Induction of Retinal Progenitors and Neurons from Mammalian Müller Glia under Defined Conditions*

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Background: Mammalian Müller glia are mitotic quiescent and committed. Loss of p53 enhances Müller glia to proliferate and become progenitor-like cells, which differentiated to photoreceptors in vitro and incorporated into retina after transplantation. Progenitor potential can be induced in mammalian Müller glia.

Significance: Induction of Müller glia stemness may serve as an exciting strategy for retinal repair and regeneration.

Vision impairment caused by loss of retinal neurons affects millions of people worldwide, and currently, there is no effective treatment. Müller glia of mammalian retina may represent an under-recognized and potential source for regeneration of a wide range of retinal cell types, including retinal ganglion cells and photoreceptors. Here, we demonstrated that mouse Müller glia cells have the capacity to be reprogrammed into the retinal neuronal cell fate and are competent to give rise to photoreceptors under a defined culture condition. Inactivation of p53 released proliferation restriction of Müller glia and significantly enhanced the induction of retinal progenitor from Müller glia in culture. Moreover, following the ocular transplantation, the Müller glia-derived progenitors were differentiated toward the fates of photoreceptors and retinal ganglion cells. Together, these results demonstrate the feasibility of using Müller glia as a potential source for retinal repair and regeneration.

The Culture of Retinal Progenitor Cells from Müller Glia—Eyeballs were enucleated from P6 mice, and the retinas were dissected free in DMEM. The retinas were digested with Papain dissociation system following the manufacturer’s instructions (dissociation solution, DMEM/F12 containing 16.5 units/ml activated papain ( Worthington, Lakewood, NJ) and 124 units/ml DNase (Sigma-Aldrich) at 37 °C for 15 min and maintained in stationary culture in 10% serum supplemented

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FIGURE 1. The phase contrast imaging of wt-MGCs (passage 1; A) and p53−/− MGCs (passage 3; B) before induction and their corresponding progenitor-like cells after induction for 6 days (C and D) are shown. Spindle morphology was observed in p53−/− cells (D), in comparison with the cells maintained in regular Müller glia culture condition (B). E, under the progenitor induction condition, the cell doubling time was ~64 and 35 h, respectively, for wild type and p53−/− Müller glia cells. Scale bars, 100 μm.

DMEM and F12 (1:1). The removal of aggregates and cellular debris after 6–7 days yielded purified Müller glial cell cultures, which could be maintained or passaged in DMEM/F12 with 20 ng/ml GGF, 20 ng/ml EGF, N2, and 0.2% BSA. For the induction of retinal progenitors from cultured Müller glial cells, the cells were grown in retinal progenitor induction medium, which is composed of DMEM/F12, 5% knock-out serum replacement, N2, 5 mM glutamine, 2 mM nicotinamide, 11.25 μg/ml linoleic acid, 1 μM Y27632, and supplemented with 20 ng/ml basic fibroblast growth factor, 20 ng/ml EGF; 10 ng/ml leukemia inhibitory factor. The confluent monolayer culture was dissociated with StemPro Accutase (Invitrogen) and can be passaged onto Matrigel-coated (BD Biosciences) plates.

The Photoreceptor Differentiation—Müller glia-derived retinal progenitors (MRPs)3 were cultured in photoreceptor differentiation medium (DMEM/F12:Neurobasal medium 1:1, N2, B27, 0.05% BSA fraction V; 2 mM Glutamax, 50 mM docusa-hexanoic acid, and supplemented with 1 μM IWP2, 10 μM N-[3,5-difluorophenyl]acetyl]-l-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester, 100 nM purmorphine, 100 nM retinoic acid, 100 μM taurine, 10 ng/ml basic fibroblast growth factor) for 2 weeks.

