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Lin28a regulates neurogliogenesis in mammalian retina through the Igf signaling

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ABSTRACT

In the developing central nervous system (CNS) the majority of neurons are born before the generation of glia. Emerging evidence implicates heterochronic gene, *Lin28* in the temporal switch between two distinct lineages. However, the respective contributions of *Lin28a* and *Lin28b* in neurogliogenesis remain poorly understood. Here, we have examined the relative involvement of *Lin28a* and *Lin28b* in mammalian retina, a simple and accessible CNS model where neurogliogenic decision largely occurs postnatally. Examination of Lin28a/b involvement during late histogenesis by the perturbation of function approaches revealed that while Lin28b did not influence differentiation in general Lin28a facilitated and antagonized the generation of neurons and glia, respectively. Silencing of Lin28a expression in vitro and its conditional deletion in vivo during early histogenesis led to premature generation of glia. The instructive role of Lin28a on neuronal differentiation was revealed by its influence to suppress glial-specific genes and directly differentiate glia along the neuronal lineage. This function of Lin28a is likely mediated through the Igf signaling, as inhibition of the pathway abrogated Lin28a-mediated neurogliogenesis. Thus, our observations suggest that Lin28a is an important intrinsic factor that acts in concert with cell-extrinsic factors like Igfs, coordinating the developmental bias of the progenitors and niche, respectively, for the successive generation of neurons and glia.

1. Introduction

During the development of the vertebrate central nervous system (CNS), neurons and glia are generated in an evolutionarily conserved temporal sequence wherein neurogenesis precedes gliogenesis (Bayer and Altman, 1991; Miller and Gauthier, 2007). However, the mechanism by which the differentiation of neural progenitor cells (NPCs) is switched from one lineage to another remains poorly understood. Homologues of heterochronic genes *Lin28* and *let-7* miRNA, which regulate the temporal progression of developmental stages in nematodes (Rehfeld et al., 2015), have recently been implicated in the temporal aspect of vertebrate neurogenesis (Kawahara et al., 2012, Lang et al., 2012; La Torre et al., 2013). Evidence suggests that Lin28a/b, an RNA binding protein that executes its heterochronic function by inhibiting the processing of *let-7*, represents a conserved bi-stable molecular axis that regulates progenitors' progression through successive stages during neurogenesis. For example, in an in vitro model of neural development using the P19 cells, a mouse embryonic carcinoma cell line, it was observed that Lin28a or Lin28b expression was preferentially associated with the generation of neurons, but was incompatible with gliogenesis (Balzer et al., 2010). The differential effect of Lin28a/b on neuronal versus glial differentiation was found to be independent of *let-7*. By contrast, another study demonstrated, using NPCs isolated from either human pluripotent cells or fetal tissues, that *let-7* alone could regulate the fate of NPCs along neuronal and glial lineages (Patterson et al., 2014). Moreover, they observed that *let-7* influenced these divergent fates through Hmga2, a DNA architecture protein, contradicting the notion that it regulates self-renewal of progenitors (Nishino et al., 2013; Parameswaran et al., 2014). Another study using NPCs isolated from human embryonic stem (ES) cells demonstrated that *let-7b* and *let-7i* inhibited differentiation of neurons by targeting proneural transcripts, *Ascl1* (Cimadamore et al., 2013). Whether or not *let-7b*/*let-7i* influenced differentiation of progenitors along the glial lineage was not reported. A more recent study using transgenic knockout (Lin28a/Lin28b) and knock-in (Lin28a) mouse technology observed that Lin28a/b promotes NPCs proliferation (Yang et al., 2015). This finding is in conflict with Balzer et al., 2010, who observed that ectopic expression of Lin28a/b facilitated neuronal differentiation without affecting cell division (Balzer et al., 2010). The exact role of Lin28a/b in neurogliogenesis

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thus remains vague, presumably due to the diverse models and approaches used to decipher it.

Here, we examined Lin28 involvement in the neurogliogenesis of mammalian retina, a simple and accessible model of the CNS. The retina consists of seven different cell types generated by the multipotential retinal progenitor cells (RPCs) in two distinct evolutionarily conserved stages of histogenesis (Young, 1985; Rapaport et al., 2004). In rodent retina, retinal ganglion cells (RGCs), horizontal cells (HCs), cone photoreceptors (CPs), and amacrine cells (ACs) are born during early histogenesis, between the embryonic days (E) 11.5 and E18 and the majority of rod photoreceptors (RPs), bipolar cells (BCs) and Müller glia (MG) are generated during late histogenesis, between E18 and postnatal day (PN) 6 (Young, 1985; Rapaport et al., 2004). During late histogenesis, RPs, the predominant cells in the rodent retina, are born significantly earlier than BCs, the majority (95%) of which are generated earlier than MG (95%) (Rapaport et al., 2004). In the developing retina *let-7* and *Lin28b* have been implicated in the temporal identity of RPCs, however, their respective involvement in the out put of neurons versus glia remained undefined (La Torre et al., 2103). We recently demonstrated that Hmga2, a target of *let-7*, regulates self-renewal of RPCs (Parameswaran et al., 2014) and that *let-7* plays an important role in the differentiation of RPCs into neurons and MG during late histogenesis, without preference to a particular lineage (Xia and Ahmad, 2016a). We observed that the positive influence of *let-7* on neuronal and glial differentiation is due to *let-7*-mediated degradation of Hmga2 transcripts, and that this degradation tips the balance from maintenance of RPCs in favor of their differentiation (Xia and Ahmad, 2016a). Here, we demonstrate that Lin28a and Lin28b facilitate RPC proliferation, but that the expression of Lin28a additionally facilitates neuronal differentiation and is incompatible with glial differentiation of late RPCs. Conditional deletion of *Lin28a* during early retinal histogenesis leads to premature generation of glia. Its ectopic expression also converts MG into neurons, positing it as an important factor in the regulation of RPCs' differentiation in deference to neurons. Our results further suggest that the influence of Lin28a on neuronal differentiation is mediated through Igf-2 signaling, thus demonstrating interplay between cell-intrinsic and cell-extrinsic factors during neurogliogenic decision.

