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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.

2.7. RNA-—Seq and data analysis

NPCs were treated with (+)-JQ-1 or (−)-JQ-1 at 0.2 μM or 0.5 μM in differentiation medium for 12 and 24 h. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) and sent to Beijing Genomics Institute (BGI) Tech Solutions for library construction and sequencing (HiSeq2000 sequencing platform, Illumina, CA, USA). Following quality control to raw data using "fastqc" (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), RNA-seq reads were aligned to mouse genome (mm9) using TopHat2 (ref: 23618408). The gene expression was quantified using RPKM values from cufflink analysis using NCBI RefSeq gene annotation. Hierarchical clustering and principal component analysis were performed to all samples based on the similarity of their expression profiles. Using these clustering, we examined the effects of treatments on gene expression. The differentially expressed genes (DEGs) between samples with (+)- JQ1 or (−)-JQ-1 treatment were found using cuffdiff. The thresholds for the DEGs were: Fold change ≥ 1.5 and FDR ≤ 0.05. Then, DEGs from each treatment were applied for functional enrichment and pathway analysis using MetaCore (https://portal.genego.com). Using all expressed genes as the background, DEGs were evaluated for their enrichment to biological processes and pathways. At a cutoff of $p \le 0.01$, we selected the enriched ones and used these terms to describe the functional or processes association.

2.8. qRT-PCR of total RNA

RNA from NPC samples was subject to RT-qPCR as previously described (Xu et al., 2013). The primer sequences were listed in Supplemental Table S2.

2.9. Quantification and statistical analysis

The studies were carried out in at least 3 independent experiments and/or 3 replicates. For quantification of ShRNA knockdown effect on NPC differentiation, immuno-stained cells were imaged from 27 fields per shRNA construct. Each field had 400–600 GFP^+ cells. The numbers of $Tuj1^+$, $GFAP^+$, or $Olig2^+$ cells were counted among the total number of GFP^+ cells in each field. The data were presented as mean ± SEM in the figures. Unpaired student's *t*-test or two-way ANOVA with Bonferroni post-tests were used to evaluate the significance of differences between means. In all cases, significance was noted at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Neurogenesis phenotypic screening identified BET-bromodomain inhibitors

To identify small molecules that selectively direct NPC differentiation into neurons, we previously developed a high-content imaging assay based on the induction of neuronal marker Tuj1 expression in differentiated NPCs, and validated it using GSK 3β inhibitors (Kim et al., 2012). Primary embryonic mouse cortical NPCs proliferate in vitro when cultured in serum-free basal media containing FGF. $Nestin^+$ primary NPCs were passaged twice to expand the limited amount of starting material and to enrich self-renewing $Nestin^+$ progenitor population while depleting any differentiated cells that remained after dissection. Upon removal of FGF, $Nestin^+$ NPCs cease to proliferate, and undergo spontaneous differentiation giving rise to neurons, and glial cells including astrocytes and oligodendrocytes (Kim et al., 2012), which could be identified using the cell type specific markers, Tuj1, GFAP and Olig2, respectively. In this assay that had been optimized for high-throughput chemical genomics screen, small molecule compounds were added to NPCs at the time of FGF withdrawal. Upon 5 days of NPC differentiation, the cells were labeled with Tuj1 antibody to detect neurons, scanned and quantified using Acumen or Cellomics automated high-content imaging and analysis platform (Fig. 1A). The percentage of $Tuj1^+$ cells in total cell number ($Tuj1^+$%) was quantified as the readout for neurogenesis. As commonly observed in cell-based high-throughput assays, variations in the assay readout in 0.01% DMSO vehicle controls were observed among individual wells, plates, cell preparations, and cell batches. Therefore, the average of $Tuj1^+$% for 0.01% DMSO vehicle controls per 384-well plate was used as the baseline reference. All compound data were normalized and shown as the fold change of Tuj1% relative to DMSO control.

