Role of HMG-transcription factor Sox2 in the mouse retinal development

Sox2 転写因子のマウス網膜発生過程の役割解析

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Abstract

Sox2, a transcription factor, plays important roles in both human and mouse retinal development. Several studies show that loss-of-function mutations in Sox2 have been studied in mice; however, gain-of-function experiments in the neural retina have been found still lacking. In my study, the gain-of-function experiment was applied, and the detailed expression pattern of Sox2 in the developing mouse retina was examined by immunohistochemistry. The result indicated Sox2 was expressed throughout the neuroblastic layer in the embryonic retina, but only in the inner nuclear layer in the mature retina. In addition, double immunostaining revealed that Sox2 was expressed in Müller glial cells and in a subset of amacrine cells. Then, in order to examine its role in retinal development, Sox2 was expressed in a retinal explant culture prepared from E17 mouse embryos by retrovirus-mediated gene transfer. Forced expression of Sox2 in a mouse retinal explant culture resulted in the dramatic accumulation of amacrine cells in the inner nuclear layer; in addition, cells expressing amacrine cell markers were also found on the innermost side of the outer nuclear layer. The expression of Pax6, which plays an important role in amacrine cell differentiation, was observed in Sox2-expressing cells. Using luciferase analysis, I found that Sox2 activated the Pax6 promoter to drive luciferase expression in Y79 cells. A decrease in retinal progenitor cell proliferation accompanied these effects. Lastly, shRNA was used to suppress Sox2 in a retinal explant using a retrovirus-mediated system. The suppression of Sox2 expression resulted in a decreased number of cells in the inner nuclear layer. Therefore, I concluded ectopic Sox2 expression can induce amacrine cells in the mouse retina from stage E17 onward, possibly by facilitating cell cycle exit.

Introduction

The vertebrate central nervous system (CNS) consists of a complex variety of neuronal cell types. This diversity of neurons is produced from the multipotent progenitor cells in an orderly manner during neural development (ref. 1); however, the mechanisms involved in the generation of different cell types had remained largely unknown at the molecular level. Until recently, studies on how neural progenitor cells develop into different neuronal cell types have became noted. Especially the neural retina, a part of the central nervous system, which can be used as an ideal model system for studying the mechanisms of the generation of different cell types from common progenitor cells, because it has a comparatively simple structure and can mimic normal retinal development in isolated explant culture system (ref. 2, 3, 4, 5, 6).

Retinal development starts with specification of the eye primordia during early stages of embryogenesis. In vertebrates, the eye development arises from three embryonic parts: the optic vesicle (OV), which is a protrusion of the diencephalic neuroepithelium of the neural tube, the surrounding mesenchyme and the overlying surface ectoderm (SE). The eye development is coordinated by the successive signals between these tissue components (ref. 7). At embryonic day 8.5, a lateral evagination extending from the wall of the diencephalon will develop into the optic vesicle. The optic vesicle contacts the surface ectoderm and induces the signals to surface ectoderm that leads to the thickening of surface ectoderm and the forming of lens placode (LP) which develops into the mature lens. The optic vesicle folds inwards and forms a bilayered cup, the optic cup. In the cup the inner layer develops into the neuroretina (NR) and the outer layer becomes the retinal pigmented epithelium (RPE) (Fig. 1) (ref. 7, 8, 9, 10).

The vertebrate neural retina consists of six major neuronal cell types (cone photoreceptors, rod photoreceptors, horizontal cells, bipolar cells, amacrine cells and ganglion cells) and one type of glia (Müller cells) that produce from a common population of undifferentiated multipotent retinal progenitor cells (RPCs). During retinogenesis, these different cell types are generated in a

conserved birth order from the multipotent retinal progenitor cells (RPCs) residing in the inner layer of the optic cup (ref. 11, 12). These functionally different cells form three cellular (nuclear) layers and two synaptic (plexiform) layers in the mature retina. The outer nuclear layer (ONL) contains rod and cone photoreceptor cells; the inner nuclear layer (INL) contains bipolar, horizontal and amacrine cells and Müller glia cells. The ganglion cell layer (GCL), which is located in most basal side, contains ganglion cells and displaced amacrine cells (Fig. 2B). The synaptic connections of these neuronal cells are localized in the outer plexiform layer (OPL) and the inner plaxiform layer (IPL) (ref. 9, 13).

In mice, retinal cell differentiation is initiated in the inner layer of the central optic cup, and progenitors start to differentiate at embryonic day (E) 10.5. Retinal ganglion cells and horizontal cells are generated first, followed in overlapping phase by cone photoreceptor cells, amacrine cells, and rod photoreceptor cells, while bipolar cells and Müller gilal cells appear last (ref. 8, 11, 13); however, the retinogenesis in mice is completed at approximately postnatal day 10 (P10) (Fig. 2A).

Like many other central nervous system structures, the retina has a diversity of neuronal types. Mammalian retinas contain about fifty-five distinct types of neurons; however, each of these cell types has a different physiological function. Each major retinal cell consists of multiple cell types distinguished by morphology, connectivity and light response properties (ref. 6, 14). The retinal image process is composed of five major kinds of neurons within a layered structure (Fig. 2B). Rod and cone photoreceptor cells convert light to chemical and electrical signals that are relayed to interneurons in the outer retina. Bipolar cells combine and convey photoreceptor signals to retinal ganglion cells (RGCs) and amacrine cells. Horizontal cells perform lateral processing by interacting with bipolar and photoreceptor cells. Amacrine cells can modulate signals between the bipolar and ganglion cells. Finally, light signals leaves the retina through axons of the optic nerve to the brain (ref. 14, 15). However, there are many specialized subcircuits in the vertebrate retina, which work together in parallel to perform different properties of the image. The rod photoreceptor cells are sensitive to low light levels and a rod-driven circuit pathway mediates the night vision. In

vertebrates, the rod-driven pathway implicates connections between rod photoreceptor cells, rod bipolar cells and the AII amacrine cells. Cone photoreceptor cells contact a diversity of cone bipolar cells, some of which are depolarized (ON) and others are hyperpolarized (OFF) by increased illumination. ON- and OFF- cone bipolar cells contact retinal ganglion cells, which response to change in illumination according to their bipolar cells input. All rod and cone photoreceptor cells receive feedback from horizontal cells; however, the number of these cells are generally less than 5% of cells of the inner nuclear layer (ref. 6). The general function of horizontal cells is to feed back to photoreceptor cells or bipolar cells. Amacrine cells are important interneurons existent in the INL and GCL that modulate the synaptic connection between bipolar and ganglion cells (ref. 6, 16, 17). Some of them are located in the GCL (displaced amacrine cells) and others are in the inner region of INL. They have different morphological and functional subtypes. In mammals, amacrine cells can be further classified into twenty-nine different amacrine subtypes, such as sub-laminar localization (the inner plaxiform layer, the ganglion cell layer and the inner part of the inner nuclear layer), morphology (starburst, parasol or midget) and neurotransmitter type (GABAergic, glycinergic, dopaminergic or serotoninergic) (ref. 17).

Amacrine cells can be divided into two major nonoverlapping subpopulations classified by neurotransmitter production. In mouse retina, GABAergic amacrine cells comprise ~35% and glycerinergic amacrine cells comprise ~40% of all amacrine cells (ref. 18, 19). During mouse retinogenesis, amacrine cells are born early, starting from embryonic day (E) 11 to postnatal day (P) 4 (ref. 8). The retinal ganglion cell is the only retinal neuron that projects and conveys visual information to the brain. However, the only one type of glial cells (Müller cells), which span the depth of the retina, provide important structural and functional support for the retinal neuron (ref. 14).

Retinal progenitor cells are multipotent and can give rise to different cell types. The development process of retinal progenitor cells into the mature retina includes several stages: cell division, exiting the cell cycle, taking on a specific cell fate and performing the differentiation

program for this cell type (ref. 20). Recent misexpression and loss of function studies have indicated that cell-extrinsic signals, such as transcriptional factors, and cell-extrinsic signals, such as neurotrophic factors, play important roles in progenitor cell fate determination and their subsequent differentiation during retinogenesis. Intrinsic and extrinsic factors, such as EGFs, FGFs, shh, retinoic acid, and Notch/Delta singnaling molecules, are involved in the determination and affect the cell fate of progenitors (ref. 21, 22, 23, 24, 25). In addition to the signaling factors, many transcription factors have been found to serve as intrinsic factors and are involved in cell fate identity, such as the basic-helix-loop-helix (bHLH) factors Mash1, Math3, Math5, NeuroD, Hes1 (ref. 26, 27, 28, 29, 30, 31) and homeobox-type transcription factors Pax6, Chx10, Crx and Prox1 (ref. 11, 27, 32, 33, 34). These combinations of bHLH and homebox genes may be important for determination of cell types. For example, the bHLH gene NeuroD and the homebox gene Crx regulate generation of photoreceptor cells (ref. 34, 35) and the bHLH gene Mash1 or Math3 and the homebox gene Chx10 regulate the specification of bipolar cells (ref. 30, 32, 36). Co-expression of the bHLH genes Math3 or NeuroD with the homebox gene Pax6 promotes amacrine cell genesis (ref. 28).

Although many transcriptional factors have been identified as important regulators in mammalian retinal development, the mechanisms that regulate the differentiation of specific neuron types still need to be defined.

The HMG-box transcription factor Sox2 is a B1-subgroup Sox gene family member and a common marker for multipotential neural stem cells and progenitor cells in the central nervous system (CNS) including the neural retina (ref. 37, 38). In the CNS, Sox2 is expressed in proliferating progenitors; its expression is downregulated as the progenitors exit the cell cycle. In accordance with this expression pattern, constitutive Sox2 expression inhibits neuronal differentiation, resulting in the maintenance of progenitor characteristics (ref. 39). The involvement of Sox2 in eye development has been studied in several animal models. During chick eye development, Sox2 is initially expressed in the anterior neural plate and optic vesicle. Its

expression is subsequently restricted to the progenitor cells of the neural retina and then to amacrine cells (ref. 40, 41). The inhibition of Sox2 signaling by the injection of Sox2 antisense morpholinos into *Xenopus* embryos results in reduced or missing eyes (ref. 42). In Sox2 mutant mice, the precise regulation of Sox2 expression is critical for the temporal and spatial regulation of retinal progenitor cell differentiation (ref. 43). In Sox2 hypomorphic/null mice, a reduction in Sox2 expression causes microphthalmia (small eye) as a result of aberrant neural progenitor differentiation and reduced proliferation caused by a lack of Notch1 activation (ref. 43). In accordance with this observation, mutations in Sox2 in humans are associated with retinal and ocular malformations, such as anophthalmia (absent eye) and severe microphthalmia (ref. 44). Other studies have revealed that Sox2 deficiency causes neurodegeneration and impaired neurogenesis in adult mice (ref. 45). However, the molecular mechanisms by which Sox2 participates in eye development are still poorly understood. I have used a gain-of-function analysis and found that Sox2 promotes amacrine cell differentiation in a retinal explant culture prepared from an E17 mouse embryo. The induction of Pax6 by Sox2 was also observed. However, proliferation of retinal progenitor cells was decreased by ectopic Sox2 expression, suggesting that during early differentiation of the retina, Sox2 may promote amacrine cell differentiation partly through the induction of Pax6 and by facilitating cell cycle exit of retinal progenitor cells.

