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The role of Zic family zinc finger transcription factors in the proliferation and differentiation of retinal progenitor cells

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ABSTRACT

Members of the Zic family of zinc finger transcription factors play critical roles in a variety of developmental processes. Using DNA microarray analysis, we found that Zics are strongly expressed in SSEA-1-positive early retinal progenitors in the peripheral region of the mouse retina. Reverse-transcription polymerase chain reaction using mRNA from the retina at various developmental stages showed that Zic1 and Zic2 are expressed in the embryonic retina and then gradually disappear during retinal development. Zic3 is also expressed in the embryonic retina; its expression level slightly decreases but it is expressed until adulthood. We overexpressed Zic1, Zic2, or Zic3 in retinal progenitors at embryonic day 17.5 and cultured the retina as explants for 2 weeks. The number of rod photoreceptors was fewer than in the control, but no other cell types showed significant differences between control and Zic overexpressing cells. The proliferation activity of normal retinal progenitors decreased after 5 days in culture, as observed in normal *in vivo* developmental processes. However, Zic expressing retinal cells continued to proliferate at days 5 and 7, suggesting that Zics sustain the proliferation activities of retinal progenitor cells. Since the effects of Zic1, 2, and 3 are indistinguishable in terms of differentiation and proliferation of retinal progenitors, the redundant function of Zics in retinal development is suggested.

1. Introduction

The vertebrate neural retina is organized into a laminar structure comprising six types of neurons and glial cells, Müller glia, and astrocytes. In mice, 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 [1]. In vertebrates, retinal ganglion cells (RGCs) differentiate first as a wave across the neuroepithelium of the optic cup. RGCs, cone photoreceptors, amacrine cells, and horizontal cells differentiate at relatively early stages, mainly before birth, while bipolar and rod cells are mainly generated at later stages after birth. Both the progression of retinal neurogenesis, and retinal cell fate specification or differentiation, have been shown to be controlled by intrinsic cues such as transcription factors, as well by as extrinsic signals [2,3].

Cell surface antigens are powerful tools for isolating specific subsets of retinal cells during development from cell mixtures without damaging the cells, which 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 and c-kit as early and late progenitor markers, respectively [4,5]. SSEA-1 marks retinal progenitor cells in the peripheral region of the retina at around E14–E16 [4]. In the later stage of embryogenesis, SSEA-1 disappears and c-kit expression is observed in the retinal progenitor cells in the central region of the retina [5]. We compared the gene expression patterns of regionally and temporally different subsets of retinal progenitor cells, SSEA-1-positive cells at E14, c-kit positive cells at P1, and differentiated c-kit negative cells at P1 using a microarray [6]. We found the Zic family of zinc finger transcription factors to be strongly expressed in SSEA-1-positive cells.

Zic family genes are vertebrate homologs of odd-paired, the *Drosophila* pair-rule gene, which was also discovered as transcription factors controlling neuroectodermal differentiation in *Xenopus* embryos [7]. Studies on various vertebrates, including humans and mice, showed critical roles for Zics in a variety of developmental processes [8]. Mutations in Zic genes in humans were implicated in a wide variety of congenital malformations, including Dandy–Walker malformation, holoprosencephaly, neural tube defects, and heterotaxy [9]. Experimental evidence also suggests that

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Zic genes have multiple roles in neural development. They control ectoderm differentiation into neuroectoderm, neural crest development, and formation of the cerebellum [8]. Mutation of Zics result in neural tube defects at different rostrocaudal levels depending on which Zic gene subtype has been affected [7]. Holoprosencephaly, forebrain anomalies, and cerebellar dysgenesis were also observed in Zic mutations, indicating the role of Zics in region-specific morphogenesis of the central nervous system.

In the visual system, Zic2 is essential for proper development of the ipsilateral projection at the mammalian optic chiasm midline [10,11]. Zic2 is sufficient to change the trajectory of RGC axons from crossed to uncrossed and regulates the expression of EphB1, which is essential for RGCs [12]. In the retina, Zic1 and Zic2 are expressed in the peripheral region at around E16, and Zic3 is very weakly expressed in the periphery at the same time [13]. However, their roles in retinal development are not known. Here, we found Zic genes strongly expressed in immature retinal progenitor cells, and their roles in retinal differentiation and proliferation are suggested by gain-of-function analysis.

