The early retinal progenitor-expressed gene Sox11 regulates the timing of the differentiation of retinal cells

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SUMMARY
Sry-related HMG box (Sox) proteins, Sox11 and Sox4 are members of the SoxC subtype. We found that Sox11 was strongly expressed in early retinal progenitor cells and that Sox4 expression began around birth, when expression of Sox11 subsided. To analyze the roles of Sox11 and Sox4 in retinal development, we perturbed their expression patterns in retinal explant cultures. Overexpression of Sox11 and Sox4 in retinal progenitors resulted in similar phenotypes: an increased number of cone cells and dramatically decreased numbers of rod cells and Müller glia. Birth-date analysis showed that cone cells were produced at a later developmental stage than that in which cone genesis normally occurs. Sox11-knockout retinas showed delayed onset and progress of differentiation of subsets of retinal cells during the embryonic period. After birth, retinal differentiation took place relatively normally, probably because of the redundant activity of Sox4, which starts to be expressed around birth. Overexpression and loss-of-function analysis failed to provide any evidence that Sox11 and Sox4 directly regulate the transcription of genes crucial to the differentiation of subsets of retinal cells. However, histone H3 acetylation of some early proneural genes was reduced in knockout retina. Thus, Sox11 may create a epigenetic state that helps to establish the competency to differentiate. Taking our findings together, we propose that the sequential expression of Sox11 and Sox4 during retinogenesis leads to the fine adjustment of retinal differentiation by helping to establish the competency of retinal progenitors.

KEY WORDS: Sox family transcription factor, Retina, Progenitor cells, Mouse

INTRODUCTION
The vertebrate neural retina is organized into a laminar structure comprising six types of neurons and glial cells, Müller glia and astrocytes. In the mouse, these major retinal cell classes are generated from a common population of multipotent retinal progenitor cells between embryonic day (E) 11 and postnatal day (P) 10, in a conserved temporal order (Marquardt and Gruss, 2002). In vertebrates, retinal ganglion cells (RGCs) differentiate first, as a wave across the neuroepithelium of the optic cup. Ganglion cells, amacrine cells, cone photoreceptors and horizontal cells differentiate at relatively early stages primarily before birth, whereas bipolar cells and rod cells are mainly generated at later stages, after birth. It has been shown that both the progression of retinal neurogenesis and retinal cell fate specification or differentiation are controlled by intrinsic cues, such as transcription factors, as well as by extrinsic signals (Cepko, 1999; Harris, 1997).

To understand how this works, cell surface antigens are powerful tools for isolating specific subsets of retinal cells during development from cell mixtures without damaging the cells. This makes it possible to characterize their properties and identify genes that regulate their proliferation and differentiation. By screening retinal cells from mice at various developmental stages for their reactivity with over 150 different antibodies against various cell-surface antigens, we identified SSEA-1 (Fut4 – Mouse Genome Informatics) and Kit as early and late progenitor markers, respectively (Koso et al., 2006; Koso et al., 2007). SSEA-1 marks retinal progenitor cells in the peripheral region of the retina. In later stages of embryogenesis, SSEA-1 disappears, and Kit expression is observed in the retinal progenitor cells in the central region of retina. Using microarrays, we compared the gene expression patterns of regionally and temporally different subsets of retinal progenitor cells, SSEA-1-positive cells at E14 and Kit-positive cells at P1, and of differentiated cells, Kit-negative cells at P1. We found Sox11 to be strongly expressed in SSEA-1-positive cells.

The Sry-related box (Sox) genes encode a group of transcription factors with a high mobility group (HMG)-type DNA-binding domain (Schepers et al., 2002). Based on their sequence homology, Sox proteins have been subdivided into groups A to J (Schepers et al., 2002). Mammalian SoxC proteins comprise Sox4, Sox11 and Sox12 (Schepers et al., 2002). All SoxC proteins are widely expressed during embryogenesis in neuronal progenitors and in mesenchymal cells in many developing organs (Dy et al., 2008; Hosher et al., 2008). The functions of SoxC genes as regulators of cell fate, proliferation and survival in major physiological and pathological processes have been reported in many organ lineages. However, the expression and function of SoxC in the developing retina is unknown. Sox11 knockout (KO) mice has been reported (Sock et al., 2004), with the eyes showing microphthalmia and anterior segment dysgenesis such as Peter’s anomaly (Wurm et al., 2008). By gain-of-function analysis of Sox11 and Sox4, as well as a detailed examination of Sox11-KO retinas, we are able to describe the roles of Sox11 and Sox4 in retinal development.
MATERIALS AND METHODS

Mice and reagents

Sox11-knockout (Sox11-KO; Sox11<sup>+/−</sup>lacZ/+ [Sox11-LacZ/+] mice (Sock et al., 2004). ICR mice were obtained from Japan SLC. All animal experiments were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo and conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research. Microarray analysis was carried out using Affymetrix GeneChip Mouse Genome 430 2.0 using total RNA from retinas of Sox11-KO at E18 and littermate. Microarray data are available in the GEO database (Accession Number GSE43089). shRNA plasmid for Sox4 (pGFP-V-RS shRNA) was purchased from Origene, and efficiency was examined by RT-PCR in NIH3T3 cells. The target sequence was 5′-CCGCTGACGTTCTCTCCAGAGCAAGCT-3′. Retinal explants and retroviral infection were performed as described elsewhere (Tabata et al., 2004). In vitro electroporation was carried out as described (Iida et al., 2011). The electroporated retinas were cultured at 34°C on a chamber filter (Millicell).

