Stem Cell Research 23 (2017) 20–32

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Evidence for a retinal progenitor cell in the postnatal and adult mouse

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ARTICLE INFO

Article history:
Received 14 February 2017
Received in revised form 16 June 2017
Accepted 19 June 2017
Available online 24 June 2017

Keywords:
Progenitor cells
Retina
c-Kit
Lineage tracing

ABSTRACT

Progress in cell therapy for retinal disorders has been challenging. Recognized retinal progenitors are a heterogeneous population of cells that lack surface markers for the isolation of live cells for clinical implementation. In the present application, our objective was to use the stem cell factor receptor c-Kit (CD117), a surface marker, to isolate and evaluate a distinct progenitor cell population from retinas of postnatal and adult mice. Here we report that, by combining traditional methods with fate mapping, we have identified a c-Kit-positive (c-Kit$^+$) retinal progenitor cell (RPC) that is self-renewing and clonogenic *in vitro*, and capable of generating many cell types *in vitro* and *in vivo*. Based on cell lineage tracing, significant subpopulations of photoreceptors in the outer nuclear layer and bipolar, horizontal, amacrine and Müller cells in the inner nuclear layer are the progeny of c-Kit$^+$ cells *in vivo*. The RPC progeny contributes to retinal neurons and glial cells, which are responsible for the conversion of light into visual signals. The ability to isolate and expand *in vitro* live c-Kit$^+$ RPCs makes them a future therapeutic option for retinal diseases.

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1. Introduction

Visional signals are mediated by photoreceptors, bipolar cells and ganglion cells, and the loss of these neurons leads to irreversible blindness (de Jong, 2006; Friedman et al., 2004; Hartong et al., 2006; Quigley and Broman, 2006; Ramsden et al., 2013; Thylefors et al., 1995). Photoreceptors are the light sensor of the outer nuclear layer (ONL) of the retina, and degeneration and death of these cells occur with retinitis pigmentosa, a disease that affects 1/3000–4000 individuals younger than 60 years of age (Hartong et al., 2006). Age-related macular degeneration (AMD), a major cause of blindness in people over 50 years, is characterized by dysfunction of the retinal pigment epithelium (RPE) followed by apoptosis of photoreceptors (de Jong, 2006; Friedman et al., 2004). The high ocular pressure of glaucoma (Quigley and Broman, 2006), or the ischemic retinopathy of diabetes (Antonetti et al., 2012), also results in the loss of functional retinal cells leading to impaired vision. Bipolar cells of the retinal inner nuclear layer (INL) transmit the visional signal from the photoreceptors to the ganglion cells and then into the brain. In addition to neurons, RPE and Müller glial cells support the nutrition and homeostasis of the retina (Goldman, 2014; Ramsden et al., 2013; Reichenbach and Bringmann, 2013).

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been used to generate RPE for the treatment of AMD, preventing its progression by protecting the underlying photoreceptors(Mead et al., 2015). However, the photoreceptors that have been lost in AMD still require replacement. The use of retinal progenitor cells (RPCs) for neuronal regeneration is an active area of investigation; although progress has been challenging due to the lack of specific surface markers for isolation of RPCs, and the fact that previously identified RPCs are a heterogeneous population of cells(Ahmad et al., 2000; Cepko, 2014; Gualdoni et al., 2010; MacLaren et al., 2006; Tropepe et al., 2000). Thus, the differentiation potential of individual progenitor cells has been difficult to evaluate. ESCs and iPSCs are capable of generating precursors of photoreceptors *in vitro*, but transplantation of these precursors *in vivo* has had limited success (Binder, 2011; Mead et al., 2015; Schwartz et al., 2012). Moreover, ESCs and iPSCs need to be

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http://dx.doi.org/10.1016/j.scr.2017.06.010
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differentiated prior to implantation *in vivo*, to avoid the risk of tumorigenesis (Cui et al., 2013; Shirai et al., 2016; West et al., 2012). Following lineage specification, ESCs and iPSCs lose their integration capacity and their multipotent phenotype, which limits their therapeutic potential(West et al., 2012). To our knowledge, a population of cells that expresses a stem/progenitor cell antigen and maintains the self-renewing and multipotent characteristics of a stem/progenitor cell *in vivo* is critical. The lack of a specific surface marker that allows for isolation and expansion of live progenitor cells from the eye, has been an issue preventing identification of a primitive cell capable of regulating physiologic cell renewal and organ reconstitution following injury.

