Transplantation of Neuro2a Cells into the Developing Postnatal Mouse Eye

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The present study aimed to investigate the influence of the host retinal microenvironment on cell migration and differentiation using Neuro2a (N2a) cells transduced with green fluorescent protein. N2a cells were transplanted into the vitreous cavities of developing mouse eyes (*C57BL/6*) on postnatal days 1, 5, and 10 (P1, 5, and 10). To analyze the effects of the host microenvironment on neural differentiation of N2a cells *in vitro*, cells were treated with a conditioned medium (CM) collected from retinal cells cultured at each developmental stage. We observed that numerous cells transplanted into P5 mice eyes migrated into all layers of the host retina, and the presence of processes indicated morphological differentiation. Some transplanted N2a cells expressed several neural markers. However, cells transplanted into the P1 and 10 mice eyes only proliferated within the vitreous cavity. Neurite length increased in N2a cells treated with CM collected from the cultured retinal cells from P5 and 10 mice, while western blotting revealed that the levels of proteins related to neural differentiation were not significantly altered in N2a cells treated with CM. We show that the migration and differentiation capacities of transplanted cells were differentially influenced by the microenvironment of the retinal postnatal ontogeny.

Key words: Neuro2a cell, postnatal retina, transplantation, differentiation, migration

I. Introduction

In transplantation, the microenvironment of host retinas is a key issue for cell differentiation and migration [18]. Cytokines and factors expressed during neurogenesis play a role in determining cell fate and differentiation through various signaling pathways. Therefore, it is particularly important to maximize the effectiveness of transplantation while considering these effects [11]. Indeed, the host microenvironment in the developmental stage encourages the transplanted cells to integrate and differentiate better into the host retina [15, 23, 24, 31, 36].

Retinal neurogenesis mainly occurs during the embryonic and perinatal stages in mammals; however, neurogenesis continues in the postnatal retina of many mammalian species. Postnatal retinal neurogenesis is a complex, finely tuned, and multi-step developmental process that regulates proliferation, migration, morphological and molecular differentiation, and neural fate specification to the distinct retinal cell types [19, 35].

Although we transplanted stem cells into the eyes of developing mice in our previous study, the ability of cells to migrate into the retina and differentiate into retinal cells was restricted [15, 36]. Because cellular or extracellular matrix components of the inner limiting membrane hindered cell migration after intravitreal cell transplantation, the transplanted mesenchymal stem cells (MSCs) and adipose-derived stromal cells (ADSCs) were unable to migrate and integrate within the host tissue. Overcoming this migration limitation would require a dramatic ability for cell migration itself. We therefore investigated the effectiveness of transplantation using Neuro2a (N2a) cells

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(a murine neuroblastoma cell line). A large number of N2a cells proliferate under standard culture conditions, and these cells can differentiate into specific neural cells, such as dopaminergic neurons or neurons possessing dendrite-like processes, under specific conditions [5, 29, 33, 34]. N2a cells are therefore widely used as models for studying neural differentiation and its mechanisms *in vitro*. Although N2a cells are not directly applicable to cell grafting for safety reasons, we were only interested in the potential abilities for high proliferation, migration, and differentiation of N2a cells.

In the present study, we evaluated the potential of intraocularly grafted N2a cells to migrate into the developing mouse retina and differentiate into various retinal cell types, and identified a suitable developmental micro-environment for postnatal transplantation. Furthermore, to confirm the changes of specific factors related to neural differentiation within the microenvironment of the developing postnatal mouse eye for N2a cell transplantation, we performed a growth factor analysis in N2a cells using conditioned medium (CM) obtained from cultured postnatal mouse retinal cells. The present study demonstrates that differentiation of N2a cells is affected by the host micro-environment, which is adjusted differently in each developmental stage of the retina.

N2a cells must behave very differently from stem cells and precursor cells generally used for cell transplantation/replacement approaches. Although N2a cells are inapplicable to the current cellular treatment models, we determined the efficient stage for cell grafting using developing postnatal mice. The results of this study reaffirmed similar results reported in previous studies using stem cells. Here, we demonstrate that the developmental stage of the retinal host microenvironment is critical for cell transplantation in mice.

II.   Materials and Methods

Animals

In our experiments, we used *C57BL/6* mice kept under a 14/10 hr light/dark cycle without distinction of sex. The date of birth was designated as postnatal day 1 (P1), and pups were used at P1, 5, and 10. All animal experiments were performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO), and were approved by the committee of Kyungpook National University.

N2a cell culture and labeling

N2a cells (Cat# CCL-131, American Type Culture Collection, Manassas, Virginia, USA) were cultured in Dulbecco's Modified Eagle Medium (Gibco, Frederick, Maryland, USA), 10% fetal bovine serum (Invitrogen, Frederick, Maryland, USA), and 1% Antibiotic-Antimycotic (Gibco). To chase the transplanted cells, we labeled N2a cells using the Lenti-hCMV-GFP-IRES-Puro (Macrogen

Inc., Seoul, Korea). N2a cells were transduced with a stock of lenti-virus at a multiplicity of infection (MOI) of 1:20.