2. Materials and methods

2.1. Animal maintenance and use

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center (protocols: #95-005-09 and #97–100-08). Animals (male and female rats and mice) were housed in the Department of Comparative Medicine at UNMC. Timed pregnant Sprague Dawley rats were purchased from SASCO and Charles River Laboratories to carry out all experiments. Transgenic mice were purchased from the Jackson Laboratory to carry out conditional knockout experiments. Rats and mice were euthanized by CO_2 exposure followed by decapitation with sterile surgical scissors to ensure death.

2.2. Generation of conditional knockout mice

To study the role of Lin28a, we mated the *Lin28a*^{flox} mouse line (Stock# 023913) with the *CAGGCre-ER*^{TM} transgenic mouse line (Stock# 004682). Heterozygous mice were used to obtain *Lin28a*$^{flox/flox}$; *CAGGCre-ER*^{TM} animals. Tamoxifen was delivered by intraperitoneal (IP) injection at 11, 12, 13, and 14 days post-conception to generate *Lin28a*$^{CKO/CKO}$ embryos.

2.3. Neurosphere assay

Retinae from embryonic day 18 (E18) rats were dissected and dissociated as previously described (Xia and Ahmad, 2016a). Neurospheres were generated from dissociated E18 retinal cells by culturing for 5 days in RCM (DMEM-F12, N2 supplement, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin) and FGF2 (10 ng/ml). On the fifth day, generated neurospheres were collected and transferred to Poly-D-Lysine- and laminin-coated plates and cover slips. Neurospheres were cultured in RCM:E18 conditioned medium (1:1) supplemented with 2% Knockout Serum (KOS), 1 mM Taurine, 3 µM DAPT, 500 nM retinoic acid, and 15 ng/ml BMP4 to facilitate differentiation. Differentiation was terminated 5 days after plating. To examine the influence of IGF on neurogliogenesis Igf-1 (100 ng/ml) and Igf-1 plus Igf pathway inhibitor, piropodophyllin (PPP; 500 nM) were added to the differentiation medium. E18 retinal conditioned medium was prepared as described (Xia and Ahmad, 2016a); E18 retinal cell dissociates were plated at a density of 1×10^5 cells/cm^2 in RCM with 2% KOS and after 3 days conditioned medium was collected, centrifuged, and filtered using 0.2 µm filters, and stored in − 80 °C until use.

2.4. Retinal explant culture

Retinal explant cultures were performed as previously described (Xia and Ahmad, 2016a). E18/E14 retinae were placed on a 0.4 µm semi-permeable membrane (Millipore, Temecula, CA), with RGC layer side up, and cultured for 10 days with RCM and 10% fetal bovine serum (FBS).

2.5. Viral vectors

Viral constructs for Lin28a overexpression (pMSCV-mLin28a; control, pMSCV) and Lin28b overexpression (pBABE-hLin28b; control, pBABE) and CAG-GFP retrovirus construct were obtained from Addgene. The piLenti-Lin28a siRNA and piLenti-Lin28b siRNA for Lin28a/b knockdown and control piLenti-siRNA-GFP constructs were obtained from Applied Biological Materials (BC, Canada). The backbone of piLenti-Lin28a siRNA, piLenti-Lin28b siRNA, and piLenti-siRNA-GFP construct was the dual promoter viral plasmid, wherein siRNA and *GFP* expression was driven by U6 and CMV promoters, respectively.

2.6. Virus preparation and transduction

Lentivirus and retrovirus preparation and transduction were carried out as previously described (Xia and Ahmad, 2016a; Das et al., 2007). Recombination lentiviral particles were generated through transient transfection of T293 cells, using the ABM lentivirus packaging system (BC, Canada). Recombination retroviral particles were generated in BOSC-23 cells using Fugene transfection reagent (Promega, Madison, WI). Viral particles were concentrated using a BioVision PEG lentivirus precipitation kit (Milpitas, CA). Virus titers were determined using the ABM lentivirus titration kit. Retinal dissociates, neurosphere cells, and explants were transduced with lentiviruses with multiplicity of infection (MOI) of 4. Twelve hours after transduction, the virus-containing medium was replaced by fresh medium. Transduction efficiency was determined 48 h post-transduction by direct observation and/or FACS of GFP$^+$ cells. Perturbation experiments were carried out three times in triplicate groups, as follows: 10–14 E18 embryos per group (*in vitro* perturbation) and 9 retinae per group (*ex vivo* perturbation).

2.7. Enrichment and neural induction of MG

MG were enriched as previously described (Das et al., 2006; Xia and Ahmad, 2016b). Eyes from 10 PN10 rats were isolated and incubated overnight in DMEM, transferred into dissociation solution, and incubated at 37° for 1 h. Retinae were dissected out, mechanically dissociated, and cultured in DMEM with 10% FBS for 8–10 days.

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