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Sphere-Induced Rejuvenation of Swine and Human Müller Glia Is Primarily Caused by Telomere Elongation

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ABSTRACT

Müller cells are the major supportive and protective glial cells in the retina with important functions in histogenesis and synaptogenesis during development, and in maintenance of mature neurons as they show to secrete various cytokines and manifest potentials of self-renewal and transdifferentiation into retinal neurons following injury in the vertebrate retinas. The swine retina has a visual streak structure similar to the human macular where cone photoreceptors are highly concentrated, thereby can serve as a better model for studying retinal diseases and for formulating cell-based therapeutics than the rodent retinas. Like most differentiated somatic mammalian cells, the isolated swine and human Müller glia become senescent over passages in culture, which restricts their potential application in basic and clinic researches. Here, we demonstrate that the senescence of swine and human Müller cells is caused by telomere attrition upon multiplications in vitro; and the senescent cells can be rejuvenated by sphere suspension culture. We also provide evidence that sphere-induced extension of telomeres in swine and human Müller glia is achieved by alternative lengthening of telomeres or/and by telomerase activation. STEM CELLS *2017;35:1579–1591*

SIGNIFICANCE STATEMENT

Müller cells are the major supportive and protective glia in the retina with important functions in maintenance of mature neurons; they manifest potentials of self-renewal and transdifferentiation into retinal neurons in the vertebrate retinas. This is the first report that mammalian Müller glia can be rejuvenated to keep their original properties and reprogrammed into an immortalized cell line with stem-like cell properties and that mammalian Müller glia can adapt alternative lengthening of telomeres- or/and telomerase-based mechanisms to extend their telomeres. We believe that our sphere-induced cell rejuvenation and reprogramming protocol would represent a more general method to be used in future regenerative medicine.

INTRODUCTION

Müller cells are the major glia in the retina supporting and protecting retinal neuron functions in converting light signal to electric pulse to the brain of vertebrates [1]. The retina is part of the central nerve system (CNS) and thereby vulnerable to neuron-specific degeneration disorders such as age-related macular degeneration (AMD) in seniors, which are not curable and often not treatable in the clinic [2]. Cell-based regenerative medicine has paved certain avenues leading to some clinic procedures in treating these degenerative diseases with promising effectiveness [3]. Animal models are attractive for cell transplant basic researches and preclinic trials [3, 4]. Compared to the most popular rodents, the swine retina contains a cone-dominant central visual streak analogous to the human macula, thereby is an ideal model for simulating human retinal diseases [4]. Nevertheless, identifying a right cell source is critical for successful retinal cell-based treatments [5]. It has been shown that Müller glia can divide and dedifferentiate into cells manifesting characteristics similar to retinal progenitor/stem cells following retinal injury, and then migrate, and transdifferentiate into cells with neuronal phenotype to substitute or compensate functionally for the degenerated neurons [6–10]. However, mature somatic mammalian cells including Müller glia are of restricted potential in proliferation [11] due to their telomere attrition upon DNA

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replication [12]. The degree to which Müller cells contribute to replacement of the damaged neurons is thereby dependent on their capacity in proliferation and transdifferentiation [1]. The first problem for Müller glia in cell-based application is therefore to overcome cell proliferation-induced replicative senescence (RS) when expanding in culture.

RS is caused by telomere shortening [12] when DNA damage-initiated activation of the tumor suppressor P53 provokes expression of cyclin-dependent kinase inhibitor (CDKI) P21 [12, 13] that reduces/prevents phosphorylation and thereby activation of another major tumor suppressor RB1 to arrest cell division [13, 14]. Elongating the shortened telomere(s) is essential to prevent such tumor repression pathways leading to RS. In majority of cell types, activation of telomerase/telomere reverse transpcriptase (TERT) to extend telomere length is the key to circumvent the telomere shortening-induced RS [12], whereas the alternative lengthening of telomeres (ALT) has also been discovered to elongate telomeres in minority of cell types that are TERT low/negative [15]. Although the ALT activity is hardly seen in normal mature somatic cells, it is observed high in the early developmental embryo prior to the blastocyst and in a few of tumors [15]. The simplest way to create an immortalized cell line not only capable of endless proliferation, but also keeping similar/identical phenotype to their parental cells, is to directly introduce a gene(s) both to activate TERT expression and to evade signaling toward tumor formation [12]. Expression of TERT by viral transduction immortalizes most cells [12], but possesses a potential risk of tumor formation in vivo because possible mutation(s) caused by viral random insertion may result in uncontrolled cell proliferation thereby cancerous transformation.