Materials and Methods

Isolation of Retina from Mice

ICR mice were obtained from Japan SLC Co. and Japan Clea Co. The day that a vaginal plug was observed was considered to be embryonic day 0 (E0), and the day of birth was marked as postnatal day 0 (P0). All animal experiments were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Analysis of transcripts using reverse transcription (RT)-PCR

Total RNAs were prepared from mouse retinal stem cells and mouse different developmental stages of retinas (TRIzol reagent; Invitrogen-Gibco, Carlsbad, CA). The cDNA were synthesized from total RNA using reverse transcriptase Superscrip II (Invitrogen-Gibco, Carlsbad, CA). The cDNA were then subjected to PCR amplicification using ExTaq polymerase (TaKaRa) and sox2 specific primers which were designed as previously described (ref. 60). The primers for sox2 were: the forward 5'-ACCTACAGCATGTCCTACTCG-3' and the reverse 5'-CTTCTCCAGTTCGC AGTCCA-3'. The condition of PCR was as follow: 94℃ for 30 seconds, 50℃ for 30 seconds, and 72℃ for 30 seconds for 35 cycles.

The primer sets were tested over a range of thermal cycles using rTaq (Takara, Shiga, Japan), and the semiquantitative cycle number was determined for each primer set. Bands were visualized with ethidium bromide.

Plasmids Construction

The mouse Sox2 gene was kindly provided by Hisato Kondoh (University of Osaka, Japan) and subcloned into *Eco*RI/*Xho*I sites of pMX-IRES-EGFP retrovirus vector (internal ribosomal entry site-enhanced green fluorescent protein). The s-opsin promoter region (-523 to first ATG in the

exon)(ref. 46) was amplified by PCR, and inserted into *Hind*III and NcoI sites of pGL3basic vector (Promega, Madison, WI). Primer sequences were as follows; 5'-AAGCTTGGCAGGATGCAGTT GTTTCT-3' and 5'-CCATGGCCCGCTTGGGATGCCCTCACTA-3'. The Pax6α-enhancer-P0 promoter was amplified from the genomic DNA of Pax6 α-cre transgenic mice (ref. 11) by PCR. Next, an *Nco*I site was created at the translation initiation site, ATG, of the Pax6 P0 promoter by PCR mutagenesis. The resultant fragment was subcloned into the *Nco*I site of pGL3-Basic (Promega). Primer sequences used were as follows: Pax6 α enhancer-5': 5'-TCAAGCT ACCCTG AAAACGCA-3', Cre-3': 5'-CCTGTTTTGCACG TTCAC-3', Pax6 P0 (ATG-*Nco*I)-3': 5'-TTCT TGGCCATGGTCGACCT-3'. Oligonucleotide-directed mutations were introduced in the conserved Sox2 binding motif of the Pax6 α -enhancer element using PCR mutagenesis. The primer sequences used were as follows: Pax6 α -enhancer Sox2mt 5': 5'-GCACTGTCCTGCAGTGACA AGGC-3'; Pax6a-enhancer Sox2mt 3': 5'-GCCTTGTCACTGCAGGA CAGTGC-3'.

The mutated Pax6 α -enhancer-P0 promoter fragment was subcloned into the *Nco*I site of the pGL3-Basic luciferase reporter vector (Promega).

Retrovirus production

Transient retrovirus packing cell line PLAT-E (ref. 47) was transfected with retrovirus vectors containing various genes by using FuGene 6 transfection reagents (Roche) according to the manufacturer'fs instructions. Two days after transfection, cell supernatants containing retrovirus were harvested and concentrated by centrifugation in a centrifugal filter device (Millipore). Two days from the initiation of cultures, the retinal explants were exposd to the virus solution, and then the cells were washed with medium. Explants were harvested at day 14, and frozen sections were prepared and immunostained with appropriate antibodies to examine the expression of marker proteins.

Retinal Explants and Retrovirus Infection

Retinal explant cultures were prepared as previously described (ref. 48). Briefly, the neural retina of E17.5 ICR mice (Japan, SLC Co.) without pigmented epithelium was isolated on a chamber filter (Millicell; Nihon-Millipore, Tokyo, Japan; Diameter 30 mm, pore size 0.4 mm) and placed with the ganglion cell layer face-up (fig.3). The filters were inserted into six-well plates and cultured in 1 mL of explant culture medium (50 % MEN with Hepes, 25 % Hank'fs solution, 25 % heat-inactivated HS, 200 mM L-glutamine, and 5.75 mg/ml glucose, 100 U/ml penicillin and 100 U/ml streptomycin). Explants were cultured at 34 ℃ in 5% CO2, and the medium was changed every other day. Then the cells were harvested and fixed with 4% PFA and immunostained with appropriate antibodies.

Immunohistochemical Analysis

Immunohistochemistry of retinal explants were carried out as described previously (ref. 48). Briefly, retinal explants were fixed with 4 % paraformaldehyde (PFA) and soaked in 30 % sucrose. For immunostaining of normal mouse retinal sections, embryos were collected at various developmental stages and eye cups were picked up from embryos. Following fixation in 4 % PFA and soaked in 30 % sucrose. Then, the samples were frozen-sectioned (10 μm thick) in OCT compound (Miles) and pre-incubated in a blocking solution (2 % BSA in PBS solution). The primary antibodies used are anti-GFP (BD-Clontech Laboratories, Palo Alto, CA), anti-HuC/HuD neuronal protein (Invitrogen-Molecular Probes, Eugene, OR), anti-islet1 (Developmental Studies Hybridoma Bank, Iowa City, IA), anti-glutamine synthetase (GS, Chemicon Int., Temecula, CA), anti-protein kinase C (PKC; Oncogene Research Products, San Diego, CA), anti-calbindin-D-28K (Chemicon Int.), anti-Pax6 (Convance), anti-Pax6 (Developmental Studies Hybridoma bank), anti-ki67 (BD Bioscience, San Diego, CA), anti-Sox2 (Abcam, Cambridge, MA), anti-choline acetyltransferase (ChAT; Chemicon Int.), and anti-calretinin (Chemicon Int.) antibodies. The primary antibodies were visualized by appropriate second antibodies conjugated with Alexa Fluor 488 or 546 (Molecular Probes). All samples were sealed with mounting medium (VectaShield;

Vector Laboratories, Burlingame, CA) containing DAPI (4`, 6-diamidino-2-phenylindole dihydrochloride for nuclear staining), and analyzed (Axioplan microscope; Carl Zeiss Meditec, Inc. or a fluorescence dissection microscope MZFL III; Leica, Bannockburn, IL). Images were processed by using Adobe Photoshop (Adobe Systems).

To evaluate the staining patterns of Sox2 and the other markers, we counted more than 200 cells from multiple pictures taken during independent experiments.

RNAi Design and Construction of Plasmids for shRNA Synthesis

Target sequence for RNA interference of Sox2 was as described previously (ref. 49, 59). For shRNA expression, a double-stranded oligonucleotide covering shRNA sequences was first subcloned into the *Bbs*I and *Eco*RI sites of a pU6 vector (ref. 50) and then the U6 promoter-shRNA cassettes were further subcloned into the *Bam*HI and *Eco*RI sites of pSSCG vector (Fig. 4). The efficiency of shRNA was examined by Western blot analysis in 3T3 cells expressing Sox2. The target sequence used for RNAi of Sox2 was as follows: 5'-GGAGCACCCGGATTATAAATA-3'. The oligonucleotides used for shRNA synthesis were as follows : 5'-TTTGGAGTACCTGGA TTGTAAATAAATATTTATAATCCGGGTGCTCCTTTTTG-3' and 5'-AATTCAAAAAGGCA CCCGGATTATAAATATTTTCTATTTA TAATC CGGGTGCTCC-3'.

Oligonucleotides were cloned into pU6 cassette vector (BbsI and EcoRI sites), then subcloned into pSSCG vector, which expresses 21-nucleotide hairpin-type short hairpin RNAs (shRNA) with a 5-nucleotide loop. All sequences were analyzed by SiDirect search to ensure that they did not have significant sequence similarity with other genes.

Western blot analysis

The NIH3T3 cells were plated at 6cm plate (5 X 10^5 cells/plate). After 24 hours cultured, 2 μg Sox2 constructs and co-transfected with pU6 control plasmid and pU6-Sox2-siRNA plasmidby lipofection using FuGENE6 (Roche). After 2 days, the transfected cells were harvested and lysed

in 200 μl of lysis buffer (1 % Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA). For Western blotting of total cell lysates, picked up 3 μl and mixed with a buffer containing 50 mM Tris-HCl, pH 6.8, 2 % sodium dodecyl sulfate (SDS), 10 % glycerol, 1 % 2-mercaptothanol, and 5 μg/ml of bromophenol blue, and boiled before loading onto SDS-polyacrylamide gel. The samples were separated by electrophoresis through a polyacrylamide gel containing 10% SDS-PAGE, then electrophoretically transferred to immobilon polyvinylidene difluoride membrane (Millipore) in a tank transfer system using transfer buffer (25 mM Tris, 14.4 % glycine, 20% methanol). The membrane was blocked in TBST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05 % Tween-20) containing 4 % BSA for 1hr at room temperature and then incubated with and the primary antibody rabbit polyclonal anti-Sox2 antibody. After washed by TBST, the blots were incubated with the secondary antibody horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibodt (Amersham, Buckinghamshire, 10000-fold dilution). The immunoreactive bands were visualized by the Lumi-Light Western Blotting system (Roche) according to the manufacturer'fs instructions.

Luciferase analysis in Y79 cells

Y79 (Riken Cell Bank, Japan) cells were maintained in RPMI1640, 10% FCS. The Y79 cells were plated at 24-wall plates (1 X 10^5 cells/wall), 0.5 μg pGL3-Pax6 and pGL3-Opsin promoter constructs were cotransfected with 0.5μg pBS (control) or pCMV-Sox2 plasmids (GeneJuice Transfection Reagent; Novagen, San Diego, CA). After 25 hours' culture, the transfected cells were harvested and lysed in 10μL luciferase assay lysis buffer. Protein concentration was determined by BCA protein assay (Pierce, Rockford IL). Luciferase activity was measured with a luciferase assay substrate (Promega, Madison, WI) and detected by luminometer (model LB 9501; Berthold Lumat Co. Ltd., Japan). Relative light units (RLU) were normalized to protein concentration.

Results

Expression of Sox2 during retinal development

Previously, Sox2 expression was examined in mice in which the coding region of Sox2 was replaced with EGFP (ref.43); however, no detailed examination was made of the endogenous Sox2 gene or protein. Thus, I first examined endogenous Sox2 in retinas of wild-type mice with respect to both mRNA transcription and protein expression. Specifically, changes in the mRNA expression of Sox2 over time in the mouse neural retina were examined at various developmental stages using semiquantitative RT-PCR (Fig. 5A). Sox2 mRNA was expressed at E16 and after birth; thereafter, its expression gradually decreased, but persisted until adulthood. I next examined the spatiotemporal expression of Sox2 at various developmental stages by immunostaining of frozen retinal sections of wild-type mice. At E17, ganglion cells had started to form the innermost layer, whereas most other areas of the retina were occupied by immature progenitor cells collectively known as the neuroblastic layer (NBL). Sox2 expression was observed in the NBL, except in the outermost region (Fig. 5B, arrowheads) at E17. At P5, Sox2 expression was dramatically downregulated in the NBL and was observed in the ganglion cell layer (GCL) and on the innermost side of the NBL, corresponding to the inner nuclear layer (INL), which had just begun to form (Fig. 5B), suggesting that Sox2 is expressed in differentiating cells. Approximately 2 weeks after birth (Fig. 5B, P15), Sox2 expression was restricted to the GCL and INL. In the adult retina, Sox2 expression was clearly observed in the INL, which consisted of horizontal, amacrine, bipolar, and Muller glia cells, and was weakly detected in the GCL, which consisted of retinal ganglion cells and displaced amacrine cells. This pattern of expression is quite similar to that reported for the Sox2 promoter- EGFP mouse (ref. 43).

