin A and at 9 DIV with 50 or 100 ng/mL activin A, and collected samples at 18 or 24 DIV. When treatment began at 9 DIV, 50 ng/mL activin A resulted in changes in *Pax6*, *Vsx2*, and *Otx2* but no increase in *Crx* (Figures 5B and 5C). At 100 ng/mL activin A was able to induce changes in all four genes after 9 days and 15 days of treatment (Figures 5B and 5C). Treatment with 50 ng/mL beginning at 3 DIV reduced *Pax6* and *Vsx2* and elevated *Crx* and *Otx2* expression by 18 DIV (after 15 days of treatment) (Figure 5B). Prolonged activin A exposure (beginning at 3 DIV for 21 days) resulted in recovery of *Pax6* and *Vsx2* expression (Figure 5C). These results show that the effects of activin signaling are similar between different pluripotent cell



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lines, although some cell line-specific variations in the timing and dose need to be taken into consideration.

SMAD2/3 Are Downstream Effectors of Activin Signaling in RPCs

The effects of activin treatment were detectable when added at 9 DIV but not at 18 DIV (Figure 2A), so we examined phosphorylation and nuclear localization of SMAD2/3 after treatment at these time points to determine whether this difference could reflect differences in the activin signaling pathway. Following activin A treatment at 9 DIV, phosphorylation of both SMAD2 and SMAD3 increased and peaked at 0.5–1 hr before gradually returning to baseline after 6 hr (Figures 6A and 6B). Non-SMAD-mediated TGF-β signaling pathways ERK and AKT (Zhang, 2009) were not activated during this time period (Figure 6C). SMAD2 and SMAD3 were also phosphorylated following activin A treatment at 18 DIV (Figures 6D and 6E). Because of the transient response to activin A, we tested whether a pulse was sufficient to induce gene expression changes. Cells were exposed to activin A for 6 hr before they were washed and grown in medium without activin A for 15 days. Expression of *Pax6*, *Vsx2*, *Otx2*, and *Crx* were unchanged between untreated and pulsed cells (Figure 6F). This suggests that phosphorylation of SMAD2/3 following a single pulse of activin A exposure is not sufficient to change gene expression.

To identify the cells that respond to activin A, we treated cultures for 1 hr and immediately fixed them for detection of nuclear SMAD2/3. The majority of cells that exhibited nuclear localization of SMAD2/3 were PAX6$^+$ and PCNA$^+$ at 9 DIV. When the same experiment was repeated at 18 DIV, the proportion of cells that were PAX6$^+$/SMAD2/3$^+$ decreased from 63.1% at 9 DIV to 5.5% at 18 DIV. Similarly, the proportion of cells that were PCNA$^+$/SMAD2/3$^+$ was reduced from 86.2% at 9 DIV to 58.6% at 18 DIV (Figures 6G and 6H). This indicates that the stage-specific changes in gene expression in response to activin signaling correlate with SMAD2/3 activation in RPCs.

To further validate whether activin A operates through SMAD2/3 activation to increase photoreceptor precursor yield, we used 10 μM SB431542 (SB), an inhibitor of type I activin and TGF-β receptors (Inman et al., 2002), and examined whether it could suppress activin-induced SMAD2/3 phosphorylation in ESC-derived retinal cultures. Treatment with 10 μM SB alone beginning at 9 DIV for 15 days resulted in levels of phosphorylated SMAD2 (pSMAD2) below those of control cultures. Addition of SB to culture medium before and during activin A treatment was able to bring pSMAD2 back to the control state, although it did not completely inhibit phosphorylation (Figures 6I and 6J). Treatment with both SB and activin A resulted in a significant, but not complete, abolition of the effect of activin A on *Crx* expression by 9 and 15 days post treatment (Figure 6K). Similar results were found after quantification of CRX$^+$ cells by immunofluorescence at 9 days (activin 32.7%, SB + activin 22.7%) and 15 days post treatment (activin 30.3%, SB + activin 20.9%) (Figures 6L and 6M). These data show that activin A signals through a SMAD2/3-dependent mechanism in RPCs.