2. Materials and methods

2.1. Mice and reagents

ICR mice were obtained from Japan SLC Co. 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 done using Affymetrix GeneChip Mouse Genome 430 2.0 using total RNA from c-kit positive (P1), c-kit negative (P1), and SSEA-1 positive (E14) cell populations. GSK-3β inhibitor BIO was from Calbiochem, recombinant BMP4 was from Wako, and LDN-193189 was from Stemgent.

2.2. DNA construction

Full-length cDNAs encoding mouse Zic1, Zic2, and Zic3 open reading frames (ORFs) were kindly provided by Dr. Aruga (RIKEN BRI). Full length cDNAs were isolated by *Sal*I, and the fragments were inserted at *Xho*I site of pMX-IRES-EGFP retrovirus vector.

2.3. RT-PCR

Total RNA was purified from mouse retinas using RNeasy Plus Micro (QIAGEN), and cDNA was synthesized using Superscript II (Invitrogen-Gibco). For semi-quantitative PCR, the primer sets were tested over a range of thermal cycles using Blend Taq Plus (TOYOBO), and the cycle number was determined for each primer set. Bands were visualized with ethidium bromide. Quantitative PCR (qPCR) was done by 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). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as the internal control.

2.4. Retinal explants, retrovirus infection, and electroporation

Retinal explants were prepared as previously described [14]. Briefly, neural retinas were isolated on a chamber filter (Millicell; Nihon-Millipore, Tokyo, Japan) and placed with the ganglion cell layer face up. The filters were inserted into six-well plates and cultured in 1 mL of explant culture medium [14]. Retroviral infection was performed as described elsewhere [14].

2.5. Immunostaining

Immunostaining of sections was done as described previously [14]. Primary antibodies used were as follows: mouse monoclonal antibodies against PNR (ppmx), glutamine synthetase (GS, Chemicon), Ki67 (BD Bioscience), BrdU (Roche); rabbit polyclonal antibodies against GFP (Clontech), RXRγ (Santa Cruz Biotechnology); goat polyclonal antibody Otx2 (R&D Systems); rat monoclonal antibody against GFP (Nakarai). The first antibodies were visualized by using appropriate Alexa-488 or Alexa-594-conjugated second antibodies (Molecular Probes). Samples were mounted in Vecta-Shield (Vector Laboratories) and analyzed by using a Zeiss Axio Vision 4.6 microscope.

2.6. BrdU labeling

For retinal explants, BrdU was present in the medium at a final concentration of 1.5 μg/ml at 24 h before fixation. Then, the explants were frozen sectioned, and immunostaining was done to detect incorporated BrdU.

3. Results and discussion

3.1. Expression of Zics during retinal development

So far, five members of the Zic family in mice have been reported, and we found significant expression of Zics 1–3 in SSEA-1-positive early retinal progenitor cells in the retina at E14 using microarray analysis (Table 1). Values of these three Zics decreased in c-kit-positive cells at P1 and further decreased in c-kit-negative post-proliferation cells at P1. We found only negligible values of Zic4; Zic5 was weakly expressed in SSEA-1- and c-kit-positive retinal progenitor cells and the value increased in c-kit-negative postmitotic cells (Table 1). Since SSEA-1-positive retinal progenitor cells are localized to the peripheral region of the retina [4], this is consistent with a previous paper describing the spatial expression of Zic in the peripheral region of the retina in early development [13]. We then examined a more detailed time course of the expression of Zics 1–3 by semiquantitative reverse-transcription-polymerase chain reaction (RT-PCR) using cDNA from the mouse retina at various developmental stages. Zic1 expression was weak and decreased gradually, while Zic2 was expressed until P3 and then decreased sharply after P5 (Fig. 1). Zic3 was expressed in all the examined stages, and its expression gradually decreased during development.

The transition of Zic expression in retinal explants was examined by microarray. Retinal explants were prepared using retina at E15. After culturing for 5 or 14 days, the explants were harvested

Table 1
DNA microarray analysis of retinal progenitor cells at different developmental stages in compare with post mitotic cells. Retinal progenitor cells at E14 (SSEA-1 positive cells), at P1 (c-kit positive cells), and post mitotic cells at P1 (c-kit negative cells) were purified by a cell sorter, and DNA microarray was done.