The stem cell factor receptor c-Kit, also known as tyrosine-protein kinase Kit or CD117, is a protein involved in the development, maturation, and survival of neurons (Hirata et al., 1993; Jin et al., 2002). Both c-Kit and Kit ligand, stem cell factor, are present on cell surface membranes of neuronal cells in the central nervous system, including retinas of mice and humans (Das et al., 2004; Hasegawa et al., 2008; Koso et al., 2007; Mochizuki et al., 2014; Morii et al., 1994; Zhou et al., 2015), and the peripheral nervous system (Goldstein et al., 2015; Guijarro et al., 2013; Sachewsky and Morshead, 2014). c-Kit-positive (c-Kit$^+$) cells have also recently been identified from the retinal neuroblast layer of human eyes (embryonic weeks 12–14), and are being proposed as RPCs with the potential for application in retinal degeneration without tumorigenesis (Chen et al., 2016; Zhou et al., 2015). However, it is not known whether c-Kit$^+$ cells with progenitor cell properties exist in the postnatal or adult retina, and whether progeny of these cells contribute to the architecture of the retina. The expression of c-Kit has been employed previously for the identification and characterization of hematopoietic, cardiac, and lung stem/progenitor cells (Bolli et al., 2011; Itkin et al., 2012; Kajstura et al., 2011), suggesting that the presence of c-Kit may uncover a pool of resident RPCs critical for the maintenance of neuronal cells responsible for vision.

Here, we report for the first time that the mouse eye possesses a primitive c-Kit$^+$ cell that is self-renewing, clonogenic and multipotent, the three critical identifiers of tissue specific stem/progenitor cells (Weissman, 2000). In addition, lineage tracing techniques *in vivo* demonstrate that the major cell types in the ONL and INL of the adult retina are progeny of c-Kit$^+$ cells. The identification of this class of resident progenitor cells in the postnatal and adult eye will help to advance our understanding of neuronal regeneration and tissue repair in disorders of the retina.

2. Materials and methods

2.1. c-Kit lineage tracing

A lineage tracing model in mice, c*Kit*$^{CreERT2+}$, was utilized for genetic fate mapping studies as previously described (Goss et al., 2016; Hatzistergos et al., 2015). In brief, c*Kit*$^{CreERT2+}$ mice contain a *Cre-ERT2* construct inserted in the first exon of c-Kit. Upon transcription from the c-Kit locus, *Cre-ERT2* is expressed and remains in the cytoplasm. In the presence of tamoxifen (TAM), the *ERT2* receptor is activated and *Cre* is translocated to the nucleus, where it promotes recombination. The c*Kit*$^{CreERT2+}$ mice were bred to *IRG* reporter mice (De Gasperi et al., 2008). The mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at the University of Miami, Miller School of Medicine, and procedures were performed using Institutional Animal Care and Use Committee-approved protocols according to NIH standards.