DNA construction

Full-length cDNAs encoding mouse Sox4, and Sox11 open reading frames (ORFs) were isolated by reverse transcription (RT)-PCR using mouse retinal RNA and then cloned into pGem-T-Easy vector (Promega). Sox4 and Sox11 fragments were subcloned into pMX-IRES-EGFP retrovirus vector using BamHI/XhoI (Sox11) and EcoRI (Sox4) sites and pCAG vector using EcoRI (Sox11) and EcoRV/XhoI (Sox4).

RT-PCR

Total RNA was purified from mouse retinas using RNasy Plus Micro (QIAGEN), and cDNA was synthesized using SuperScript II (Invitrogen-Gibco). For semi-quantitative PCR, Blend Taq Plus (TOYOBO) was used, and bands were visualized with ethidium bromide. Quantitative PCR (qPCR) was carried out using the SYBR Green-based method using the Roche Light Cycler 1.5 apparatus and analyzed by the Second Derivative Maximum Method for quantification (Roche Diagnostics). The sequences of PCR primers are listed in supplementary material Table S2.

In situ hybridization of Sox11 and Sox4

In situ hybridization was performed according to a standard protocol previously described (Koso et al., 2008) using digoxigenin-labeled RNA probes. As the coding regions of Sox11 and Sox4 have high homology in the 5′ half, we used the 3′ half and the 3′ untranslated region of cDNA using a common Apol site in the middle of the Sox11- and Sox4-coding regions.

Immunostaining

Immunostaining of sections was carried out as described previously (Tabata et al., 2004). The first antibodies were visualized by using appropriate Alexa Fluor-conjugated second antibodies (Molecular Probes). Samples were mounted in VectaShield (Vector Laboratories) and analyzed using a Zeiss Axio Vision 4.6 microscope. The primary and secondary antibodies are listed in supplementary material Table S1.

Fluorescence-activated cell sorting (FACS)

Retinas electroporated with plasmids containing EGFP were digested with trypsin (0.25%) at 37°C for 10 minutes. PBS containing FCS (20%) and DNase I (0.2%) was added, and the cells were mechanically dissociated into a single-cell suspension by gentle pipetting. Sorting was carried out using a MoFlo (DakoCytomation).

Chromatin immunoprecipitation (ChIP) assay

Mouse retinas crosslinked with 1% formaldehyde were suspended in 1% SDS lysis buffer and sonicated to shear genomic chromatin. The lysate was incubated for 1 day with the antibody-bound Dynabeads-Protein G (Invitrogen). Eluted immune complex was incubated at 65°C overnight and proteins were eliminated by proteinase K (Wako). DNA was purified with a QIAquick PCR purification kit (QIAGEN). Real-time PCR was carried out using a Roche Light Cycler 1.5 apparatus. The sequences of PCR primers are listed in supplementary material Table S2. The abundance of target genome DNA was normalized relative to that of input. For all ChIP experiments, independent experiments were carried out at least twice, and essentially the same results were obtained. One representative set of data are shown. Control IgG experiments gave only negligible values.

Brdu labeling and birth-date analysis

For pulse labeling with BrdU to detect S-phase RPCs, 100 μg of bromodeoxyuridine (BrdU; Sigma-Aldrich) per gram of body weight was injected intraperitoneally into pregnant females 1 hour before being euthanized. Embryonal heads were fixed in 4% PFA and frozen sectioned. For retinal explants, after 3 days of culture, BrdU was mixed into medium at a final concentration of 1.5 μg/ml at 24 hours before fixation. For birth-date analysis, pregnant Sox11-LacZ/+ mice were intrauterineinjected with BrdU (100 μg per gram of body weight) at 11, 13 or 15 days of pregnancy, and sacrificed at day E18. Retinas were frozen sectioned and immunostained. For Sox11 overexpression, pMX-Sox11-EGFP was electroporated in <i>vitro</i> into isolated retina from normal embryos at E14 and cultured as explants. BrdU was present in the first, second, third, fourth, fifth and sixth 24 hours of explant culture, and retina was harvested at the 14th day and frozen sectioned. Sections were immunostained with antibodies as indicated.

