Stem Cell Research 15 (2015) 554–564

Contents lists available at ScienceDirect

Stem Cell Research

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

Runx1 promotes proliferation and neuronal differentiation in adult mouse neurosphere cultures

T.T. Logan, M. Rusnak, A.J. Symes *

Department of Pharmacology and Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

ARTICLE INFO

Article history:
Received 23 June 2014
Received in revised form 21 September 2015
Accepted 26 September 2015
Available online 3 October 2015

Keywords:
Runx1
AML1
Adult neural stem cells
Neurosphere
Proliferation
Neuronal differentiation

ABSTRACT

Traumatic brain injury alters the signaling environment of the adult neurogenic niche and may activate unique proliferative cell populations that contribute to the post-injury neurogenic response. Runx1 is not normally expressed by adult neural stem or progenitor cells (NSPCs) but is induced in a subpopulation of putative NSPCs after brain injury in adult mice. In order to investigate the role of Runx1 in NSPCs, we established neurosphere cultures of adult mouse subventricular zone NSPCs. We show that Runx1 is basally expressed in neurosphere culture. Removal of the mitogen bFGF or addition of 1% FBS decreased Runx1 expression. Inhibition of endogenous Runx1 activity with either Ro5-3335 or shRNA-mediated Runx1 knockdown inhibited NSPC proliferation without affecting differentiation. Lentiviral mediated over-expression of Runx1 in neurospheres caused a significant change in cell morphology without reducing proliferation. Runx1-overexpressing neurospheres changed from floating spheres to adherent colonies or individual unipolar or bipolar cells. Flow cytometry analysis indicated that Runx1 over-expression produced a significant increase in expression of the neuronal marker TuJ1 and a minor increase in the astrocytic marker S100β. Thus, Runx1 expression drove adult NSPC differentiation, predominantly toward a neuronal lineage. These data suggest that Runx1 could be manipulated after injury to promote neuronal differentiation to facilitate repair of the CNS.

Published by Elsevier B.V.

1. Introduction

Neural stem and progenitor cells (NSPCs) in the hippocampus and subventricular zone are a constant source of new neurons in the adult mammalian brain (Ming & Song, 2011). Following various types of brain injuries, including traumatic brain injury, these NSPCs proliferate and generate new neurons at an increased rate (Yu et al., 2008; Enikolopov & Chen, 2009; Richardson et al., 2010; Zheng et al., 2013; Chen et al., 2003; Chirumamilla et al., 2002; Sun et al., 2007). This injury-induced neurogenesis may contribute to post-injury maintenance and recovery of cognitive ability (Blaiss et al., 2011), and to the repopulation of neurons in damaged areas (Yu et al., 2008; Arvidsson et al., 2002; Sohur et al., 2006). However, the regulatory signals that control injury-induced neurogenesis and the stem cell population on which they act are poorly understood.

Injury alters the signaling environment of the neurogenic niche (Logan et al., 2013; Villapol et al., 2013) and may also activate unique proliferative cell populations that contribute to the post-injury neurogenic response (Lopez-Juarez et al., 2013). However, the changes in composition of the heterogeneous cell population within the neurogenic niches of the adult brain with injury are not well understood. We recently found that expression of the transcription factor Runx1 is induced in a subpopulation of proliferative putative adult NSPCs in the SVZ (subventricular zone) and dentate gyrus after traumatic brain injury in adult mouse brain, areas where Runx1 is not normally expressed (Logan et al., 2013). The function of Runx1 induction post-injury in the neurogenic niche is not known.

Runx1 was originally identified in cases of acute myeloid leukemia, where *Runx1* chromosomal fusions lead to acute leukemia (Miyoshi et al., 1991; Ito, 2008). Subsequently, Runx1 was found to be essential for definitive hematopoiesis, regulating the transcription of several critical genes for hematopoietic development (Friedman, 2009). In hematopoietic stem cells (HSCs), over-expression of full-length Runx1 causes murine HSCs to exit the cell cycle and become quiescent, whereas over-expression of an endogenous truncated dominant negative Runx1 (Runx1a) leads to increased proliferation (Tsuzuki et al., 2007; Challen & Goodell, 2010). In numerous other cell types, Runx1 regulates cell proliferation, sometimes promoting, and sometimes inhibiting, cell division (Friedman, 2009; Scheitz & Tumbar, 2013; Blyth et al., 2005;

Abbreviations: TBI, traumatic brain injury; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; NSPC, neural stem and progenitor cell; FBS, fetal bovine serum; HSCs, hematopoietic stem cells; MOI, multiplicity of infection; DCX, doublecortin; SVZ, subventricular zone.