Stem cells, particularly embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) possess an unlimited potential in proliferation and differentiation both in vitro and in vivo, and thereby frequently are used in cell-based therapies after differentiated into a specialized cell type. The problems associated with their application in the clinic are inefficiency in such a directed differentiation in vitro and potential tumor formation in vivo if undifferentitated cells are retained or/and a viral vector is used in generation of iPSC [16]. The ideal cell sources therefore are those adult stem cells/progenitors that can self-renew for extended passages, more efficiently give rise to one or more restricted cell types, and never form tumors in recipients [17]. Müller glia are considered as a retinal stem cell to be such an ideal source for potentially compensating or/and replacing damaged retinal neurons though with restriction in self-renewal [6–10]. We developed a sphere-induced rejuvenation protocol to revive cultured swine and human Müller glial cells in laboratory based on our previous work [18], and provided evidence here that sphere formation in suspension culture can rejuvenate senescent swine and human Müller glia by elongating shortened telomeres through the mechanisms of ALT or/and activation of TERT.

Materials and Methods

Müller Cell Isolation and Culture

Adult swine eyes were collected from a local slaughter house. Adult human eyes were obtained from the eye bank of Kentucky Lions Eye Center at Louisville. This study was approved by the Institutional Review Board of University of Louisville and adhered to the tenets of the Declaration of Helsinki and a written informed consent was obtained. After removal of the connective tissue around eyeball, the eyeballs were placed in phosphate buffered saline (PBS) with 1% penicillin and 0.1% gentamycin for about 10 minutes. The anterior segments including the cornea and the lens were removed, the vitreous and the retina were separated. The retinas were rinsed in PBS twice and minced thoroughly, and digested with Worthington Papain Kit (Worthington Biochemical Corporation, Lakewood, NJ, www.worthington-biochem.com) according to the manufacturer's instruction. Large tissue debris were removed through a gauze mesh. Dissociated cells were collected in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin and cultured in 100-mm plates coated with 0.1% gelatin. The medium was changed every 5 days. Adherent cells became relatively pure Müller glia identified by cell morphology and immunostaining with glutamine synthetase (GS) after passaging at the ratio of 1:3 for two times.

Cell Spheres and Sphere-Derived Cell Preparation

Adherent cells were continuously passaged upon confluence until the occurrence of senescent sign at primary passage 10 (P10) for swine Müller glia or P18 for human Müller glia. The confluent adherent cells were scrapped off the plates and transferred to 60-mm ultra-low attachment plates (VWR International, Radnor, PA, www.vrw.com) for 3 days to form spheres in suspension. The 3-day spheres were placed back to the gelatin-coated culture plates in DMEM with FBS to produce sphere-derived Müller cells for further post-sphere passages (PSPs).

Photoreceptor Differentiation

Swine sphere-induced Müller glial stem cells (siMGSC) at PSP18 were used for photoreceptor differentiation as detailed previously [19].

DNA, RNA, and Protein Extraction

Cell genomic DNA and total RNA were extracted using DNeasy and RNeasy kits (Qiagen, Hilden, Germany, www.qiagen.com) according the manufacturer's instructions, and their quantity and quality were assessed by Nanodrop (Thermo Fisher Scientific, Waltham, MA, www.thermofisher.com). Total proteins were extracted by collecting cultured cells in the lysis buffer (50 mM Tris/HCl, pH7.4, 100 mM NaCl, 0.5% Triton X-100, 0.5% NP-40). The supernatants were used as total proteins after centrifugation at 13,000 rpm for 10 minutes.

Relative Telomere Length Detection

The relative telomere length was assessed by a monochrome multiplex quantitative polymerase chain reaction (MMQPCR) method [20] with minor modification in PCR program. See Supporting Information Methods.

Gene Expression Profiling by Real-Time Quantitative PCR

cDNA was synthesized using the Invitrogen RT Kit (Thermo Fisher Scientific, Waltham, MA, www.thermofisher.com) and qPCR was detailed previously [19]. PCR primer sequences were shown in Supporting Information Table 1 and PCR amplicons were validated by size on 1.5% agarose gels.