RESULTS

Sox11 is expressed in the retina during early developmental stages and Sox4 at a later stage

We previously found that SSEA-1 and Kit mark subsets of retinal progenitor cells in early and late embryonic stages, respectively (Koso et al., 2006; Koso et al., 2007). Using microarrays, we then searched for genes that are specifically expressed in retinal progenitor cells. We found that Sox11 was more strongly expressed in SSEA-1-positive cells than in Kit-positive cells and that Sox4 showed the reverse pattern (supplementary material Table S3). Other microarray analyses comparing the gene expression pattern of E15 retinas with that of explant retinas cultured for 5 days showed that expression of Sox11 decreased as retinal development proceeded; by contrast, expression of Sox4 increased (supplementary material Table S4). Another member of the SoxC group, Sox12 (Bowles et al., 2000), showed only negligible expression in all subsets/developmental stages examined (supplementary material Tables S3, S4). We then examined the time course of Sox11 and Sox4 expression in more detail by semi-quantitative RT-PCR. Sox11 was strongly expressed in E14 and E16 retinas, with the expression level becoming slightly weaker at E18 and decreasing sharply after birth (Fig. 1A). Sox4 was only weakly expressed in E14 retinas; its expression subsequently increased gradually, peaking at E18 (Fig. 1A). Expression continued until P12 and became faint by around P15.

We next examined the spatial patterns of Sox11 and Sox4 expression by in situ hybridization (Fig. 1B). Sox11 was expressed throughout the retina at E11 and became stronger in the inner half of the retina at E13. At E17, strong expression of Sox11 was observed in the ganglion cell layer (GCL). From P3 onwards, we could not detect Sox11 (Fig. 1B). We observed a faint Sox4 signal in the central region of the retina at E11; at E13, expression was detected in the GCL (Fig. 1B). At E17, a pattern similar to that at E13 was noted; between P3 and the adult stage, expression of Sox4 occurred in the GCL and the inner nuclear layer (INL) (Fig. 1B).

Sox11 is expressed in proliferating cells and in early retinal cells

We next examined the expression of Sox11 in detail by immunostaining lacZ (β-galactosidase) in Sox11-lacZ mice carrying a lacZ gene in the Sox11 locus (Sock et al., 2004). First,
we confirmed that the gross structure of the retina and the expression patterns of various markers of retinal cell subtypes in mature retinas of Sox11-lacZ heterozygous (lacZ/+) mice were indistinguishable from those in wild-type retinas (data not shown). Next, we confirmed that the lacZ expression pattern was similar to that obtained by in situ hybridization of Sox11 (data not shown, Fig. 1C-F). We next conducted double staining of lacZ and retinal subtype markers using retinal sections derived from Sox11-lacZ/+ mice. At E11, βIII-tubulin started to be expressed, and some Sox11-expressing cells in the central region exhibited βIII-tubulin signals (Fig. 1C). At E12, Sox11 was mainly expressed in Ki67-positive retinal progenitor cells. βIII-tubulin signals became stronger, and a subset of the βIII-tubulin-positive cells expressed Sox11 (Fig. 1D). At that time, Brn3b (Pou4f2 – Mouse Genome Informatics), a marker of retinal ganglion cells, began to be expressed; some Brn3b-positive cells expressed Sox11 (Fig. 1D). At E14, Sox11 and Sox4 signals in the innermost region merged with those for Brn3b and HuC/D, the latter of which labels ganglion and amacrine cells (Fig. 1E). Diffuse and weak expression in the outer one-third of the region coincided with Ki67 (Fig. 1E). At E18, strong Sox11 and Sox4 expression in the GCL coincided with the expression of Brn3b and Islet1, the latter of which is expressed in ganglion and amacrine cells (Fig. 1F). Most outer cells were positive for RXRγ and most cells in the middle part were positive for NF160, suggesting that they are cone and horizontal cells, respectively (Fig. 1F, arrowheads, arrows). In adults, weak expression of β-gal was observed in the GCL (Fig. 1G, arrow). We next examined whether Sox11 and Sox4 were co-expressed during

Fig. 1. Expression of Sox4 and Sox11 during retinal development.

(A) Expression of Sox11 and Sox4 mRNA in retinas was examined at various developmental stages, as indicated by semi-quantitative RT-PCR of Sox4 and Sox11. G3PDH was used as a control.

(B) In situ hybridization of Sox11 and Sox4 was carried out using retinal sections at various stages as indicated.

(C-G) Expression of Sox11 or Sox4 and various retinal markers at indicated developmental stages. Sox11 expression was visualized by immunostaining using anti-β-galactosidase antibody on frozen retinal sections from Sox11-lacZ/+ mice. Sox4 protein expression was shown by immunostaining of Sox4 antibody.

Antibodies used to visualize retinal subsets were βIII-tubulin (neuronal marker), Ki67 (progenitor marker), Brn3b (ganglion cell marker), HuC/D (ganglion and amacrine cell marker), RXRγ (cone photoreceptor marker), Islet1 (ganglion and amacrine cell marker) and NF160 (horizontal cell marker). INBL, inner neuroblastic layer; ONBL, outer neuroblastic layer; GCL, ganglion cell layer; NBL, neuroblastic layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 50 μm. Nuclei are visualized using DAPI (blue).
retinal development by immunostaining Sox4 and β-gal. Sox4 showed an expression pattern similar to that produced by in situ hybridization (data not shown, Fig. 1E-G), and co-immunostaining of Sox11-lacZ/+ retinas with β-gal showed that Sox11 and Sox4 were co-expressed in cells in the INL at E14 and cells of the INL and GCL at E18 (Fig. 1H, arrows).