Gene	Probe No.	Unigene No.	SSEA1+/E14	c-kit+/P1	c-kit-/P1
Zic1	1423477_at	Mm.2719.1	1618.0	587.4	216.4
Zic2	1421301_at	Mm.5047	160.6	95.0	82.2
Zic3	1423424_at	Mm.4265.1	364.5	108.0	79.5
Zic4	1421539_at	Mm.8062	4.7	4.6	2.7
Zic4	1456417_at	Mm.8062.2	48.9	13.1	9.8
Zic5	1456140_at	Mm.157721.2	78.6	77.8	205.5
Msx1	1417127_at	BC016426	6.3	5.4	2.5
Msx1	144861_at	BC016426	33.2	14.2	32.9
ID3	1416630_at	Mm.110	1123.8	402.9	39.5
ID2	1422537_at	Mm.1466	628.8	527.9	69.2
ID4	1423259_at	Mm.28223	654.6	496.8	395.4

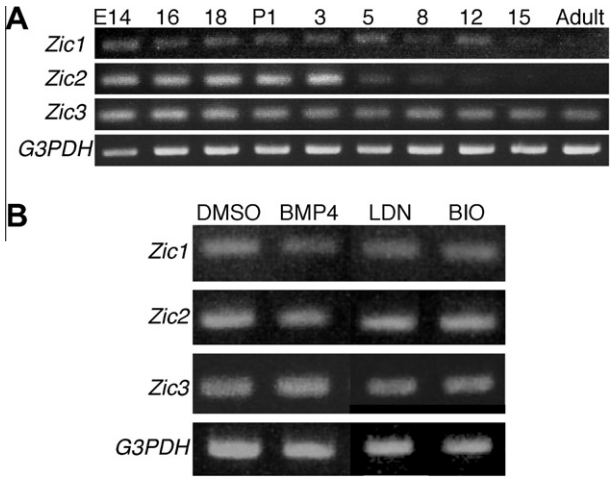

Fig. 1. Expression of Zic family genes during retinal development. (A). Expression of mRNA of Zics in retinas was examined at various developmental stages as indicated. Total RNA was extracted from retinas at indicated developmental ages, and semi-quantitative RT-PCR of Zic1, Zic2, and Zic3 was conducted. G3PDH was used as a control. (B). Retinas of E13 embryos were isolated and prepared explants. The explants were cultured in the presence of indicated drugs, DMSO (1 μM), BMP4 (1 μM), LDN 193189 (150 nM), BIO (5 μM) and harvested after 24 h. RNA was extracted and RT-PCR was done.

and used for a DNA-microarray (Table 2). The expression of Zics 1, 2, and 3 was decreased by culturing, suggesting that the temporal transition of expression of Zics is similar to that of the *in vivo* developmental process. Previous work on *in situ* hybridization showed the expression of Zics 1–3 in the most peripheral retinal regions, and the expression of Zics was most strongly observed at E13.5, with diminished expression at E16.5 [13]. The time course of Zic expression examined by RT-PCR did not show such a sharp decrease in mRNA levels between E14 and E16, suggesting that the expression disappeared from the periphery but remained in the central region of the retina.

The involvement of various signaling pathways in regulating the expression levels of Zics was reported [8]. BMP is a negative regulator of Zic family gene expression during neural development [7,15], and Msx1, an immediate-early response gene to BMP signaling in *Xenopus* ectodermal cells, negatively affects Zic1 [16]. We examined the role of BMP4, which is critical in retinal development, in the expression of Zics. Addition of BMP4 or inhibition of BMP signaling by the specific inhibitor LDN-193189 did not affect the expression of Zics (Fig. 1B), suggesting that BMP may not play a role in the regulation of Zic expression in the retina. This notion was further supported by the observation that the expression level of Msx1 did not show a negative correlation with Zics in our microarray analysis (Table 1). SSEA-1-positive cells are positively regulated by Wnt signaling in the retina [4]. We next asked whether enhancement of Wnt signaling by BIO, a Gsk-3β inhibitor, affects Zic expression. We did not observe any modulation of Zic expression by BIO (Fig. 1B), suggesting that Wnt may not have a direct effect on Zic expression.