RNA Isolation, Reverse Transcription, and Quantitative PCR Analysis—Total cellular RNA was isolated from cells using the RNeasy kit (Qiagen), and cDNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad), both following the manufacturer’s instructions. The transcripts were amplified for 40 cycles using Power SYBR Green PCR Master Mix on 7500 Real Time PCR System (Applied Biosystems). Measurements were performed in triplicates and normalized to GAPDH levels. Gene-specific primer sequences were used as follows: Pax6, 5′-CTGAGGAACAGAGAAGACAGG-3′ (forward) and 5′-CTGGAGAGGTGATGTAAGGAG-3′ (reverse); Chx10, 5′-CCACCTTCTTGGAGAGTGTG-3′ (forward) and 5′-AGTCACTGGAGGAGGTAGTCG-3′ (reverse); Pax2, 5′-CAGCCTTTCACACCAAGC-3′ (forward) and 5′-GTGGCCG-CGTAGGCAGCAG-3′ (reverse); GFAP, 5′-CACCTACAGGGAAATTGCTGGAGG-3′ (forward) and 5′-CCACGATGTTCCTCTTTGAGTG-3′ (reverse); vimentin, 5′-CGGAAAGTGGAATCTTGGTCA-3′ (forward) and 5′-CACATCGATGTTCACATGCTGT-3′ (reverse); Gadd45g, 5′-GGGAAAGACCTGCAGGA-3′ (forward) and 5′-AGCACGCAAAAGTCACATTG-3′ (reverse); glutamine synthetase 5′-CTGATGGAAGGCCCTGCTTCTGAC-3′ (forward) and 5′-CATGGAAGCATGTAAGCAGATCGC-3′ (forward) and 5′-TCCTCAGATGTCAGCAGCAGTCAGCTTG-3′ (reverse); Crx, 5′-TATGCACGTGGAGGTTGTGCT-3′ (forward) and 5′-TGTTCTGCAATTTCGCCCT-ACG-3′ (reverse); Nr2e3, 5′-GCCTTATACCCGGCCAAACATTG-3′ (forward) and 5′-CATGGATGGCATCCAGACTGCA-3′ (reverse); Pde6b, 5′-TGGAGAACAGCATAGACATCGC-3′ (forward) and 5′-TCCTCAGATGTCAGCAGCAGTCAGCTTG-3′ (reverse); arrestin, 5′-CTGTTGGAAGGTTGGCTCCACCCTCAAGTG-3′ (forward) and 5′-CCACCTTCTTGGAGAGTGTG-3′ (forward) and 5′-AGTCACTGGAGGAGGTAGTCG-3′ (reverse).

Immunofluorescence Staining—For immunocytochemistry, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS for 5 min twice, and blocked with 5% normal donkey serum in PBST (0.3% Triton X-100), followed by an overnight incubation in primary antibody solutions at 4 °C. After three washes in PBS, cells were incubated with Alexa Fluor fluorescently conjugated secondary antibodies for another 90 min. After rinses and washes in PBS, cell nuclei were counterstained with Hoechst 33342 (100 ng/ml) for 10 min. Primary antibodies and their working dilutions were as follows: rabbit anti-Pax6 (1:600), mouse anti-Ki65 (1:100, BD Biosciences), rabbit anti-β-catenin (1:100, Cell Signaling), rabbit anti-GFAP (1:1000), mouse anti-recoverin (1:2000, Covance), rabbit anti-β-arrestin (1:2000, Santa Cruz Biotechnology), and mouse anti-RFP (1:500, Pierce Biotechnologies). 3 The abbreviations used are: MRP, Müller glia-derived retinal progenitor; IRBP, interphotoreceptor retinoid binding protein; RFP, red fluorescent protein.
nology), rabbit anti-GFP (1:1000, Invitrogen), or chicken anti-GFP (1:400, Invitrogen). The secondary antibodies used were the corresponding Alexa Fluor 488, 555, 633, or 647 fluorescently labeled antibodies (1:1000, Invitrogen). Labeled cells were imaged with a laser-scanning confocal microscope (Olympus FV1000). The specific immunoreactivity of each antibody was confirmed by immunostaining with appropriate retinal tissues as a positive control under the same conditions.