I next examined the retinal subtypes of Sox2-expressing cells by examining the expression of various retinal cell−specific markers. Sox2 was expressed in Müller glial and amacrine cells based

on the coexpression of GS and HuC/HuD (Fig. 6). While most of the GS-positive cells also expressed Sox2, less than 30% of the HuC/HuD-positive amacrine cells expressed Sox2. These results suggest that not all amacrine cells express Sox2.

Amacrine cells can be classified into several subtypes, and so we examined the expression of amacrine cell subtype markers. In mouse retina, GABAergic neurons compose approximately 40% of amacrine cells (ref. 18). Calretinin was localized in some GABAergic neurons, and nearly all the Calretinin-positive cells coexpressed Sox2 (Fig. 6). Many GABAergic amacrine cells in the mouse retina contain other neurotransmitters such as acetylcholine and dopamine in addition to GABA. Islet1 and choline acetyltransferase (ChAT) are markers of cholinergic amacrine cells. Approximately 80% of Islet-1-positive amacrine cells coexpressed Sox2, and all of the ChAT-positive cells also expressed Sox2 (Fig. 6). These results suggested that Sox2 could also be a marker for cholinergic amacrine cells. The Sox2 and Islet-1 double-positive cells in the GCL were assumed to be displaced amacrine cells (Fig. 6). Although I could not obtain clear antibody staining against another amacrine cell subset marker that localizes to 30% of all amacrine cells (ref. 18), all the Sox2-positive cells in the row of amacrine cell nuclei of the developing retina expressed GABAergic neuron markers. Therefore, I assume that Sox2 is a marker for this subset of amacrine cells.

Effect of Forced Sox2 Expression on Proliferation in E17.5 Retinal Explant Cultures

I next examined whether Sox2 overexpression affects retinal development using *in vitro* retinal explant cultures. Retroviruses encoding Sox2-IRES-EGFP or IRES-EGFP (control) were used to infect retinal explant cultures prepared from an E17.5 mouse eye (Fig. 7A). Because the retrovirus infects only mitotic cells, retinal progenitor cells are assumed to be the major target for gene transfer. Explanted retinal cells differentiate in culture as during *in vivo* development; thus, the localization and morphology of the virus-infected cells can be determined by monitoring EGFP

expression. After two weeks of culture, uninfected cells and cells infected with Sox2 and control viruses were immunostained with anti-Sox2 antibody to compare their levels of Sox2 expression. Sox2 virus−infected cells were identified by EGFP signals, and these cells (Fig. 7B, arrows) exhibited a higher level of Sox2 protein than did noninfected cells (Fig. 7B, bottom) and control virus-infected cells, which showed no detectable Sox2 protein expression (Fig. 7B, top. The red signal was intensified to examine expression in EGFP-positive cells, and this intensity was not comparable to, and cannot be compared with, the Sox2-overexpressed samples that appear in the bottom panels). I then examined the distribution of infected cells in the retinal sublayers of the explants by immunostaining a frozen, sectioned explant with anti-GFP antibodies (Fig. 7C). In the control explant, most of the EGFP-positive cells were localized in the outer nuclear layer (ONL), as previously observed (ref. 51); however, when Sox2 was expressed, several EGFP-positive cells were observed in the INL (Fig. 7C). Furthermore, the EGFP-positive cells in the ONL were localized on the innermost side (Fig. 7C). The quantification of the distribution of EGFP-positive cells in the sublayers of the retina showed that Sox2 expression increased the population of retinal cells in the INL from < 20% in the control to nearly 60% in the Sox2- expressing samples (Fig. 7D). In contrast, the population of EGFP-positive cells in the ONL decreased from 80% in the control to 40% in the Sox2-expressing cells (Fig. 7D).

Given the location of the amacrine cells within the inner half of the INL, I speculated that the forced expression of Sox2 may be inducing progenitor cell differentiation into amacrine cells. To confirm this possibility, double-immunostaining was conducted using anti-EGFP antibodies with anti-HuC/ HuD or anti-Pax6 antibodies (Figs. 8A, 8B), both of which label amacrine cells. The forced expression of Sox2 dramatically increased the number of HuC/HuD- and Pax6-positive cells from 10% in controls to 60% in Sox2-expressing cells (Fig. 10). When I examined markers for amacrine cell subtypes, the number of cells expressing GABA and/or calretinin, both of which are markers of GABAergic amacrine cells, was enhanced (Figs. 8C, 8D). However, the increase in the number of GABA and calretinin positive cells was less than that of HuC/HuD-positive cells (Fig.

10). This suggests that the remaining HuC/HuD positive/GABA-negative cells were glycinergic cells. Therefore, Sox2 is not specifically an enhancer for the differentiation of the GABAergic subtype of amacrine cells. Alternatively, these cells are amacrine cell precursors that have fail to differentiate.

I also examined the expression of various markers for retinal subtypes other than amacrine cells among Sox2-expressing cells. The number of GS-positive Müller glial cells was increased (Fig. 9A), whereas the number of rhodopsin-positive rods was decreased dramatically among Sox2-expressing cells (Fig. 10). The numbers of PKC-positive bipolar cells and calbindin-positive horizontal cells were unaffected by the expresion of Sox2 (Figs. 9B, 9C, 10). These data indicate that the forced expression of Sox2 in retinal explants promotes the differentiation of amacrine cells and Müller glial cells and suppresses that of photoreceptor cells. I also observed several unidentified cells at the border between the ONL and the outer plexiform layer (OPL) in retinal explants infected with Sox2-IRES-EGFP (Fig. 7B, arrows). These cells expressed HuC/HuD, but not rhodopsin, suggesting that they may have amacrine cell characteristics, but that they failed to migrate to the appropriate location for amacrine cells in the INL.

During proliferation, retinal progenitor cells are assumed to move vertically between apical and basal sides of the retina (ref. 52), and cells in the same column are thought to derive from a single common progenitor cell. When I examine cells in individual columns, the number of cells in the control EGFP sample is clearly greater than the number of cells observed in the Sox2/EGFP samples, suggesting that forced expression of Sox2 suppresses the proliferation of retinal progenitor cells. Therefore, I next asked whether Sox2 forces retinal progenitor cells to stop dividing, leading to their differentiation into amacrine cells. Thus, I examined the mitotic status of Sox2 expressing cells in retinal explants by analyzing the expression of Ki-67, a nuclear cell proliferation−associated antigen expressed during the active stages of the cell cycle (ref. 53). Explants prepared from the retinas of E17 embryos were harvested after 3 or 5 days of culture, and the frozen sections were stained with anti-Ki67 antibodies to analyze cell proliferation (Fig. 11, left). The proportion of

Ki67-positive proliferating cells was approximately 20% after 3 days of culture and decreased to approximately 10% after 5 days of culture in the control sample. In contrast, the number of proliferating cells in the Sox2- IRES-EGFP−infected population was half that in the control samples. In the samples that were cultured for 5 days, the number of Ki67-positive cells among the Sox2-expressing population was still lower than in the control. I tested our results by using a BrdU incorporation assay and obtained essentially the same data (Fig. 11, right). Thus, it is possible that Sox2 promotes amacrine cell differentiation by altering the exit of the cells from the cell cycle.

Sox2 Enhancement of Pax6 Enhancer Activity

Because I observed the expression of Pax6 in Sox2-expressing cells (Figs.10, 12A), I examined whether Pax6 induces Sox2. Pax6 was overexpressed in a retinal explant using a retrovirus, and the expression of Sox2 was examined by immunostaining. Sox2 expression was not induced by Pax6 (Fig. 12B). These results suggest that Sox2 acts upstream of Pax6. Thus, I asked whether this effect occurs via transcriptional activation or another mechanism. Using the retina-specific Pax6 enhancer region (Pax6α-enhancer-P0 promoter)(ref. 11) fused with a luciferase reporter gene, we examined the effects of Sox2 on luciferase activity in Y79 retinoblastoma cells. A mouse S-opsin promoter−luciferase construct was used as the control. Sox2 strongly activated Pax6-promoter luciferase activity in Y79 cells; in contrast, s-opsin-luciferase activity was suppressed by the expression of Sox2 (Fig. 14B). There is one putative Sox2 binding site in the Pax6-enhancer that is conserved between both human and mouse enhancers (Fig. 14A), and so we constructed a Pax6 promoter with a mutation in this putative Sox2 binding site and cloned it into the luciferase reporter construct. However, luciferase activity driven by this construct was only slightly less than that produced from the wild-type Pax6 enhancer construct (Fig. 14B). This result suggested that the putative conserved site may not be the only one; multiple binding sites could be involved in enhancing Pax6 transcription. I next examined whether the expression of Pax6 and Sox2 overlaps

during amacrine cell development by immunostaining of frozen sections. In retinal sections at E14.5, both Pax6 and Sox2 were strongly expressed. A relatively strong signal was observed in the outer and inner regions for Sox2 and Pax6, respectively (Fig. 13). Most of the Sox2- positive cells on the inner side were also Pax6-positive. Double staining for HuC/HuD and Pax6 or Sox2 revealed several positive cells in the inner half of the neuroblastic layer in both cases (Fig. 13). These results suggest that Sox2 and Pax6 are coexpressed at an early stage of amacrine cell development.

Effect of Inhibition of Sox2 Function on the Number of Cells in the Inner Nuclear Layer (INL)

I next investigated the effect of a loss of function of Sox2 at the stage used in our overexpression experiments. I first used RNA interference (RNAi) to reduce the expression of Sox2. The ability of the Sox2-RNAi construct to knock down Sox2 mRNA expression was examined in NIH3T3 cells by the cotransfection of a pU6-Sox2-specific shRNA and a Sox2-expressing construct (Fig. 4). Western blot analysis showed that Sox2 expression was significantly decreased by the expression of the shRNA (Fig. 15). A retrovirus encoding Sox2-RNAi was then used to infect retinal explants prepared from an E17.5 mouse eye. After two weeks, the localization and morphology of virus-infected cells were determined by examining EGFP-positive cells. The number of Sox2-downregulated cells was decreased in the INL and slightly increased in the ONL (Figs. 16A, 16B). To determine whether the cells missing from the INL due to Sox2-downregulation were amacrine cells, I examined the expression of the amacrine cell markers Pax6 and HuC/D in both control and Sox2-shRNA expressing retinal samples (Figs. 17A, 17B). The number of Pax6- or HuC/D-positive cells decreased with the expression of Sox2-shRNA, suggesting that suppression of Sox2 leads to a decrease in the number of amacrine cells (Fig. 17C).

I tried to determine whether a specific loss of GABAergic amacrine cells occurred but the number of cells was too small to obtain statistically significant results.