Table 2
DNA microarray analysis of retinal cells of explant cultures. Mouse retina at E15 was isolated and cultured as explants. Then, retinas were harvested after 5 days of culture and analyzed gene expression pattern by DNA microarray.

Gene	Probe No.	Unigene No.	E15	5 days	14 days
Zic1	1423477_at	Mm.2719.1	582.0	190.2	40.1
Zic2	1421301_at	Mm.5047	67.0	55.6	15.7
Zic3	1423424_at	Mm.4265.1	153.0	65.9	18.1
Zic4	14521539_at	Mm.8062	7.5	6.1	3.3

3.2. Gain-of-function analysis of Zics showed altered retinal progenitor differentiation

To delineate the functions of Zics in retinal development, we conducted a gain-of-function analysis using retrovirus-mediated gene transfer into retinal explant cultures [14]. A retrovirus encoding Zic1-, Zic2-, or Zic3-IRES-EGFP or control EGFP was used to infect retinal explants prepared from embryos at E17, and the retinal sub-layer distribution of Zic-overexpressing cells was examined after 2 weeks of culture by immunostaining of frozen sections (Fig. 2A, B). The number of Zic1 and Zic2 overexpressing cells in the outer nuclear layer (ONL) increased slightly, while that in the inner nuclear layer (INL) decreased slightly, but these differences were not statistically significant (Fig. 2A). The distribution of Zic3 in the layers was similar to the control (Fig. 2A). Further examination of the sub-layer distribution of EGFP-positive cells in the ONL showed that Zic2 overexpressing cells tended to be located on the outer one-third of cells (Fig. 2B).

We then examined the retinal subtypes of Zic-overexpressing cells by immunostaining of various markers specific for retinal subtypes. Rod photoreceptors are the most abundant cell type in the retina, and when we examined the number of rod photoreceptor cells by immunostaining of PNR, we found that PNR-positive rod cells were significantly decreased by Zic1, Zic2, and Zic3 overexpression (Fig. 2C and D). Since the total number of cells in the ONL was comparable in Zic-overexpressing cells, as in the control, we suspect that the number of cone cells, which are also located in the ONL, may have increased. However, immunostaining of the cone specific marker, RXRγ, suggested that the number of cones was unchanged by Zic overexpression (Fig. 2E and H). Therefore, we suggest that progenitor cells migrate into the ONL, but do not differentiate into rod cells. In other words, continuous expression of Zic may suppress final differentiation. We also examined other cell types – Müller glia by glutamine synthetase (GS), horizontal cells by Prox1 (data not shown), and amacrine and horizontal cells by Pax6 (data not shown), and none of them showed significant differences in cell number between the control and Zic-overexpressing cells (Fig. 2F and H, and data not shown). All criteria showed similar activities of Zic1, Zic2, and Zic3, suggesting that Zics are redundant in terms of retinal cell differentiation.

Otx2 is a transcription factor that plays essential roles in photoreceptor cell differentiation [17]. We next asked whether Zics suppress photoreceptor cell differentiation in the early phase by examining Otx2 expression by immunostaining. At day 5, Otx2 signals were seen in most regions except the ganglion cell layer, and expression of Otx2 in EGFP-positive cells was comparable in control and Zic overexpressing cells (Fig. 2G and H). At day 14, Otx2 signals were observed strongly in the INL and weakly in the ONL, as previously reported [18]. The number was comparable between the control and Zic-overexpressing samples (Fig. 2G and H).

Nr2e3 and Nrl are transcription factors that play roles in rod photoreceptor development at stage later than Otx2 [17] and we examined the effects of Zic on the expression of these genes by RT-PCR. Zic2 was expressed in the retina at E17 by retroviral gene transfer and infected explants were harvested after 5 days of culture. EGFP-positive cells were purified using a cell sorter and RT-PCR was performed. We previously showed Id3 enhanced SSEA-1 positive retinal progenitor cells [6], and the expression of Id3 was upregulated in the retina by Zic2 overexpression (Fig. 2I). Otx2 was slightly upregulated, but not significantly, and Nr2e3 and Nrl were suppressed by Zic2 (Fig. 2I). Taken together, these results suggest that Zic may not affect the fate determination of retinal progenitor cells into photoreceptors, but suppresses the final differentiation of rod photoreceptors probably by affecting Nr2e3 and Nrl expression.