Transplantation of N2a cells into the developing mouse eye

Centrifuged N2a cells were resuspended in Earle's Balanced Salt Solution (Invitrogen). Pups of ages P1, 5, and 10 were anesthetized individually with ethyl ether and then received transplantation of N2a cells. Approximately 1 μl of cell suspension (~50,000 cells/μl) was slowly injected into the vitreous cavity using a 30-gauge Hamilton syringe (Hamilton Co., Reno, Nevada, USA), and animals were monitored daily after the procedure. Mice were sacrificed after 7, 14, and 28 days post-transplantation (DPT), and their tissues were analyzed using immunohistochemistry.

Tissue processing and immunohistochemistry

After an appropriate survival period, the heads of the pups or the eyes of young mice were removed, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), and then cryo-protected in a series of 10, 20, and 30% sucrose in 0.1 M PB. Tissue was embedded in Tissue-Tek O.C.T. compound (VWR International, West Chester, Pennsylvania, USA), frozen at −80°C, and sectioned coronally at a thickness of 20 μm using a microtome cryostat HM 525 (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA). For immunohistochemistry, we used the following primary antibodies: mouse anti-microtubule associated protein 2ab (MAP2ab; mature neuronal cell marker, 1:200, Sigma-Aldrich, St. Louis, Missouri, USA), rabbit anti-glial fibrillary acidic protein (GFAP; glial cell marker, 1:200, DakoCytomation, Glostrup, Copenhagen, Denmark), rabbit anti-calbindin D28K (CB; horizontal and amacrine cell marker, 1:200, Sigma-Aldrich), rabbit anti-calretinin (CR; amacrine and ganglion cell marker, 1:200, Millipore, Bedford, Massachusetts, USA), and chicken anti-green fluorescent protein antibody (GFP; to increase fluorescence intensity, 1:200, Abcam, Cambridge, Massachusetts, USA). We used the following secondary antibodies: Cy3-conjugated donkey anti-mouse and anti-rabbit (1:200, Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-chicken (1:200, Jackson Immuno-Research Laboratories, Inc.). Finally, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:500, Invitrogen). Labeled tissues were coverslipped with Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, California, USA). Negative controls were prepared in parallel during all immunohistochemical experiments by omitting the primary or secondary antibodies. No antibody labeling was observed in the control experiment. Fluorescent labeling was examined and photographed using a Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss Meditec, Inc., Jena, Germany).

Preparation of primary retinal cell CM

To prepare mouse primary retinal cell cultures, the

eyes were collected from P1, 5, and 10 *C57BL/6* mice. The retinas were isolated and placed in Hanks' Balanced Salt Solution (Gibco) with 1% Antibiotic-Antimycotic. They were then dissociated with papain (Worthington, Lakewood, New Jersey, USA) according to the instructions of the manufacturer. Mouse retinal cells, including both neurons and glial cells, were seeded at a density of 3.5×10^6 cells/ml in culture dishes coated with poly-D-lysine (10 mg/ml, Sigma) and laminin (2 μg/ml, Sigma). Mouse retinal cells were incubated in Neurobasal-A Medium (Gibco) supplemented with 1% ITS (insulin-transferrin-sodium selenite media supplement, Sigma-Aldrich), 2% B27 (Gibco), 50 ng/ml brain-derived neurotrophic factor (BDNF; PeproTech, Rocky Hill, New Jersey, USA), 10 ng/ml ciliary neurotrophic factor (CNTF; Life technologies, Frederick, Maryland, USA), 10 ng/ml forskolin (Sigma-Aldrich), and 1% Antibiotic-Antimycotic. To prepare the CM, the medium including secreted factors was harvested twice at an interval of 24 hr. N2a cells were seeded and incubated for 3 hr to allow attachment and stabilization. The culture medium of N2a cells was then replaced with the CM, and cells were incubated for 12 and 24 hr.

Measurement of neurite length

To investigate the effect of CM collected from the cultured postnatal mouse retinal cells at each specific developmental stage, differentiation of the N2a cells was investigated by measuring the total neurite length of the N2a cells. Treated N2a cells (1×10^5 cells per dish) were fixed with 4% paraformaldehyde for immunocytochemistry. To visualize the neurites of the cells, primary mouse anti-neuron-specific beta-III tubulin (TuJ1; R&D Systems, Minneapolis, Minnesota, USA) and secondary Cy3-conjugated anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used. TuJ1-positive cells were examined using a Zeiss Axioplan microscope with a Zeiss Plan-Apochromat 40× objective and a Zeiss AxioCam HRc digital camera (Carl Zeiss Meditec, Inc.). The cells were viewed on a computer monitor, and the length of the TuJ1-positive neurites was measured from the soma to the dendritic tip. All visible neurites were measured regardless of their number, except those that were shorter than the diameter of the soma. The measurement was repeated three times independently using Image J software (US National Institute of Health, Bethesda, Maryland, USA).