Gain-of-function analysis of Sox11 and Sox4 revealed altered retinal differentiation

To delineate the functions of Sox11 and Sox4 in retinal development, we conducted a gain-of-function analysis by retrovirus-mediated gene transfer into retinal explant cultures (Tabata et al., 2004). A retrovirus encoding Sox11-IRES-EGFP or Sox4-IRES-EGFP or control EGFP was transduced into retinal explants at E17 and cultured for 2 weeks. Frozen sections were made and stained with antibody against EGFP (A-C) or with EGFP in combination with various retinal subtype-specific markers (D-F). Arrowheads indicate double-positive cells (D).

Population of EGFP-positive cells in retinal sublayers (ONL, INL, GCL) is shown in B. The ONL was divided horizontally into three regions and EGFP-positive cells in each subregion are shown in C. (F) Population of EGFP and marker double-positive cells out of EGFP single-positive cells is shown. (G-I) Proliferation-related indicators were examined 72 hours after retrovirus infection. In H, BrdU was present during the last 24 hours of culture. The average of three independent experiments with standard deviation is shown. **P<0.01, *P<0.05, calculated using Student’s t-test. Scale bars: 50 μm. N.S., not significant.

Fig. 2. Gain-of-function analysis of Sox11 and Sox4 resulted in enhancement of cone and suppression of rod and Müller glia retinal subtypes. (A-F) Retrovirus encoding Sox11-IRES-EGFP, Sox4-IRES-EGFP or control EGFP was transduced into retinal explants at E17 and cultured for 2 weeks. Frozen sections were made and stained with antibody against EGFP (A-C) or with EGFP in combination with various retinal subtype-specific markers (D-F). Arrowheads indicate double-positive cells (D). The populations of EGFP-positive cells in retinal sublayers (ONL, INL, GCL) is shown in B. The ONL was divided horizontally into three regions and EGFP-positive cells in each subregion are shown in C. (F) Population of EGFP and marker double-positive cells out of EGFP single-positive cells is shown. (G-I) Proliferation-related indicators were examined 72 hours after retrovirus infection. In H, BrdU was present during the last 24 hours of culture. The average of three independent experiments with standard deviation is shown. **P<0.01, *P<0.05, calculated using Student’s t-test. Scale bars: 50 μm. N.S., not significant.
However, these cells expressed neither cone arrestin 4 nor M-opsin (supplementary material Fig. S1), suggesting that final maturation did not occur with these cells. By contrast, expression of Sox4 or Sox11 dramatically suppressed rod cell differentiation (Fig. 2F, PNR, rhodopsin), suggesting that the increase in the number of cones occurs at the expense of rod cells. All criteria showed similar activities for Sox11 and Sox4, suggesting that Sox11 and Sox4 are redundant in terms of retinal cell differentiation.

The Notch signaling pathway affects the differentiation of Müller glia and cone photoreceptors in positive and negative ways (Furukawa et al., 1997; Hojo et al., 2000; Jadhav et al., 2006; Yaron et al., 2006; Riesenberg et al., 2009). We examined whether Sox4 and Sox11 affect Notch signaling by analyzing RBP-jκ-luciferase, a reporter for Notch signaling (Minoguchi et al., 1997). No effect was observed (supplementary material Fig. S2).

As altered timing of exit from the cell cycle often results in perturbation of differentiation, we next examined the effects of Sox11 and Sox4 on retinal cell proliferation by assessing BrdU incorporation and the expression of Kip1/p27 and Ki67. None of these parameters showed significant differences in Sox11- or Sox4-overexpressing cells compared with control cells (Fig. 2G-I), suggesting that Sox11 and Sox4 may not play important roles in the proliferation of retinal cells.

Loss of Sox11 function in retinas delayed the initiation of neurogenesis and differentiation of ganglion and cone cell subtypes