Discussion

The forced expression of Sox2 promoted the differentiation of amacrine cells from mouse retinal progenitor cells. Sox2 was previously reported to be expressed in proliferating CNS progenitors, and evidence is mounting to indicate that Sox2 maintains their progenitor properties (ref. 39, 45). Furthermore, the expression of Sox2 in differentiated cells such as oligodendrocyte precursors converts them to multipotent neural stem-like cells that are capable of generating neurons and glial cells (ref. 54). My findings concerning Sox2 expression in the retina differed from previous findings in that Sox2 expression promoted progenitor cell differentiation. Currently, I do not have a rational explanation for these contradictory results; however, I surmise that they were not caused by a difference in the species or tissue used because the suppression of proliferation by the ablation of Sox2 was also observed in the retina, suggesting that Sox2 maintains the progenitor character of the retina (ref.43). One possible explanation is that Sox2 has different functions depending on the developmental stages of the retina (i.e., it sustains the character of the progenitor cells during early retinal development and promotes amacrine cell differentiation later on). A similar conjecture can be applied to Pax6, which is a key regulator of eye development. Pax6 encodes a transcription factor that has two DNA-binding motifs: a paired domain and a paired-type homeodomain. During early retinal development, Pax6 is expressed in the progenitor cells; thereafter, as retinal differentiation progresses, Pax6 expression is restricted to retinal ganglion, amacrine, and horizontal cells (ref. 55, 56, 57). In accordance with this expression pattern, Pax6 is important during early retinal development, especially the proliferation of retinal progenitor cells; however, at later stages, it functions in amacrine cell differentiation (ref. 11). A similar change in function during retinal development may occur for Sox2.

My results indicate that Sox2 induces Pax6 expression by transcriptional activation. In addition, because Pax6 could not induce Sox2 under the same experimental condition, Sox2 likely acts upstream of Pax6. I found a putative Sox2-binding domain in the Pax6 promoter region. Mutational

analysis of the domain reduced, but did not abolish, Sox2-induced luciferase activity (Fig. 17), suggesting that multiple binding sites are coordinately involved in the activation of the Pax6 promoter by Sox2. However, I cannot exclude the possibility of an indirect effect by Sox2 on Pax6 induction. Sox2 binding to a lens-specific enhancer of Pax6 has been reported (ref. 58); nevertheless, to our knowledge, no such observation has been made in the retina. I also observed strong induction of the *Pax6* promoter by Sox2 in NIH3T3 cells (Fig. 18), suggesting that retina-specific molecule(s) are not required for induction. I must take into account accumulating evidence suggesting the existence of a complex network of molecules, including Pax6 and Sox2. As different physiological roles have been proposed for Pax6 during early and late development, Pax6 expression may also be differentially regulated during the early and late phases of retinal development. Moreover, Sox2 may contribute to Pax6 induction during the late phase only.

Amacrine cells are a class of retinal interneurons of the INL and GCL that modulate the synaptic activity between bipolar and ganglion cells. Some are located in the GCL as displaced amacrine cells, whereas others are found in the inner region of the INL. My current work reveals that Sox2 is expressed in the GABAergic subset of amacrine cells. Sox2 is expressed only in the subset of the cells that are positive for the general amacrine marker HuC/HuD, even at E17. This suggests that the commitment to differentiate into the Sox2 subset of amacrine cells may have already occurred by E17. However, although forced expression of Sox2 promoted the development of new amacrine cells, I could not obtain any conclusive results to determine whether Sox2 specifically differentiated the GABAergic subset of amacrine cells. Furthermore, the idea that Sox2 may activate the expression of specific molecules in GABAergic neurons is an interesting concept that needs to be clarified by future extensions of this study. Amacrine cells emerge relatively early during mouse retinal development (ref. 8), which raises the possibility that Sox2 promotes amacrine cell differentiation by inducing retinal progenitor cells to exit the cell cycle prematurely. This notion is supported by my finding that retinal cell proliferation was reduced by the expression of Sox2. However, because I found that Sox2 induces Pax6, we propose the specific induction of amacrine

cells by Sox2. Both the modification of the timing of cell cycle exit and the specific activation of the Pax6 gene may contribute to this phenomenon.

Forced Sox2 expression also promotes Müller glial cell fate slightly. Previous studies showed that overexpression of Sox2 in *Xenopus* retinal progenitors promotes an increase in Müller glial cell differentiation (ref. 42), suggesting that Sox2 may regulate progenitors toward the Müller glial cell fate in mouse retina too. Ablation of Sox2 expression from neural progenitors decreased the expression of Notch1 and Hes5 (ref. 43). Since Notch signali also plays roles in Müller glial cell differentiation through the Hes1 and Hes5 bHLH transcription factors (ref. 27), the combined results suggested the possibility that Sox2 promotes Müller glial cells through promotion of the Notch1 and Hes5 genes.

The siRNA knockdown of Sox2 in the progenitor cells of the neural retina of an E17 mouse resulted in a decreased number of cells in the INL. However, a recent study showed that decreased Sox2 expression in vivo using a cre/lox system leads to a loss of retinal ganglion cells and disrupted cell layering (ref. 43). The latter study used a Pax6 retina-specific enhancer, which acts from around E13; thus, the remarkable defect in the Sox2 mutants may be caused by the early disruption of Sox2 by the Pax6 promoter. In contrast, I suppressed Sox2 at a much later stage (i.e., E17.5, which may have led to the difference in the observed phenotype). Taken together, my results suggest that a stage-specific mechanism may contribute to this specific phenomenon.

It should be noted that Sox2 may induce amacrine cells not simply by the induction of Pax6, as Pax6 alone does not suffice to induce amacrine cells (ref. 28). Also, various other transcription factors could affect, or be affected by, the expression of Sox2 (ref. 43). Using the in vitro luciferase reporter system, I found that Sox2 induces the NeuroD promoter in Y79 cells (data not shown). However, I were unable to detect any augmentation of NeuroD protein in a retinal explant by immunostaining (data not shown). Therefore, the contributions of even undetectable levels of inducers or activators to the expression of NeuroD or other molecules may still influence cellular activities or possibly even, as suggested herein, Sox2-induced amacrine cell differentiation.

Furthermore, if Sox2 is specifically involved in the differentiation of the GABAergic subset of amacrine cells, my findings provide several insights helpful in the study of the differentiation of those amacrine cell subtypes, whose molecular and cellular pathways are not well understood.

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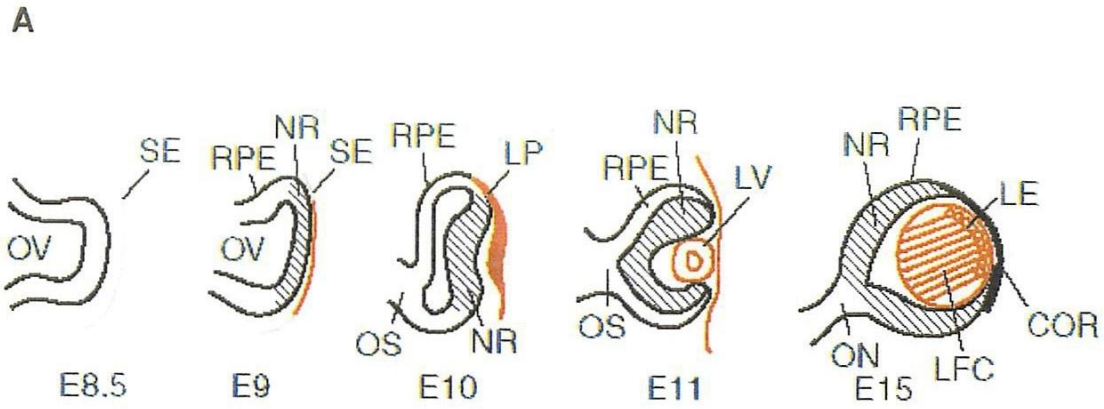

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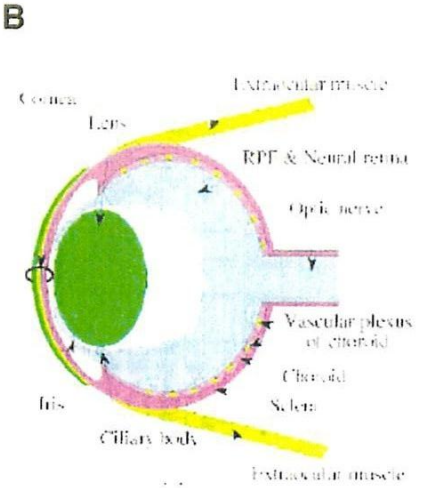

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Figure 1 Development of the vertebrate eye.
(A) Schematic illustration of eye development in the mouse. (B) Schematic structure of the adult eye. OV: optic vesicle; SE: surface ectoderm; RPE: retinal pigmented epithelium; NR: neuroretina; LP: lens placode; LV: lens vesicle; OS: optic stalk; LE: lens epithelium; ON: optic nerve; COR: cornea.

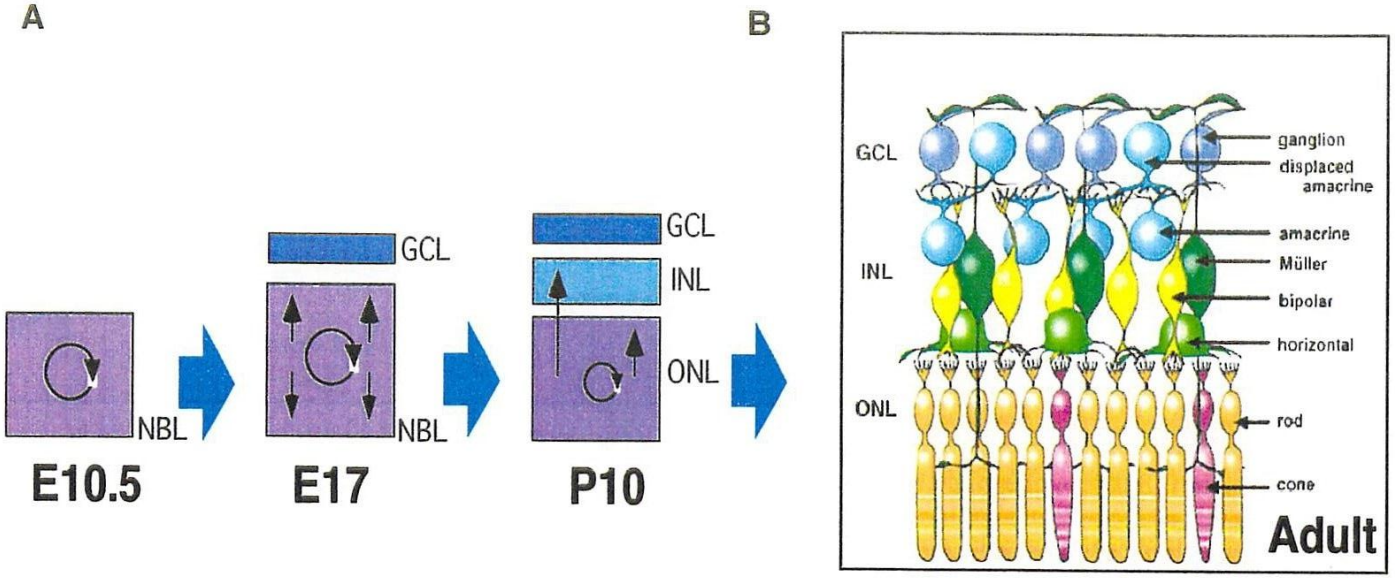

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Figure 2 Retinal development and bHLH gene expression during retinogenesis.
(A)Retinal differentiation from common progenitor cells in an order conserved progress among many species : ganglion cells first and Muller glial cells last. (B) Schematic structure of the neural retina. GCL, ganglion cell layer; INL, inner nucclear layer; ONL, outer nuclear layer; NBL, neuroblastic layer.

