Maintenance of retinal stem cells by *Abcg2* is regulated by notch signaling

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Summary

ABCG2 belongs to the ATP-binding cassette superfamily of transmembrane proteins and is ubiquitously expressed in stem cells including those in the developing nervous system. The ability of ABCG2 to preferentially exclude DNA-intercalating dyes is regarded to be the basis for the enrichment of stem cells or progenitors as dye^{low} side population (SP) cells. However, the role of ABCG2 in neural stem cells remains speculative and poorly understood. Here, we demonstrate using retinal stem cells, that ABCG2 is the molecular determinant of SP cell phenotype of neural stem cells and plays an important role in their maintenance. Overexpression of ABCG2 prevents the SP cell phenotype and adversely affects the lineage commitment of retinal stem cells. By contrast, targeted attenuation of ABCG2 depletes retinal SP cells and promotes their differentiation along pan neural and retinal lineages. In addition, we demonstrate for the first time that ABCG2 is a target of Notch signaling, and as such, constitutes one of the genes in the regulatory network of Notch signaling, involved in the maintenance of stem cells.

Supplementary material available online at
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Key words: Retina, Stem cells, Progenitors, Notch, ABCG2

Introduction

One of the emerging universal characteristics of stem cells or progenitors is the preferential expression of ABCG2, a half transporter belonging to ATP-binding cassette (ABC) superfamily of transmembrane proteins (Doyle and Ross, 2003). These transporters mediate efflux of a broad spectrum of substrates including cytotoxic drugs, peptides, steroids, ions and phospholipids (Krishnamurthy and Schuetz, 2006). Their ability to preferentially exclude the DNA-intercalating dye, Hoechst 33342, is regarded to be the molecular basis for the enrichment of stem cells as side population (SP) cells by the Hoechst dye efflux assay (Goodell et al., 1996; Abbott, 2003; Doyle and Ross, 2003; Bunting, 2002). The assay involves incubation of cells with the Hoechst dye followed by dual-wavelength fluorescence-activated cell sorting (FACS), which identifies a small side population of cells with low dye accumulation, possessing stem cell properties and potential (Goodell et al., 1996). The notion that SP cell phenotype is mediated by ABCG2 was supported by observations that the perturbation of *Abcg2* expression by retrovirus-mediated transduction or gene deletion affected the number of SP cells present in the hematopoietic compartment (Kim et al., 2002; Scharenberg et al., 2002; Zhou et al., 2002).

The conservation of *Abcg2* expression in SP cells, enriched from a wide range of tissues including blood (Zhou et al., 2003), muscle (Meeson et al., 2004), heart (Martin et al., 2004), gonad (Lassalle et al., 2004), lung (Summer et al., 2003), intestine (Staud and Pavek, 2005) and cornea (Budak et al., 2005) suggests that ABCG2 has an important role in stem cells. Molecular studies carried out on the hematopoietic compartment have begun to shed light on the involvement of *Abcg2* in stem cells. For example, the highly regulated expression of *Abcg2* during hematopoiesis, i.e. high levels of *Abcg2* transcripts in stem cells and their sharp decline during lineage commitment, and the blocking of lineage commitment in response to ectopic expression of *Abcg2* indicates a role for ABCG2 in the maintenance of hematopoietic stem cells (Zhou, S. et al., 2001; Scharenberg et al., 2002).

The expression of *Abcg2* and SP cell phenotype are also characteristics of neural stem cells, derived from different brain regions, including the retina (Hulspas and Quesenbury, 2000; Bhattacharya et al., 2003; Ahmad et al., 2004; Mouthon et al., 2006). However, little is known about the role of *Abcg2* in neural stem cells and their lineage commitment. In addition, we do not know how *Abcg2* expression is linked with the maintenance of stem cells. We have addressed these issues in developing retina, a suitable and accessible model of the central nervous system, where seven different types of cells are generated in an evolutionarily conserved temporal sequence by multipotential retinal stem cells (Rapaport et al., 2004). Our results demonstrate a regulated expression of *Abcg2* during retinal histogenesis; levels of *Abcg2* transcripts decrease with the onset of lineage commitment. This decrease in *Abcg2* expression correlates with the progressive depletion of SP cell population as retinal histogenesis ensues. When *Abcg2* is overexpressed in retinal progenitors, using retrovirus-mediated transduction, the differentiation is blocked, accompanied by an increase in the expression of stem cells markers and SP cell phenotype. By contrast, siRNA-mediated silencing of *Abcg2* expression in retinal progenitors depletes SP cell population and promotes differentiation. In addition, we observed that *Abcg2* expression and SP cell phenotype are influenced by Notch signaling, a key regulator of retinal stem cells. Our results demonstrate for the first time that ABCG2 is a

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downstream target of Notch signaling. Together, these observations suggest that *Abcg2* participates in the maintenance of retinal stem cells or progenitors, under the regulation of Notch signaling.

Results

Abcg2 expression is developmentally regulated and associated with SP cell phenotype of retinal progenitors

To examine the involvement of ABCG2 in the regulation of retinal progenitors, we first studied the temporal pattern of expression during retinal development. Retinal cells are born in an evolutionarily conserved temporal sequence; retinal ganglion cells, cone photoreceptors, horizontal cells and the majority of amacrine cells are born during early histogenesis whereas rod photoreceptors, bipolar cells and Müller glia are generated during late histogenesis (Rapaport et al., 2004). We examined *Abcg2* expression during late histogenesis for the following reasons: first, because rod photoreceptors constitute ~80% of all cells in the rodent retina, the majority of retinal cells differentiate during this stage, and second, the number of progenitors is significantly higher at this stage, compared with those in early histogenesis where tissue is a limiting factor for SP cell analysis. RT-PCR analysis revealed an inverse correlation between expression of *Abcg2* transcripts and the process of histogenesis; levels of *Abcg2* transcripts decrease at each stage studied (Fig. 1A,B). The levels of *Abcg2* transcripts were lowest at PN9, when the generation of ~95% of all retinal cell types have been completed (Rapaport et al., 2004). Such a temporal pattern of expression suggested that *Abcg2* expression is associated with proliferating progenitors and the attenuation in *Abcg2* expression reflected a decrease in the number of such cells as differentiation proceeded. To test this premise, we examined the spatial and cell-specific expression of *Abcg2* in postnatal day 1 (PN1) retina. Immunohistochemical examination of retinal sections revealed that ABCG2 immunoreactivities were predominantly distributed in the outer neuroblastic layer, where progenitors and precursors are preferentially localized in the developing retina (Fig. 1C,D). To determine whether or not ABCG2-expressing cells are proliferating, cells from PN1 retina were dissociated, chased with BrdU and subjected to double immunocytochemical analysis. We observed ABCG2$^+$ cells, expressing BrdU immunoreactivity, demonstrating their proliferative nature (Fig. 1E,F).

Next, we examined the temporal association of *Abcg2* expression with SP cell phenotype of retinal progenitors. We have previously demonstrated that both early and late retinal progenitors display SP cell phenotype (Bhattacharya et al., 2003; Ahmad et al., 2004). We have also demonstrated that SP cell phenotype was retina specific and not due to contamination with blood during the isolation of retinal cells (Bhattacharya et al., 2003). Since SP cell phenotype is dependent on *Abcg2* expression (Zhou et al., 2002), we were interested to know whether a temporal decline in *Abcg2* expression was correlated with a decrease in SP cells during late retinal histogenesis. We carried out Hoechst dye efflux assay on fresh retinal dissociates obtained from PN1, PN3 and PN6 rats and observed that the proportion of SP cells progressively decreased over time such that at PN6 only ~0.002% of cells could be characterized as SP cells, compared with ~0.1% SP cells at PN1 (Fig. 2A-D,E). To determine the phenotypes of SP and non-SP (NSP) cells during late histogenesis we examined the expression of transcripts corresponding to cell-specific markers (Fig. 2F).