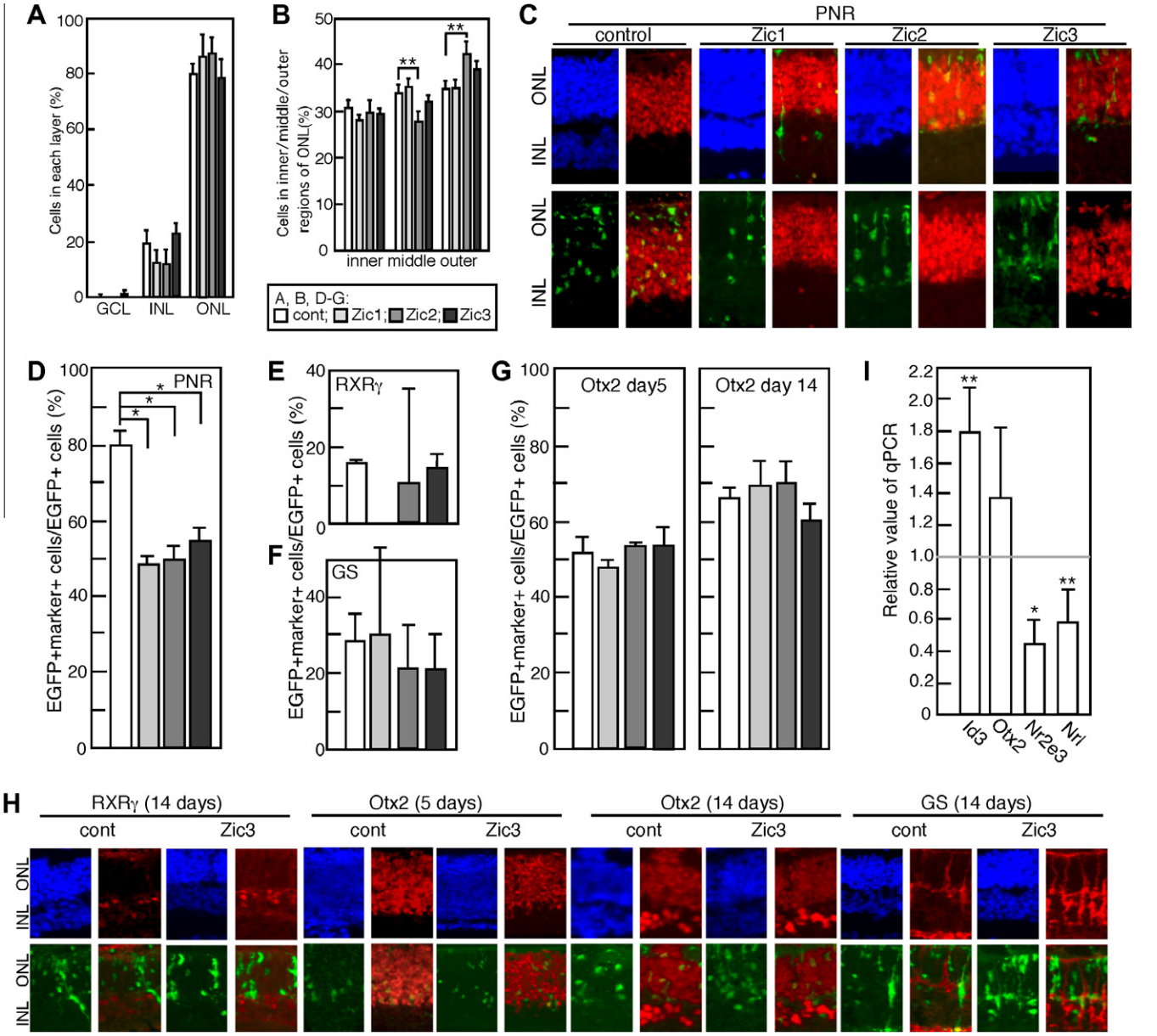

Fig. 2. Gain-of-function analysis of Zics in retinal progenitor cells. (A–H). Retrovirus encoding Zic1-, Zic2-, or Zic3-IRES-EGFP, or control EGFP was transduced into retinal explants at E17 and cultured for 2 weeks. Frozen sections were made and stained with antibody against EGFP in combination with antibodies anti various retinal subtype specific markers. Population of EGFP positive cells in retinal sub-layers (ONL, INL, and GCL) is shown in A. ONL region was divided horizontally into 3 regions, and EGFP positive cells in each sub-region are shown in B. (C–H). Population of EGFP and marker double-positive cells in EGFP positive cells is shown (D–G). Anti-EGFP and -PNR double immune-staining patterns are shown in C. Immunostaining pattern of Zic3 overexpressed samples are shown in H as representative results (H). Retrovirus encoding Zic2 was infected into retinal explants prepared from E17 eye, and the explants were cultured for 5 days. Cells were harvested, and RT-PCR was done (I). Values of qPCR are shown as relative values to control virus infected samples. The average of at least 5 (A–H) and 3 (I) independent experiments with standard deviation is shown. Cell number was calculated from more than 20 sections. *p* value; $^{**}<0.05$, $^{*}<0.01$, was calculated by Student's *t*-test.

3.3. Overexpression of Zic sustained proliferation activity of retinal progenitor cells