Western blotting

N2a cells incubated in CM were suspended in RIPA lysis buffer with protease inhibitors. Equal amounts of proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to nitrocellulose membranes. After a blocking with 5% skim milk powder, membranes were incubated in the primary rabbit anti-transforming growth factor beta 2 (TGFβ2; Santa Cruz Biotechnology, Dallas, Texas, USA), and rabbit anti-fibroblast growth factor-2 (FGF-2; Abcam) antibodies. To ensure equal protein loading, blots were probed with mouse anti-β-actin antibody (Santa Cruz Biotechnology). The primary antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology, Danvers, Massachusetts, USA). We revealed the HRP signal using enhanced chemiluminescence (ECL kit; Elpis-Biotechnology, Inc., Daejeon, Korea) according to the instructions provided by the manufacturer. Densitometry for bands on western blots was determined by Image J software.

Statistical analysis

Significant differences between groups were determined using a one-way ANOVA via the Statistical Package for the Social Sciences (SPSS; International Business Machines Corporation, Armonk, New York, USA). A value of $p<0.05$ was considered statistically significant.

III. Results

To investigate the ability of N2a cells to survive, migrate, and differentiate in the developing retina, we transplanted N2a cells into the vitreous cavity of the postnatal mouse eye. Hosts at these postnatal ages were selected based on previous studies that investigated the developmental stages that are suitable for cell survival and differentiation using mouse and rat neural progenitor cells (NPCs) for transplantation into the postnatal Brazilian opossum (*Monodelphis domestica*) eye [23, 24, 31]. Based on cellular differentiation and lamination patterns, P1, 5, and 10 mice developmentally corresponded to P12–14, 20–25, and 34–35 of the Brazilian opossum, respectively [9].