Cell Transplantation—We performed transplantation experiments following published protocols with some minor modifications (13, 14). Briefly, RFP-labeled MRP derivatives were dissociated into single cells with StemPro Accutase (Invitrogen) and concentrated to a density of $5 \times 10^4/\mu l$ in balanced salt solution (Alcon). With a glass micropipette, 1–2 $\mu l$ of cell suspension was slowly injected into the vitreous and subretinal spaces of eyes of 15-day-old NSG mice (The Jackson Laboratory, Bar Harbor, ME). At different time points post-transplantation, the animals were sacrificed, and the eyes were enucleated and embedded in tissue freezing medium, cryosliced, and co-immunostained as described above. Animal procedures were conducted with the approval and under the supervision of the Institutional Animal Care and Use Committee at the University of California, San Diego.

RESULTS

Dedifferentiation of Müller Glia to Retinal Progenitor-like Cells—As demonstrated recently, loss of $p53$ could enhance the proliferation of Müller glia (6) and enhance induced pluripo-
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FIGURE 3. Photoreceptor precursors are induced from p53<sup>-/-</sup> MRP. More than 80% of cells expressed Crx, a photoreceptor progenitor specific marker, after induction for 5 days (A and B, green), cell nuclei were counter stained with Hoechst 33342 (B, blue). After further culturing in photoreceptor differentiation medium for ~10 days, photoreceptor specific markers such as recoverin (C) with nuclei counterstaining (D), rhodopsin (E) and red/green opsins (R/G opsin; F) were detected by immunostaining. IRBP-GFP reporter expression was barely visible before the photoreceptor differentiation of p53<sup>-/-</sup> MRPs (G) but was brightly visible and showed photoreceptor-like morphology after differentiation for 10 days (H). Scale bars, 25 μm (A–H). I, quantitative PCR analysis of gene expression in photoreceptor precursors derived from p53<sup>-/-</sup> MRP after 2 weeks of differentiation in culture. Induced expression of photoreceptor markers such as precursor transcription factors Crx and Nr2e3, cone cell-specific marker Arrestin, and rod-specific markers Pde6b and Gnat1 was detected. The error bars represent S.E. *, p ≤ 0.05.

tent stem cell reprogramming (8). To test whether p53 null Müller glia could be readily converted into progenitor-like cells in culture, we isolated the Müller glial cells from the neural retina of p53 knock-out mice. In agreement with previous report (6), we found that p53<sup>-/-</sup> Müller glia maintains its proliferative state through the culture in vitro and can be propagated for generations (Fig. 1, A and B). Therefore, we used p53<sup>-/-</sup> Müller glia to ask whether once reentering cell cycle, mammalian Müller glia is more likely to gain progenitor-like characteristics and can be further directed toward a specific neuronal fate under a defined condition. To test this hypothesis, neural retinas of postnatal day 6 eyes of p53<sup>-/-</sup> mice were dissected and dissociated to single cell suspension, followed by seeding on a Matrigel-coated plate. After a week culturing in regular Müller glia growth medium, the cells proliferated and formed a confluent monolayer. The proliferating p53<sup>-/-</sup> Müller glia could be continuously passaged in vitro for at least 10 passages. Following the establishment of primary Müller glia culture, the cells were subjected to dedifferentiation in a serum-free and chemical defined medium. After three to four days, the cells rapidly formed a confluent monolayer and displayed a spindle-like morphology (Fig. 1D). The calculated doubling time for the p53<sup>-/-</sup> MRPs is ~35 h, which is similar to the doubling time 31~36 h reported previously for p53<sup>-/-</sup> Müller glia culture (6). The p53<sup>-/-</sup> MRP continued to be proliferative as indicated by numerous Ki67-positive cells in the culture (Fig. 2A). To explore the plausible involvement of some key stem cell/progenitor signaling pathways, such as Wnt and Hippo signaling pathways (15–17) in the conversion of Müller glia to retinal progenitor, we performed immunocytochemistry to examine the subcellular localization of key players in these two pathways. Interestingly, we found that β-catenin, a major player of Wnt pathway, and Yap, a major downstream effector of Hippo signaling pathway, were both accumulated in nucleus (Fig. 2, B and C) even at high cell density. The staining result suggested that these two well documented progenitor-promoting factors may be actively involved in induction of retinal progenitor-like cells from Müller glia. In addition, the elevated expression of retinal progenitor markers such as nestin, Pax6, and Chx10 was detected after the induction (see Fig. 2, E–G, respectively). Although the loss of p53 usually elevates GFAP expression in Müller glia (6), under our serum-free and defined condition for progenitor induction, p53<sup>-/-</sup> MRPs showed the significant down-regulation of GFAP (Fig. 2, D and H). The expression of a typical glial transcription factor Pax2 and Müller glial cell markers such as Gadd45g, glutamine synthetase, and vimentin were also decreased significantly (Fig. 2H).

