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BET bromodomain inhibition promotes neurogenesis while inhibiting gliogenesis in neural progenitor cells

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ABSTRACT

Neural stem cells and progenitor cells (NPCs) are increasingly appreciated to hold great promise for regenerative medicine to treat CNS injuries and neurodegenerative diseases. However, evidence for effective stimulation of neuronal production from endogenous or transplanted NPCs for neuron replacement with small molecules remains limited. To identify novel chemical entities/targets for neurogenesis, we had established a NPC phenotypic screen assay and validated it using known small-molecule neurogenesis inducers. Through screening small molecule libraries with annotated targets, we identified BET bromodomain inhibition as a novel mechanism for enhancing neurogenesis. BET bromodomain proteins, Brd2, Brd3, and Brd4 were found to be downregulated in NPCs upon differentiation, while their levels remain unaltered in proliferating NPCs. Consistent with the pharmacological study using bromodomain selective inhibitor (+)-JQ-1, knockdown of each BET protein resulted in an increase in the number of neurons with simultaneous reduction in both astrocytes and oligodendrocytes. Gene expression profiling analysis demonstrated that BET bromodomain inhibition induced a broad but specific transcription program enhancing directed differentiation of NPCs into neurons while suppressing cell cycle progression and gliogenesis. Together, these results highlight a crucial role of BET proteins as epigenetic regulators in NPC development and suggest a therapeutic potential of BET inhibitors in treating brain injuries and neurodegenerative diseases.

1. Introduction

Remarkable progress has been made in the research and application of neural stem cells (NSCs) and progenitor cells (NPCs) demonstrating their tremendous potential for stem-cell based cell therapy or targeting endogenous NPCs to treat CNS injury and neurodegenerative diseases (Gage and Temple, 2013; Goldman et al., 2012; Gupta et al., 2012; Lu et al., 2012; Ming and Song, 2011). New opportunities have emerged to discover neural regenerative therapeutics towards significant unmet medical needs. However, many challenges still remain. For example, in injured CNS and under disease conditions, transplanted NPCs preferentially become astrocytes (Aboody et al., 2011; Reekmans et al., 2012; Robel et al., 2011). Small molecules that modulate the developmental processes of NSCs or NPCs towards desired cell fate not only offer significant opportunities for therapeutic drugs targeting endogenous NPCs for repair and regeneration, but also could enhance the efficacy of NSC transplantation for neuronal replacement (Li et al., 2013).

Once committed to a certain cell fate, NPCs undergo cell cycle arrest and terminal differentiation leading to the exhibition of cell-type-specific features. NPC differentiation including neurogenesis and gliogenesis is a highly orchestrated process that is tightly regulated via both extrinsic environmental signals and intrinsic changes in gene expression and epigenetic regulation. Several crucial signaling pathways including Wnt, Notch and the bone morphogenetic proteins (BMPs) pathway have been identified in regulating the development of NPCs (Faigle and Song, 2013; Kriegstein and Alvarez-Buylla, 2009). The interplay of transcription factors and epigenetic modifiers, including histone modifications, DNA methylation and microRNAs during development is essential for NPCs to control self-renewal, fate

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specification, and differentiation (Hirabayashi and Gotoh, 2010; Juliandi et al., 2010). Suppression of astrocytic lineage genes during the neurogenic phase is one of the key cell-intrinsic epigenetic mechanisms underlying fate specification (Kanski et al., 2014; Sun et al., 2001). Recent studies have identified many different types of epigenetic regulators, including polycomb group and trithorax group proteins, DNA-damage inducible protein 45b, methyl-CpG-binding protein MBD1, DNA methyltransferases, histone deacetylases (HDACs) and acetyltransferases (HATs), which are involved in the tight regulation of the proliferation and specification of NPCs or the differentiation and maturation of newborn neurons (Lim et al., 2009; Ma et al., 2010; Wu et al., 2010; Zhao et al., 2003). In the case of histone modifications, extensive studies have now illustrated the important role of the histone code in methylation and acetylation, epigenetic writers (HATs) and erasers (HDACs) in neurogenesis (Hsieh et al., 2004; Merson et al., 2006; Montgomery et al., 2009; Prozorovski et al., 2008; Yu et al., 2009). However, little is known about epigenetic readers in the development of NPCs and neurogenesis.

The bromodomain and extraterminal (BET) family of bromodomain-containing proteins (Brds), including Brd2, Brd3, Brd4, and testis-specific BrdT, are epigenetic readers of the acetylation histone code on chromatin. The two tandem bromodomains of BET proteins bind acetylated lysine in histone N-terminal tails. The binding is proposed to assist the recruitment and passage of RNA polymerase II co-regulatory complexes facilitating transcription of target genes (Belkina and Denis, 2012; Filippakopoulos and Knapp, 2014; Loven et al., 2013; Nicodeme et al., 2010; Shi and Vakoc, 2014). BET proteins regulate expression of multiple genes of therapeutic relevance, including those involved in tumor cell growth, inflammatory response, and cardiac hypertrophy (Anand et al., 2013; Delmore et al., 2011; Filippakopoulos et al., 2010; Nicodeme et al., 2010). The understanding of BET biology has been greatly accelerated by the discovery of selective, small-molecule inhibitors of BET bromodomains that specifically disrupt the interaction between BET proteins and acetylated histones (Chung et al., 2011; Filippakopoulos et al., 2010; Gosmini et al., 2014; Nicodeme et al., 2010). BET bromodomain inhibitors (I-BET) have been used to probe BET function in a number of developmental and disease contexts, such as spermatogenesis, infection, cancer, and heart failure (Anand et al., 2013; Asangani et al., 2014; Dawson et al., 2011; Henssen et al., 2013; Matzuk et al., 2012; Nicodeme et al., 2010; Puissant et al., 2013; Wyce et al., 2013). Recently, I-BET, included in a small-molecule cocktail that can directly convert fibroblasts into neurons, was proposed to play a role in disrupting the fibroblast-specific program during reprogramming (Li et al., 2015). However, the direct role of BET bromodomain proteins in NPC development, particularly NPC differentiation, remains less well characterized.