To analyze the in vivo role of Sox11 in retinal development, we examined retinas from Sox11-lacZ/lacZ homozygous null (Sox11-KO) mice (Sock et al., 2004). Analysis of the gross morphology of the eyes in Sox11-KO mice at E18 showed microphthalmia and anterior segment dysgenesis, as previously reported (Wurm et al., 2008). We next examined the retinal phenotype in more detail by immunostaining with antibodies specific for markers of retinal cell subtypes. The first retinal neuron ganglion cells appear in the dorso-central retina, and neurogenesis spreads peripherally (Hufnagel et al., 2010). At E11, βIII-tubulin and Brn3b were expressed in the dorso-central retina, but were not detected in the Sox11-KO retina (Fig. 3A). At E12, ganglion cells started to differentiate in the control retina. By contrast, only a few Brn3b- or βIII-tubulin-positive cells were observed in the Sox11-KO retina (Fig. 3A,B). At E13, the areas in which βIII-tubulin and Brn3b were expressed expanded, but only the central part of the retina expressed these proteins in Sox11-KO retinas. However, at E14, the βIII-tubulin- and Brn3b-positive areas in the Sox11-KO retina started to expand (Fig. 3A,B). Quantification of the peripheral spread of...
neurogenesis by measuring the outer edges of the βIII-tubulin domains in the central retinal sections confirmed the delay of neurogenesis in the Sox11-KO retina until E13 (Fig. 3C). Cone photoreceptors are one of earliest differentiated retinal cell subtypes. At E13, signals for the cone marker RXRγ were observed in the control retina. By contrast, the RXRγ signal in the Sox11-KO retina was weak, and the number of positive cells was about half that in the control retina (Fig. 3D). At E18, numbers of RXRγ-positive cells were comparable in Sox11-KO and control retinas (Fig. 3D). Similarly, HuC/D-positive amacrine cells and NF160-positive horizontal cells, which are absent at around E12 in Sox11-KO retinas, were present in similar numbers in Sox11-KO and control retinas at E18 (data not shown).

Sox11-KO retinas develop relatively normally at the postnatal stage

Because Sox11-KO mice die at birth (Sock et al., 2004), we examined postnatal retinal development by explant culture. E18 retinas were isolated and cultured as explants for 2 weeks. Frozen sections were then immunostained. HuC/D, calbindin 28k (horizontal cells), RXRγ, PNR, Chx10 (bipolar cells), GS and cyclin D3 (Müller glia) showed similar expression patterns in Sox11-KO and control retinas (supplementary material Fig. S3), suggesting that postnatal differentiation of Sox11-KO retinal cells was normal.

Proliferation of retinal cells was only slightly perturbed in Sox11-KO retinas

As Sox11-KO retinas showed microphthalmia, we next examined proliferation in Sox11-KO retinas by measuring BrdU incorporation and expression of phospho-histone H3, an M-phase marker (Fig. 4A). Although the diameters of Sox11-KO retinas were much smaller than those of control retinas, numbers of BrdU-positive cells (Fig. 4A,B) and phospho-histone H3-positive cells (Fig. 4A,C) were comparable at E11-E13. Numbers of phospho-histone H3-positive cells were also comparable at E14 and E18 (data not shown). We then examined BrdU and phospho-histone H3 double-positive cells at E12 after in utero BrdU labeling for 2 or 4 hours (Fig. 4D). Incorporation of BrdU was comparable in control and Sox11-KO retinas (Fig. 4D,E), and the number of BrdU- and phospho-histone H3-double-positive cells was also comparable (Fig. 4F). Taking these results together, we conclude that ablation of Sox11 delayed retinal cell differentiation without perturbing cell proliferation. We also investigated apoptotic cells...
by examining active caspase 3-positive cells (Fig. 4G,H). The number of apoptotic cells was higher in Sox11-KO retinas at all stages we examined (E12, E14 and E18), suggesting that Sox11 may play important roles in cell survival during retinal development. The microphthalmia observed in Sox11-KO retinas may be partly explained by increased apoptosis. No difference was seen in the number of apoptotic cells when Sox11 was overexpressed (Fig. 4I). We surmise that high expression of endogenous Sox11 in the early developmental period may explain why we did not observe fewer apoptotic cells when Sox11 was overexpressed.

Birth dates of cone and ganglion cell were affected by the level of Sox11

As modulating the level of Sox11 perturbs the differentiation of retinal cell subtypes, we examined whether birth date of these cells was affected by Sox11. We first examined whether the generation of cone and ganglion cells was affected in Sox11-KO retinas by pulse-labeling with BrdU at different embryonic stages. BrdU was present every 24 hours, as indicated schematically in G. (H) Retinas were harvested after 2 weeks. The sections were triple immunostained with anti-EGFP, anti-BrdU and anti-RXRγ. (I) The percentage of RXRγ and EGFP double-positive cells in EGFP single-positive cells over a total of 6 days' labeling are shown. BrdU-positive and -negative populations in RXRγ/EGFP cells are shown in different colors. Percentage of RXRγ/EGFP and BrdU-positive cells in EGFP cells are calculated in each sample and a summary of the results is shown. (J) The percentage of triple-positive cells out of double-positive cells on each BrdU labeling day. **P<0.01, Student’s t-test. Data are mean±s.d.

