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Lin28b stimulates the reprogramming of rat Müller glia to retinal progenitors

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ABSTRACT

In lower-order vertebrates, Müller glia exhibit characteristics of retinal progenitor cells, while in higher vertebrates, such as mammals, the regenerative capacity of Müller glia is limited. Recently, we reported that Lin28b promoted the trans-differentiation of Müller cells to rod photoreceptor and bipolar cells in the retina of retinitis pigmentosa rat model, whereas it is unclear whether Lin28b can stimulate the reprogramming of Müller glia *in vitro* for transplantation into a damaged retina. In the present study, Long-Evens rat Müller glia were infected with Adeno-Lin28b or Adeno-GFP. Over-expression of Lin28b in isolated rat Müller glia resulted in the suppression of GFAP expression, enhancement of cell proliferation and a significant increase of the expression of retinal progenitor markers 5 days after infection. Moreover, Lin28b caused a significant reduction of the Let-7 family of microRNAs. Following sub-retinal space transplantation, Müller glia-derived retinal progenitors improved b-wave amplification of 30d Royal College of Surgeons retinitis pigmentosa model (RCS-P +) rats, as detected by electroretinography (ERG) recordings. Taken together, these data suggest that the up-regulation of Lin28b expression facilitated the reprogramming of Müller cells toward characteristics of retinal progenitors.

1. Introduction

Retinal degeneration (RD) is a major cause of permanent vision loss, of which age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are the most prevalent diseases [1]. RP mostly affects the young, is caused by photoreceptor loss, and is characterized by retinal pigment deposits [2]. Conversely, AMD mainly affects the elderly and is characterized by drusen or choroid neovascularization formed underneath the macula [1,3].

The Müller glia extend through the entire retina, support the structure of the retina and transfer information between neurons as messengers [4]. Additionally, Müller glia are critical for the maintenance of homeostasis in the retinal extracellular milieu [5]. In teleost fish, when retina constantly expanding, progenitors in the retinal periphery can produce all neurons except rods. The rods in the outer nuclear layer are generated by a dedicated lineage of cells known as the rod lineage with the apex of Müller glia. Furthermore, when retina injured, Müller glia will dedifferentiate into multipotent progenitors and responsible for regenerating all of the major retinal neurons [4,6,7]. However, in mammals, Müller glia have very limited potential for cell cycle reentry, and do not contribute to a rod lineage. When retinal injured, Müller glia are activated through transiently de-differentiation process, ultimately gliosis happened, which is a double edged sword for retina [5,6,8,9]. Many studies have been carried out to address how to promote the generation of retinal neurons from Müller glia in mammals [10–15]. These studies suggest that under appropriate conditions, Müller glia can be reprogrammed to multipotential stem cells to generate retinal neurons. However, the mechanism of action remains unclear.

Lin28a and Lin28b (collectively referred to as the RNA-binding protein Lin28) have been shown specifically to block Let-7 microRNAs to regulate the pluripotency of stem cells [16–18]. During development of the central nervous system, expression of Lin28b completely blocks gliogenesis, while neurogenesis is increased [16]. Additionally, in mouse hematopoietic stem cells (HSCs), self-renewal activity and Hmga2 levels are elevated after Lin28 over-expression [19]. Furthermore, in teleost fish, pluripotency factor Lin28 is necessary for Müller glia de-differentiation, and lin28b is up-regulated within 6 h when the retina is injured [20]. Our recent results demonstrated that ectopic expression of Lin28b in the rat retinitis pigmentosa model

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(RCS-P+) would promote Müller glia to trans-differentiate into rhodopsin-positive rod photoreceptor-like cells and PKCα positive bipolar cells [21]. Based on this evidence in the RCS-P+ rat model, we hypothesized that Müller glia could be reprogrammed under no pathological condition in vitro.

In this study, adenovirus was used to over-express Lin28b in isolated rat Müller glia. The GFAP level, proliferation ratio and characteristics of retinal progenitor were detected in vitro. The Ad/Lin28b reprogrammed progenitors were transplanted into the subretinal space of RCS-P+ rats to explore its regeneration effect.

2. Materials and methods

2.1. Animals and ethical statement

Royal College of Surgeons rat (RCS-P+) at postnatal day (PND) 30 and Long-Evans rat (LE) at PND 8–10 were used in this study. The rats were housed in a temperature-controlled room at 12-h light-dark cycle in the Laboratory Animal Unit of the Third Military Medical University. Three groups of the RCS-P+ rats were divided for treatment: Ad/GFP infected Müller cells treated RCS-P+ rats and Ad/Lin28b infected Müller cells treated RCS-P+ rats and untreated RCS-P+ rats. All experimental and animal handling procedures complied with the Association for Research in Vision and Ophthalmology Statement and were also reviewed and approved by the Faculty Committee on the Use of Live Animals in Teaching and Research, Third Military Medical University.