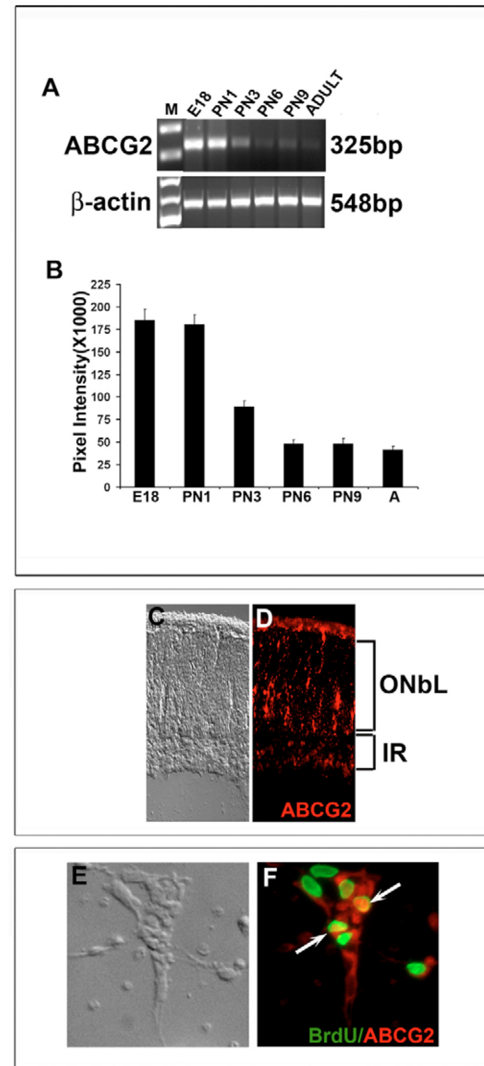

Fig. 1. Temporal and spatial patterns of *Abcg2* expression in the developing retina. (A,B) RT-PCR analysis carried out on cDNA obtained from different stages of late retinal histogenesis and adult retina revealed that levels of transcripts corresponding to *ABCG2* decreased as differentiation progressed. (C,D) ABCG2 immunoreactivity in a PN1 retinal section is predominantly confined to the outer neuroblastic layer. (E,F) In dissociated PN1 retinal cells pre-exposed to BrdU, ABCG2 is expressed in cells that also incorporated BrdU (arrows). ONbL, outer neuroblastic layer. IR, inner retina. Magnification, 200×.

Embryonic day (E)18 retinal cells were examined as the representative stage. We observed that SP cells predominantly expressed progenitor-specific transcripts, i.e. *Nestin*, *Notch1*, *Pax6* and *Sox2*, in addition to those corresponding to *Abcg2*. These transcripts were detected at relatively low levels in NSP cells. The NSP cells expressed retinal cell-type-specific transcripts, i.e. rhodopsin kinase (rod photoreceptors), *mGluR6* (bipolar cells) and *Brn3b* (retinal ganglion cells). Such transcripts were not detected in SP cells. These observations suggested that SP cells are uncommitted retinal progenitors. The association of *Abcg2* expression with retinal SP cells and the progressive decline in the SP cell population in parallel

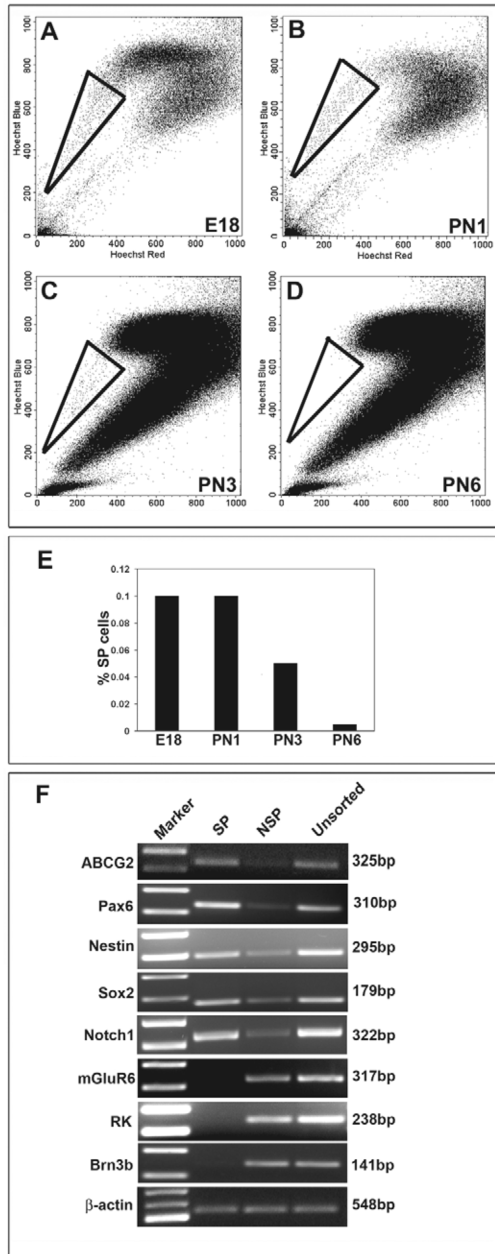

Fig. 2. Temporal decrease in SP cells in the developing retina. (A-E) Hoechst dye efflux assay, carried out on cell dissociates from different stages of late retinal histogenesis, revealed a temporal decrease in the proportion of SP cells (within triangle). (F) RT-PCR analysis carried out on SP and non-SP (NSP) cells from E18 retina revealed that the former predominantly expressed transcripts corresponding to *Abcg2*, *Nestin*, *Notch1*, *Pax6* and *Sox2* whereas the latter expressed those corresponding to differentiated cell markers, i.e. rhodopsin kinase (rods), *mGluR6* (bipolar cells) and *Brn3b* (retinal ganglion cells).

with the decrease in *Abcg2* expression suggested that SP cell phenotype is due to *Abcg2*. To test this notion further we examined the effect of perturbation of *Abcg2* expression on SP cell phenotype of retinal progenitors. Late retinal progenitors were enriched as neurospheres from E18 retina (E18 neurospheres) and transduced with *Abcg2* retrovirus vector (G1-ABCG2) to overexpress ABCG2, followed by Hoechst dye efflux assay to enrich SP cells (Bhattacharya et al., 2003). Since the gain-of-function approach is thought to carry a risk of non-specific results (Zhou, C. et al., 2001; Zhou et al., 2002), we examined the effects on SP cell phenotypes and maintenance of progenitors in response to acute decrease in *Abcg2* expression, using siRNA-mediated gene silencing. To attenuate *Abcg2* expression, E18 neurospheres were transfected with pSuper expression vector containing *Abcg2* siRNA sequence (pSuper-ABCG2$_{siRNA}$). Controls included neurospheres transduced with either empty retrovirus or transfected with pSuper containing scrambled *Abcg2* siRNA sequence. We observed a remarkable increase (~14-fold) in the proportion of SP cells in *Abcg2* retrovirus-transduced neurospheres, compared with controls (14.5±3.7 vs 0.9±1.9; $P<0.001$) (Fig. 3A-C). By contrast, there was a significant decrease (~tenfold) in the proportion of SP cells in neurospheres expressing *Abcg2* siRNA, compared with controls (0.1±0.19 vs 1.1±0.46; ***$P<0.001$) (Fig. 3D-F). The specificity of siRNA-mediated attenuation in gene expression was demonstrated by a decrease in the levels of *Abcg2* transcripts (Fig. 3G), ABCG2 protein in western blot analysis (Fig. 3H) and immunocytochemical analysis (Fig. 3I-L) compared with controls. Together, these observations demonstrated that *Abcg2* expression is developmentally regulated and is the molecular determinant of SP phenotype of retinal stem cells during late histogenesis.