Since altered timing of exit from the proliferation phase of retinal progenitor cells often results in perturbation of differentiation [19,20], we next examined the effects of Zics in retinal cell proliferation by BrdU incorporation and expression of Ki67, which is a nuclear cell proliferation-associated antigen that is expressed during the active stages of the cell cycle [21]. Retroviruses encoding Zics-IRES-EGFP were infected into retinal progenitor cells at E15, and the infected retina was cultured as explants for 3 days, with BrdU present in the culture for the last 24 h. The explants were frozen and sectioned, and BrdU incorporation and Ki67 expression were examined by immunostaining (Fig. 3A and B). As shown in Fig. 3C, the incorporation of BrdU in control and Zic-overexpressing cells was comparable at day 3. Over the same days of culture, the expression of Ki67 was slightly higher in Zic-overexpressing cells (Fig. 3D). We then extended the period of culture to 5 and 7 days. At day 5, incorporation of BrdU in Zic1-overexpressing samples was higher than in controls, and values in Zic2 and Zic3 overexpressing cells were slightly higher, although not significantly (Fig. 3C). The number of Ki67 positive cells in Zic1-, Zic2-, or Zic3-overexpressing samples was significantly higher than in the control (Fig. 3D). At day 7, both BrdU incorporation and Ki67 expression were higher in Zic1-, Zic2-, and Zic3-overexpressing samples (Fig. 3C and D). Therefore, Zics may not enhance