SMAD2/3 Binds to Regulatory Regions of RPC and Photoreceptor Marker Genes

pSMAD2/3 regulates gene expression by binding to SBEs. We identified potential SBEs in the ±2,500-bp region

Figure 6. Activin A Treatment Activates SMAD2 and SMAD3 in ESCs Undergoing Retinal Differentiation

(A) Western blot of SMAD2 and SMAD3 in cells treated with 50 ng/mL activin A at 9 DIV.

(B) Quantification of (A). One-way ANOVA with Newman-Keuls post hoc test.

(C) Western blot of AKT and ERK in cells at 9 DIV treated with control medium or 50 ng/mL activin A. Positive controls are MDA-MBA-231 (second lane from right) and MOLT4 (rightmost lane) lysates.

(D) Western blot of SMAD2 and SMAD3 in cells treated with 50 ng/mL activin A at 18 DIV.

(E) Quantification of (D). One-way ANOVA with Newman-Keuls post hoc test.

(F) qRT-PCR analysis of *Pax6*, *Vsx2*, *Otx2*, and *Crx* expression in untreated (in control medium) or treated cells (50 ng/mL activin A for 6 hr at 9 DIV) collected at 24 DIV. Student's t test.

(G) Quantification of immunofluorescence in cells treated for 1 hr with 50 ng/mL activin A at either 9 DIV or 18 DIV. Quantification: number of double-labeled nuclei/number of SMAD$^+$ nuclei, Student's t test.

(H) Representative images of cultures quantified in (G). PAX6 (green), PCNA (green), and SMAD2/3 (red). Scale bar, 10 μm.

(I) Western blot of pSMAD2 in cells treated with control medium, 50 ng/mL activin A alone, 10 μM SB alone, or pretreated for 30 min with SB followed by activin A addition to medium containing SB at 9 DIV and collected at 24 DIV.

(J) Quantification of (I). One-way ANOVA with Newman-Keuls post hoc test.

(K) qRT-PCR analysis of *Crx* expression in cells treated with 50 ng/mL activin A at 9 DIV and collected at either 18 or 24 DIV; one-way ANOVA with Newman-Keuls post hoc test.

(L) Cells were treated at 9 DIV, dissociated at 15 DIV, and fixed at either 18 DIV or 24 DIV for immunofluorescent detection of CRX.

(M) Representative images of cultures quantified in (L); CRX (red), Hoechst (blue). Scale bar, 50 μm.

Quantification: number of primary antibody-labeled nuclei/number of Hoechst-labeled nuclei, one-way ANOVA with Newman-Keuls post hoc test, n = 3 independent experiments, *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant; error bars denote SEM.