* Corresponding author at: Department of Pharmacology, Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD, 20814.
E-mail address: aviva.symes@usuhs.edu (A.J. Symes).

http://dx.doi.org/10.1016/j.scr.2015.09.014
1873-5061/Published by Elsevier B.V.

Hoi et al., 2010). Runx1 is primarily a sequence-specific DNA binding protein. Runx1 stability and DNA binding is enhanced approximately ten-fold through complexing with the protein, CBFβ (Bravo et al., 2001; Tang et al., 2000a; Tang et al., 2000b). Both proteins are necessary for normal Runx1 function in vivo, as demonstrated by the similar phenotype of mice that are null for either Runx1 or CBFβ (Wang et al., 1996).

Runx1 also controls the proliferation and neuronal differentiation of certain neural progenitor cell populations. In embryonic olfactory bulb progenitor cells in vivo, as well as in cultures of either embryonic olfactory progenitor cells or embryonic cortical NSPCs, Runx1 increases cell proliferation and also increases the expression of the neuronal marker protein NeuroD (Theriault et al., 2005). However, Runx1 inhibits the proliferation of embryonic microglia (Zusso, 2012) and olfactory ensheathing cells (Murthy et al., 2014). Given its ability to regulate stem and progenitor cell proliferation and differentiation, induction of Runx1 in some NSPCs after injury (Logan et al., 2013) suggests that Runx1 could be regulating the injury-induced proliferation or neuronal differentiation of a specific subpopulation of adult NSPCs.

We therefore investigated the expression and function of Runx1 in adult NSPCs, using neurosphere cultures of adult mouse NSPCs. We demonstrate that Runx1 is expressed in adult neurosphere cultures, and expression decreases in different culture conditions. We found that inhibiting Runx1 activity can inhibit adult NSPC proliferation, and that induction of Runx1 through lentiviral mediated over-expression pushes NSPCs predominantly toward a neuronal lineage. Thus, Runx1 may play an important role in the injury-induced neurogenesis seen in the adult mammalian brain after models of traumatic brain injury.

2. Materials and methods

2.1. Adult neurosphere cultures

All animal care and procedures were approved by the Institutional Animal Care and Use Committee at Uniformed Services University and performed in accordance with their guidelines. Adult male C57BL/6 mice (NCI, Frederick, MD), approximately 9 weeks of age, were deeply anesthetized by isoflurane inhalation and euthanized by decapitation. Brains were excised, placed in ice-cold PBS, and the tissue adjacent to the anterior portion of the lateral ventricles containing the SVZ carefully dissected out. This tissue was homogenized (Miltenyi neural tissue dissociation kit, Miltenyi Biotec, MA), passed through a 70 μM filter to obtain a single cell suspension, and plated in Neurocult complete culture media (Stem Cell Technologies, WA, 05702) supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, and 2 μg/mL heparin ('growth' media). These primary cultures were grown initially for 7 days. Cells were then passaged every 5 days for subsequent passage numbers. Cells from passages 3–5 were used for all experiments. For treatment of neurosphere cultures with the Runx1 inhibitor Ro5-3335 (Calbiochem, MA, no. 219506), Ro5-3335 was resuspended at 250 mM in DMSO, and further diluted in sterile PBS for treatments. The highest concentration of inhibitor resulted in a 0.01% DMSO concentration. Thus, 0.01% DMSO was used as a control. For treatment with Ro5-3335, cells were plated at a density of 6×10^5 cells/100 mm dish. For lentiviral transduction experiments, cells were plated in 48 well culture dishes at a density of 2000 cells/well.