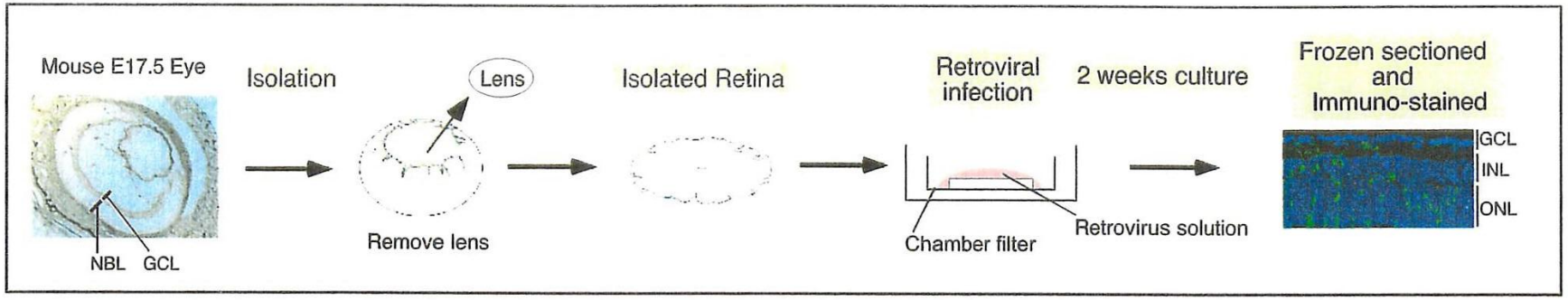


Figure 3 Neural retina explant culture methods.
Neural retina of mouse embryo (E17.5) was isolated and cultured for 2 weeks on the surface of chamber filter. During the 2 weeks culture, precursor cells in the retinal explant differentiate into neurons and glial cells and form layered structure which is typical of mature retina. After isolation of neural retina, retrovirus solution was added on the top of retinal explant. Since retroviruses infect only to mitotic cells, retinal precursor cells are assumed to be the major targets.

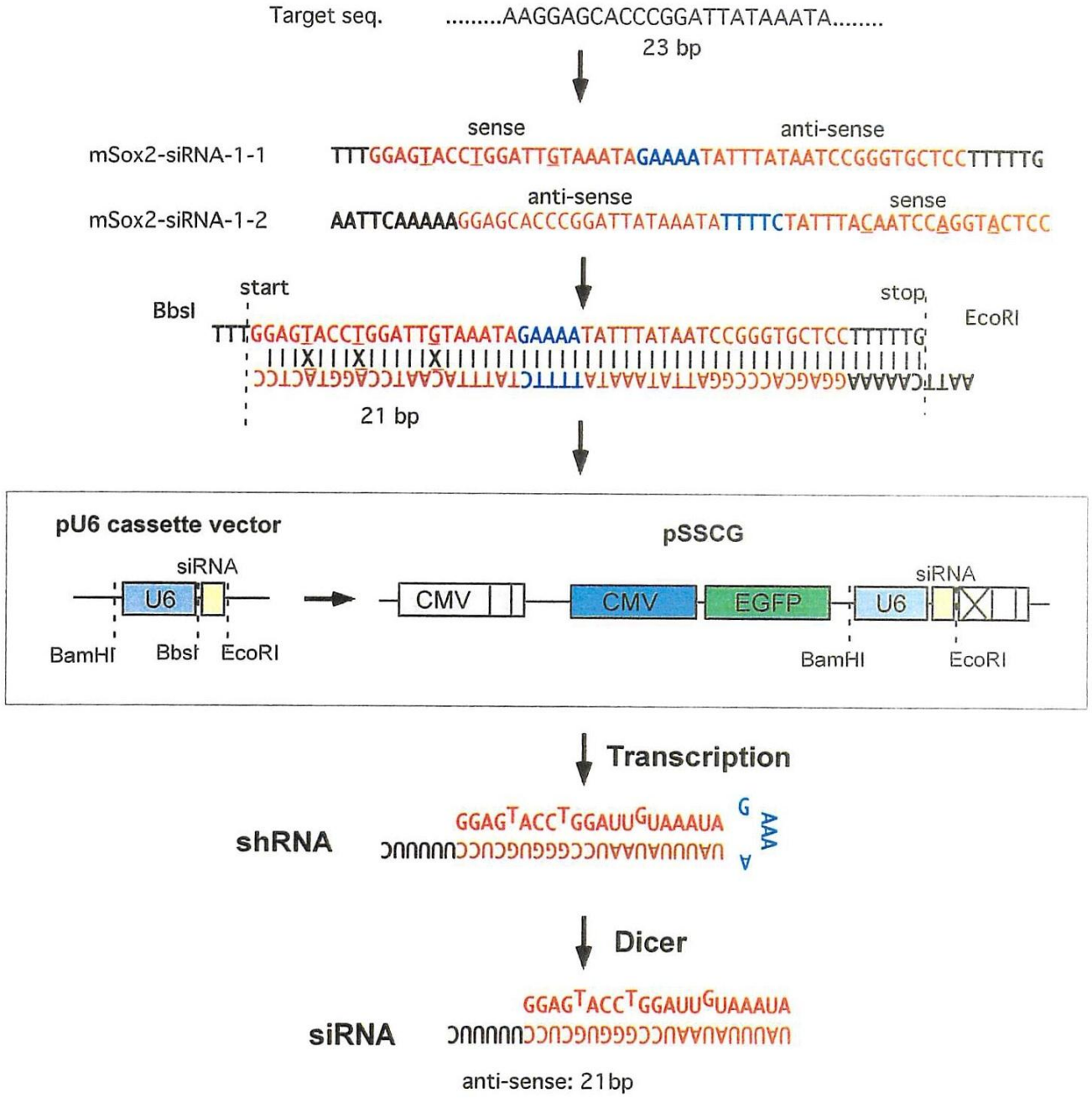


Figure 4 The schematic drawing of the progress of the production of Sox2 RNAi construct. The dsRNA were designed from the target sequence of Sox2 then cloned into pSSCG expression vector. The predicted structure of pSSCG- Sox2 RNAi transcript was produced from this vector and the synthetic siRNA used to target sequence was shown.

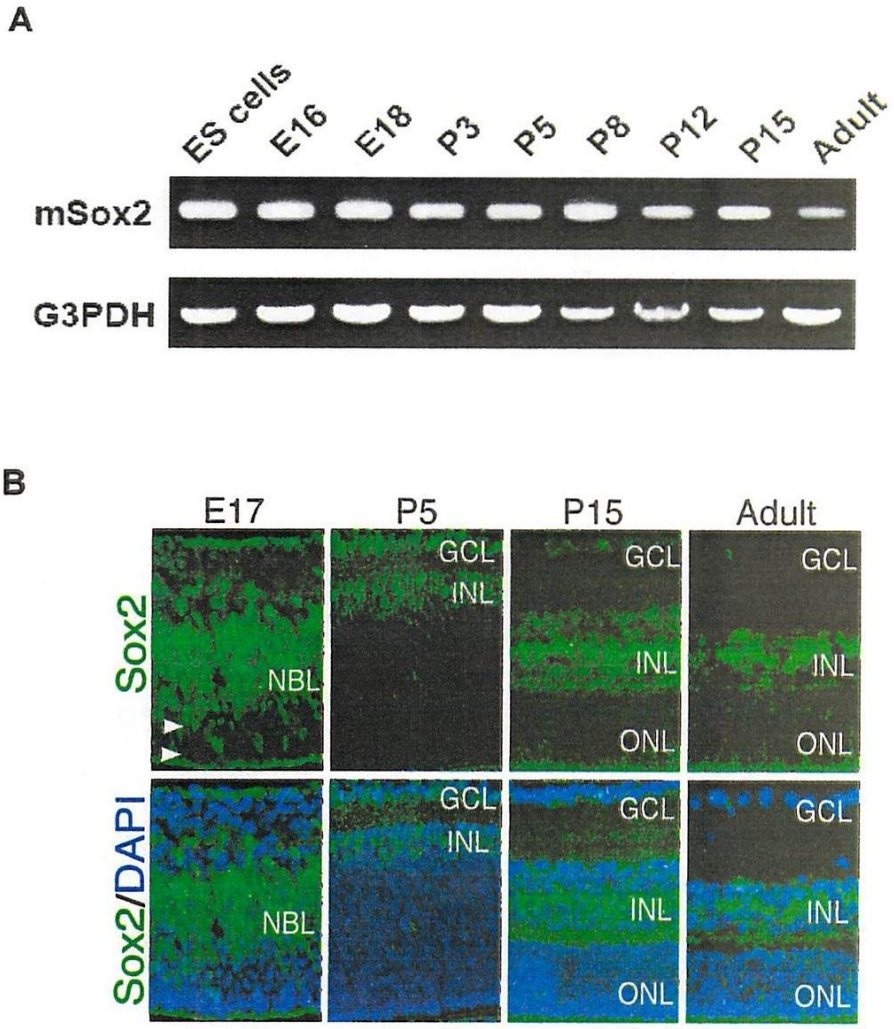


Figure 5 Expression of Sox2 in developing mouse retina.
(A) Sox2 mRNA expression in developing mouse retina was examined by semiquantitative RT-PCR. G3PDH was used as the control. Total RNA isolated from mouse retina at the indicated developmental stages was used as the template. (B) Immunostaining of Sox2 was conducted of frozen, sectioned retinas at various developmental stages. The sections were stained with anti-Sox2 antibody, and signals were visualized with secondary antibody conjugated with Alexa488. DAPI was used to stain the nuclei.