**Induction of Photoreceptor Precursors from p53<sup>-/-</sup> MRPs**—To determine whether Müller glia-derived retinal progenitor-like cells have the potential to differentiate toward photoreceptor fate in vitro in a similar fashion as seen in cases of photoreceptor induction from primary retinal progenitors and embryonic stem cell-derived retinal progenitors (18, 19), we cultured p53<sup>-/-</sup> MRPs in a defined medium that is favorable for photoreceptor induction. Differentiation was initiated by withdrawal of EGF and LIF. Previous studies demonstrated that inhibition of Notch and Wnt signaling is important for retinal progenitor differentiation. In addition, sonic hedgehog (shh) and retinoic acid were used along with N-[3,5-difluorophenyl]-acetyl]-1-allyl-2-phenylglycine-1,1-dimethyl ester, a γ-secretase inhibitor that could significantly increase the formation of photoreceptor precursors by suppressing Notch signaling, to direct the photoreceptor lineage commitment of retinal progenitors in culture (20, 21). Therefore, we included small molecular inhibitors IWP2 and N-[3,5-difluorophenyl]-acetyl]-1-allyl-2-phenylglycine-1,1-dimethyl ester for suppressing Wnt and Notch signaling during the photoreceptor induction. Moreover, based on the previous reports (19, 22), we used purmorphamine, a small molecular agonist of Hedgehog pathway, retinoic acid, and taurine to promote neuronal fate restriction toward photoreceptor in culture. After 5 days in culture, >80% cells expressed Crx, a key transcription factor for initiating photoreceptor lineage differentiation (Fig. 3, A and B), indicating an massive induction of photoreceptor precur-
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Differentiation of Photoreceptors from Photoreceptor Precursors—Next, we tested whether the MRP-derived Crx^+ photoreceptor precursors could be further differentiated to more mature photoreceptors containing visual pigments. The photoreceptor precursors were continuously cultured in the photoreceptor differentiation medium. After 10 more days, ~20% cells started to exhibit characteristic neuronal morphology and showed positive staining for various photoreceptor markers, including recoverin of immature photoreceptors (Fig. 3C), red/green opsin (Fig. 3F) of cone photoreceptors, and rhodopsin (Fig. 3E) of rod photoreceptors. To determine whether photoreceptors differentiated from p53^-/- MRP s in vitro also express interphotoreceptor retinoid binding protein (IRBP), a marker of both rod and cone photoreceptors (23), we infected MRPs with an IRBP-GFP lentivirus and differentiated the transduced cells toward the photoreceptor fate. The IRBP-GFP labeling approach has been used to specifically marked photoreceptors in transgenic mice and in human, mouse, and chick retinal explants (24, 25). After 2 weeks of differentiation, >90% cells became GFP-positive. By day 20, clusters of bright IRBP-GFP-expressing photoreceptors (20% of population) displayed typical morphological features such as a short inner process and long extended outer process, similar to an outer segment (Fig. 3H). Moreover, quantitative PCR analysis showed significant up-regulation of photoreceptor specific markers after the 15 days of induction. In comparison with mouse neural retina at embryonic day 17.5, the cultured photoreceptors showed ~78- and 22-fold increase of expression of rod specific proteins Gnat1 and Pde6b, respectively, and ~5.4-fold increase of cone arrestin expression (Fig. 3I). We noticed that the photoreceptor differentiation showed the preference toward the fate of rod, as evidenced by the relatively high expression level of rod specific markers Gnat1 and Pde6b (Fig. 3I) but relatively small increase of cone marker arrestin.

