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Tauroursodeoxycholic Acid Promotes Neuronal Survival and Proliferation of Tissue Resident Stem and Progenitor Cells in Retina of Adult Zebrafish

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Regenerative medicine aims to replenish damaged tissue. Boosting the capacity of intrinsic stem cells to proliferate is one key for successful regeneration. Adult zebrafish possess tissue resident stem and progenitor cells, which contribute to homeostatic growth and tissue regeneration. In the intact retina, Müller glia sporadically divide to generate fate restricted, proliferative precursors. Cell death reprograms Müller glia into stem cells that divide and produce multi-potent retinal progenitors. Using zebrafish, we evaluated the effect of taurine-conjugated bile acid, Tauroursodeoxycholic acid (TUDCA) on retinal regeneration. In the intact retina, treatment with TUDCA significantly promotes proliferation of the fate restricted precursors, but has no effect on Müller glia. Following constant light exposure, TUDCA attenuates photoreceptor death, indicating that TUDCA is neuroprotective. Following a stab wound, which initiates death of retinal neurons and reprogramming of Müller glia, treatment with TUDCA significantly increases the number of proliferating cells. In the intact retina, TUDCA-induced proliferation was accompanied by decreased expression of cell cycle inhibitors. These results suggest that TUDCA promotes proliferation of actively-cycling stem and progenitors, identifying TUDCA as a potential reagent to promote regeneration of retinal neurons.

Key words bile acid, stem cells, Müller glia, cyclin dependent kinase inhibitor

INTRODUCTION

The goal of regenerative medicine is to replace damaged tissues and organs and to restore their functions. Current approaches employing transplantation of stem cells or engineered tissues and gene therapy surgically deliver cells, tissues, and materials that promote protective and mitogenic events.[1)] Although these approaches hold great promise, such therapies are invasive. In contrast, regenerative pharmacology aims to regenerate tissues and organs *in situ*.[2,3)] It utilizes native cells that are capable of producing and enhancing protective and mitogenic events to modulate, accelerate, and improve functional outcomes.[2,3)]

In the teleost fish, tissue resident stem cells reside *in situ* throughout the life to contribute continuous growth of organs.[4)] In retina of adult zebrafish, Müller glia, which normally function to maintain structural and physiological homeostasis of retina, sporadically divide to generate fate-restricted rod precursors.[5-7)] Genesis of rod precursor peaks during the juvenile stage and as fish mature, mitotic activity of Müller glia declines.[8)] In damaged retina by light or needle stab, Müller glia serve as the intrinsic stem cells and generate retinal tissues including neurons.[8,9)] In response to cell death, Müller glia dedifferentiate, enter the cell cycle, and undergo a single asymmetric division to produce multipotent retinal progenitors.[10-13)] Although in mammals the capacity of Müller glia to proliferate and regenerate retinal neurons is very limited, genetic and pharmacological modulations can enhance production of new neurons that can integrate into mature retina.[14,15)]

In traditional Chinese medicine, Tauroursodexycholic acid (TUDCA), one of the secondary bile acids, has been used to treat numerous diseases.[16)] In liver, TUDCA promotes growth and regeneration via activating receptors that drive proliferation of Mesenchymal stem and hepatic stellate cells.[17–19)] Recent study also demonstrates that in the neurogenic niches of adult rat brain, administration of TUDCA induces *in vivo* proliferation of neural stem cells,[20)] suggesting that TUDCA can elicit proliferation of tissue resident stem and progenitor cells in the central nervous system.

Using adult zebrafish, we investigate the effect of TUDCA on proliferation of retinal stem and progenitors. In the intact retina, TUDCA enhanced proliferation of actively cycling progenitors, but not quiescent stem cells. Following constant light lesion, TUDCA exerted protective effect and attenuated loss of photoreceptors. Importantly, following stab wound lesion, TUDCA significantly increased the number of BrdU-labeled proliferative cells derived from reprogrammed Müller glia. These results highlight TUDCA as a potential pharmacological substance that promotes neural survival and proliferation of tissue resident stem and progenitor cells.

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MATERIALS AND METHODS

Animals Adult wild-type zebrafish (RIKEN-WT, 4-6 month old) were raised at 28.5°C under a 14 h light:10 h dark cycle with standard husbandry procedures.[21] All experiments were performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Ophthalmic and Vision Research and were approved by the Gifu Pharmaceutical University Committee on the Use and Care of Animals.