To identify the compounds that direct neuronal differentiation, we screened >8000 compounds using highly selective compound libraries with known target associations and broad target coverage at 1 μM screening concentration in triplicates. These compounds were derived from different libraries including Biologically Diverse Compound Set, which is structurally enriched to cover various target classes. Positive hits were defined as the compounds that enhanced $Tuj1^+$ cell formation by at least 3 SD above the mean of DMSO control (Fig. 1B). Among the primary hits, 30 actives were further confirmed by concentration-response analysis that showed a concentration-dependent and reproducible effect, representing a hit rate of ~0.37%. Out of 30 confirmed hits, 21 hits were annotated as BET-bromodomain inhibitors (I-BET). I-BET is a novel class of chemical tools recently identified to selectively inhibit the binding of bromodomain of BET family bromodomain proteins to acetylated lysines in histone resulting in alterations on chromatin recognition and transcription through modulating the epigenetic function of the acetyl-lysine reading process. The neurogenic effect and the concentration-response of representative I-BET compounds are shown in Fig. 1C. The EC_{50}s of active exemplar compounds was in the range of 80 nM and 100 nM. To examine whether the neurogenic effect of I-BET in NPCs correlates with its biochemical binding affinity of BET proteins, we prioritized 17 compounds that have diverse structures and showed activity within a 2 log range of pEC50 potency in independent experimental repeats for evaluation in a time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Chung et al., 2011). The pIC50s of I-BET in TR-FRET assay were determined. The pEC_{50} of BET inhibitors in neuronal differentiation showed a significant correlation with the pIC50 of I-BET in binding analysis ($R^2 = 0.57$, p-value = 0.00043, Fig. 1D). Collectively, using a chemical genomic screen we identified a number of novel selective BET inhibitors as effective neurogenesis inducers.

3.2. The expression of Brd2, Brd3, and Brd4 is down-regulated in NPC upon differentiation

To understand how Brds are involved in NPC development, and inhibition of BET bromodomain induces neuronal differentiation, we examined the expression of Brds in NPCs. Given BrdT is specifically expressed in testis (Shang et al., 2004), we assessed the expression of Brd2, Brd3, and Brd4 of BET family in the proliferating and differentiating NPCs. Brd2, Brd3 and Brd4 were highly expressed in proliferating NPCs. There was no significant change in the expression of Brds in proliferating NPCs (Fig. 2A), while the expression level of Brd2, Brd3, and Brd4 were markedly decreased upon differentiation and had a trend of continuous decrease with time (Fig. 2B). These results indicated that the down-regulation of BETs is associated with the initiation and progression of NPC differentiation.

3.3. BET inhibition promotes neurogenesis while blocking gliogenesis

To validate the role of Brds in NPC development, we generated and validated lentiviral vectors expressing small hairpin RNAs (shRNAs) that target Brd2, Brd3, and Brd4 respectively (Zuber et al., 2011) to knock-down its expression in mouse NPCs (Fig. S1). The lentiviral vector expressing irrelevant shRNA was the control. These viral vectors co-expressed green fluorescent protein (GFP) for the identification