Fig. 5. Birth-date analysis of loss- and gain-of-function of Sox11. (A-F) For BrdU labeling to Sox11-KO embryo, pulse labeling of BrdU was conducted by injection of BrdU into pregnant females intraperitoneally at timing indicated in A, and embryos were sacrificed at E18. Eyes were frozen sectioned and double stained with anti-BrdU and markers (Brn3b or RXRγ). Marker and BrdU double-positive cells (C,D), BrdU-positive cells (E) and double-positive cells out of total BrdU-positive cells (F) are shown. (G-J) Birth-date analysis was carried out for gain-of-function Sox11 retina. At E14, retinas were isolated and transfected with Sox11-EGFP expression plasmid. Then retinas were cultured as explants, and BrdU was present every 24 hours, as indicated schematically in G. (H) Retinas were harvested after 2 weeks. The sections were triple immunostained with anti-EGFP, anti-BrdU and anti-RXRγ. (I) The percentage of RXRγ and EGFP double-positive cells in EGFP single-positive cells over a total of 6 days' labeling are shown. BrdU-positive and -negative populations in RXRγ/EGFP cells are shown in different colors. Percentage of RXRγ/EGFP and BrdU-positive cells in EGFP cells are calculated in each sample and a summary of the results is shown. (J) The percentage of triple-positive cells out of double-positive cells on each BrdU labeling day. **P<0.01, Student’s t-test. Data are mean±s.d.
immunostaining (Fig. 5H). We first confirmed that cones were produced in greater numbers when Sox11 was overexpressed, but the numbers were not as high as when Sox11 was introduced at E17 (Fig. 5I, blue empty bar; RXRγ–EGFP+/EGFP+). This may be explained by high endogenous expression of Sox11 and ongoing cone genesis in retina at E14, such that ectopic Sox11 expression might provide only a moderate boost. Triple staining with BrdU, RXRγ and EGFP showed that for all the BrdU-labeling periods examined, the number of labeled cone cells was higher than that of control (Fig. 5I), suggesting that Sox11 overexpression leads to cone genesis, even after the normal cone genesis. We surmise that the non-staining cone population may correspond to cones born during the initial 24 hours of culture. We first determined the RXRγ–EGFP+BrdU+/EGFP+ population in each labeled sample (five samples) and determined the sum of these values (Fig. 5I, green region). The value of the non-stained cell population (Fig. 5I, non-colored values), which represents RXRγ–EGFP+BrdU cells in the total EGFP+ cell population, was about 6% in both control and Sox11-overexpressing samples (Fig. 5I, non-colored values of blue bars). This suggests that at E14, cone genesis is comparable in control and Sox11-overexpressing samples. Cone genesis rapidly declines thereafter in controls, whereas it continues in Sox11-overexpressing samples. Therefore, with Sox11 overexpression, cone genesis began at the normal time but did not cease at the appropriate time, instead continuing through to a later stage, resulting in an excess number of cone cells.

**Overexpression of Sox11 or Sox4 did not induce expression of genes, with the exception of βIII-tubulin**

We next tested whether Sox11 and Sox4 induce Math5 (Atoh7 – Mouse Genome Informatics), Ngn2 (Neurog2 – Mouse Genome Informatics) and Math3 (Neurod4 – Mouse Genome Informatics) expression. As a positive control, we first examined βIII-tubulin, which is known to be a direct target of Sox11 and Sox4 (Bergsland et al., 2006; Dy et al., 2008). Sox11-IRES-EGFP or Sox4-IRES-EGFP was introduced into retinas at E16, and after 4 days of culture, βIII-tubulin and EGFP were immunostained (Fig. 6A). Sox11 and Sox4 each increased the number of βIII-tubulin-positive cells (Fig. 6A,B). After 2 days of culture, βIII-tubulin expression was examined by qPCR (Fig. 6C). As expected, βIII-tubulin mRNA expression was induced both by Sox11 and Sox4. Using the same samples, we tested whether Sox11 and Sox4 promote neurogenesis by examining the expression of genes related to neural fate. Expression of the proneural bHLH genes – Math5, Ngn2, Mash1 (Ascl1 – Mouse Genome Informatics), NeuroD (Neurod1 – Mouse Genome Informatics) and Math3, all of which are expressed by retinal precursor cells as intrinsic regulators of retinal cell fate decision (Akagi et al., 2004; Cepko, 1999) – was examined, the number of labeled cone cells was higher than that of control (Fig. 5I), suggesting that Sox11 overexpression leads to cone genesis, even after the normal cone genesis. We surmise that the non-staining cone population may correspond to cones born during the initial 24 hours of culture. We first determined the RXRγ–EGFP+BrdU+/EGFP+ population in each labeled sample (five samples) and determined the sum of these values (Fig. 5I, green region). The value of the non-stained cell population (Fig. 5I, non-colored values), which represents RXRγ–EGFP+BrdU cells in the total EGFP+ cell population, was about 6% in both control and Sox11-overexpressing samples (Fig. 5I, non-colored values of blue bars). This suggests that at E14, cone genesis is comparable in control and Sox11-overexpressing samples. Cone genesis rapidly declines thereafter in controls, whereas it continues in Sox11-overexpressing samples. Therefore, with Sox11 overexpression, cone genesis began at the normal time but did not cease at the appropriate time, instead continuing through to a later stage, resulting in an excess number of cone cells.