2.2. Tamoxifen administration

For lineage tracing studies, *Cre-ERT2* was activated by intraperitoneal injections of 100 μL of TAM (Sigma-Aldrich), dissolved in peanut oil (Sigma-Aldrich) at a concentration of 20 mg/ml, or 400 mg/kg in food at designated time points, as described previously (Goldstein et al., 2015; Hatzistergos et al., 2015). In the 4-day treatment group, the 6-month-old mice received a daily injection of TAM for 4 consecutive days and the eyes were harvested on day 10 from the initial injection. For treatment groups of 1 and 3.5 months, the 6-week-old mice received TAM from food followed by the harvesting of eyes. One eye was used for flow cytometry assays and the other was for immunohistochemical staining. Mice carrying only the *cKit*$^{CreERT2+}$ and *cKit*$^{+/+}$*IRG* alleles, receiving the same TAM treatment as *cKit*$^{CreERT2+}$*IRG* mice, and the *cKit*$^{CreERT2+}$*IRG* mice without TAM treatment were used as controls.

2.3. Isolation and in vitro culture of c-Kit$^+$ cells

Cell culture was performed as previously described (Klassen et al., 2004; Li et al., 2013), with modifications. Mice were sacrificed on postnatal day 1 (P1), The retinas were dissociated in PBS containing collagenase I (10 mg/ml) and collagenase II (25 mg/ml, Worthington Biochemical). The dissociated cells were cultured in dishes pre-coated with laminin (20 μg/ml, Sigma-Aldrich), in growth medium, containing DMEM/F12 medium (Lonza) supplemented with murine basic fibroblast growth factor (bFGF, 20 ng/ml, PeproTech), murine epidermal growth factor (EGF, 20 ng/ml, PeproTech), B27 (1:50, Gibco), N2 (1:100, Gibco), insulin/transferrin/sodium selenite (1:500, Lonza) and leukemia inhibitor factor (LIF, 10 ng/ml, Chemicon). After expansion of the cells, c-Kit$^+$ cells were sorted using anti-mouse c-kit (CD117) MicroBeads (Miltenyi Biotec, Supp Table 1).

2.4. Differentiation of c-Kit$^+$ RPCs

Cell differentiation assays were performed as previously described (Kelley et al., 1994; Li et al., 2013), with modifications. c-Kit$^+$ cells were cultured in differentiation conditions using DMEM/F12 medium supplemented with bFGF (10 ng/ml) and B27 (1:50) for the next 2 days. To promote neural cell differentiation, including Müller cells and bipolar cells, the cells were next switched to the differentiation medium plus N2 (1:100) for another 6 days. For amacrine cell differentiation, cells were cultured in the differentiation medium plus JAG1 (40 nM, AnaSpec, Inc.) for 6 days. For horizontal cell differentiation, cells were cultured in the differentiation medium plus nerve growth factor (NGF, 10 ng/ml, Sigma-Aldrich) and insulin-like growth factor 1 (IGF-1, 10 ng/ml, Sigma-Aldrich) for 6 days. For photoreceptor differentiation, cells were cultured in the differentiation medium plus N2 (1:100), Docosahexaenoic acid (DHA, 50 nM, Sigma-Aldrich), Retinoic acid (RA, 2 μM, Sigma-Aldrich), γ-secretase inhibitor (DAPT, 10 μM, Sigma-Aldrich) for 2 days, and then changed to medium containing DMEM/F12 with B27 (1:50), NGF (10 ng/ml), IGF-1 (10 ng/ml) and brain-derived neurotrophic factor (BDNF, 10 ng/ml, Sigma-Aldrich) for another 4–6 days.

2.5. Limiting dilution and clone formation

Limiting dilution for clone formation was performed as we previously described (Liu et al., 2015). Briefly, 100 c-Kit$^+$ cells were plated in 100 mm diameter dish (the density, ≈1 cell/60 mm^2) to obtain multicellular clones derived from a single founder cell. The clones formed over 2–3 weeks in growth medium (see isolation and *in vitro* culture of c-Kit$^+$ cells), were then imaged by phase contrast microscopy and fluorescent microscopy for c-Kit immunostaining (see immunocytochemistry staining).

2.6. Flow cytometry

For the surface marker, cells dissociated from retinal tissue or cells detached from culture dishes were blocked with CD32/16 for 15 min at room temperature (RT). Then, the cell suspensions were incubated

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