Abcg2 participates in the maintenance of retinal progenitors

The expression of *Abcg2* in proliferating progenitors and its temporal decrease during histogenesis suggested that ABCG2 is involved in the maintenance of these cells. Maintenance of stem cells entails the maintenance of progenitor properties and the uncommitted state. To determine the influence of *Abcg2* on the retinal progenitor properties, we examined their proliferation and the expression of pan neural (Nestin) and retinal (Pax6) stem-cell-specific markers in response to perturbation of *Abcg2* expression in E18 neurospheres, as described above. Neurospheres were cultured in proliferating conditions for 4 days following transduction of G1-ABCG2 virus and transfection of pSuper-ABCG2$_{siRNA}$. Neurospheres were exposed to BrdU in the last 24 hours, to tag proliferating cells, followed by immunocytochemical and RT-PCR analyses of cell-specific markers. We observed a significant increase in the proportion of BrdU$^+$ cells expressing Nestin or Pax6 immunoreactivities in *Abcg2* retrovirus-transduced neurospheres, compared with controls (BrdU$^+$ Nestin$^+$ cells: 79.21±4.6 vs 46.9±2.8; BrdU$^+$ Pax6$^+$ cells: 73.45±3.7 vs 38.8±3.2; $P<0.001$) (Fig. 4A-H,I). By contrast, there was a significant decrease in the proportion of BrdU$^+$ cells expressing Nestin or Pax6 immunoreactivities in neurospheres transfected with pSuper-ABCG2$_{siRNA}$, compared with controls (BrdU$^+$ Nestin$^+$ cells: 22.24±2.8 vs 55.81±4.6; BrdU$^+$ Pax6$^+$ cells: 19.72±1.9 vs 47.35±3.2, $P<0.001$) (Fig. 5A-H,I). RT-PCR analysis carried out on similarly treated and cultured neurospheres revealed an increase and decrease in levels of transcripts corresponding to *Nestin*, *Pax6* and *Sox2*, in *Abcg2* retrovirus-transduced and siRNA-treated neurospheres, respectively, compared with controls, thus corroborating the immunocytochemical results (Fig. 4 and Fig. 5J). Together,

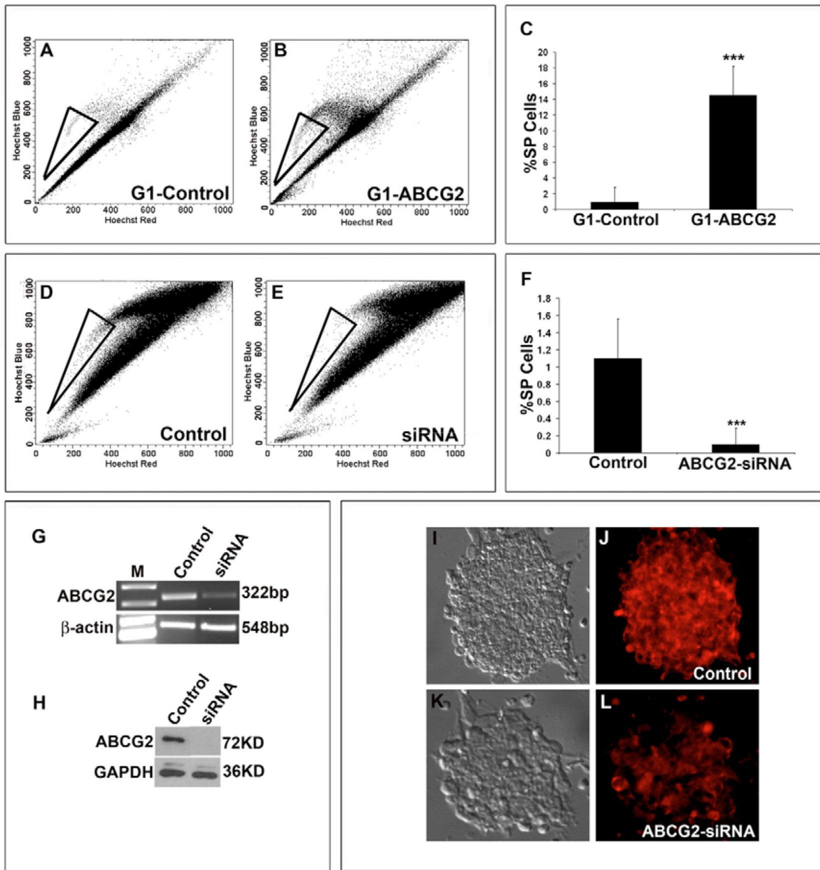

Fig. 3. Perturbation of *Abcg2* expression affects SP cell phenotype of late retinal progenitors. (A-C) E18 retinal progenitors, enriched as neurospheres, were transduced with ABCG2 retrovirus (G1-ABCG2) or empty retrovirus (G1-Control), followed by Hoechst dye efflux assay. There was a significant increase in the proportion of SP cells in G1-ABCG2 retrovirus-transduced neurospheres, compared with controls. (D-F) Neurospheres were transduced with retrovirus to express ABCG2 siRNA (pSuper-ABCG2$_{siRNA}$) or missense siRNA (pSuper-ABCG2$_{missense}$) as controls, followed by Hoechst dye efflux assay. There was a significant decrease in the proportion of SP cells in pSuper-ABCG2$_{siRNA}$ retrovirus-transduced neurospheres, compared with controls. (G-L) The specificity of siRNA-mediated attenuation of *Abcg2* expression is demonstrated by a decrease in levels of *Abcg2* transcript (G) by RT-PCR analysis and ABCG2 protein by western blot analysis (H) and by immunocytochemical analysis (I-L) in pSuper-ABCG2$_{siRNA}$-transduced neurospheres, compared with controls. Data are expressed as the mean ± s.e.m. from triplicate cultures of two different experiments. ***$P<0.005$ compared with levels in the control. Magnification, 200×.

these results suggested that *Abcg2* expression contributes towards the maintenance of retinal progenitor properties.

One of the mechanisms for the maintenance of retinal progenitors is to keep them from lineage commitment. Therefore, we were interested to determine whether *Abcg2* expression has a negative influence on the differentiation of retinal progenitors. We addressed this question by examining the effects of perturbation of *Abcg2* expression on pan neural

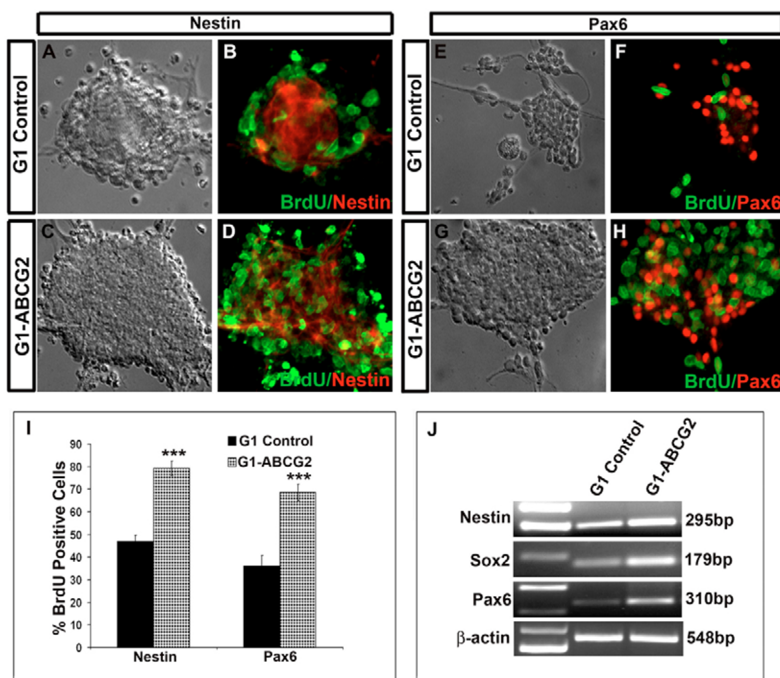

Fig. 4. Ectopic *Abcg2* expression promotes pan neural and retinal progenitor properties in late retinal progenitors. (A-I) E18 neurospheres, transduced with G1-ABCG2 retrovirus to overexpress ABCG2 or empty retrovirus as controls. There was a significant increase in the proportion of BrdU^{+} cells expressing pan neural progenitor marker, Nestin (A-D,I) and retinal marker, Pax6 (E-H,I) in G1-ABCG2 retrovirus-transduced neurospheres, compared with controls. (J) RT-PCR analysis revealed an increase in levels of transcripts corresponding to Pax6, Nestin and Sox2 in G1-ABCG2-transduced cells, compared with controls, corroborating the immunocytochemical analysis. Data are expressed as the mean ± s.e.m. of triplicate cultures from two different experiments. ***$P<0.005$ compared with levels in the G1-control. Magnification, 200×.