Retinal Transplantation of p53^-/- MRP Derivatives—Next, we investigated whether MRP-derived differentiated cells are able to survive and differentiate into different types of retinal neurons after being injected into the rodent eye. To track the fate of transplanted cells in vivo, the cells were transduced with a RFP-expressing lentivirus. To avoid immune rejection of transplanted cells, the NSG immunodeficiency SCID mice were used for cell grafting. ~5 x 10^5 cells in 1–2 µl volume were injected into the subretinal as well as epi-retinal spaces of 2-week-old animals. Overall, six eyes were injected and enucleated at 3 weeks post-transplantation. In the subretinal region, majority of grafted RFP^+ cells were found near the injection site. Although many of these did not migrate into the photoreceptor layers, they exhibited neuronal characteristics such as elongated cell processes and continuously expressed nestin, an immature neuronal marker (Fig. 4A). A close examination of retinal sections of grafted eye, we observed that some RFP^+ cells moved into the outer segment layers were also positive for rhodopsin immunostaining (Fig. 4, B–E). The data indicates that MRP-derived precursors may survive and commit to the photoreceptor fate in the grafted tissue and are capable of expressing rod-specific proteins. Moreover, we observed the transplanted cells attached onto the retina to form an epi-retinal tissue (Fig. 5). Some of these RFP-tagged cells were co-staining for Islet1 and Brn3, two transcription factors known for their role in driving retinal ganglion cell formation (Fig. 5, A–D). In addition, migration of transplanted cells into the RGC layer was observed (Fig. 5, H–J). These results indicate that p53^-/- MRP has the ability to commit to different retinal cell fates in vivo.

DISCUSSION
Unlocking mammalian Müller glia regenerative potential to replace lost retinal cells has long been a fascinating topic. In this report, we investigated whether mouse Müller glia could be redirected to the different fates of retinal neurons and serve as a potential source for retinal regeneration. We found that Müller glia can be reversely dedifferentiated to retinal progenitors under a serum-free, chemically defined condition. It has been well known that proliferation of mammalian Müller glia is slow

sors from MRPs can be achieved efficiently in a monolayer culture system.

FIGURE 4. Transretinal transplantation of p53^-/- MRPs in immunodeficient mice. A, RFP-tagged grafted cells (red) were detected in the subretinal space 3 weeks post transplantation, and a majority of these were still positively stained for nestin (RFP Nest; green), a marker for immature progenitors, and localized next to the photoreceptor layer. B–E, a subset of incorporated cells expressed rhodopsin (green), a rod photoreceptor-specific marker. Scale bars, 25 µm (A) and 20 µm (B–E).
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Müller glia cell is the last differentiated cell type during retinal neurogenesis after differentiation of all of retinal neurons during mammalian retinal development (27). A similar scenario exists in the adult mammalian brain in which glial cells, the equivalent of Müller glia, is the last differentiated cell type during neurogenesis after differentiation of all cortical neurons. It has been found that radial glial-like cells of hippocampus behave similar to resident, dormant adult neural stem cells and can be activated upon stimulation or injury (28).

Using pluripotent stem cells to regenerate and repair damaged tissues holds great promise; however, it also faces significant safety hurdles such as tumor formations and genomic mutations (29). Endogenous adult stem cells provide an alternative source of regeneration, as they will not have issues of tumor formation or transplant rejection. In this regard, glial cells could be an ideal source of neural repair and regeneration.

Our results provide a new perspective on the role of p53 in restricting mammalian Müller glia potential for retinal neuronal regeneration, and form a basis toward in vivo repair and regeneration of photoreceptors as well as retinal ganglion cells by Müller glia cell-based therapy.

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