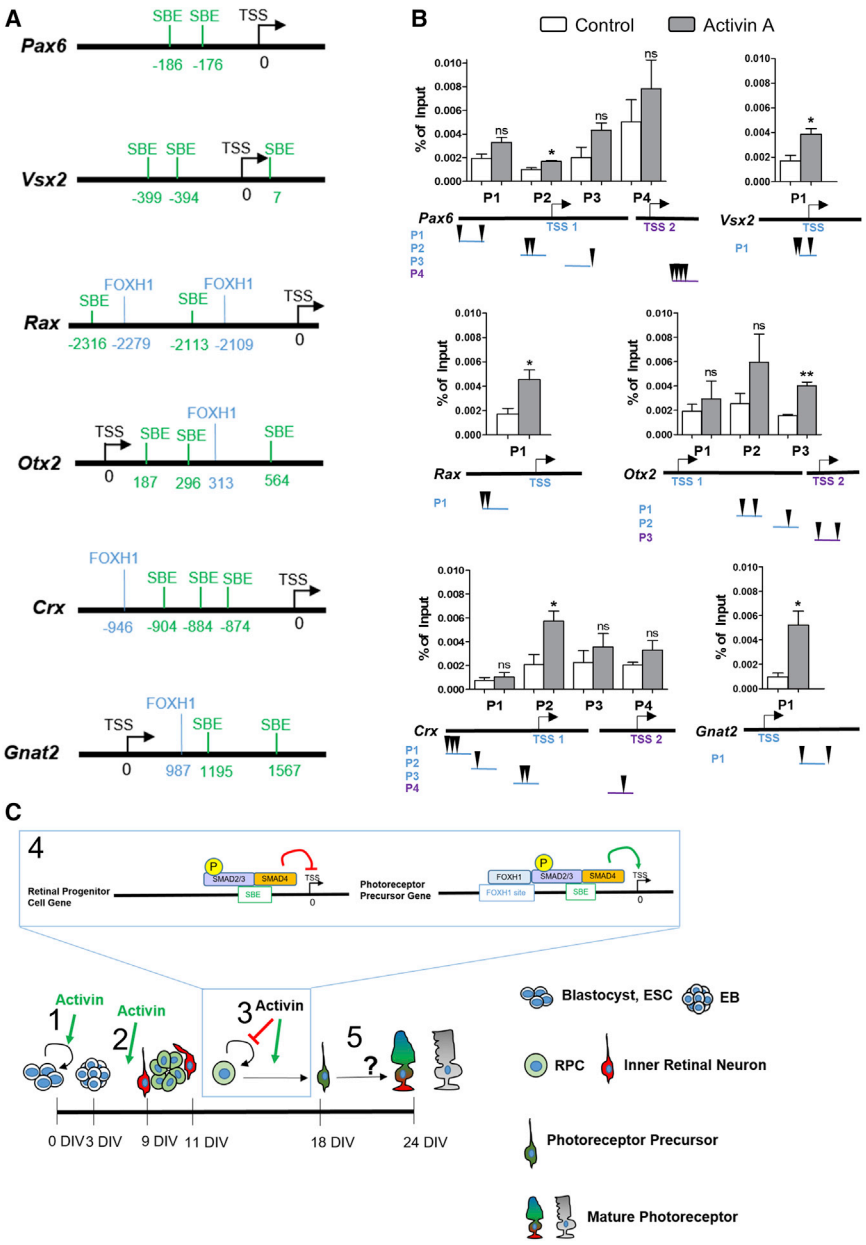

Figure 7. SMAD2/3 Binds to Promoter Regions of Key RPC and Photoreceptor Genes

(A) Line schematics show positions of select potential SBEs (green) and proximal FOXH1 (blue) consensus sites in relation to the TSS (black). Segments represent regions of mouse genome that show significant enrichment in (B).

(B) Cells were treated with 50 ng/mL activin A beginning at 9 DIV and harvested at 18 DIV for ChIP-qPCR normalized to sample input. n = 3 independent experiments, Student's t test; *p < 0.05, **p < 0.01; ns, not significant; error bars denote SEM. Downward arrowheads indicate location of SBE conserved between species. Schematics are not drawn to scale.

(C) Proposed model for role of activin signaling at different stages during retina development. For robust photoreceptor specification to occur, activin needs to be inhibited in blastocysts and ESCs (1) and activated to promote eye field and RPC generation (2), after which it causes differentiation of RPCs into photoreceptor precursors (3) through regulation of gene expression by SMAD2/3 (4). Photoreceptor maturation is likely regulated by other mechanisms (5).

See also Figure S3 and Table S2.

surrounding the TSS of *Pax6*, *Vsx2*, *Rax*, *Otx2*, *Crx*, and *Gnat2* by searching for repeats of the SBE sequence AGAC or its palindrome GTCT. Several potential SBEs were found in all the genomic segments studied and some of these SBEs were in proximity to FOXH1 consensus binding sequences (Figure 7A). We treated ESC-derived retinal cultures with activin A for 9 days, and cells were collected for anti-SMAD2/3 chromatin immunoprecipitation and qPCR (ChIP-qPCR).

As a positive control, we quantified SMAD2/3 binding to a segment of *Nanog* promoter containing a known SBE (Greber et al., 2010). There was significant enrichment in SMAD2/3 binding in undifferentiated ESCs compared with neonatal mouse retina (Figure S3A). This binding is specific to the differentiation state of the sample because the same region failed to be enriched following activin A treatment on differentiated cultures (Figure S3B). Similarly, SMAD2/3 binding is specific to retinal genes at the time points studied because SBE-containing segments in promoters of ubiquitously expressed gene *Gapdh*, endoderm gene *Gata6*, and telencephalon marker genes *Emx1* and *Tbr1* failed to exhibit increased binding (Figures S3B and S3C).