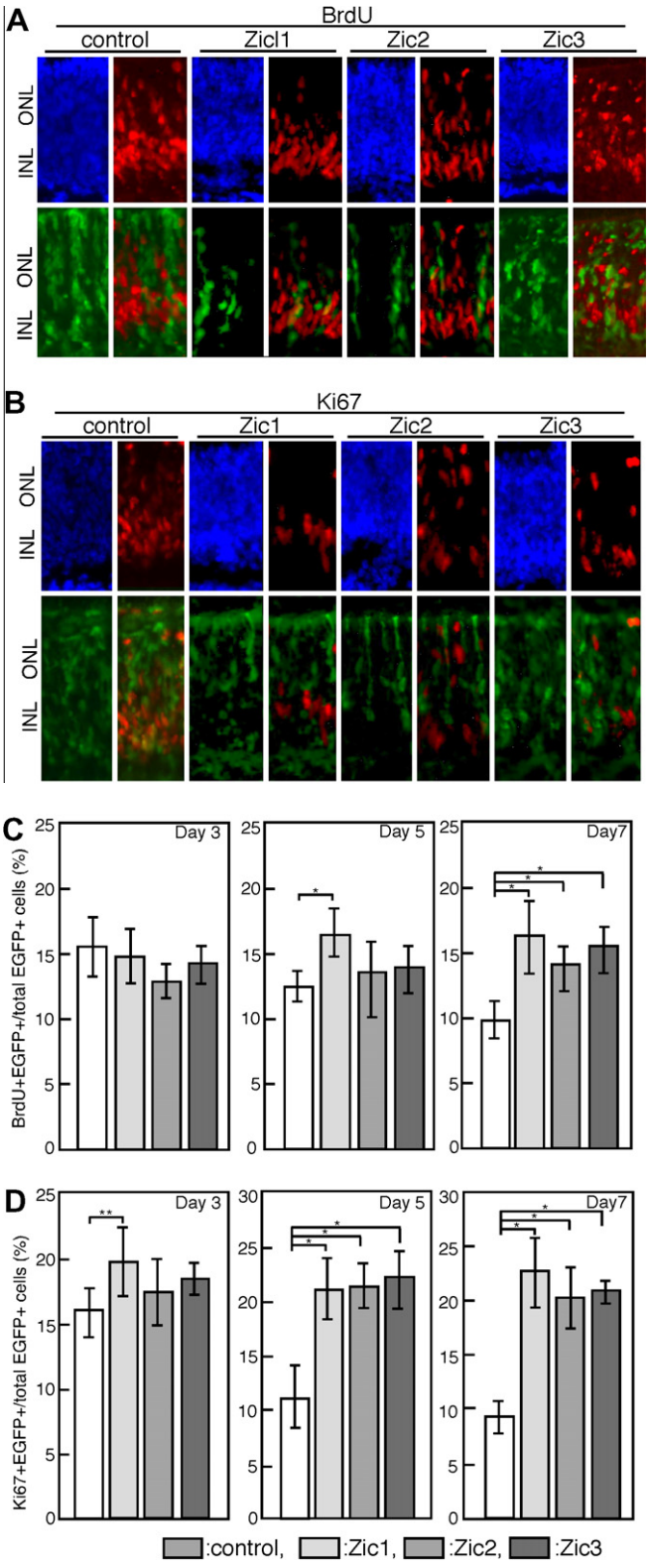

Fig. 3. Proliferation activities of Zics overexpressed cells. Retrovirus encoding Zic1, Zic2, or Zic3 followed by IRES-EGFP were infected into retina at E15, and retinas were cultured indicated days as explant cultures. BrdU was present at the last 24 h of cultures, and incorporation of BrdU and expression of Ki67 were examined by immunostaining of frozen sections. Immunostaining patterns are shown in A and B, and calculated results are shown in C and D. The average of more than 20 sections of 5 independent experiments with standard deviation is shown in C and D. $^{*}P < 0.01$, $^{**}p < 0.05$ was calculated by Student's *t*-test.

proliferation but sustain the proliferation activity of retinal progenitor cells.

We found that Zics 1, 2, and 3 have indistinguishable effects on retinal development (sustaining proliferation and modulating rod photoreceptor differentiation in the retina) when they are overexpressed in retinal progenitor cells. The expression domain of the Zics overlap in the developing retina [13]. In addition, our data suggest that continuous expression of Zics prevents final differentiation of rod photoreceptors. These results suggest that Zics play important roles in retinal development, but whether Zics are essential for retinal development need to be examined by triple inactivation of Zics in the retina.

Although Zic proteins enhance differentiation into neuroectoderm in the early phase of neural development, they inhibit further neuronal differentiation [10]. Zic proteins are present in immature neural cells, and their expression decreases during neuronal differentiation and increases again in mature neurons. Therefore, we speculate that the negative effect on neural maturation is a common feature of Zic in neurons and that the downregulation of Zics in the late phase of retinal development is purposeful. In neurons, shh inhibits Zic expression in the ventral neural tube [10]. In the retina, shh is expressed in ganglion cells in the early retina [22]. Ganglion cells start to differentiate from the central region of retina, and since Zic expression is in the peripheral retina, expression of Zics and shh appear to be complementary. Therefore, negative regulation of Zic by shh is also feasible in the retina.

We found that Inhibitor of DNA binding (Id) 3 was enhanced by the expression of Zic2. Id3 genes have similar expression patterns to Zics (Table 1); other Id genes are also expressed strongly in SSEA-1-positive cells, but their expression was sustained even in c-kit-negative cells. Therefore, Zics may exert their activities through Id gene expression. Zic1 misexpression leads to Notch expressing domain Hes1 enhancement [7]. In the retina, activation of Notch signaling leads to glial differentiation, but no such phenotype was observed, suggesting at least at E14-17, Zics do not induce Notch signaling. To identify targets of Zics, the next step is to reveal the functions of Zics by loss of function analysis of family genes in retinal development.

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