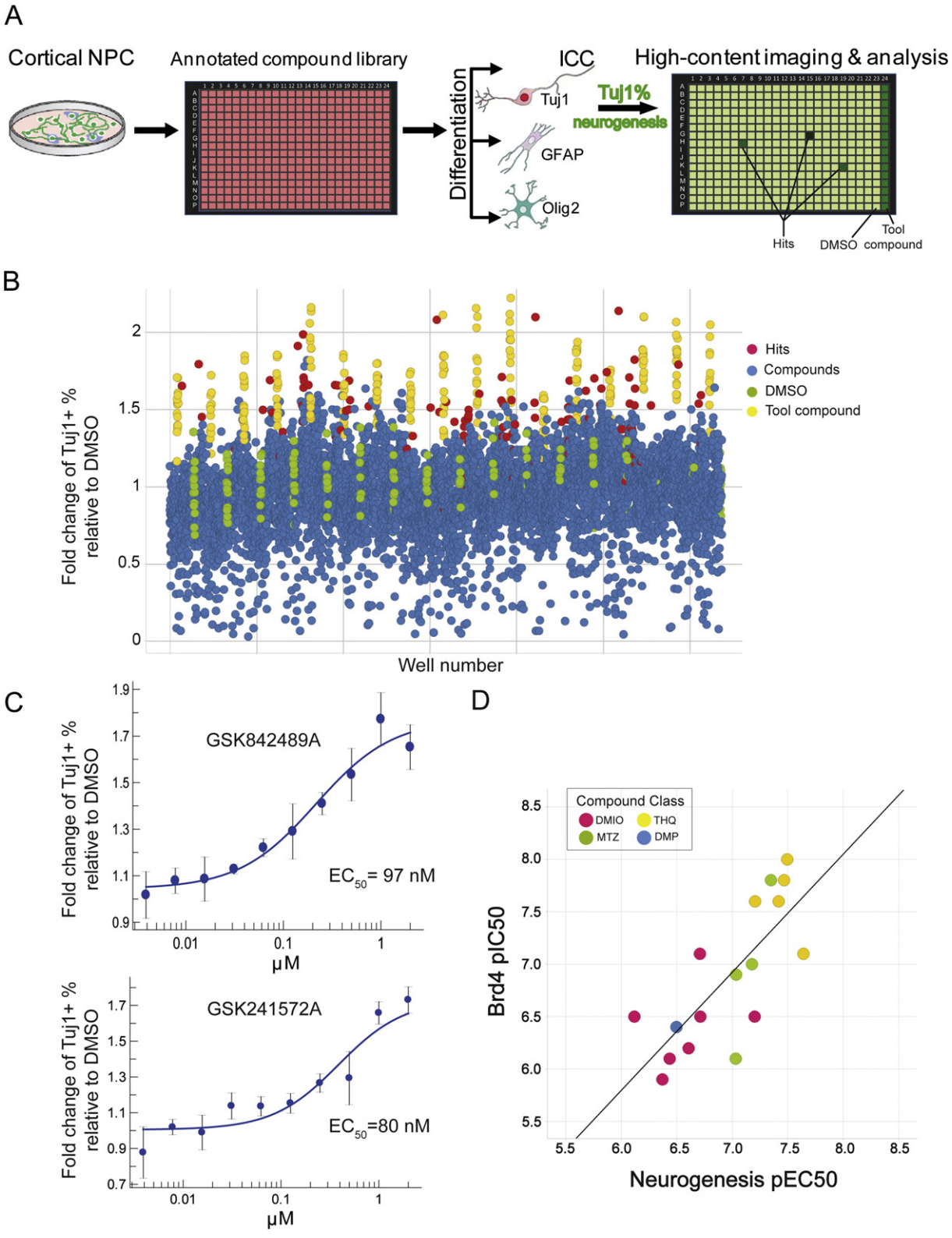

Fig. 1. Identification of BET-bromodomain inhibitors as the hits for neurogenesis through a NPC based phenotypic screen. A) Overview of the assay concept and the workflow of neurogenesis phenotypic screen. ICC, Immunocytochemistry. B) Scatter plot of primary screening results shown as the fold change of differentiated neuronal population ($Tuj1^{+}$%) compared to DMSO vehicle control (green). The primary hits, which increased neuronal population by at least 3 SD, are shown in magenta. C) Concentration-response curves of two exemplar BET-bromodomain inhibition hit compounds. EC_{50} values are indicated. D) Neurogenesis potency correlates with in vitro binding affinity of BET proteins. Plot of mean neurogenesis assay pEC_{50} against plC 50 of Brd4 bromodomain fluorescence resonance energy transfer binding assay, for 17 compounds with diverse structures tested in both assays, showing excellent correlation ($R^2 = 0.57$, p-value = 0.00043). DMIO, dimethyl isoxazole derivatives; THQ, tetrahydroquinoline derivatives; MTZ, methyltriazole derivatives; DMP, dimethyl pyrazole derivatives.