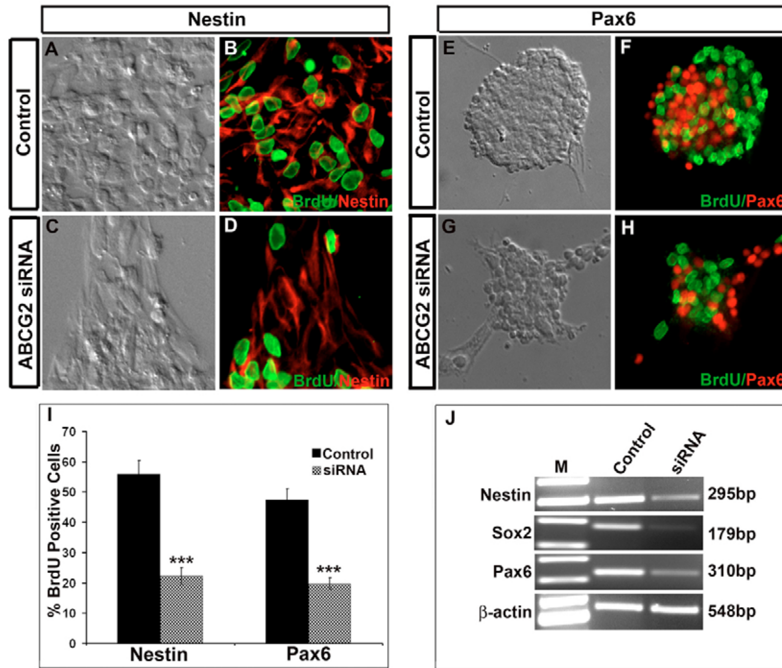

Fig. 5. Attenuation of *Abcg2* expression adversely affects pan neural and retinal progenitor properties in late retinal progenitors. (A-I) There was a significant decrease in the proportion of BrdU$^+$ cells expressing the neural progenitor marker, Nestin (A-D,I) and retinal marker, Pax6 (E-H,I) in pSuper-ABCG2$_{siRNA}$ retrovirus-transduced neurospheres, compared with controls. (J) RT-PCR analysis revealed a decrease in levels of transcripts corresponding to Pax6, Nestin and Sox2 in pSuper-ABCG2$_{siRNA}$-transduced neurospheres compared with controls, corroborating the immunocytochemical analysis. Data are expressed as the mean ± s.e.m. of triplicate cultures from two different experiments. Magnification, 200×.

differentiation of retinal progenitors. E18 retinal progenitors, enriched as neurospheres, were transduced with G1-*Abcg2* retrovirus or transfected with pSuper-ABCG2$_{siRNA}$ as described above. Instead of culturing them in proliferating conditions, neurospheres were shifted to differentiating conditions (1% serum, minus mitogen) and cultured for 5 days, followed by immunocytochemical and RT-PCR analyses of the expression of neuronal (Map2) and glial (GFAP) specific markers. In both experimental and control groups a subset of cells was detected expressing Map2 or GFAP immunoreactivities. However, there was a significant decrease in the proportion of Map2$^+$ as well as GFAP$^+$ cells in *Abcg2* retrovirus-transduced neurospheres, compared with controls (Map2$^+$ cells: 73.27±3.2 vs 41.23±2.8, $P<0.001$; GFAP$^+$ cells: 41.05±2.3 vs 20.21±2.8; $P<0.001$) (Fig. 6A-H,M). By contrast, the proportion of Map2$^+$ and GFAP$^+$ cells increased significantly in siRNA vector-transfected neurospheres, compared with controls (Map2$^+$ cells: 63.1±3.7 vs 81.01±4.6, $P<0.001$; GFAP$^+$ cells: 32.05±2.8 vs 54.19±3.61; $P<0.001$) (Fig. 7A-H,M). Examination of levels of transcripts corresponding to *Map2* and *Gfap* revealed that their levels decreased and increased in neurospheres transduced with *Abcg2* and transfected with siRNA, respectively, compared with controls, corroborating immunocytochemical results (Fig. 6 and Fig. 7N). Together, these observations suggested

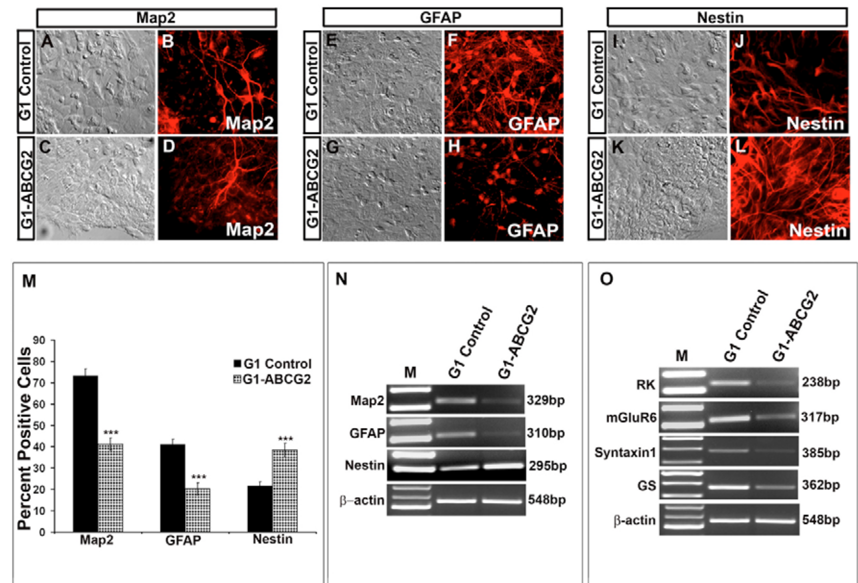

Fig. 6. Ectopic *Abcg2* expression adversely affects differentiation of late retinal progenitors. (A-M). There was a significant decrease and increase in the proportion of Map2$^+$ (A-D,M)/GFAP$^+$ (E-H,M) and Nestin$^+$ (I-L,M) cells, respectively, in G1-ABCG2 retrovirus-transduced neurospheres, compared with controls. (N) RT-PCR analysis revealed a decrease and increase in levels of transcripts corresponding to Map2/GFAP and Nestin, respectively, in G1-ABCG2-transduced neurospheres, compared with controls, corroborating the immunocytochemical analysis. (O) RT-PCR analysis revealed a decrease in the expression of transcripts encoding the specific retinal cell markers, rhodopsin kinase (RK, rods), mGluR6 (bipolar cells), Syntaxin1 (amacrine cells) and glutamine synthetase (GS, Müller cells) in G1-ABCG2 transduced neurospheres compared with controls, demonstrating the effects of ABCG2 on retinal-cell-specific differentiation. Data are expressed as the mean ± s.e.m. of triplicate cultures from two different experiments. ***$P<0.005$ compared with levels in the control. Magnification, 200×.