N2a cells transplanted into the P5 mouse eye

Figure 1 shows the viability of transplanted N2a cells in P5 mice retinas on 7 DPT. As shown in Figure 1, the transplanted N2a cells migrated into the several layers of the host retina. As the injected cells actively proliferated, the vitreous cavity was filled with GFP-expressing N2a cells, thereby making the boundary between the ganglion cell layer (GCL) and vitreous body (VB) indiscriminately defined (Fig. 1A and C). To distinguish between the GCL and VB, we additionally stained the inner limiting membrane using antibody against GFAP. Labeling of GFAP is restricted to the Müller cells located at the inner limiting membrane of the retina of the wild-type mouse [27]. In addition, the nuclei sizes of the injected cells were bigger than those of host retinal cells, which is characteristic of neuroblastoma cells. In the Figure 1A and B, normal retina was labeled with the GFAP to mark the inner limiting membrane. We also found the gap between the GCL and VB in DAPI stained images, and this gap matched up to the inner limiting membrane labeled with GFAP (Fig. 1C and D). Surprisingly, numerous transplanted N2a cells migrated spontaneously into the GCL, inner nuclear layer (INL), and
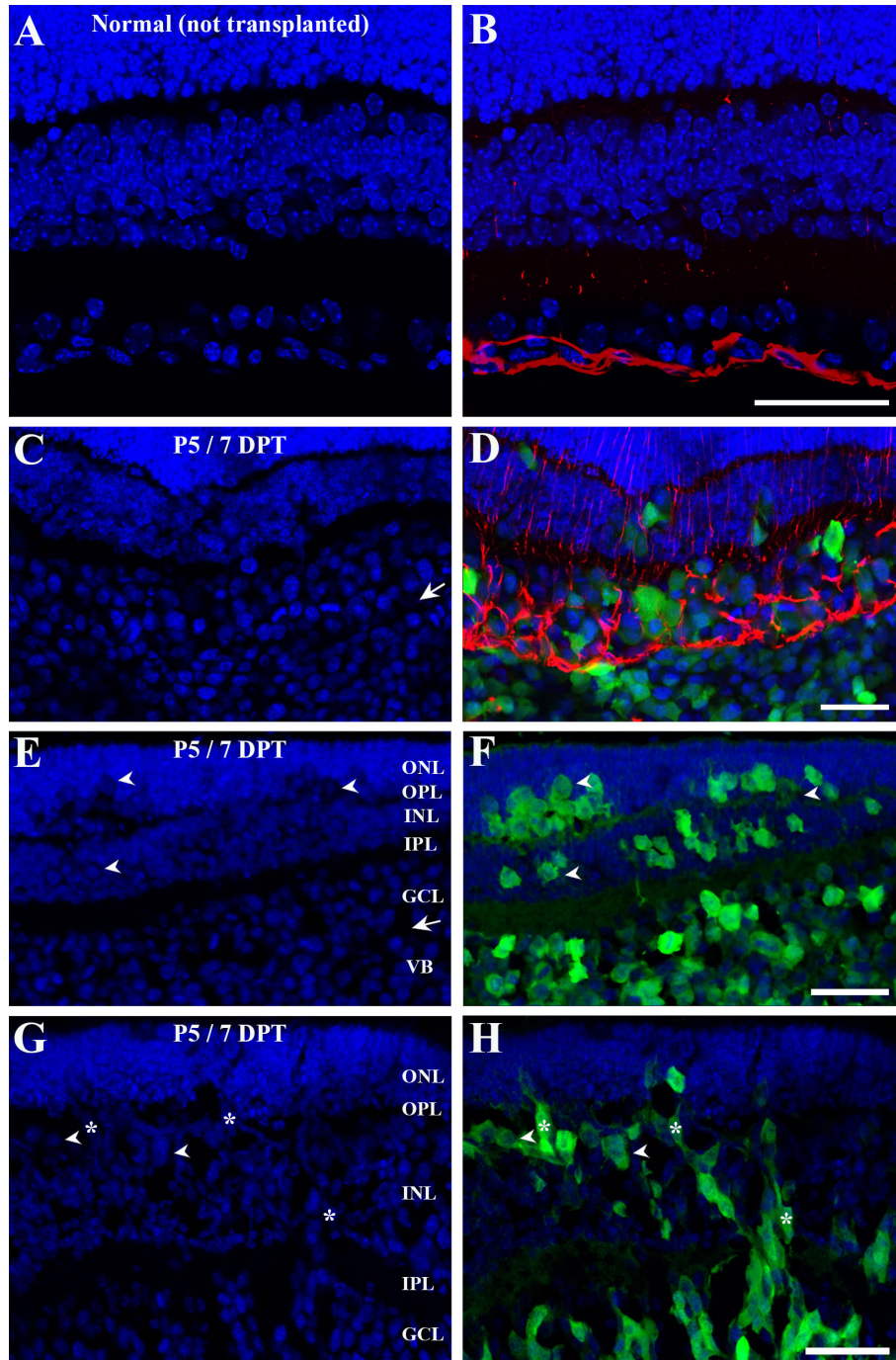
Fig. 1. Fluorescence images showing the migration of N2a cells transplanted into the VB of P5 mice on 7 DPT. (**A, B**) Fluorescence image of normal retina showing the nuclei stained with DAPI (blue), and merged image showing colocalization of DAPI (blue) with GFAP staining (red). These figures show that GFAP was immunoreactive for the inner limiting membrane in normal retina. (**C**) Fluorescence image of P5 mice retinal section on 7 DPT showing the gap between the GCL and VB (arrow). (**D**) Merged image showing that the gap marked in (**C**) match up to the GFAP labeling (red). This image shows the colocalization of GFAP immunoreactivity (red), GFP-expressing grafted cells (green), and DAPI (blue). (**E, F**) Transplanted cells migrated into the GCL, INL, and even into the ONL of the host retina. (**G, H**) Transplanted cells migrated into the GCL and INL of the host retina. Arrowheads indicate the cell bodies of GFP-expressing N2a cells. Morphologically differentiated transplanted cells with some neurites are indicated with asterisks. DPT, day post-transplantation; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; P, postnatal day; VB, vitreous body. Bar=50 μm.
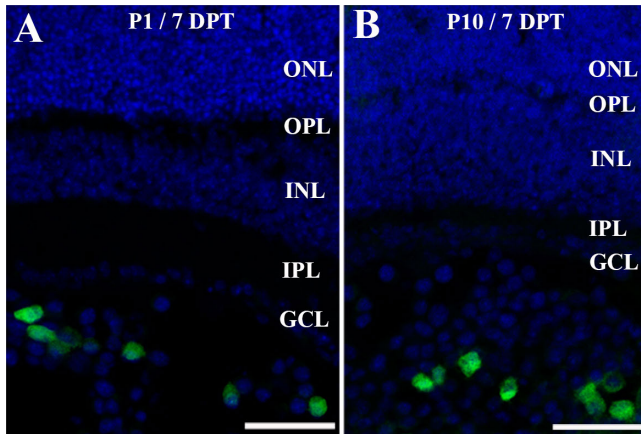
Fig. 2. Fluorescence images of N2a cells transplanted into the vitreous cavity of the developing eye of P1 and 10 mice. On 7 DPT, GFP-expressing N2a cells were only observed within the vitreous cavity of P1 (**A**) and 10 (**B**) mice. DPT, day post-transplantation; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; P, postnatal day. Bar=50 μm.

even into the outer nuclear layer (ONL) in the retinas of P5 mice (Fig. 1E and F). Neural processes or dendrites of some migrated cells were also observed in the INL (Fig. 1G and H). However, these cells did not seem to morphologically differentiate into typical retinal cells such as amacrine, bipolar, and horizontal cells. Two weeks after transplantation, all layers of the host retina were histologically disrupted by the actively proliferating N2a cells (data not shown).

N2a cells transplanted into P1 and 10 mouse eyes

One week after transplantation, the transplanted N2a cells were unambiguously identified by GFP expression. Figure 2 shows that transplanted N2a cells survived in P1 (Fig. 2A) and 10 mouse eyes (Fig. 2B). As can be seen from Figure 2, transplanted N2a cells were generally observed within the vitreous cavity without any migration into the layers of the host retina, and morphological differentiation such as neurite development was not visible.