TUDCA Treatment Zebrafish (six animals) were immersed in 1.5 L of system water containing 500 µM TUDCA. The solutions were replaced every 24 h. The previous study showed that intraventricular infusion of 300 µM TUDCA increased neural stem cell pool in adult rat brain.[20] Here, zebrafish were treated TUDCA by dissolved system water. Then, the final concentration at retina is probably lower than topical administration. Since we chose 500 µM TUDCA to investigate the effects on proliferation of zebrafish retina. We confirmed TUDCA did not affect fish survival rate at this dose.

Constant Light Lesion Constant light lesions were performed as previously described.[22] Briefly, following 14 d of dark adaptation, 12 free-swimming zebrafish were placed in a 3 L transparent tank and were exposed to a constant white light with an intensity of 16,000–20,000 lux (measured at the outside surface of the tank), using two halogen lamps at a distance of 30 cm from the tank. To maintain water temperature, a fan was placed behind the tank. Following light lesions, fish were kept in the dark until they were sacrificed.

Stab Lesion Stab lesion was performed as previously described.[9] Briefly, after anesthesia with 0.1% phenoxyehanol (Wako Pure Chemical Industries, Ltd, Osaka, Japan), fish were placed in a wet Kim towel. A 30G needle was inserted through the sclera to a depth of bevel at each quadrant of the right eye.

5-Bromo-2'-Deoxyuridine (Brdu) Incorporation To label proliferative cells, zebrafish were intraperitoneally injected with 30 µL of 20 mM bromodeoxyuridine (BrdU) in phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) 3 h prior to fixation. The number of BrdU positive cells in the serial transverse sections were counted using the ImageJ software.

Tissue Preparation The eye balls were enucleated and were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer at 4°C overnight. Tissues were then soaked in 5% and 12.5% sucrose for 1 h. Following immersion in 20% sucrose and mixed liquor, containing an optimum cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and 20% sucrose at ratio of 1:2, tissues were embedded in OCT compound. Serial transverse sections were made at a thickness of 12 µm using a cryostat and mounted on glass slides (MASCOAT; Matsunami Glass Ind. Ltd., Osaka, Japan)

Immunohistochemistry For antigen retrieval, slides were treated with 2M HCl containing 0.3% Triton-X at room temperature for 30 min, followed by 10 min incubation in 0.1% trypsin solution at 37°C. Sections were blocked in 10% normal goat serum in PBS (Vector Laboratories Inc., Burlingame, CA, USA). Rat monoclonal anti-BrdU antibody (1:500; Abcam, Cambridge, UK) and Alexa Fluor® 546 goat anti-rat IgG (1:1000; Thermo Fisher Scientific, Waltham, MA, USA) were used. After nuclear staining with Hoechst 33342 (Thermo Fisher Scientific), sections were mounted in Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA). Images were captured with a Fluorescence Microscope BZ-X700(Keyence, Osaka, Japan).

RNA Isolation and Quantitative Real-Time PCR (qPCR) RNA isolation and qPCR were performed according to the manufacturer's protocol. Briefly, total RNA was isolated from whole retinas using a NucleoSpin® RNA kit (Takara, Shiga, Japan). After measurement of RNA concentration in a NanoVue Plus (GE Healthcare Japan, Tokyo, Japan), RNAs were reverse transcribed into cDNA using a PrimeScript RT reagent kit (Perfect Real Time; Takara). qPCR was performed using SYBR Premix Ex TaqTM II (Takara), TP 8000 Thermal Cycler Dice Real Time system (Takara), and following primers.

p21; F: 5'-CCGCATGAAGTGGAGAAAAC-3',
R: 5'-ACGCTTCTTGGCTTGGTAGA-3'
p27; F: 5'-TGAAGCCTGGAACTTCGACT-3',
R: 5'-TGTGAATATCGGAGCCCTTC-3'
p53; F: 5'-GCTTGTCACAGGGGTCATTT-3',
R: 5'-ACAAAGGTCCCAGTGGAGTG-3'
gapdh; F: 5'-ATGACCCCTCCAGCATGA-3',
R: 5'-GGCGGTGTAGGCATGAAC-3'

The *glyceraldehyde-3-phosphate dehydrogenase (gapdh)* was used as an internal standard, and ΔΔCt method was used for the quantitative. Nine to ten independent biological samples containing 2 retinas from a single fish were analyzed at each group.