Activin A treatment increased SMAD2/3 binding to promoter regions in all six of the retinal genes we studied. This included enrichment at selected promoter regions of *Pax6* and *Vsx2* (Figure 7B), which are both downregulated by activin signaling, as well as binding to promoter regions of *Rax*, *Otx2*, *Crx*, and *Gnat2* (Figure 7B), which were upregulated following treatment (Figure 2C). More detailed examination of the enriched loci revealed that the SMAD2/3-bound regions in *Rax*, *Otx2*, *Crx*, and *Gnat2* contain FOXH1 consensus sequences in proximity to the SBEs while enriched regions in *Pax6* and *Vsx2* do not contain these sequences (Figure 7A). In agreement with the lack of effect on *Rho* expression, there was no significant difference in binding to any of the putative SBE-containing *Rho* loci studied (Figure S3D). This increased binding of SMAD2/3 is specific to select SBE-containing sequences within the promoter regions of retinal genes and does not bind to regions of these genes that do not contain SBE sequences (Figure S3E).

Taken together, our data indicate that SMAD2/3 directly bind to regulatory regions of RPC and photoreceptor precursor genes at an *in vitro* time frame comparable with early embryonic retinogenesis, during which it may inhibit *Pax6* and *Vsx2* expression and activate *Rax*, *Otx2*, *Crx*, and *Gnat2* transcription.

DISCUSSION

The sequential steps of mouse ESC differentiation mimic the sequence of *in vivo* retinal development (Figures 1B and 1C). For example, 9 DIV in ESC-derived retinal cultures corresponded to early embryonic retina, during which RPCs are abundant and inner retinal cell types begin to appear. We have demonstrated that activin increased the generation of photoreceptor precursors from ESC-derived retinal cultures by activating SMAD2/3 in RPCs. Previous studies have focused on the role of activin signaling in the early stages of eye field and RPC generation as well as in the late maturation of retinal cell types, while our work has examined an intermediate stage, the transition of RPCs into photoreceptor precursors.

Activin A expression has been examined in several *in vivo* animal models and was detected in extraocular tissues, RPE, and presumptive ganglion cells in developmental ages spanning the equivalent of embryonic day 12.5 (E12.5) and neonatal mice (Belecky-Adams et al., 1999; Davis et al., 2000; Feijen et al., 1994). The outer neuroblast layer, which consists of RPCs and photoreceptor precursors, displayed little or no expression of activin A but did begin to express activin receptors during the periods studied. This suggests that RPCs can respond to activin A secreted by adjacent tissue, such as RPE, during early embryonic retinogenesis *in vivo*.