In this study, we discovered BET bromodomains as a novel target for neurogenesis through a NPC phenotypic screen aimed to identify drug-like small molecules inducing neuronal differentiation. We provide evidence that BET bromodomain proteins are critical players in NPC development including cell cycle progression, fate specification and cell differentiation. BET bromodomain inhibition induces a broad but specific transcription program promoting neurogenesis while simultaneously inhibiting gliogenesis. Our study establishes a rationale for using BET inhibitors to enhance neuronal differentiation efficacy in regenerative cell therapy.

2. Materials and methods

2.1. NPC culture, proliferation and differentiation assay

Mouse NPCs were isolated, cultured and passaged as described in Kim et al. (Kim et al., 2012). Briefly, NPCs from E12 mouse cortices were isolated and expanded in DMEM/F12 containing 2% of B27-supplement and 10 ng/ml of basic fibroblast growth factor (bFGF). NPCs of passage 3 were used in all assays.

For proliferation assay, NPCs were expanded in the presence of bFGF (10 ng/ml) refilled daily to the culture medium. For differentiation assay, NPC were allowed to differentiate spontaneously in the absence of bFGF for 5 days. Cells were then subject to Immunocytochemistry staining. Neurons, astrocytes and oligodendrocyte lineage were immunno-labeled with Tuj1, GFAP and Olig2 antibody, respectively.

Immunocytochemistry images were scanned and analyzed by using Cellomics (Target Activation V4 algorithm, ArrayScan VTI 700, Thermo Fisher Scientific, MA, USA), or Acumen (Composite population manager, Explorer X3, TTP LabTech, Royston, UK) automated high content imaging and analysis platforms.

2.2. Compound Screening, dose-response series and curve fitting

GlaxoSmithKline's focused compound libraries (~8300 compounds with target annotation) were used for NPC differentiation phenotypic screen. Primary screen was carried out in single shot (1 μM of each compound in 0.01% DMSO) and 3 replicates in the format of 384-well plates. Each plate had tool compound and 0.01% DMSO as positive and negative control, respectively. Primary hits were defined as the compounds that promote neuronal differentiation by at least 3 SD above the fold change of $Tuj1^{+}$ cell percentage relative to control DMSO. Primary hits were subject to 8-point or 10-point dose-response studies with series dilution of compound in triplicates. Dose-response curves were generated from XL fit (IDBS, Burlington, MA, USA) using the dose-response onsite formula as follows: fit = (A + (B / (1 + ((x/C)^D)))); inv = ((((B/(y – A)) – 1)^(1/D)) ∗ C); res = (y − fit).

2.3. Time-resolved fluorescence resonance energy transfer titrations (TR-FRET)

Test compounds were titrated against pre-coupled Bromodomain fragment and tetra-acetylated histone H4 (1−21) peptide as previously described (Chung et al., 2011). The interaction of Brds and histone peptide was determined using Time Resolved FRET.

2.4. Immunocytochemistry

Cells were fixed by 4% paraformaldehyde for 20 min, washed 3 times with phosphate-buffer (PBS) and proceeded as described in Xu et al. (Xu et al., 2013). The following primary antibodies were used: anti-Tuj1(Millipore, MAB1637), anti-GFAP(DAKO, Z0334), and anti-Olig2 (Millipore, MABN50).

2.5. Immunoblotting

Western Blotting was performed using the following antibodies: Anti-Brd2 (Bethyl Laboratories, A302-583A), anti-Brd3 (Bethyl Laboratories, A302-368A), and anti-Brd4 (Bethyl Laboratories, A301-985A), monoclonal anti-β-actin-peroxidase clone AC-15 (Sigma, A3854).

2.6. Construction of Brd shRNA, generation of lentivirus and NPC infection

Mouse Brd2, 3 and 4 small hairpins RNA (shRNA) (Zuber et al., 2011) DNA constructs (Supplemental Table S1) were cloned in pLVX-shRNA2 vector (Clontech, Mountain View, CA, USA). The knock-down constructs with the following specific sequences for Brd2 (5′-GAAACATCGTGGCCGAATT-3′), Brd3 (5′-TTTATGATAATCAGGCAGGTTC-3′), and Brd4 (5′-TTTGTTGATATCTAGACTTAGC-3′) were validated and selected by DNA sequencing and western blotting. Lentivirus production and titer test were carried out following the previously published protocol (Crittenden et al., 2007). Infections were carried out in proliferating NPC at a M.O.I of 15 for 48 h. Media containing virus was then removed and changed to differentiation media. All the virus related experiments were approved by the Genetically Modified Organism Management Committee of GSK R&D China.