2.2. Primary Müller glia culture

The enrichment of Müller glia was performed according to previously described methods [22]. Briefly, eyes from Long-Evens (LE) rats at PND 8–10 were enucleated and incubated for 6–8 h in DMEM. The eyes were subsequently transferred to dissociation solution (DMEM containing 0.1% Trypsin and 0.1% collagenase IV) and incubated at 37 °C in a CO_2 incubator for 40 min. The eyes were washed with DMEM containing 20% FBS, 1% antibiotic-antimycotic mixture and 1% Glutamax. Retinas were dissected to avoid retinal pigment epithelium (RPE), lens and ciliary epithelium contamination carefully. The retinas were mechanically dissociated into small aggregates and cultured in DMEM containing 20% FBS, 1% antibiotic-antimycotic mixture and 1% Glutamax in 6-well plates for five to seven days, while removing the floating retinal aggregates and debris, leaving target cells attached to the bottom. The cells were trypsinized and cultured in DMEM containing 20% FBS for five days to further purify the cell population. Cells of the third passage were used for immunocytochemistry and infection. The purified Müller glia were detected by immunofluorescence staining co-labeled CRALBP/GS and CRALBP/GFAP.

2.3. Quantitation using real-time PCR for mRNA and microRNAs

Total RNA was isolated from each cell group (control, Ad/GFP, Ad/Lin28b on 3, 5, 7 days) using Trizol according to the manufacturer's protocol. cDNA from mRNA was synthesized using the SYBR Premix Ex Taq II (Takara) and cDNA from microRNAs was synthesized using the miDETCT A Trackt™ microRNA qRT-PCR start kit (Ruibo) and the specific primers are given in Table 1. A change in the target gene expression of each group relative to the control was calculated using the following formula: fold change=$2^{-(\Delta CT,Tg\Delta CT,control)}$.

2.4. Western blot analysis

The protein levels of GFAP were detected by western blot [23]. Proteins were extracted from cells using Radioimmuno Precipitation Assay (RIPA) buffer (Beyotime) containing 1:100 protease inhibitor.

Table 1
Real-time PCR primers.

Gene	Primer sequence (5′-3′)	
sox2	TGCTGCCTCTTTAAGACTAGGGCT	(Forward primer)
	GGGCGAAGTGCAATTGGGATGAAA	(Reverse primer)
oct4	AGGTGTTCAGCCAGACAACCATCT	(Forward primer)
	ACGGTTCTCAAGCTAGTCCGCTT	(Reverse primer)
PAX6	GACATTTCCCGAATTCTGCAGACC	(Forward primer)
	ACTCTTGGCTTACTGCCTCCGATT	(Reverse primer)
Lin28b	CAGATGTGGACTGTGAGAGAAG	(Forward primer)
	GGAGGTAGACCGCATTCTTTGG	(Reverse primer)
GAPDH	AGACAGCCGCATCTTCTTGT	(Forward primer)
	TGATGGCAACAATGTCCACT	(Reverse primer)
Vsx2	TACCCCACCACTCTAGGCA	(Forward primer)
	TCTCAGGGATGAAATGGCAGG	(Reverse primer)
Nestin	GGGGTTCCTGTACTATCTGAGC	(Forward primer)
	GGGTGTTGGCTCTCCTCTTT	(Reverse primer)
microRNA		
Universal primer	GAATCGAGCACCAGTTACGC	
U6	TGGCCCCTGCGCAAGGATG	
let-7a-5p	TGAGGTAGTAGGTTGTATAGTT	
let-7b-5p	TGAGGTAGTAGGTTGTGTGGTT	
let-7c-5p	TGAGGTAGTAGGTTGTATGGTT	

The cells were fully lysed then centrifuged at 12,000 bpm at 4 °C for 10 min. The protein concentration was determined by a bicinchoninic acid assay. The lysates were separated using a 12% gel in SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Beyotime). After blocking with 5% nonfat milk in Tris Buffered Saline Tween (TBST), membranes were incubated with rabbit anti-GFAP (Abcam, Cambridge, MA, USA) and mouse anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After several washes, membranes were incubated with horseradish peroxidase–conjugated sheep anti-mouse (1:3000; Santa Cruz Biotechnology) or sheep anti-rabbit immunoglobulin G (Santa Cruz Biotechnology) as secondary antibodies for 2 h. A chemiluminescence substrate (Beyotime) was used to detect protein bands. The data were analyzed by ImageJ software using GAPDH as internal controls.

2.5. Immunocytochemistry

For immunocytochemistry [21,22], cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS for 10 min, blocked with 3% BSA in PBST (0.3% TritonX-100) for 1 h at 37 °C, and incubated with primary antibodies overnight at 4 °C. After three washes in PBS (5 min each), cells were incubated with Alexa fluorescent-conjugated secondary antibodies for another 1 h at room temperature. After rinsing and washing three times in PBS (5 min each), cell nuclei were counterstained with DAPI for 10 min. Primary antibodies and their working dilutions were as follows: rabbit anti-GS (Abcam, Cambridge, MA, USA), rabbit anti-GFAP (Abcam, Cambridge, MA, USA), mouse anti-CRALBP (Abcam, Cambridge, MA, USA), rabbit anti-Sox2 (Abcam, Cambridge, MA, USA), rabbit anti Ki67 (Abcam, Cambridge, MA, USA), mouse anti-PAX6 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-CHX10, (Chemicon international, USA), mouse anti-Nestin, (Abcam, Cambridge, MA, USA), Fluorescence-conjugated secondary antibodies were Alexa Fluor 568 or 488 (Invitrogen).

2.6. Fluorescence-activated cell scanning (FACS) analysis of the cell cycle

Fluorescence aided cell scanning (FACS) was used to analyze the cell cycle of Lin28b infected Müller glia. Cells were harvested using 0.25% trypsin, centrifuged at 1500 rpm for 5 min, and washed once with PBS then suspended in 50 PBS at a minimum density of per tube. FACS analysis of the cell cycle was applied according to previously