Activin A treatment of differentiating ESCs caused increased expression of eye field and RPC markers (Bertacchi et al., 2015). We investigated the effect of activin signaling on ESC-derived cultures that had reached the RPC stage, a time frame that corresponds to *in vivo* dates at which expression of activin A and its receptors were detected in the embryonic eye and retina. In control conditions, RPCs declined after 18 DIV (comparable with neonatal retina, Figure 1C), probably because they differentiated into post-mitotic retinal cells. This also occurred in cultures treated with activin A, but the reduction in RPCs was more pronounced. We suggest that activin A promotes the transition of RPCs into photoreceptor precursors that then rapidly become post-mitotic (Figure 3). Treatment at a later time point did not induce changes in gene expression likely because fewer RPCs, the cells that respond by becoming photoreceptor precursors, were present. Previous work in neonatal and postnatal retina has shown that CRX^+ cells are post-mitotic in rodents (Garelli et al., 2006). However, the majority of CRX^+ cells are mitotically active in 10- to 13-week-old normal human fetal retina, which are comparable with E12–E15 mouse retina (Glubrecht et al., 2009). We suggest that *in vitro* ESC-derived photoreceptor precursors progress through a CRX^+/$PCNA^+$ phase, but become CRX^+/$PCNA^-$. Notably, activin A seems to drive this transition as the majority of CRX^+ cells in untreated cultures continued to express PCNA even at 24 DIV (Figures 3C and 3G). Our results are similar to those from E18 rat cultures, comparable with E16 in mouse, which found that activin A decreased neuroblasts and increased number of cells expressing markers of mature rod photoreceptors (Davis et al., 2000). Because the effects of activin A are dependent on the time point at which it is introduced, we propose a schematic of development in which activin signaling first maintains pluripotency and therefore needs to be inhibited for differentiation to begin (Wong et al., 2015; Zhou et al., 2015) (Figure 7C1). Following this, it promotes eye field formation and generation of RPCs (Bertacchi et al., 2015) (Figure 7C2). After the RPC pool has been established, our work shows that activin supports the transition from RPCs into photoreceptor precursors (Figure 7C3 and C4). Interestingly, a high dose of activin A at 9 DIV increased EFTF expression even after the initial period of eye field formation. Because EFTFs such as *Rax* are expressed in RPCs as well as post-mitotic cells including photoreceptor precursors and inner retinal neurons throughout the duration of retina development (Irie et al., 2015), this effect might not be specific to RPCs or photoreceptor precursors so the majority of our experiments, such as those addressing cell yield and mechanism, were performed with 50 ng/mL activin A.

Following activin A treatment, CRX$^+$ cells appeared in clusters indicative of a clonally derived population (Figures 3E and 3G). This suggests that while activin signaling generally decreases the number of mitotic cells, it allows or promotes expansion of specific RPCs that are competent to generate photoreceptor precursors. In the case of midbrain differentiation, Activin might act either to promote the subset of differentiating ESCs that are capable of generating retinal cells that are present even when the majority of cells have been directed into dopaminergic lineage or shift differentiation toward retinal lineage. The clustered morphology indicates that the generated CRX$^+$ cells can potentially be isolated via dissection to obtain a purified culture. This would be similar to the isolation of retinal morphologies composed of multiple retinal cell types used in organoid induction approaches that can recapitulate much of the architectural arrangement of the *in vivo* retina. However, the adherent differentiation approach used in this work is adapted from a protocol that yields 70%–80% RPCs (Lamba et al., 2006). This allows for study on a relatively pure RPC population while circumventing the steps of formation and isolation of invaginated retinal structures that limit the efficiency of many retinal organoid techniques (Eiraku and Sasai, 2012; Hiler et al., 2015). The requirement for continuous exposure to activin A, as opposed to a 6-hr pulse (Figure 6F), suggests that the RPCs are an asynchronous cell population and continuous ligand exposure increases the probability of pathway activation in individual RPCs that are progressing through an activin-responsive state. An alternative reason for the need for continuous activin A exposure is that multiple downstream interactions need to be fulfilled before the observed changes can occur. This might account for the partial effect of SB inhibition of SMAD2/3 on CRX expression. Our finding that activin A causes similar effects on some, but not all, non-retinal populations suggests that activin alone is insufficient to induce these gene expression changes (Figure S1). Other studies on neuralization and eye field specification in differentiating ESCs have shown that activin signaling coordinates with bFGF, BMP, and Wnt pathways (Bertacchi et al., 2015; Lupo et al., 2013).

Photoreceptor precursors can be identified by coexpression of TFs such as *Rax*, *Otx2*, *Crx*, and *Prdm1*. In addition to these TFs that are common to cone and rod photoreceptor precursors, a combination of TFs including *Nrl*, *Nr2e3*, and *Neurod1* make up a gene regulatory network that in concert with the temporal stage of the retina determines whether the precursor becomes a cone or rod photoreceptor (Swaroop et al., 2010; Wang and Cepko, 2016). Activin did not directly cause changes to the TFs involved in cone versus rod specification (Figure 2C), suggesting that it plays a limited role in the subsequent maturation of developing photoreceptors. Therefore, it was not surprising that activin A treatment did not result in changes in markers associated with phototransduction, which is dependent on several other signaling cascades including Notch, retinoic acid, thyroid hormone, and protein kinase C (Kelley et al., 1994; Pinzon-Guzman et al., 2011; Roberts et al., 2006; Völkner et al., 2016; Zhang et al., 2004) (Figure 7C5).