Molecular differentiation of transplanted N2a cells

One week after transplantation, antibodies against MAP2ab, CB, CR, and GFAP were used to determine whether the transplanted N2a cells were immunopositive for well-known markers of retinal cell types following an induction by the retinal microenvironment. We observed that some transplanted N2a cells seemed to be neuronal and glial in morphology. In P5 mouse retinas, a subpopulation of N2a cells that migrated into the retina clearly expressed MAP2ab (Fig. 3A–C) and GFAP (Fig. 3J–L), which are known as markers for mature neuronal cells and glial cells, respectively. MAP2ab was clearly expressed within the cytoplasm, whereas it was lightly expressed within the nuclei and dendrites [14, 25, 26]. On the other hand, CB,

which is a marker for horizontal and amacrine cells (Fig. 3D–F) and CR, which is a marker for amacrine and ganglion cells (Fig. 3G–I), were not expressed in grafted GFP-expressing N2a cells. These results indicate that transplanted cells failed into differentiate to these types of retinal cells.

Effect of host microenvironment on the retinas of P5 mice

We predicted that the microenvironment of the P5 mouse eye might be advantageous than that of other stages for differentiation and migration of N2a cells into neural cells. To confirm the capacity of N2a cells for neural differentiation induced by the retinal microenvironment at a specific developmental stage, we measured the total neurite length of N2a cells treated with CM derived from each developmental stage of cultured retinal cells. Our findings are illustrated in Figure 4. After 12 and 24 hr of treatment, N2a cells treated with CM from cultured retinal cells from P5 and 10 mice grew significantly longer neurites than those from P1 mice. However, CM obtained from the retinal cells of P5 and 10 mice induced a similar neurite outgrowth in N2a cells *in vitro*. Significances between groups at the neurites of N2a cells treated for 12 hr were similar to those for 24 hr.

To investigate the effect of CM on N2a cells, we analyzed the level of two growth factors, TGFβ2 and FGF-2, which affect neural differentiation. TGFβ2 has been known to play a key role in multiple developmental processes, neuron survival and differentiation [13]. FGF-2 has also been shown to stimulate the division of astrocytes, and neural differentiation [10, 20]. To examine whether the factors secreted by cultured retinal cells change TGFβ2 and FGF-2 production, N2a cells were cultured with CM derived from each developmental stage of cultured retinal cells. We analyzed the expression levels of these growth factors in N2a cells treated with CM for 12 and 24 hr, and compared them with the primary retinal cell culture medium (Control) and normal medium for N2a cells (Negative) (Fig. 5). The levels of TGFβ2 and FGF-2 were not significantly altered in N2a cells treated with CM collected at all developmental stages after treatment for 12 and 24 hr. Therefore, the retinas from P5 mice, which were at a developmental stage that provides a suitable microenvironment for N2a cell transplantation, did not induce the production of TGFβ2 and FGF-2 in N2a cells *in vitro*.