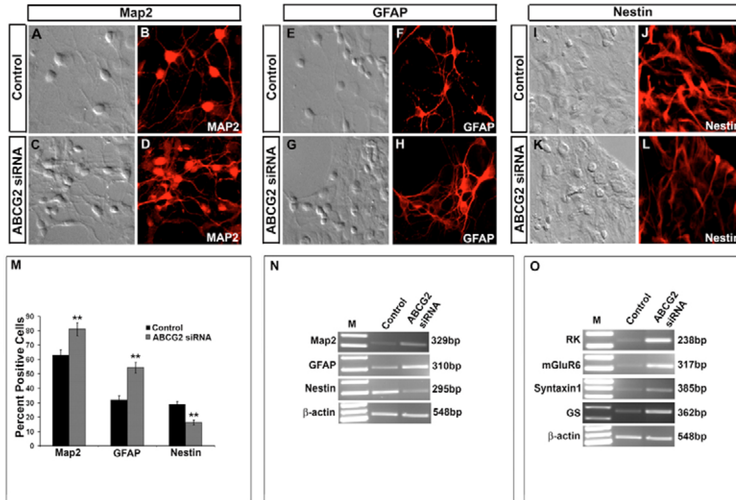

Fig. 7. Attenuation of ABCG2 promotes differentiation of late retinal progenitors. (A-M) There was a significant increase and decrease in the proportion of Map2^{+} (A-D,M)/GFAP^{+} (E-H,M) and Nestin^{+} (I-L,M) cells, respectively, in pSuper-ABCG2$_{siRNA}$ retrovirus, compared with controls. RT-PCR analysis revealed an increase and decrease in levels of transcripts corresponding to Map2/GFAP and Nestin, respectively, in pSuper-ABCG2$_{siRNA}$ retrovirus-transduced neurospheres, compared with controls, corroborating the immunocytochemical analysis (N). RT-PCR analysis revealed an increase in the expression of transcripts corresponding to specific retinal cell markers, rhodopsin kinase (RK, rods), mGluR6 (bipolar cells), Syntaxin1 (amacrine cells) and glutamine synthetase (GS, Müller cells) in pSuper-ABCG2$_{siRNA}$ retrovirus-transduced neurospheres as compared to controls, demonstrating the effects of ABCG2 on retinal cell specific differentiation (O). Data are expressed as the mean ± s.e.m. of triplicate cultures of two different experiments. Magnification, 200×.

that ABCG2 has a negative influence on pan neural differentiation of retinal progenitors. *Abcg2* expression influenced the differentiation of retinal progenitors along specific retinal lineage as demonstrated by a decrease and increase in levels of transcripts corresponding to rod photoreceptors (*Grk1*, rhodopsin kinase), bipolar cells (*mGluR6*), amacrine cells (*Stx1a,* syntaxin1) and Müller glia (*Glul*, glutamine synthetase) in neurospheres overexpressing *Abcg2* (Fig. 6O) and those treated with siRNA (Fig. 7O), respectively, compared with controls. To determine whether the effects of perturbation of *ABCG2* expression on differentiation is reflected in changes in progenitor populations, we examined the proportion of cells expressing immunoreactivities corresponding to Nestin in differentiating conditions. In an inverse relationship to cells expressing pan neural differentiation markers, the proportion of cells expressing Nestin and transcripts increased and decreased in neurospheres transduced with *Abcg2* retrovirus (Fig. 6I-L,M,N) and treated with *Abcg2* siRNA (Fig. 7I-L,M,N), respectively, suggesting that *Abcg2* influenced differentiation at the levels of progenitors.

Next, to determine the mechanism underlying the expansion of SP cell populations in response to the ectopic expression of *Abcg2*, we examined the expression of *Mash1*, a proneural gene, whose transient expression characterizes the proliferating intermediate retinal precursors during late retinal histogenesis (Ahmad et al., 1998). Levels of *Mash1* transcripts along with those corresponding to cell cycle regulators, cyclin D1, P27^{kip1} and Ki67, and the pan neuronal marker, *Map2*, were determined in SP and NSP cells in E18 neurospheres transduced with G1-*Abcg2* or empty retrovirus. *Mash1* transcripts were detected in SP and NSP cells in both conditions (Fig. 8). However, there was an increase in levels of *Mash1* transcripts in SP cells in G1-*Abcg2*-transduced neurospheres, compared with those in controls, and the increase was at the expense of levels of transcripts in NSP cells. A similar change in the expression of cyclin D1, the major D type cyclin found in retinal progenitors (Sicinski et al., 1995; Fantl et al., 1995) was observed; levels of *Ccnd1* transcripts increased in SP cells, enriched from G1-*Abcg2*-transduced neurospheres, compared with those in controls, at the expense of those in NSP cells. Transcripts producing P27^{kip1}, a cyclin kinase inhibitor that negatively regulates retinal progenitor proliferation (Ohnuma et al., 1999; Levine, 2000; Dyer and Cepko, 2001) were detected only in NSP cells and their levels decreased in NSP cells enriched from *Abcg2*-transduced neurospheres, compared with those in controls. By contrast, transcript producing Ki67, a nuclear protein characteristic of cells in S phase (Tirelli et al., 2002) was detected only in SP cells but their levels were higher in those overexpressing *Abcg2* than in controls. Together, these observations suggested that *Abcg2* favored the maintenance of proliferating progenitors over committed or differentiating precursors.

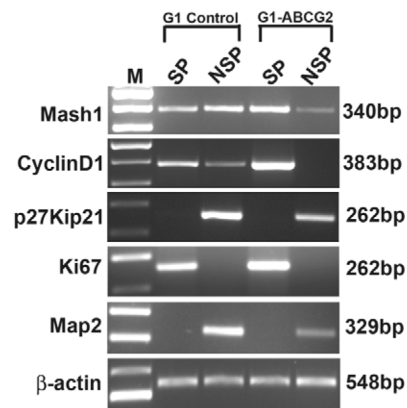

Fig. 8. Ectopic *Abcg2* expression influences *Mash1* and cell cycle regulators expression. SP and NSP cells were enriched by Hoechst dye efflux assay from neurospheres, transduced with G1-ABCG2 retrovirus or empty retrovirus, followed by examination of expression of transcripts corresponding to a proneural gene (*Mash1*), cell cycle regulators (*Ccnd1*, *Ki67*, *p27Kip1*) and pan neural marker (*Map2*). Levels of Mash1, CyclinD and Ki67 transcripts were increased in SP cells obtained from ABCG2 retrovirus-transduced neurospheres compared with those from control neurosheres. Whereas levels of *p27Kip1* and *Map2* transcripts were detected only in NSP cells and their levels decreased in those transduced with ABCG2 retrovirus, compared with those in controls.

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Notch signaling influences *Abcg2* expression and SP cell phenotype of retinal progenitors

One of the key regulators of retinal stem cells is Notch signaling, which maintains a pool of stem cells throughout histogenesis by keeping them uncommitted. The fact that ABCG2 inhibited the differentiation of retinal stem cells, while maintaining progenitor properties, suggested that *Abcg2* is one of the downstream targets of Notch signaling. We tested this premise as follows: first, we studied the effects of perturbation of Notch signaling on *Abcg2* expression and retinal progenitor properties. To activate Notch signaling, E18 neurospheres were transduced with retrovirus expressing Notch intracellular domain (NICD) and cultured in proliferating conditions for 4 days. Neurospheres were exposed to BrdU in the last 24 hours of culture to tag proliferating progenitors, followed by immunocytochemical and RT-PCR analyses of *Abcg2* expression. NICD, a product of activated Notch receptor obtained through the proteolytic activity of γ-secretase, mediates the CSL-dependent activation of Notch target genes (Mumm and Kopan, 2000). Therefore, overexpression of NICD leads to constitutive activation of Notch signaling. To attenuate Notch signaling, a batch of neurospheres was cultured under conditions promoting proliferation in the presence of DAPT and examined as described above. DAPT is an inhibitor of γ-secretase and thus attenuates Notch signaling by antagonizing the release of NICD (James et al., 2004). We observed a significant increase in the proportion of BrdU$^+$ cells expressing ABCG2 in NICD-transduced neurospheres, compared with controls (39.52±2.3 vs 20.17±1.9; $P<0.001$) (Fig. 9A-D,G). By contrast, the proportion of such cells decreased in DAPT-treated neurospheres, compared with controls (20.17±1.9 vs 17.11±1.9; $P<0.05$) (Fig. 9E-G). To ascertain that these results were not non-specific but related to changes in progenitor properties in response to Notch signaling, we examined the expression of *Nestin*, *Sox2* and *Pax6* transcripts. We observed that the changes in levels of *Abcg2* transcripts in response to perturbation in Notch signaling were accompanied by similar trends in levels of *Nestin*, *Sox2* and *Pax6* transcripts (Fig. 9H). The specificity of perturbation in Notch signaling was demonstrated by an increase and decrease in levels of Notch target gene, *Hes1* in NICD retrovirus-transduced and DAPT-treated neurospheres, respectively, compared with controls (Fig. 9H).