**Global transciptional effects of loss of Sox11 function**

To delineate the molecular mechanisms underlying the retinal action of Sox11, we performed microarray analysis of E18 retinas from wild-type and Sox11-KO mice. Genes with a greater than halving of expression are listed in supplementary material Table S5A; those with a greater than twofold increase in expression are given in supplementary material Table S5B. Expression of S-antigen (arrestin; Mm.1276), PNR (Mm.103641), and Nrl (Mm.20422) decreased by more than half; in Sox11-KO retinas, suggesting that differentiation of rod photoreceptors was delayed in Sox11-KO retinas. Expression
of Math5 (Mm.228661), Dlx1 (Mm.475101) and Dlx2 (Mm.3896), important genes in the differentiation of ganglion cells (de Melo et al., 2005; Wang et al., 2001; Yang et al., 2003), increased more than twofold in the Sox11-KO retina, showing that a delay in ganglion cell differentiation had occurred by the onset of Sox4 expression, and that related genes were expressed at E18, a stage by which they had already been shut off in normal retinas (data not shown).

**Knockdown of Sox4 in Sox11-KO retina prevented the recovery of gene expression**

The onset of βIII-tubulin protein expression was delayed in the Sox11-KO retina (Fig. 3A,C). The expression of βIII-tubulin mRNA in the Sox11-KO retina was much lower at E12 and E14. At E18, the level was comparable with that in controls (Fig. 6A), suggesting that βIII-tubulin expression is regulated by Sox11 in the early developmental stage. The proneural bHLH genes Ngn2, Math3, Mash1 and NeuroD were also expressed at lower levels in the Sox11-KO retina (Fig. 7A). At later stages, the expression of βIII-tubulin and proneural genes was restored in the Sox11-KO retina (Fig. 7A). We surmised that this recovery may have been caused by the onset of Sox4 expression. To test this possibility, we introduced a Sox4-specific shRNA (sh-Sox4) into E16 Sox11-KO retinas by electroporation and culturing the retinas for 2 days as explants. The expression of βIII-tubulin and proneural genes was reduced in sh-Sox4-treated retinas (Fig. 7B), supporting the notion that Sox4 participates in the restoration of βIII-tubulin expression.

As retina from Sox11-KO mice showed anterior segment dysgenesis (Wurm et al., 2008), it is possible that the retinal defects described in our work are secondary. We therefore expressed sh-Sox11 in normal retina, and examined its effects on gene expression by qPCR (Fig. 7C). We first confirmed suppressive effects of sh-Sox11 in retina (supplementary material Fig. S4A). We found that expression of proneural genes was reduced in the presence of sh-Sox11, but found no effect for sh-Sox4, probably because Sox4 is not significantly expressed at around the E14 stage in retina. In accordance with this observation, when we extended culture of sh-Sox11-expressing retina to 7 days, repression of the genes was not observed (data not shown) probably owing to expression of Sox4 in later stage retinal development.

Based on our finding that Sox11 expression did not induce significant gene expression, we speculated that Sox11 may help to establish competency to allow differentiation of neurons. To
examine this hypothesis, we next assessed the histone modification status in promoters of selected genes. We performed chromatin immunoprecipitation (ChIP) analysis of acetyl histone H3 (AcH3) and histone H3K4 tri-methylation (H3K4me3), both of which positively regulate transcription, using E16 retinas from Sox11-KO mice and littermates. AcH3 levels for Math5, Ngn2, Math3 and Mash1 promoters were lower in Sox11-KO (Fig. 7D). The values for H3K4me3 were comparable in Sox11-KO and control retinas (Fig. 7E), except for the NeuroD promoter, which showed a higher value in Sox11-KO retinas. We further analyzed whether the epigenetic status of genes identified in the DNA microarray analysis was modified in Sox11-KO retina. We examined Nrl, Ntind, Fbxo2, Nrze3, Bcan, Adil1, Sag, Tcfap2 and Camk2b. However, significant, reproducible differences were not observed, except for Fbxo and Adil1. In the Sox11-KO retina, acetylated histone AcH3 levels in the Fbxo locus were reduced (Fig. 7D), whereas Adil1 H3K4me3 levels increased (Fig. 7E). Finally, we examined whether overexpression of Sox11 or Sox4 affects epigenetic modification of these genes. Using EGFP to monitor transformation, we overexpressed Sox11 or Sox4 in E15 retinas, and after 2 days of culture, EGFP-positive cells were purified using a cell sorter, and ChIP analysis was conducted for AcH3 and H3K4me3 modifications in the Ngn2, Math5, Math3, NeuroD and Mash1 loci. We observed no significant changes in AcH3 and H3K4me3 levels at the Ngn2 and Math3 loci. For NeuroD and Mash1, the levels of both AcH3 and H3K4me3 were downregulated by Sox11 (Supplementary material Fig. S4B). With Sox4, AcH3 was downregulated in the NeuroD locus. As the level of AcH3 for NeuroD was increased in Sox11-KO (Fig. 7D), we inferred a role for Sox11 in the AcH3 modification of NeuroD and continued to work to uncover the mechanisms.