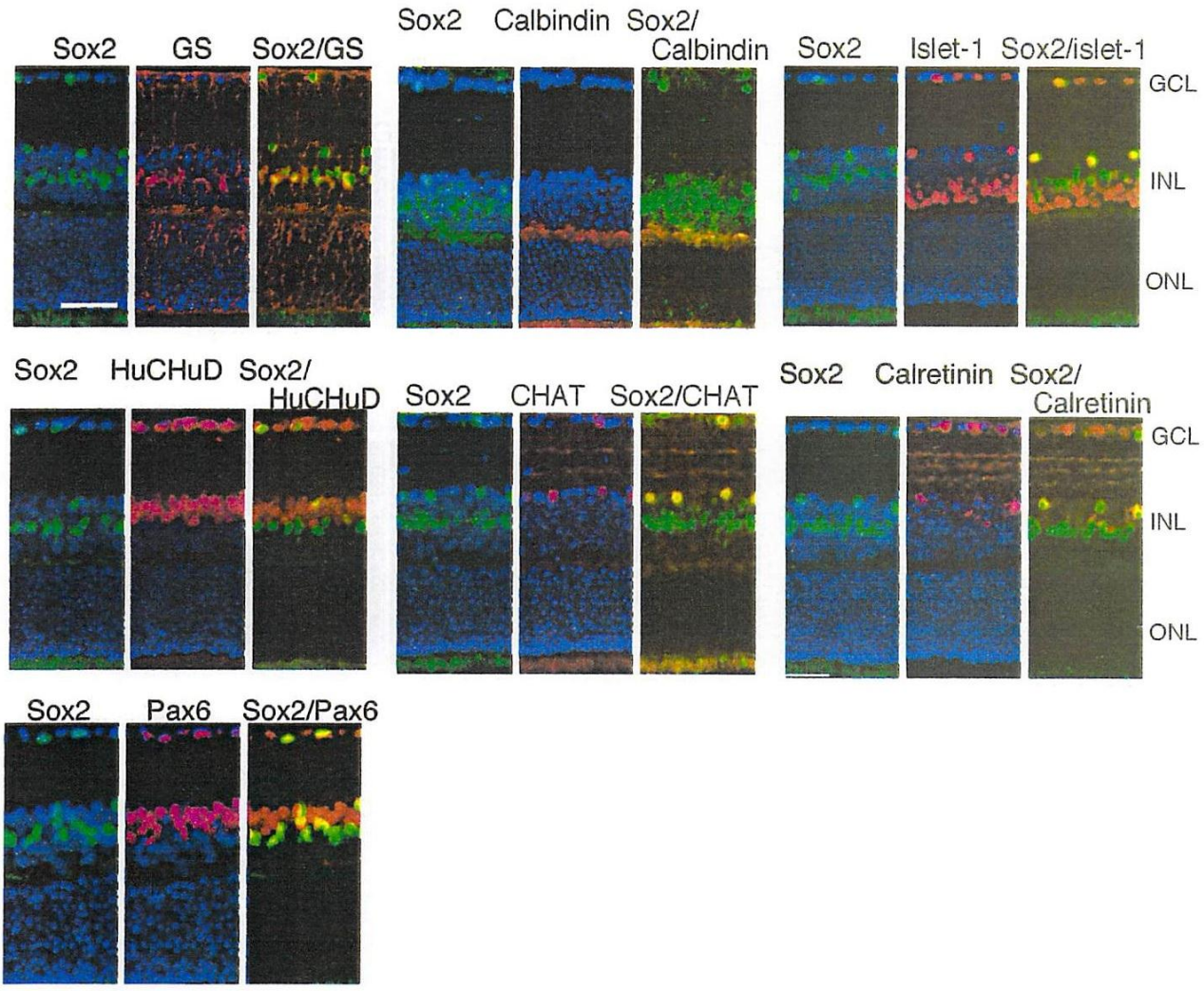


Figure 6 Sox2 was co-immunostained with other retinal markers.
Frozen sections of adult mouse retina was used for immunostaining, and signals of Sox2 and other markers were visualized with *green* (Alexa488) and *red* (Alexa546), respectively. ONL, outer nuclear layer, INL, inner nuclear layer, GCL, ganglion cell layer. Scale bar, 25 μm.

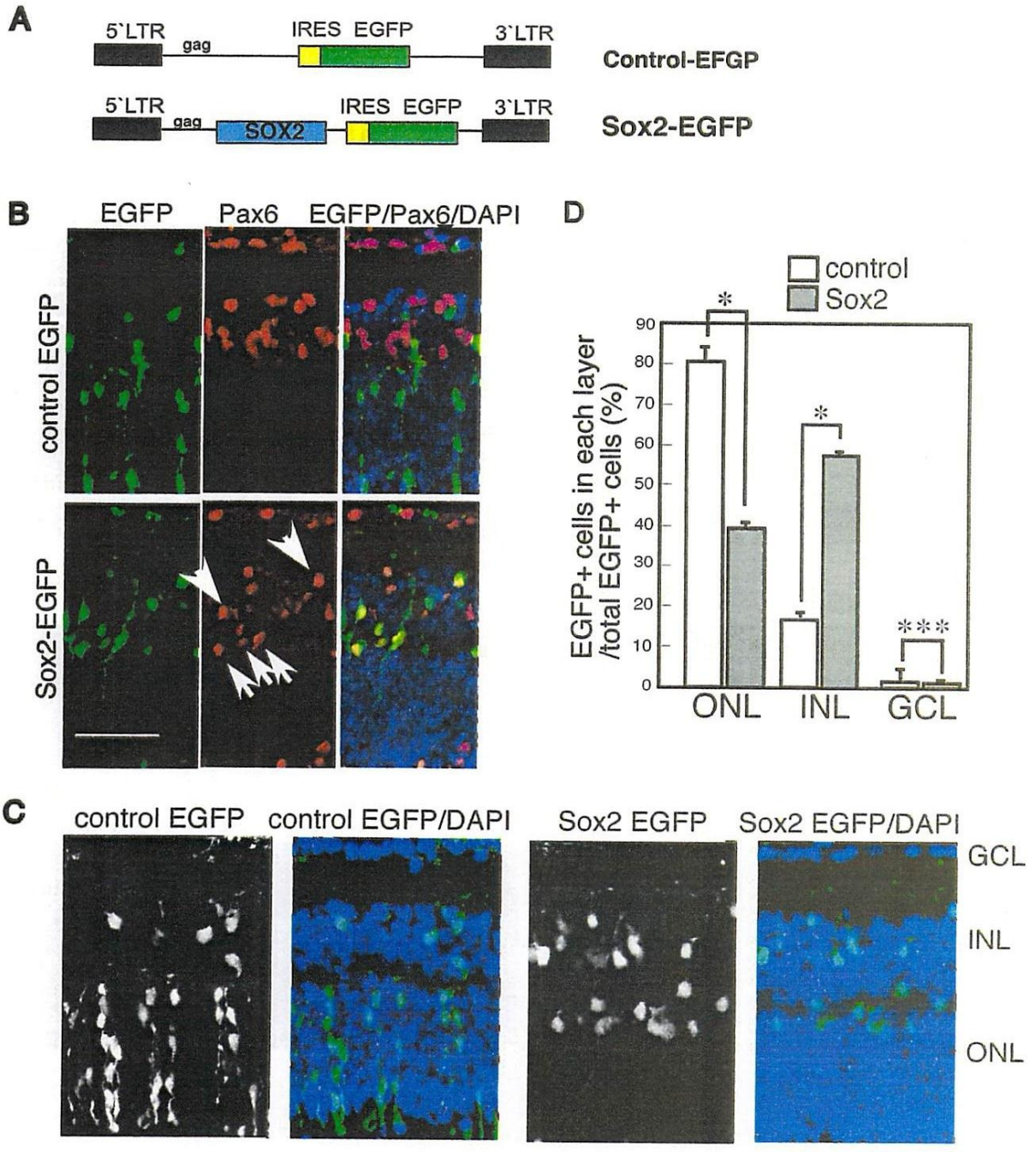
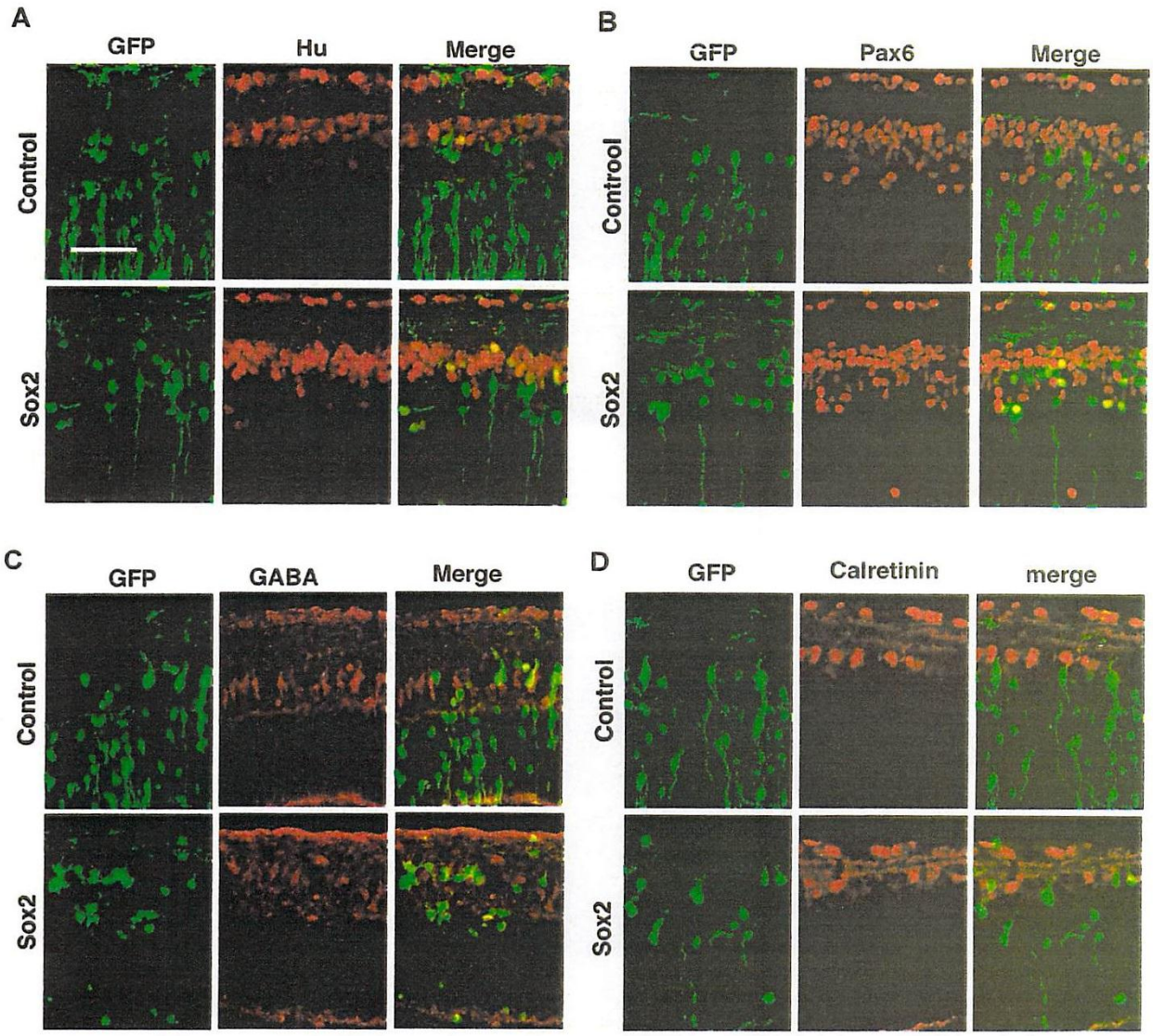


Figure 7 Overexpression of Sox2 in retinal explant culture.

(A) Schematic of the control-EGFP and Sox2-EGFP retrovirus constructs. (B) Immunostaining pattern of anti-Sox2 antibody and (C, D) sublayer distribution and morphology of virus-transduced, EGFP-positive cells in the retinal explant. Retinal explants were infected with retrovirus particles encoding control- or Sox2-IRES-EGFP, and after two weeks of culture, the explants were harvested, frozen, and sectioned. Immunostaining was performed with anti-EGFP and -Sox2 antibodies (B) or anti-EGFP antibody (C). The percentages of sublayer distribution of the virus-infected cells are shown in (D). Scale bars, 50μm. More than 200 cells were counted for each sample, and the SD was calculated from three independent experiments. (B, *arrows*: Sox2 virus-infected cells; C, *arrows*: Sox2 virus-infected cells in the innermost ONL). $*p < 0.01$, $**p < 0.05$, $***p > 0.05$.



Fifure 8 Differentiation of virus-infected cells examined by immunostaining with markers specific for subpopulations of the retina.

(A-D) Frozen sections were stained with cell type specific markers (red): anti-HuC/HuD antibody for amacrine cells (A); anti-Pax6 abtibody for amaceine cells (B); anti-GABA antibody for GABAergic amacrine cells (C); anti-calretinin antibody for GABAergic amacrine cells (D). Nuclei were stained with DAPI and infected cells were detected by anti-GFP antibody (green). Scale bar, 50 μm.