Next, we examined the effects of perturbation of Notch signaling on SP cell phenotype of retinal progenitors. We subjected neurospheres transduced with NICD retrovirus or treated with DAPT to the Hoechst dye efflux assay. We observed that the proportion of SP cells increased ~sixfold in neurospheres transduced with NICD retrovirus compared with those in untreated controls (Fig. 10A,B,D). By contrast, the proportion of SP cells was remarkably decreased in DAPT-treated neurospheres, compared with controls (Fig. 10A,C,D). The majority of cells in DAPT-treated neurospheres consisted of NSP cells. Together, these observations suggested that Notch signaling regulated expression of ABCG2 and the SP cell phenotype associated with it.

Abcg2 is the direct target of Notch signaling

Next, we wanted to know whether or not Notch signaling directly influenced the expression of the *Abcg2* gene. A prerequisite for direct activation by Notch signaling is the co-

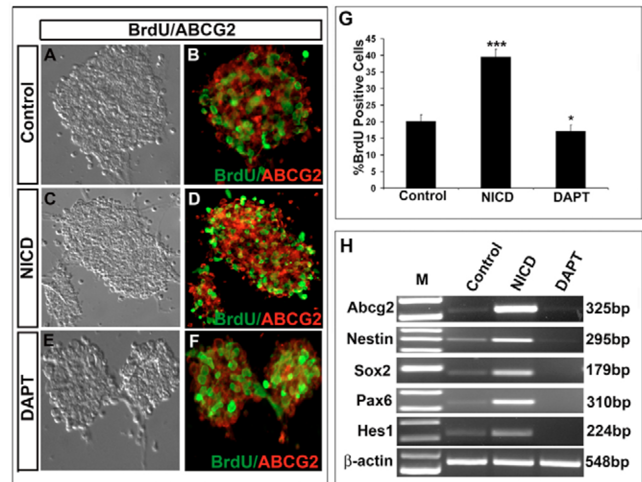

Fig. 9. Perturbation of Notch signaling affects ABCG2 expression in late retinal progenitors. (A-G). There was a significant increase and decrease in BrdU$^+$ cells, expressing ABCG2 immunoreactivities in neurospheres transduced with NICD and those cultured in DAPT, respectively. (H) RT-PCR analysis revealed that levels of transcripts encoding ABCG2, and those to progenitor markers, i.e. Pax6, Sox2 and Nestin, increased and decreased in neurospheres transduced with NICD and those cultured in DAPT, respectively, compared with controls. The specificity of the perturbation of Notch signaling was displayed by changes in the expression of Notch target gene *Hes1*. Data are expressed as the mean ± s.e.m. from triplicate cultures of two different experiments. Magnification, 200×.

expression of Notch and *Abcg2* in retinal progenitors. Double immunocytochemical analysis, carried out on E18 retinal progenitors revealed that Notch1 and ABCG2 immunoreactivities were co-localized (Fig. 11A-D). Notch signaling regulates its target genes through NICD-CSL mediated transcriptional activation. Some of the best-known Notch target genes are bHLH transcription factors belonging to hairy enhancer of split class, *Hes1* and *Hes5*. These genes contain CSL-binding elements in their promoters, which mediate the action of CSL-NICD complex. Examination of *Abcg2* promoter revealed the presence of multiple CSL-binding elements in its proximal promoter suggesting that *Abcg2* is one of the Notch target genes (Fig. 11E). To test this possibility we first tested whether or not the *Abcg2* CSL-binding element is involved in Notch-mediated transcriptional activation. *Abcg2* promoter reporter constructs containing serial deletion of *Abcg2* promoter sequence (Bailey-Dell et al., 2001) (Fig. 11E), were transfected in 293T cells, transduced with NICD retrovirus, followed by the analysis of luciferase activities. We observed a significant increase in luciferase activities (~3000-fold) in cells transfected with –1285/+362 constructs that contained CSL-binding sites compared with those transfected with the rest of the constructs that did not and demonstrated low luciferase activities (Fig. 11F). Second, we carried out a mobility shift assay to examine the ability of *Abcg2* promoter sequence containing the CSL-binding element to interact with nuclear factor(s) obtained from retinal progenitors. When the labeled wild-type oligonucleotide was incubated with nuclear extracts complexes were formed whose specificity could be shown by competitive inhibition of complex formation when an excess of unlabeled wild-type

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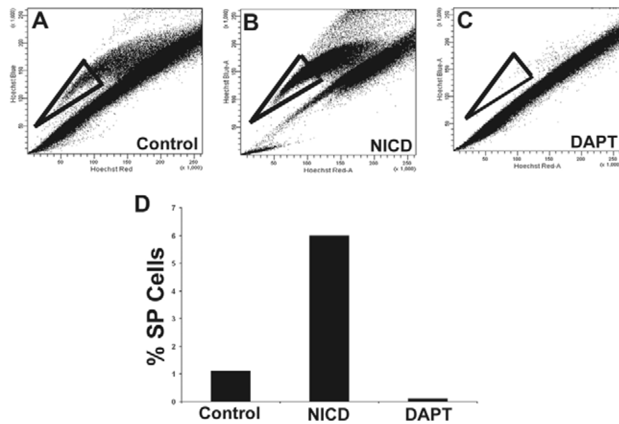

Fig. 10. Perturbation of Notch signaling affects SP cell phenotype of late retinal progenitors. To determine whether or not Notch signaling influenced SP cell phenotype of late retinal progenitors, E18 neurospheres were transduced with NICD retrovirus or cultured in the presence of DAPT to accentuate or attenuate Notch signaling, respectively. There was an increase and decrease in the proportion of SP cells in neurospheres, transduced with NICD and those cultured in DAPT, respectively, compared with levels in the controls (A-D).

oligonucleotide was included in the reaction (Fig. 11G). Complex formation was not observed when the reaction was performed with mutated oligonucleotide. To test the specificity of the complex formation, a mobility shift assay was carried out with in vitro translated CSL protein, which showed a complex formation similar to that obtained with nuclear extract obtained from retinal progenitors. Finally, to gain an insight into CSL-mediated regulation of *Abcg2* in retinal progenitors in vivo, we carried out chromatin immunoprecipitation (ChIP) on neurospheres maintained in proliferating conditions. The CSL antibody immunoprecipitated a nucleosomal complex that contained DNA sequence, amplifiable by *Abcg2* promoter-specific primers, suggesting that CSL is part of a transcriptional complex that regulates the activities of the *ABCG*2 promoter in vivo (Fig. 11H). Together, these observations suggest that the CSL-binding element, through which Notch activates its target genes, plays a role in the activation of *Abcg2* expression.

Discussion

The ubiquitous association of *Abcg2* expression with stem cells and their function in the efflux of a broad spectrum of substrates ranging from cytotoxic drugs to small molecules of differentiation suggest a role in the maintenance of homoeostasis of these progenitor cells. This notion led us to a hypothesis that the expression of *Abcg2* may constitute an integral part of the cellular mechanism that maintains retinal stem cell populations, and as such it is under the regulatory network of intercellular pathways that keep stem cells self renewing and uncommitted. The first part of the hypothesis, i.e. its role in the maintenance of retinal stem cells, is supported by temporal expression patterns of *Abcg2* during late histogenesis. We demonstrate that, as observed during the lineage commitment of hematopoietic stem cells (Zhou, S. et al., 2001), the expression of *Abcg2* is downregulated during the differentiation of retinal stem cells. This phenomenon is accompanied by a decrease in the proportion of SP cells at each successive stage of retinal histogenesis, suggesting that *Abcg2* is the regulator of retinal stem cells, which is characterized by SP cell phenotype (Bhattacharya et al., 2003). This premise was further tested by perturbation of the expression, which demonstrated that the overexpression of *Abcg2* expands retinal SP cells and at the same time attenuates the differentiation of retinal stem cells. By contrast, a targeted decrease in *Abcg2* expression depletes retinal SP cells and promotes the differentiation of retinal stem cells. Taken together, these observations suggest that *Abcg2* expression influences the maintenance of retinal stem cells. These observations including those that demonstrated the involvement of ABCG2 in lineage commitment in the hematopoietic compartment appear at odds with the lack of abnormalities in mice lacking a functional *Abcg2* gene (Zhou et al., 2002). Given the large ABC transporter family and the evidence that transporters such as *Abcg2* and *ABCB1* are co-expressed in stem cells (Zhou et al., 2003; Lin et al., 2006), the normal phenotype of *Abcg2*-knockout mice may be due to efficient functional compensation. Such functional compensation by ABC transporters is known to occur in the hematopoietic compartment. For example, the lack of effects of the absence of murine *ABCB1* genes on SP cell phenotype is regarded to be due to functional compensation by ABCG2 (Zhou, S. et al., 2001; Zhou et al., 2002).