IV. Discussion

The objective of this study was to investigate the potential of grafted N2a cells to migrate and differentiate into various retinal cell types in the developing mouse retina. Our findings demonstrate that the intraocularly injected N2a cells were localized in the vitreous chamber and several distinct retinal layers. It is interesting to note that the transplanted N2a cells migrated better into the retinal layers of P5 mice than in that of P1 and 10 mice. These
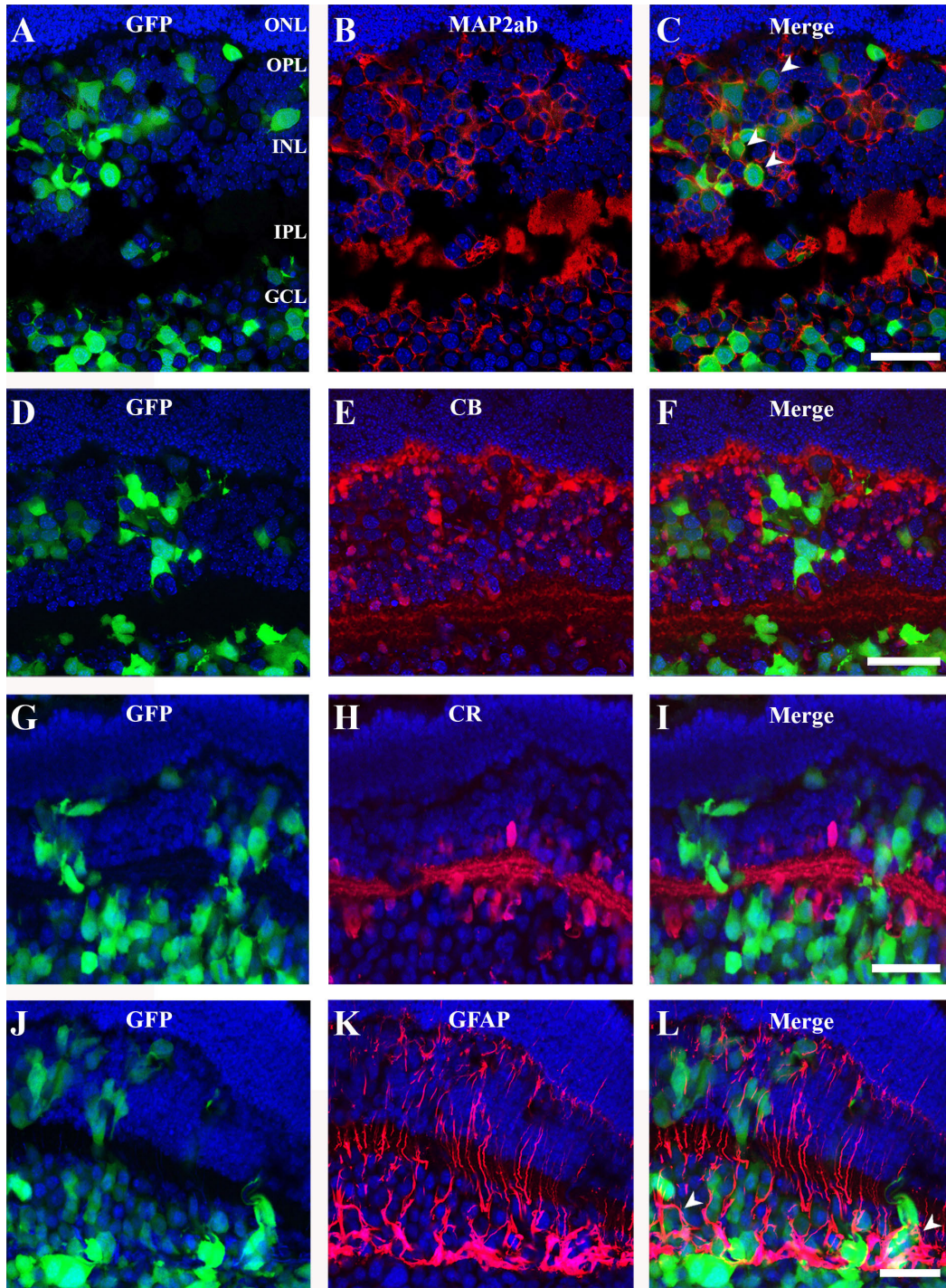
Fig. 3. Molecular differentiation of transplanted N2a cells. N2a cells grafted at P5 7 DPT into the host retina: (**A, D, G, J**) Fluorescence images of retinal sections showing the nuclei stained with DAPI (blue) and the GFP-expressing grafted N2a cells (green). (**B, E, H, K**) Grafted cells and the host retina labeled with specific primary antibodies, secondary antibodies coupled to Cy3 (red), and DAPI (blue). (**C, F, I, L**) Merged images showing the colocalization of N2a cells (green), various molecular markers (red), and DAPI staining (blue). (**A–C**) The grafted N2a cells migrated into the GCL and INL. Sections were immunolabeled with an anti-MAP2ab antibody to identify mature neurons. (**D–F**) The grafted N2a cells migrated into the GCL and INL. These cells were not immunopositive for CB, a marker for horizontal and amacrine cells. (**G–I**) The grafted N2a cells were not labeled with the anti-CR antibody, which showed that they did not differentiate into amacrine and ganglion cells. (**J–K**) The grafted N2a cells migrated into the GCL and INL, and were labeled with GFAP, a marker for glial cells. Arrowheads indicate the GFP-expressing cells that coexpressed MAP2ab (**C**) or GFAP (**L**). CB, calbindin D28K; CR, calretinin; DPT, day post-transplantation; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; IPL, inner plexiform layer; MAP2ab, microtubule associated protein 2ab; ONL, outer nuclear layer; OPL, outer plexiform layer; P, postnatal day. Bar=50 µm.
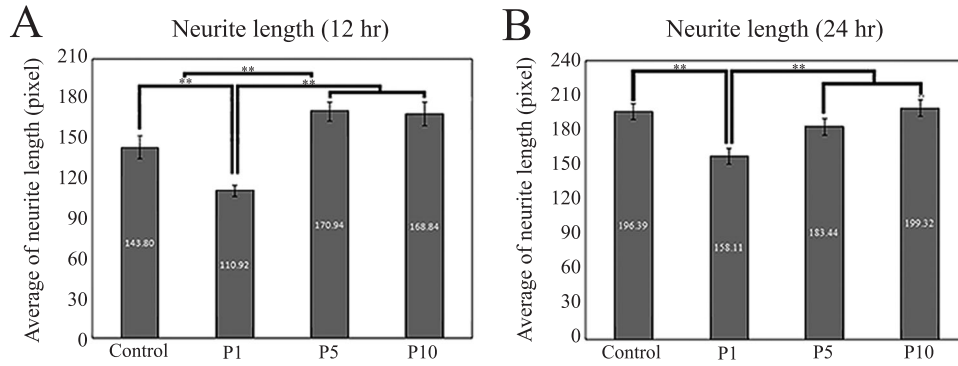
Fig. 4. Neurite length of N2a cells treated with control and CM collected from the host retinas of P1, 5, and 10 mice. The length of the neurites was measured using the Image J software. N2a cells treated with CM from the cultured retinal cells from P5 and 10 mice grew significantly longer neurites compared with those from P1 mice. There was a statistically significant difference among the group means as determined by a one-way ANOVA test (**$p<0.05$). CM, conditioned medium; P, postnatal day.
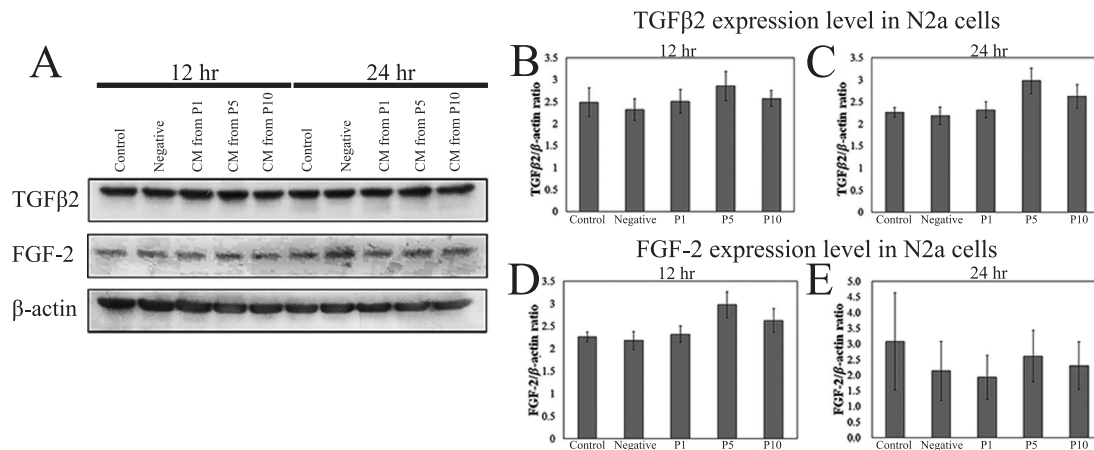