Importantly, few studies have investigated the mechanism through which activin signaling exerts its effects on retina development. We report that pathway activation causes bidirectional effects on gene expression in differentiating retinal cultures. SMAD2/3 can directly bind to putative promoter regions of key RPC and photoreceptor genes. An unbiased search of TF binding sites within the ~500-bp region surrounding the genomic sequence containing SBE with enriched SMAD2/3 binding shows that consensus sites for several TFs are selectively present in either genes upregulated or downregulated by activin A treatment (Table S2). Among these, FOXH1 binding sites were identified in close proximity to SBEs that were occupied by SMAD2/3 in regulatory regions of *Rax*, *Otx2*, *Crx*, and *Gnat2*, which all exhibited elevated expression, but not *Pax6* and *Vsx2*, whose expression was inhibited (Figure 7A). Because FOXH1 is a well-established SMAD cofactor (Silvestri et al., 2008; Yoon et al., 2011; Zhou et al., 1998), the presence or absence of its binding sequence may determine whether SMAD2/3 activates or represses transcription (Figure 7C4). This observation underscores the importance of complex regulatory networks in retina cell type specification.

We found that activin A treatment decreased the number of PCNA$^+$ cells and resulted in a 3-fold increase in photoreceptor precursor yield. This implies that manipulation of activin signaling might be useful in therapy for photoreceptor degenerative diseases including age-related macular degeneration, retinitis pigmentosa, and a host of inherited cone/rod dystrophies that cause loss of photoreceptors, eventually leading to blindness. Replacing photoreceptors can potentially restore vision in these diseases (Mandai et al., 2017; Pearson et al., 2012; Seiler and Aramant, 2012) but, while pluripotent stem cells are a robust source for cell transplants, there is a concern for teratoma formation if grafts include mitotically active cells that do not become photoreceptors (Chaudhry et al., 2009; West et al., 2012). RPE expresses activin A, so the effects of treatment likely mimic events that occur during *in vivo* photoreceptor development that are not present at sufficient levels in the post-transplant host. The different requirements for timing and dose of treatment between BK3 and HM1 cell lines indicate that pluripotent stem cells should be characterized before selecting a differentiation protocol to generate cells for therapeutic grafts. This is especially relevant for induced pluripotent stem cells from different tissues or individuals to provide a consistent yield of transplantable cells.

Taken together, our characterization of the effect of activin A in differentiating ESCs fills an important gap in knowledge regarding the transition from proliferating RPCs to post-mitotic photoreceptor precursors. Investigation into the mechanism of activin signaling reveals that it acts through SMAD2/3 effectors, which can directly bind to regulatory regions of key cell type-determining genes. This work will contribute to the improved generation of post-mitotic photoreceptor precursors that can be used for further studies in photoreceptor maturation and for cell replacement therapy in photoreceptor degenerative diseases.

EXPERIMENTAL PROCEDURES

Full details are provided in Supplemental Experimental Procedures. All animal work was approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine.

Cell Culture

Mouse ESC lines BK3 and HM1 were maintained as described by Woll and Bronson (2006) and differentiated in adherent condition with stepwise addition of DKK1, NOGGIN, IGF1, and bFGF as previously reported with additional modifications (La Torre et al., 2012). Activin A (R&D Systems, Minneapolis, MN) and was applied to cells at time points indicated.

qRT-PCR and ChIP-qPCR

cDNA as well as ChIP samples and inputs were prepared and quantified as previously described (Popova et al., 2016). See Supplemental Experimental Procedures for primer sequences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.06.021.

AUTHOR CONTRIBUTIONS

A.Q.L. and C.J.B. conceived the work, designed experiments, and wrote the manuscript. E.Y.P. provided critical intellectual input and designed the ChIP experiment. A.Q.L. performed the experiments. All authors edited and reviewed the manuscript.

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