The test of the second part of the hypothesis required the demonstration of interactions between ABCG2 and pathways that regulate the self-renewal and state of commitment of retinal stem cells. Prominent among these pathways are those mediated by Frizzled (Wnt signaling), Notch (Notch signaling), Patched (Shh signaling) and c-Kit (c-Kit signaling) (Ahmad et al., 2004). Notch signaling plays an important role in the maintenance of retinal stem cells. Attenuation in Notch signaling promotes differentiation of retinal ganglion cells (Austin et al., 1995; Ahmad et al., 1997) and rod photoreceptors (Jadhav et al., 2006) during early and late histogenesis, respectively. The temporal and spatial aspects of *Abcg2* and *Notch1* expression during retinal histogenesis betrays their possible interactions; levels of transcripts from both *Abcg2* and *Notch1* (Ahmad et al., 1995) decrease with lineage commitment and immunoreactivities corresponding to ABCG2 and Notch1 are co-localized in retinal stem cells. Our observations suggest that *Abcg2* is one of the targets of Notch signaling; like *Hes1* and *Hes5*, it is regulated through NICD-CSL-mediated transcriptional activation. The functional consequence of such regulation is demonstrated by changes in the levels of *Abcg2* expression and the proportion of SP cells in response to perturbation of Notch signaling. These changes are accompanied by changes in the differentiation status of retinal stem cells.

An interesting question remains as to how the expression of ABCG2 expands SP cells and keeps them undifferentiated. There is no definite answer as yet but it could be suggested that part of the increase in SP cells, observed in response to overexpression of ABCG2, might be at the expense of NSP cells. The NSP, besides containing post mitotic cells, includes proliferating precursors in different stages of differentiation. The developing retina contains committed precursors characterized by their ability to divide and expression of proneural genes, such as *Mash1* (Ahmad et al., 1998). These precursors are malleable and when exposed to conditions that

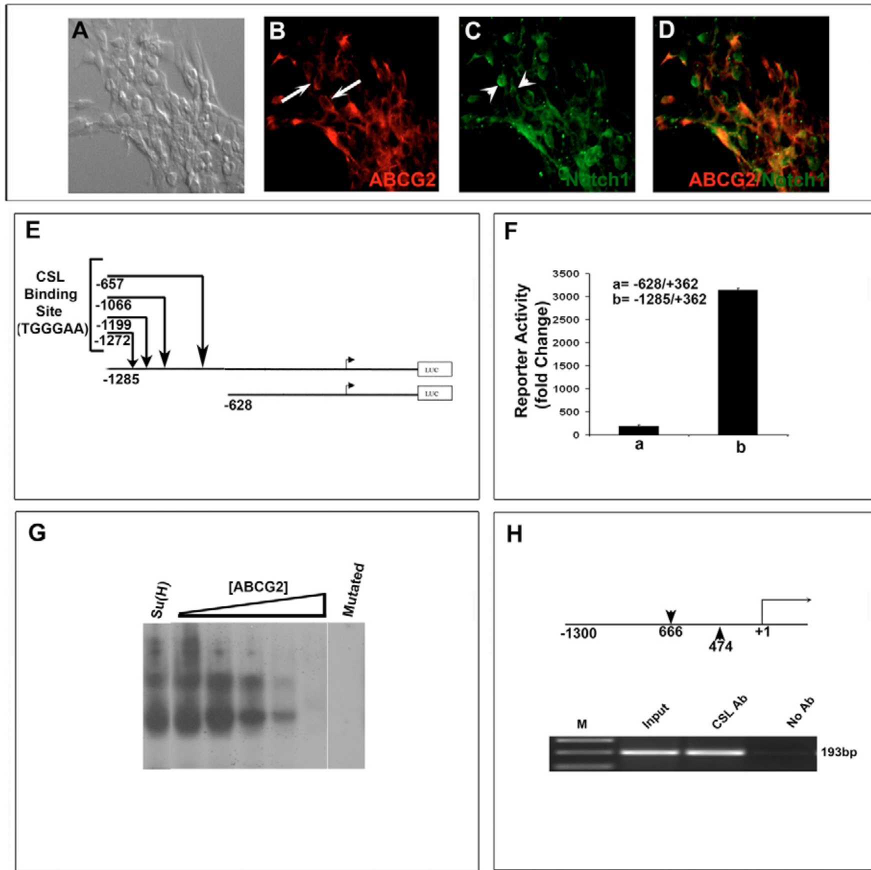

Fig. 11. Notch signaling influences *ABCG2* expression directly.
(A-D) Immunocytochemical analysis of E18 neurospheres showed that immunoreactivities corresponding to ABCG2 (arrow) and Notch1 (arrowhead) were co-localized in retinal progenitors. Examination of ABCG2 promoters revealed the presence four CSL binding sites (TGGGA) between –1285 and –657 (E). To determine the CSL-mediated effect on the activities of ABCG2 promoter, ABCG2 promoter deletion constructs were transfected into 293T cells, transduced with NICD retrovirus. (F) There was a significant increase in reporter activities with constructs containing CSL sites (b), compared to those without (a), which displayed base level reporter activities. (G) Mobility shift assay carried out with labeled wild-type ABCG2 promoter sequences containing one of the CSL sites (sequence) using HEK293T cell nuclear extracts revealed the formation of complexes, which were similar to those formed with in vitro translated suppressor of hairless (SuH) protein. These complexes could be competed with an excess of unlabeled sequences and were not formed with sequences in which the CSL site was mutated. (H) Nucleosomal DNA immunoprecipitated with CSL antibody in a ChIP assay on E18 retinal progenitors contained sequences corresponding to the *Abcg2* promoter, suggesting the presence of CSL on *Abcg2* regulatory complexes in vivo.

promote the maintenance of retinal stem cells rather than their differentiation they revert to a progenitor stage (Ahmad et al., 1998). The fact that the expression of *Mash1* increases in SP cells in neurospheres transduced with ABCG2 retrovirus compared with those in controls suggests that the Mash1-positive precursors are recruited as SP cells in response to overexpression of ABCG2 (Fig. 11). Indeed, the selective proliferation of ABCG2-expressing SP cells should also be taken into account for the expansion of retinal SP cells. The classic mechanism of the maintenance of stem cells involves the influence of the genome to promote cell proliferation, as in the case of Wnt signaling, and repress cell commitment, as in the case of Notch signaling (Ahmad et al., 2004). ABCG2, whose apparent function in stem cells is to protect them from toxins and maintain the homeostasis, might prevent cell commitment by actively extruding extrinsic small molecules that promote differentiation. Additionally, it might prevent differentiation by actively extruding key components of the differentiation-promoting signaling pathway, thus impairing it. Evidence has emerged that disparate signaling pathways are recruited for the maintenance of stem cells or progenitors and it is quite likely that disparate mechanisms, one involving an ABCG2 pump, are used to keep stem cells uncommitted to maintain their pool.