Fig. 5. Expression levels of TGFβ2 and FGF-2 in N2a cells incubated in CM. Equal amounts of N2a cell lysates (20 μg) were subjected to electrophoresis, and were analyzed by Western blotting using anti-TGFβ2 (upper part of **A**) and FGF-2 antibodies (middle part of **A**); β-actin was used as a loading control (lower part of **A**). The intensity of protein band of TGFβ2 or FGF-2 in N2a cells was assessed by densitometry. Intensity of TGFβ2 or FGF-2 bands was normalized by intensity of β-actin band (**B–E**). Protein levels of TGFβ2 and FGF-2 were not significantly altered in N2a cells after 12 (**B, D**) and 24 hr (**C, E**) incubation in the CM collected from cultured developing mouse retinal cells. Protein levels of each group were compared with the primary retinal cell culture medium (Control) and normal medium for N2a cells (Negative). There was not a statistically significant difference among the group means as determined by a one-way ANOVA test ($p<0.05$). CM, conditioned medium; FGF-2, fibroblast growth factor-2; P, postnatal day; TGFβ2, transforming growth factor beta 2.

results are in agreement with our previous studies on MSCs and ADSCs transplanted into the developing mouse retina [15, 36]. According to our previous studies, the retinas of P5 mice allowed a better morphological and molecular differentiation of migrated MSCs and ADSCs than retinas of P1 and 10 mice.

Neurogenesis in the mouse retina is a relatively fixed temporal sequence of events during which distinct cell types are produced from retinal progenitors. In the embryonic period, retinal ganglion cells differentiate first, followed by the cone photoreceptors, horizontal cells, and amacrine cells. Rod photoreceptors, bipolar cells, and Müller glia cells differentiate in the early postnatal period [1, 19, 35]. Zhang and colleagues identified gaps in gene expression patterns using microarray experiments and thus divided retinogenesis into the developmental phase (from E

12.5 to P5) and functional phase (from P7) [37]. Two developmental groups were clearly divided with transition stage P5. At transition stage, the starting point of outer plexiform layer formation and the peak of Müller glia genesis have been observed in the mouse retina [8, 37]. We assumed that the microenvironment at P5, which is a day close to the transition stage, might be effective for the migration and differentiation of transplanted cells. The potential of transplanted N2a cells may be influenced by several genes expressed in the transition stage.