2.2. Lentivirus generation and treatment

Lentiviral plasmids pLenti-suCMV(Runx1)-Rsv(GFP-Bsd) (termed LV-Runx1) and LV-PL3 (LV-RSV(GFP-Bsd) (termed LV-GFP)) were purchased from GenTarget inc (Gentarget, CA). Runx1 silencing shRNA lentiviral plasmids (GIPZ Mouse Runx1 shRNA) with clone IDs: V2LMM_70199, V3LMM_506997, V3LMM_506996, V3LMM_521271, and GIPZ non-silencing lentiviral shRNA control (RHS4348) were purchased from Thermo-Scientific (Pittsburgh, PA). Lentiviral plasmids from Gentarget, packaged with VSVG and delta 8.2 plasmids, and GIPZ Runx1 silencing and non-silencing lentiviral plasmids packaged with psPAX2 and pMD2.G plasmids were transfected into HEK 293 T cells with lipofectamine in DMEM/10%FBS. 12 h after transfection, the media was discarded and replaced by serum-free media. Media containing viral particles was collected every 12 h, up to 36 h with fresh serum-free media added to the cells after each collection. Viral containing media was stored at 4 °C until 3 sets of media were collected. Combined viral containing media was centrifuged at low speed to clear large debris and filtered through a 0.45 μM filter. The virus particles were then concentrated by ultracentrifugation at 25,000 rpm in a Beckman SW27 rotor for 2 h, and resuspended in sterile PBS. Viral titer was determined by analysis of GFP expression in HEK 293 T transduced cells, 72 h after viral transduction by flow cytometry. Neurosphere cultures were transduced by lentiviral particles at an MOI of 10, 1 day after passaging.

2.3. Protein extraction and Western blots

Neurospheres were grown in media containing the indicated growth factors for 24 h before harvest. Both floating neurospheres and loosely adherent cells were collected, pelleted by low-speed spin at 4 °C, washed with ice-cold PBS, and resuspended in 1% RIPA lysis buffer containing protease and phosphatase inhibitors. Cells were lysed before centrifugation at 1600 *g*, at 4 °C for 20 min. Protein concentration in the supernatant was determined by the BCA protein assay (Pierce, Rockford, IL, USA). 15 μg protein was combined with loading buffer, boiled for 5 min, before loading onto an SDS–polyacrylamide gel. Separated proteins were transferred onto nitrocellulose membranes and probed with rabbit anti-Runx1 antisera (1:1000, Novus, no. NBP1-89105), or rabbit anti-β-actin (1:8000, Sigma, no. A1978), followed by incubation with goat anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling, MA). Blots were developed with supersignal-enhanced chemiluminescence reagent (Thermo-Scientific) and detected using a Fuji LAS-3000 image acquisition system (Fuji, Stamford, CT, USA) equipped with a cooled CCD camera. For each experiment, blots were first probed with the Runx1 antiserum, before being stripped and reprobed for β-actin. Runx1 expression was normalized to the expression level of β-actin.

2.4. RNA isolation and qPCR

Cells were harvested, washed with ice-cold PBS, and resuspended in TRIzol reagent (Invitrogen, CA). 0.2 volumes of chloroform were added, tubes were centrifuged at 16,000 *g* for 15 min, and the aqueous layer was extracted for RNA isolation. Further purification proceeded with the RNeasy mini kit (Qiagen, MD). Isolated RNA was then incubated with RNase-free DNase according to the manufacturer's instructions and quantified with a Nanodrop spectrophotometer (Thermo Scientific, DE). RNA integrity was checked by agarose gel electrophoresis. qPCR was performed with SYBR Green qPCR MasterMix (Qiagen, CA) using the BioRad CFX96 instrument and the following gene-specific primers: Runx1; forward 5′TGGCACTCTGGTCACCGTCAT3′, reverse 5′GAAGCTCT TGCCTCTACCGC3′, cyclin D1; forward 5′AGAGGGCTGTCGGCGCAGTA3′, reverse 5′GGCTGTGGTCTCGGTTGGGC3′, p21; forward 5′TCCAGGAGG CCCGAGAACGG3′, reverse 5′CTCCGAACGCGCTCCCAGAC3′. GAPDH; forward 5′ACCATCTTCCAGGAGCGAGA3′, reverse 5′GGCGGAGATGATGA CCCT3′. Reactions consisted of 10 min incubation at 95 °C, then 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 58 °C for 10 s. Expression levels of all genes of interest were normalized to GAPDH, and relative changes in gene expression were calculated using the delta delta threshold cycle method (Susarla et al., 2011).

2.5. Cell culture imaging and quantifications

Neurospheres were treated 24 h following passage with either different concentrations of Ro5-3335 or lentiviral particles. Cells were