Materials and Methods

Cell culture

Embryos were harvested from timed pregnant Sprague-Dawley rats (SASCO), and the gestation day was confirmed by the morphological examination of embryos (Christie, 1964). Embryos were harvested in Hank's balanced salt solution (HBSS). Eyes were enucleated, and retinas were dissected and dissociated into single cells as previously described (Bhattacharya et al., 2003). Retinal dissociates were cultured in retinal culture medium or RCM (DMEM-F12, 1× N2 supplement, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) containing 20 ng/ml EGF for 5 days to generate clonal neurospheres. The neurospheres were collected, and plated on poly-D-lysine-coated and laminin-coated glass coverslips and exposed to 5-bromo-2′-deoxyuridine (BrdU) (10 μM) for the final 24 hours. Neurospheres were frozen for RT-PCR or fixed in 4% ice-cold paraformaldehyde for immunocytochemical analysis. To induce differentiation, neurospheres were washed extensively to remove BrdU and mitogen, and cultured in RCM and 1% FBS for a further 5 days, followed by collection for RT-PCR and immunocytochemical analysis, as described above.

Hoechst dye efflux assay

Retinal cells, from different stages of histogenesis were resuspended in Hoechst IMDM (10^6 cells/ml) containing 2% FCS at 4°C overnight followed by staining with Hoechst 33342 (2.0 μg/ml) at 37°C for 30 minutes. Cells were sorted on a FACStar Plus (BDIS) cell sorter. The Hoechst dye was excited at 350 nm and its fluorescence was measured at two wavelengths using a 485 BP22 (485/22 nm band-pass filter) and a 675 EFLP (675nm long-pass edge filter) optical filter (Omega Optical, Brattleboro, VT). A 610 nm short-pass dichroic mirror was used to separate these emission wavelengths (Omega Optical). The SP region was defined on the cytometer on the basis of its fluorescence emission in both blue and red wavelengths. Side population (SP) sorting gates were established after collecting 5×10^5 events within this live gate.

Immunofluorescence analysis

Detection of cell-specific markers and BrdU was performed as previously described (Bhattacharya et al., 2003). Briefly, paraformaldehyde-fixed cells were incubated in 1× PBS containing 5% NGS and 0, 0.2 or 0.4% Triton X-100 followed by an overnight incubation in antibodies against Nestin (DSHB), Pax6 (DSHB), ABCG2 (Chemicon), Map2 (Chemicon), GFAP (Sigma), Notch (Ahmad et al., 1995) and BrdU at 4°C. Cells were examined for epifluorescence after incubation in IgG conjugated to cyanin 3 (Cy3)/FITC. Images were captured using a cooled CCD camera (Princeton Instruments, Trenton, NJ) and Openlab software (Improvision, Lexington, MA). To determine the percentage of specific cell types in a particular

condition, the number of BrdU$^+$ and cell-specific antigen-positive cells was counted in 10-15 randomly selected fields in two to three different coverslips. Each experiment was repeated at least three times. Values are expressed as means ± s.e.m. Data were analyzed using the Student's *t*-test to determine the significance of the differences between various conditions.

RT-PCR analysis

Total RNA was isolated using a Qiagen RNA isolation kit. 2 μM cDNA was amplified using gene-specific primers by using the following step cycle program on a Robocycler (Stratagene): denaturation at 94°C for 30 seconds, annealing at specific temperature (see supplementary material Table S1) for 35 seconds, extension at 72°C for 40 seconds for 35 cycles followed by a final extension at 72°C for 5 minutes. PCR products were resolved on 2% agarose gels against 100bp DNA marker (MBI Fermentas). Gene-specific primers were used as listed in supplementary material Table S1.

Transduction of neurospheres

Late retinal progenitors were enriched as neurospheres from E18 retina (=E18 neurospheres) and were transduced with *ABCG2* retrovirus vector (G1-ABCG2) to overexpress ABCG2. After transduction, the medium was replaced, and neurospheres were cultured in proliferating conditions for 4 days and exposed to BrdU in the last 24 hours, to tag proliferating cells, followed by immunocytochemical and RT-PCR analyses of cell-specific markers.

siRNA-mediated gene silencing

For targeted silencing of *Abcg2* expression, ABCG2 siRNA was cloned into pSuper vector (pSuper-$ABCG2_{siRNA}$) according to the manufacturer's (Oligogene) protocol. Neurospheres in proliferating conditions were co-transfected with pSuper-$ABCG2_{siRNA}$ and pEGFP-C3 (to determine transfection efficiency) using Fugene according to the manufacturer's (Roche) protocol. A batch of neurospheres was similarly transfected with pSuper-$ABCG2_{missense}$, containing scrambled sequence, as controls. The efficiency of gene silencing was determined by immunocytochemical and RT-PCR analyses of ABCG2 protein and transcripts, respectively.

Reporter assay

HEK293T cells were plated onto 100 mm Petri dishes at a density of 8-10$\times 10^6$ cells. Cells were co-transfected with a series of *Abcg2* promoter constructs (Bailey-Dell et al., 2001) and NICD expression construct (James et al., 2004) using a calcium phosphate-based protocol. Transfection efficiency was examined by co-transfecting cells with pGFP-C3 (Clonetech). For luciferase assay, cells were lysed in 1× reporter lysis buffer (Promega) and 100 μl lysate was diluted five times using assay reagent (Promega). Diluted samples (100 μl) were analyzed for luciferase activities using a luminometer (Pharmingen). Mean fold change for the respective deletion constructs in the experimental groups was calculated with respect to controls (pGL3-basic). Values are expressed as mean fold change ± s.e.m. from three different experiments. Statistical analysis was performed using the Student's *t*-test to determine the significance between different conditions.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as previously described (Ahmad et al., 1995). Briefly, oligonucleotides corresponding to *Abcg2* promoter sequence containing the wild-type (GGTAGATGTTGGGATGGCTAC) or mutated (GGTAGATGTGAGAT-TGGCTAC) CSL binding site were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs). The binding reaction was performed in 20 μl buffer containing 20 mM Tris-HCl (pH 8.0), 75 mM KCl, 5% glycerol, 50 μg/ml bovine serum albumin (BSA), 0.025% nonidet NP-40, 1 mM EDTA, 5 mM DTT and 1 μg of poly(dI/dC). The end-labeled probe (100 pmol (20,000 cpm) was incubated on ice for 30 minutes with 5 μg of nuclear extract. Samples were loaded on a 5% polyacrylamide gel in 0.5 TBE buffer for 2 hours at 10 V/cm. The gel was dried and autoradiographed. For the competition assay, a molar excess of cold probe was added to the binding reactions.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was done using a modified procedure from Upstate Biotechnology. Briefly, E18 retinal progenitors were grown in proliferating conditions and histones were crosslinked to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% and incubating for 10 minutes at room temperature on a rocking platform. Cells were washed three times with ice-cold PBS containing protease inhibitors. Cell pellets were resuspended in pre-warmed SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). To reduce the non-specific background, the samples were pre-cleared using 80 μl salmon-sperm DNA/protein A agarose slurry at 4°C for 30 minutes. Samples were centrifuged at 100 r.p.m. for 1 minute at 4°C. Supernatants were transferred to a new tube, the immunoprecipitating antibody was added and incubation was carried out overnight at 4°C on a rocking platform. For a negative control we used no antibody or non-specific antibodies. The histone-antibody complex was precipitated using 60 μl salmon sperm DNA and protein-A-Sepharose (Upstate) for 1 hour at 4°C. Precipitates were washed sequentially at room temperature for 5 minutes, once with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl.), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) and lithium salt immune complex wash buffer (2.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid sodium salt, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and finally twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After completely removing the TE buffer, the precipitate was resuspended and extracted twice in 250 μl freshly prepared elution buffer (1% SDS, 0.1M $NaHCO_3$). To reverse the histone-DNA crosslinks, samples were heated at 65°C for 4 hours. 200 μl of initial sonicated sample was reverse crosslinked and used as input. After removing the antibodies by protease digestion of the samples, DNA was recovered and column purified. PCRs were performed using gene-specific primers (5′-GGTAGATGTTGGGATGGCTAC-3′, 5′-CCATCTACAACCCTACCGATG-3′).

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