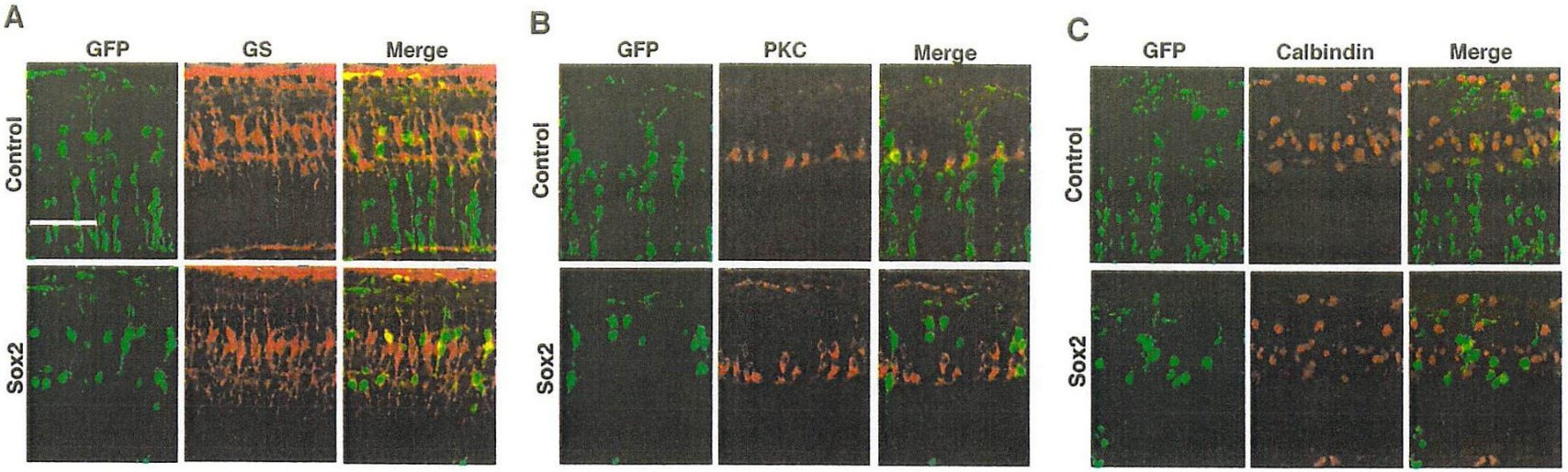


Figure 9 Differentiation of virus-infected cells examined by immunostaining with markers specific for subpopulations of the retina. (A-C) Frozen sections were stained with cell type specific markers (red): anti-glutamine synthetase (GS) abtibody for glia cells (A); anti-PKC antibody for bipolar cells (B); anti-calbindin antibody for horizontal cells (C). Nuclei were stained with DAPI and infected cells were detected by anti-GFP antibody (green). Scale bar, 50 μm.

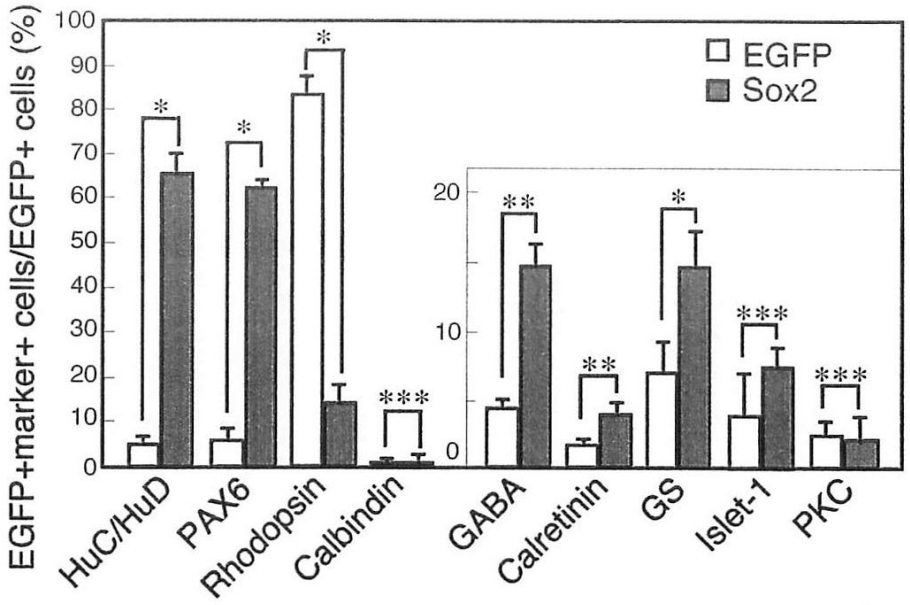


Figure 10 Differentiation of virus-infected cells examined by immunostaining with markers specific for subpopulations of the retina. The percentages of marker-positive cells in the EGFP-positive population are shown. More than 100 cells were examined for each sample, and the average value from three independent experiments is shown with the SD. $*P < 0.01$, $**P < 0.05$, $***P > 0.05$.

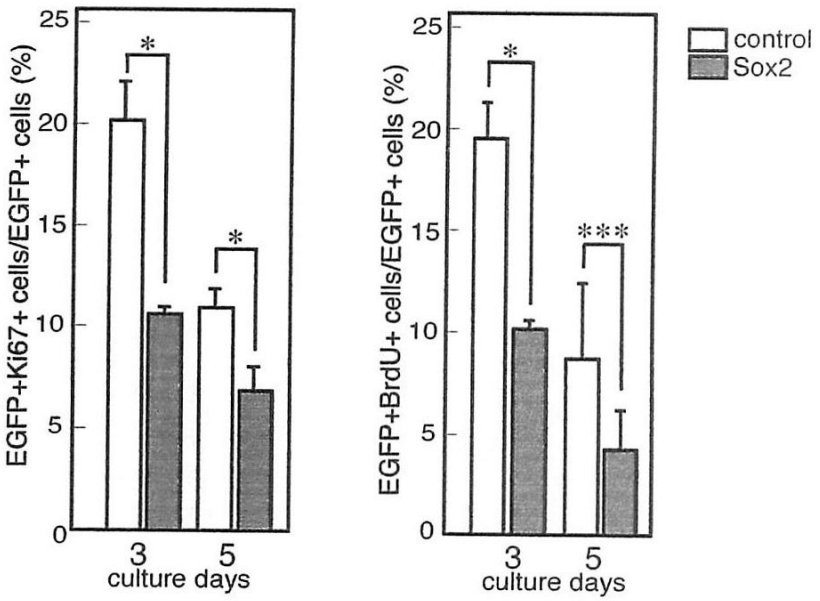


Figure 11 Effect of forced Sox2 expression on proliferation in E17.5 retinal explant cultures. Proliferation of Sox2-expressing retinal cells was examined by expression of Ki67 (*left*), a nuclear cell proliferation- associated antigen expressed during the active stages of the cell cycle, or by incorporation of BrdU (*right*). Retinal explants infected with retrovirus particles encoding Sox2-IRES-EGFP or a control construct were harvested after 3 or 5 days of culture. For BrdU incorporation assay, BrdU was present during the last 24 hours of the culture. The expression of Ki67 or incorporated BrdU was visualized by immunostaining specific for Ki67 or BrdU, respectively. More than 100 cells were examined for each sample, and the average value from three independent experiments is shown with the SD. $*P < 0.01$, $**P < 0.05$, $***P > 0.05$.

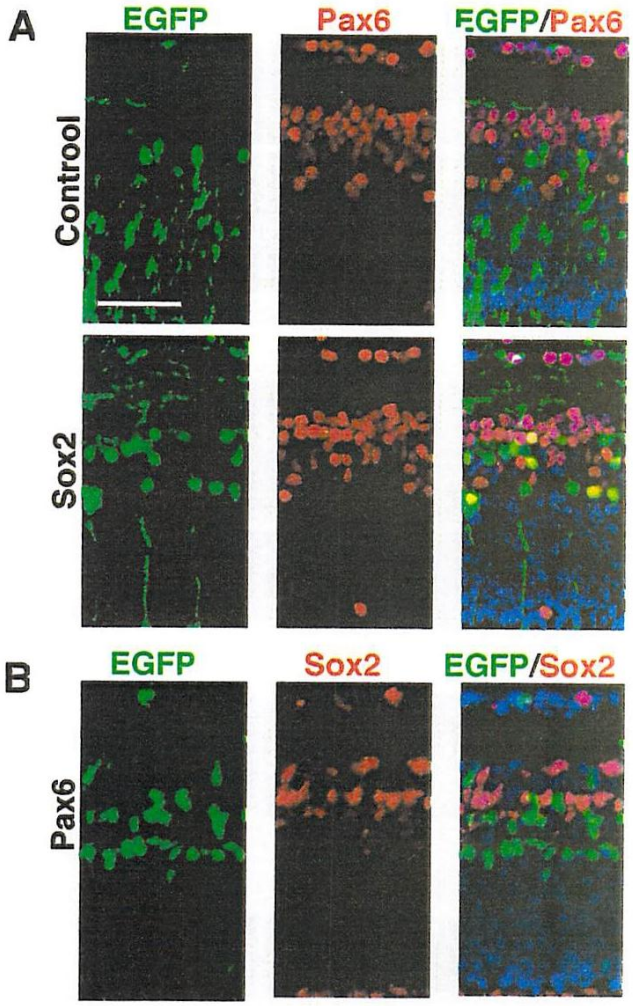


Figure 12 Interrelationship of Sox2 and Pax6 in regulating their own expression.
Sox2 (A) or Pax6 (B) were overexpressed in retinal explant prepared from E17 mouse embryos by retrovirus-mediated gene transfer, and after 14 days of culture, the explants were harvested, frozen, and sectioned. The sections were immunostained with the indicated antibodies.
Scale bars, 50 μm.

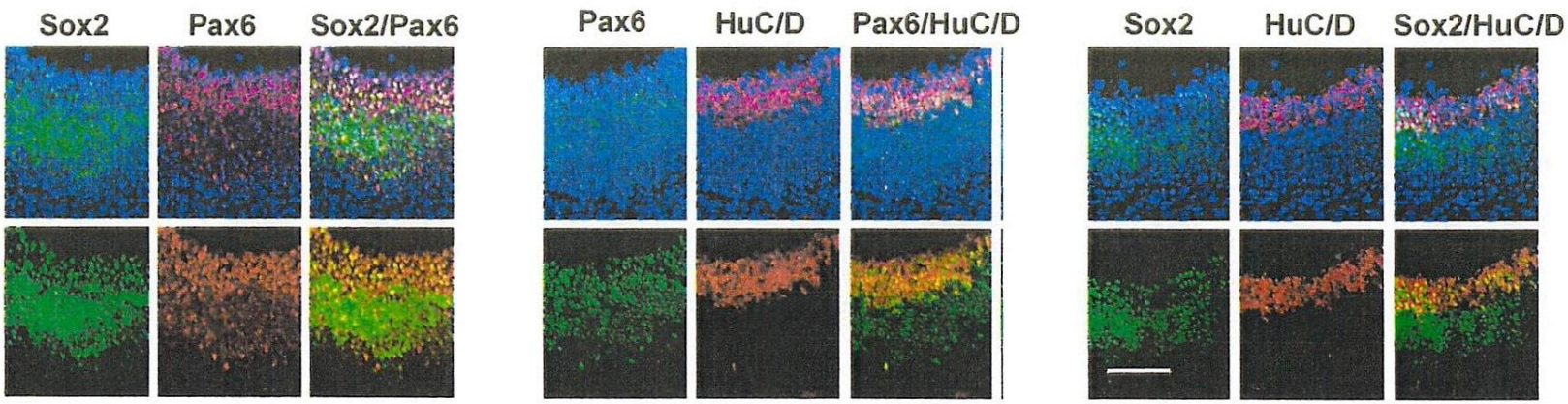


Figure 13 Immunostaining of E14.5 mouse retina with indicated antibodies on the panels. Scale bars, 50 μm.