Statistical Analysis Data are presented as means ± standard error of the mean (SEM). The statistical analyses were performed using the SPSS statistical software package (IBM, Armonk, NY, USA). We made statistical comparisons using the Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

We first evaluated the effect of TUDCA in the intact retina and asked whether TUDCA can stimulate proliferation of Müller glia and/or fate-restricted precursors (Fig. 1A). Following 48 h of treatment, TUDCA significantly increased the number of BrdU-labeled proliferative cells in the outer nuclear layer, where rod precursors divide (Fig. 1B,C). In contrast, in the inner nuclear layer, where the cell body of Müller glia reside, the number of proliferative cells was statistically indistinguishable (Fig. 1B,C). These results indicate that in adult retina TUDCA accelerates proliferation of mitotically active, cycling population of precursors, but does not stimulate proliferation of Müller glia stem cells.

To ask whether TUDCA modulates proliferation during photoreceptor regeneration, we conducted a constant light lesion, which selectively kills photoreceptors (Fig. 2A).[23] In control animals at 3 d post lesion (dpl), the death of photoreceptors is evident by the decreased thickness (Fig. 2B,C) and reduced number of nuclei in the outer nuclear layer (Fig. 2B,D). In response to cell death, Müller glia and Müller glia-derived progenitors actively proliferate in the inner nuclear layer (Fig. 2B, E,F). However, treatment of TUDCA following 24 h of light lesion attenuated the loss of photoreceptors (Fig. 2B,C) and significantly reduces the number of BrdU-positive cells (Fig. 2B,E,F). These results suggest that TUDCA is neuroprotective to photoreceptors and that cell death is necessary to initiate proliferation of Müller glia.

Stab lesion mechanically destroys all types of retinal neu-

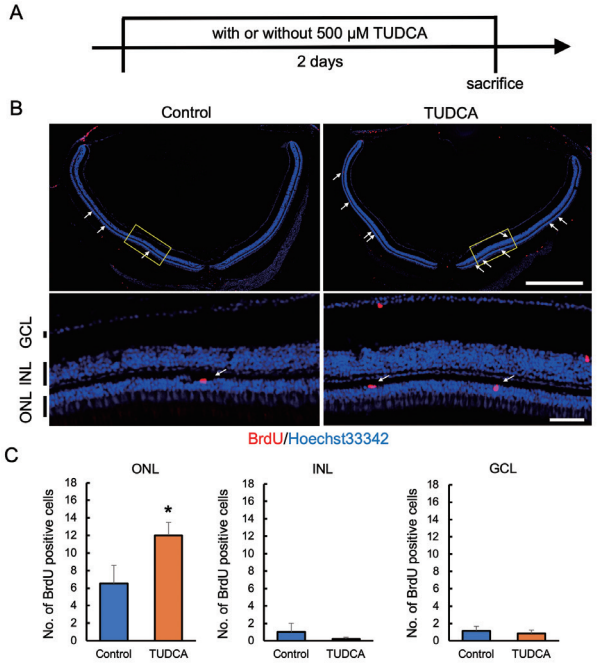

Fig. 1. TUDCA Promotes Proliferation of Rod Precursors in the Intact Retina.

(A) Experimental paradigm for TUDCA treatment. (B) Confocal microscope images of control and TUDCA-treated retinas immuno-labeled with BrdU (red). BrdU was given 3 h prior to sacrificing animals. Bottom panels show high magnification of the boxed region. White arrows indicate BrdU-positive cells in the outer nuclear layer. Nucleus were stained with Hoechst 33342 (blue). (C) Quantification of BrdU-positive proliferating cells in the outer nuclear layer, inner nuclear layer, and ganglion cell layer. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Scale bars equals 50 mm. Data are shown as means ± SEM (n=6-7). *p<0.05.

rons, therefore, it is considered as the most aggressive lesion among the injury paradigms.[24] To answer the initial question of whether TUDCA modulates proliferation, we performed stab lesions and compared the proliferative response between control and TUDCA-treated animals (Fig. 3A). In control animals following a stab lesion, the proliferation of Müller glia and Müller glia-derived progenitors peaks around 4 dpl (Fig. 3B). In the animals treated with TUDCA, the stab lesions kill retinal neurons (data not shown) and, at 4 dpl, results in significantly greater number of BrdU-labeled cells (Fig. 3B,C). These results indicate that following a stab lesion, TUDCA promotes proliferation of Müller glia and Müller glia-derived progenitors.