Previous studies on stem cell transplantation revealed that intraocularly injected cerebral or retinal progenitor cells integrated into several layers of the retina with a morphological differentiation in the developmental stage [23, 24]. Particularly, intraocularly transplanted murine cerebral progenitor cells differentiated into molecularly distinct reti-

nal cells that contained MAP2, CR, and recoverin, which are neural markers [31]. In comparison with other stem cells, the transplanted bone marrow derived MSCs were positive for neuronal and glial markers, and they did not migrate into the GCL [15]. In the present study, the transplanted N2a cells were positive for MAP2ab and GFAP and migrated into several layers of the retina. However, transplanted cells were not labeled with specific retinal cell markers such as CB and CR. These results show that N2a cells failed to differentiate into distinct retinal neurons. The results of this study are in agreement with earlier experimental stem cell studies. Transplanted retinal stem cells were negative for markers of specific retinal cells (e.g., CB and recoverin) even though some of these cells had a morphology resembling the specific retinal cells in degenerating retinas [3]. Therefore, we concluded that N2a cells are capable of migrating into several layers of the retina, albeit with a restricted capacity for differentiation into distinct retinal cells. However, N2a cells might be able to morphologically and molecularly differentiate into distinct retinal neurons if the retinal microenvironment provides appropriate stimulation.

Although N2a cells transplanted into P10 mice eye did not migrate into host retina and differentiate into the neuronal cells, CM obtained from the P10 retinal cells increased the neurite length of N2a cell in vitro. Besides, the neurite length of N2a cell induced by CM obtained from the retinal cells of P5 and 10 mice were similar to each other. Although their capacity to grow a neurite is not sufficient to directly judge their neural differentiation capacity, the neurite length measurement is not only a key method to quantify differentiation but also mainly used for assessing neurotrophic activity. Our data have shown that the capacity of N2a cells for growth of neurites in P5 and 10 mice was clearly similar *in vitro*, however, the capacity for differentiation were significantly different *in vivo*. As N2a cells in P10 mice did not migrate and those in P5 mice migrated into retina, the capacity of differentiation may be influenced by migration into retina and interaction with retina.

In this study, we demonstrated the migration and differentiation properties of N2a cells transplanted into the eyes of P5 mice. We predicted that these properties would be ascribable to the developing retinal microenvironment. Retinal cell fate specification is regulated and modulated by numerous transcription factors. In addition, several secreted factors, including neurotrophins and growth factors, are involved in the commitment and maturation of specific retinal cells. TGF, FGF, epidermal growth factor, and leukemia inhibitory factor regulate the production of specific retinal cell types [17]. In addition, several factors are neuroprotective in the developing mammalian retina and in a retinal degeneration model [12]. Intravitreal injection of BDNF reduced retinal thinning and protected the cells in the INL in an induced retinal ischemia model [30]. In addition, administration of nitric oxide induced neuronal death in retinal ganglion cells, which was rescued by BDNF [28].

Exogenous administration of nerve growth factor (NGF) in Royal College of Surgeons rats has been implicated in the enhancement of photoreceptor survival through modulated expression levels of other survival factors such as BDNF, bFGF and, TGFβ2 [16]. In order to determine whether the secreted factors in the postnatal retinal microenvironment are effective for the differentiation of N2a cells, we collected the CM by dissociating retinal neurons from postnatal retinas, and subsequently treated the N2a cells with the CM *in vitro*. However, the factors secreted by the retinal cells of P5 mice may not be responsible for neural differentiation, such as the neurite outgrowth of N2a cells. We investigated the protein expression of two growth factors in N2a cells in order to identify the direct factors targeting the differentiation of N2a cells treated with CM from the retinas of P5 mice. Among several secreted factors, we focused on TGFβ2 and FGF-2, which promote the survival and differentiation of retinal cells [4, 12, 22, 32]. TGFβ2 signaling has a neuroprotective effect in the retina, modulates the cell death of progenitors during the development of the vertebrate retina and regulates retinal thickness [2, 6]. On the other hand, FGFs are neurotropic factors that promote neuronal and glial cell maturation, retinal differentiation, and rescue the photoreceptors and neural cells from programmed or surgically induced cell death. Particularly, FGF-2 is implicated in the regression of retinal damage and maintenance of retinal thickness and morphology [7, 21]. We therefor postulated that TGFβ2 and FGF-2 may induce neural differentiation in N2a cells, and may be neuroprotective for N2a cells in the host retina. However, transplanted N2a cells in P5 retinas, considered as a stage suitable for efficient transplantation, did not induce the production of TGFβ2 and FGF-2 in N2a cells *in vitro*. Although we could not find out key factors involved directly in stimulating cell differentiation, our results indicate that the differentiation and migration of N2a cells were not directly induced by TGFβ2 and FGF-2.

In the present study, we demonstrated that the migration and differentiation capacities of transplanted cells are differentially influenced by the microenvironment of retinal postnatal ontogeny. Our study clearly shows that transplanted N2a cells can migrate into the retinal layers, and that transplantation into the retinas of P5 mice was more effective than that into the retinas of P1 and 10 mice. However, levels of TGFβ2 and FGF-2 were not significantly altered in N2a cells after treatment with CM collected from cultured mouse retinas at each developmental stage. Further studies are required to identify the molecular mechanisms responsible for the induction of migration and differentiation in transplanted cells in order to develop effective therapeutic conditions for retinal cell transplantation.

V. Acknowledgments

VI.   References

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