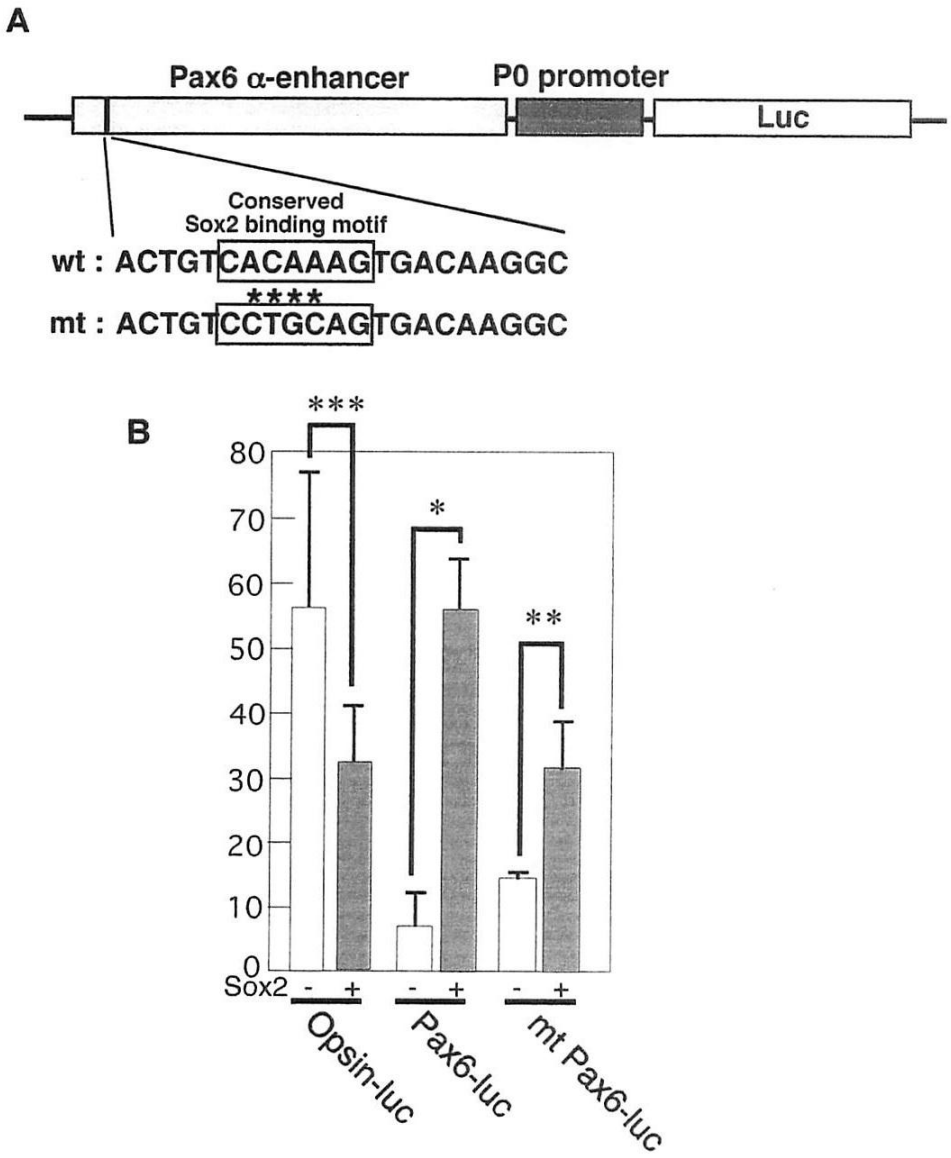


Figure 14 Sox2 enhancement of Pax6 enhancer activity.
Schematic representation of mutant Pax6 enhancer construct. *Asterisks*: changed bases from wild type Pax6 (A). Pax6-, Pax6 mutant-, or opsin-luciferase plasmids were transfected with or without Sox2 expression plasmid into Y79 retinoblastoma cells (B). After 25 hours of culture, the cells were harvested, and luciferase activities were examined. Relative luciferase activities with SD are shown. $*p < 0.01$, $**p < 0.05$, $***p > 0.05$.

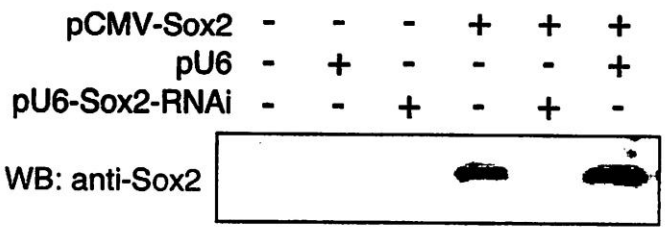


Figure 15 Suppression of Sox2 expression by shRNA in retinal explant culture.
Effects of shRNA specific for Sox2 were examined by Western blot analysis in NIH3T3 cells. Sox2 expression plasmid was transfected into NIH3T3 with or without pU6-shRNA for Sox2. After 48 hours of culture, the cells were harvested, and Western blot analysis was performed with anti-Sox2 antibody.

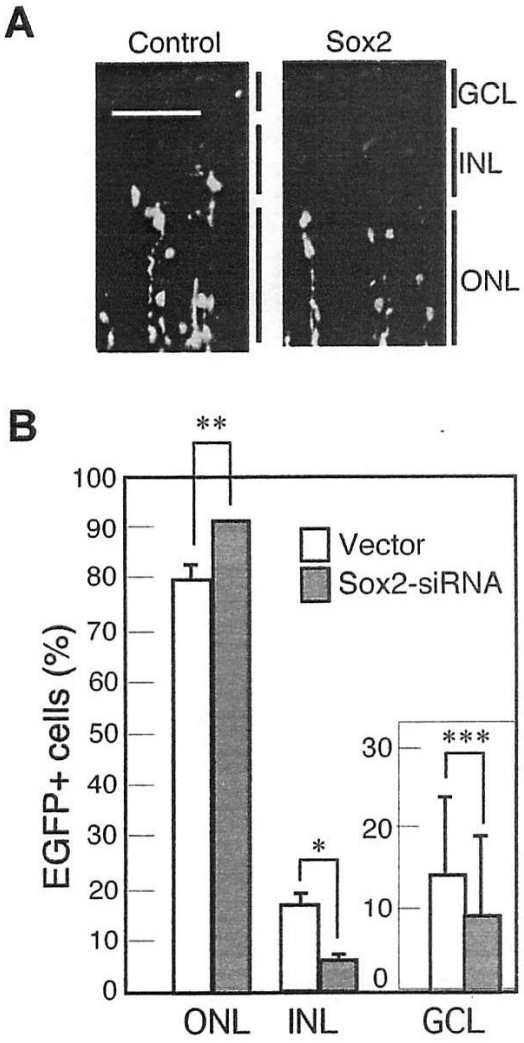


Figure 16 Suppression of Sox2 expression by shRNA in retinal explant culture.
Control EGFP or shRNA for Sox2-encoding retrovirus was infected into retinal explant culture prepared from E17 mouse embryos, and after 14 days of culture, the explants were harvested, frozen, and sectioned. Immunostaining was performed with anti-EGFP antibody, and the cellular distribution of EGFP-positive cells was examined. Fluorescence microscopic views (monochrome) are shown in (A), and the percentages of sublayer distribution of the virus-infected cells are shown in (B).

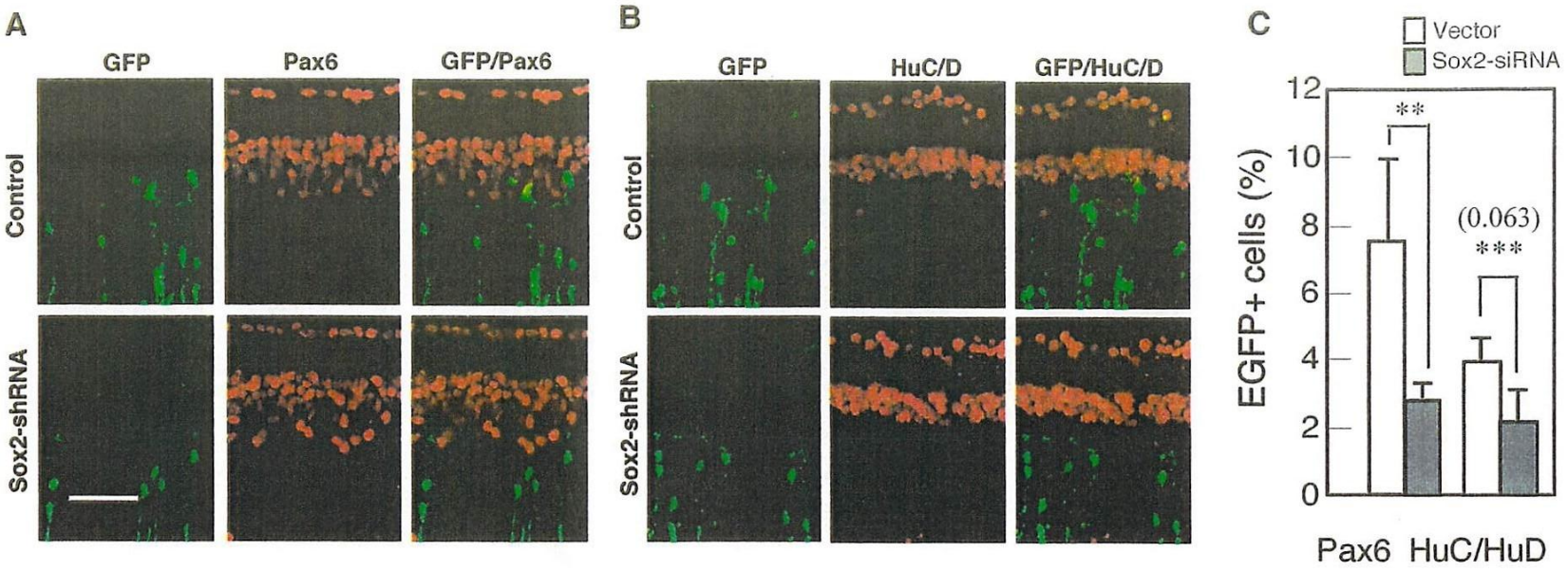


Figure 17 Effect of inhibition of Sox2 function on the number of cells in the INL.
Differentiation of virus-infected cells examined by immunostaining with markers specific for subpopulations of the retina. (A-B) Frozen sections were stained with cell type specific markers (*red*): anti-HuC/D antibody (A) and anti-Pax6 antibody (B) for amacrine cells. (C) Double immunostaining of anti-EGFP with either anti-Pax6 or anti-HuC/D was examined in control EGFP or Sox2-shRNA-expressing cells. The explants prepared from E17 mouse embryos were cultured for 14 days, frozen, and sectioned. Scale bar, 50 μm.
*$p < 0.01$, ** $p < 0.05$, *** $p > 0.05$.