During growth and regeneration of the liver, bile acids govern proliferation by negatively modulating cell cycle inhibitors.[25] Cyclin-dependent kinase inhibitors (cdkns) bind to the Cyclin/Cyclin dependent kinase complex and prevent progression of the cell cycle.[26] To gain mechanistic insight of TUDCA-mediated proliferation in the retina, qPCR was performed to determine the expression levels of the cdk inhibitors, *p21*/ *cdkn1ab*, *p27*/*cdkn1bb*, and *p53*. To exclude dying neurons, which will show apoptosis-related induction of these genes, we examined unlesioned retinas for this analysis. These experiments show that treatment with TUDCA significantly suppresses the expression *p27*/*cdkn1bb* (Fig. 4B), and *p53* (Fig. 4C). The level of *p21*/ *cdkn1ab* was not significant altered (Fig. 4A). These results suggest that in retina of adult zebrafish TUDCA may negatively regulate cdkns to enhance proliferation among the populations of mitotically active cells.

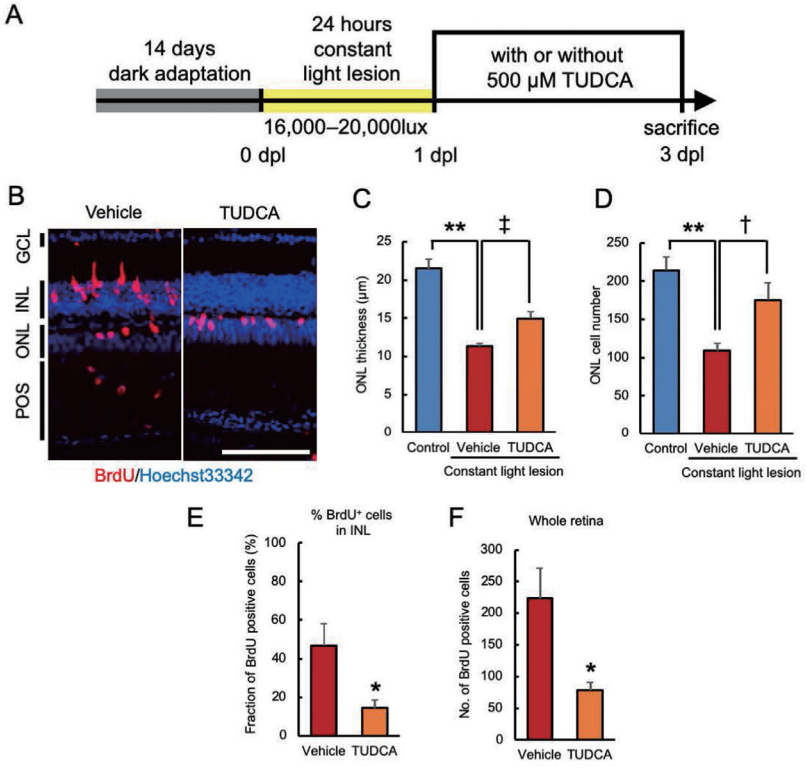

Fig. 2. TUDCA Promotes Survival of Photoreceptors Following Constant Light Lesion.

(A) Experimental paradigm of constant light lesion followed by treatment of TUDCA. (B) Confocal microscope images of control and TUDCA-treated retinas immuno-labeled with BrdU (red) at 3 dpl. BrdU was given 3 h prior to sacrificing animals. (C,D) Quantification of the thickness (C) and the number of nucleus (D) in the outer nuclear layer at 3 dpl. (E,F) Quantification of the number of BrdU-positive cells in the inner nuclear layer (E) and all retinal layers (F) at 3 dpl. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Scale bars equals 50 mm. Data are shown as means ± SEM (n=6-7). *p<0.05, **p<0.01, †p<0.05, ‡p<0.01.

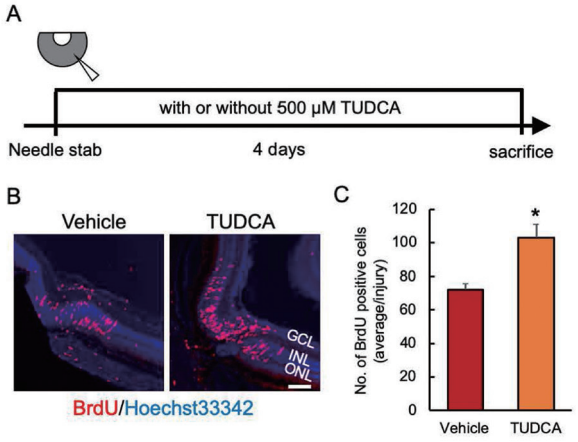

Fig. 3. TUDCA Promotes Proliferation of Reprogrammed Müller Glia and Their Progenitors Following Stab Lesion.