DISCUSSION

We found that Sox11 and Sox4 have unique patterns of expression during retinal development, and that perturbation of the expression pattern of either Sox11 or Sox4 disrupts differentiation of subsets of retinal cells without affecting the proliferative activity of retinal progenitor cells. Therefore, the altered differentiation is probably caused by the direct effects of Sox11 and Sox4 on retinal cell fate decision, rather than secondary effects resulting from perturbation of the timing of cell cycle exit. In adult mouse hippocampus, Sox4/Sox11 ablation decreased the generation of cells expressing neuron-specific proteins, without significant alterations in proliferation (Mu et al., 2012). However, evidence showing involvement of Sox11 in mantle cell lymphoma had been accumulated (Sander, 2011), and more recently, involvement of Sox4 in lung cancers was reported (Castillo et al., 2012), thus suggesting that Sox11 and Sox4 differently involve proliferation in CNS and other tissues.

Sox11 and Sox4 have remarkable identity in the HMG box DNA-binding and the C-terminal transactivation domains, and their conserved molecular properties have been demonstrated previously (Dy et al., 2008). Our results demonstrate that Sox11 and Sox4 had nearly identical effects on retinal progenitor cell behavior when they were overexpressed. Therefore, phenotypic recovery of the Sox11-KO retina at or after birth may be explained by the compensatory effects of Sox4, which starts to be expressed at around E18.

As Sox11 is known to be a transcription activator, we tried to identify targets of Sox11 in the retina. BIII-tubulin is the only one whose expression was shown to be induced by ectopic expression of Sox11 or Sox4 in the retina. Initiation of BIII-tubulin and Ngn2 expression coincide in the mouse retina, and no differentiating retinal neurons are present prior to the onset of Ngn2 and BIII-tubulin expression (Hufnagel et al., 2010). Therefore, BIII-tubulin is a marker for the beginning of neurogenesis in the retina; however, as far as we know, no report has suggested that BIII tubulin acts as a master regulator to initiate neurogenesis. Therefore, the BIII-tubulin yet not give retinal progenitors the cue to start differentiating. Recently, FezF2 and BDNF were found as targets of Sox11 in cortex and dorsal root ganglia, respectively (Salerno et al., 2012; Shim et al., 2012). Our microarray data of Sox11-KO retina showed rather elevated expression of FezF2 and BDNF (data not shown), suggesting cell type-specific activation of genes by Sox11.

Comparative microarray analysis of Sox11-KO and wild-type retinas revealed global transcriptional effects as a consequence of the loss of Sox11 function. However, overexpression experiments identified only BIII-tubulin as a possible target gene of Sox11 and Sox4. Therefore, we surmise that Sox11 may help cells to acquire competence for stage-specific differentiation, rather than directly activating the transcription of genes that are crucial to retinal differentiation. Our finding that the histone acetylation of several genes was altered in the Sox11-KO retina supports this hypothesis. Based on these results alone, we cannot attribute the gain-of-function phenotype to changes in the epigenetic statuses of the genes. We surmise that a set of genes is epigenetically regulated by Sox11, but why they are selectively regulated remains to be clarified. A recent paper (Bergsland et al., 2011) showed sequential roles of Sox3 and Sox11 in neural lineage development, and a model that bivalent histone modifications of specific genes had been established by sequential Sox protein bindings in target genes. We are currently attempting to determine the molecular mechanisms by which Sox11 and Sox4 regulate epigenetic status in the retina.

Strong suppression of differentiation of Müller glia by Sox11 and Sox4 was observed. Notch signaling is known to promote differentiation in the Müller glia lineage in the retina (Furukawa et al., 1997; Hojo et al., 2000), but our results suggest that Sox11 and Sox4 may not directly suppress Notch. Instead, they may suppress the expression of genes that play roles important in glia differentiation. Recently, the results of comprehensive analysis of expression patterns in purified Müller glia lineage cells from P0 to P21 were reported (Nelson et al., 2011). We chose several genes from the microarray result and examined their expression in Sox11- or Sox4-overexpressing retinal cells by qPCR and in the Sox11-KO retina using microarrays. We identified several genes that are up- or downregulated in the Sox11-KO retina; but, in the Sox4-overexpressing cells, only Bmpr1a expression was found to be enhanced (data not shown). The roles of BMP signals in mammalian Müller glia differentiation have been reported in chick (Huillard et al., 2005), and we are currently clarifying the participation of BMP signaling in Müller glia differentiation in conjunction with Sox11.

The forced activation of Notch signaling has been shown to suppress cone photoreceptor fate specification (Jadhav et al., 2006; Riesenberg et al., 2009; Yaron et al., 2006). However, as discussed above, direct suppression of Notch signaling by Sox11 or Sox4 is unlikely. We examined whether Sox11 and Sox4 induce the expression of cone- and photoreceptor-lineage related genes such as Nrl, Otx2, and TRP2 (Thrb2 – Mouse Genome Informatics), but no induction in the retina was observed at E17 when Sox11 or Sox4 was overexpressed (data not shown). Therefore, analysis of epigenetic regulation seems promising as an approach to learning about the mechanisms of cone induction, and further studies are currently under way.
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Competing interests statement
The authors declare no competing financial interests.

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References