(A) Experimental paradigm of stab lesion and treatment of TUDCA. (B) Confocal microscope images of control and TUDCA-treated retinas immuno-labeled with BrdU (red). BrdU was given 3 h prior to sacrificing animals. (C) Quantification of the number of BrdU-positive cells in control (red) and TUDCA (orange) treated animals. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Scale bars equals 50 mm. Data are shown as means ± SEM (n=6-7). *p<0.05.

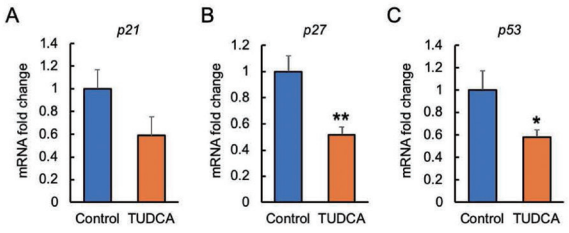

Fig. 4. TUDCA may Negatively Regulate Expression of Cyclin Dependent Kinase Inhibitors.

(A-C) Quantitative RT-PCR for selected cyclin dependent kinase inhibitors, *p21/cdkn1ab* (A), *p27/cdkn1bb* (B), and *p53* (C) in control (blue) and TUDCA treated (orange) retinas. Data are shown as means ± SEM (n=9–10). *P<0.05, **P<0.01.

DISCUSSION

Proliferation of Müller glia can be triggered by stimuli that modulate cell-cell communication, upregulation of transcription factors or exposure to growth factors and cytokines. For example, in zebrafish, pharmacological suppression of Notch signaling, together with activation of TNFa signaling, promotes proliferation by driving Müller glia reprogramming and entry into the cell cycle.[27] In mice, treatment of the histone deacetylase inhibitor, combined with overexpression of Ascl1, stimulates Müller glia to adopt a neurogenic state.[14] These events act directly on quiescent Müller glia to drive reprogramming and proliferation. While reprogramming and proliferation are coupled, they are discrete events and both are required for proper regeneration.[28] Our data demonstrate that in retina of zebrafish, TUDCA does not initiate entry into the cell cycle of quiescent stem cells. Instead, TUDCA acts on actively cycling stem and progenitor cells to promote proliferation.

Intriguing models have been proposed on how bile acids or TUDCA promote proliferation of tissue resident stem and progenitor cells during growth, homeostasis, and regeneration. In liver, increased bile acid flux activates the nuclear bile acid receptor, which targets genes regulating homeostatic liver growth. [17] In the subventricular zone of adult rat, TUDCA governs the level of mitochondria-related factors, which in turn regulate transcription of cell cycle regulators. [20,29] One of the possible mechanisms of TUDCA for stem cell proliferation is inhibition of p53 mitochondrial translocation which is first signs of differentiation-induced mitochondrial damage and then mitochondrial reactive oxygen species are suppressed. [29] Although exact mechanisms remain unknown, our data indicate that TUDCA acts as a negative regulator of the cell cycle inhibitors, *p27/cdkn1bb*, and *p53*, consistent with previous reports.

In addition to the effect on proliferation, we show that TUDCA is neuroprotective, consistent with reports that in mice systematic administration of TUDCA prevents photoreceptor death following retinal detachment, light exposure, or mutation-based degeneration.[30,31] TUDCA exerts neuroprotection in several disease models, including Alzheimer's and Huntington's disease.[32–34] In the context of photoreceptors, it remains unknown whether TUDCA inhibits cascades of cytochrome *c* release from mitochondria,[35] pro-apoptotic components of caspase and Bax,[36] and production of reactive oxygen species to inhibit apoptosis.[37]

In summary, we demonstrated that TUDCA, one of the secondary bile acids, promotes survival of retinal neurons and proliferation of resident stem and progenitor cells. These results highlight TUDCA as a potential reagent to promote regeneration of retinal neurons.

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Conflict of interest The authors declare no conflict of interest.

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