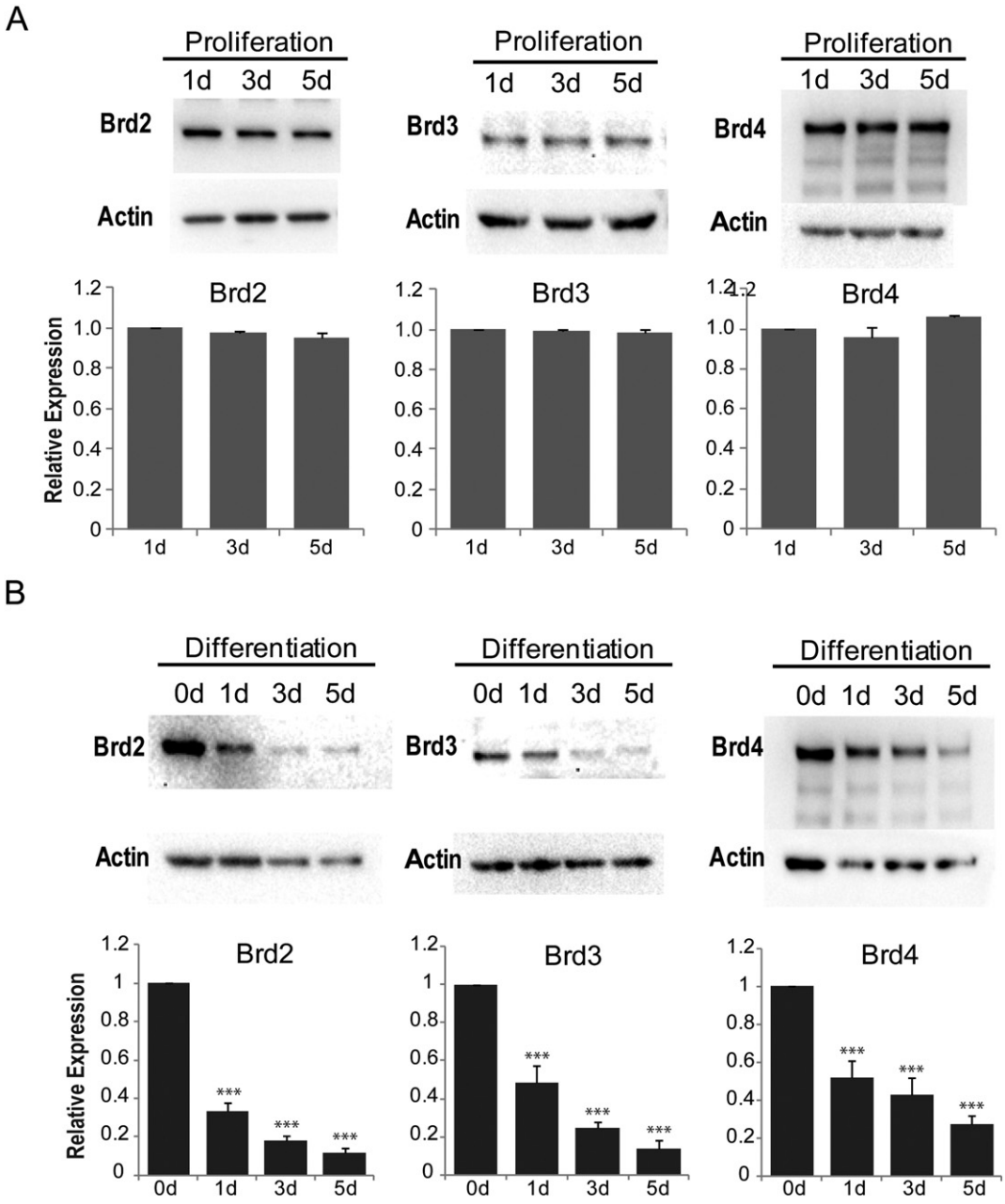

Fig. 2. Expression of Brd2, Brd3, and Brd4 during NPC proliferation and differentiation in vitro. A) Representative Western blots and relative expression quantification of BET proteins and β-actin (loading control) in proliferating NPCs at 1, 3 and 5 day in vitro. B) Representative Western blots and relative expression quantification of BET proteins in NPC upon FGF withdraw (0 d), and at 1, 3 and 5 day in vitro differentiation. Quantification data were mean ± SEM from 3 independent replicates, *P < 0.001 versus 0 d were determined by unpaired *t*-test.

and tracking of infected NPCs and their progeny. To quantify the extent of cell differentiation following the knockdown of Brds, differentiated cells infected with lentiviral vectors were immunostained for cell-linage markers. Quantification of the percentage of differentiated neurons ($Tuj1^+$), astrocytes ($GFAP^+$), or oligodendrocytes ($Olig2^+$) in the total number of GFP^+ cells revealed that the knockdown of Brd2, Brd3, and Brd4 enhanced NPC differentiation towards neurons. Interestingly, knocking down of Brds in NPCs also significantly inhibited the formation of astrocytes and oligodendrocytes after differentiation (Fig. 3). To further validate a dual role of BET silencing in gliogenesis and neurogenesis, we used (+) JQ-1, a structurally distinct widely used and now well validated tool compound for BET bromodomain inhibition, to assess the effect of BET inhibition in NPC differentiation. (+)-JQ-1 promoted neurogenesis at similar concentrations seen with other I-BET compounds, while also inhibiting the differentiation into astrocytes and oligodendrocytes reproducibly in a concentration-dependent manner (Fig. 4). (−)-JQ-1, an inactive enantiomer for BET bromodomain binding, had no significant effect on NPC differentiation (Fig. S2). Together, both genetic (shRNA) and pharmacological (JQ-1) studies confirmed our finding from the phenotypic screen and further validated the dual role of BET bromodomains in both neurogenesis and gliogenesis.

3.4. BET Bromodomain inhibition results in transcriptional changes that suppress cell cycle progression and gliogenesis while favoring neurogenesis

To determine how the inhibition of BET bromodomain regulates NPC development and differentiation, we treated the NPCs with 0.2 μM or 0.5 μM of (+)- JQ-1 or (−)-JQ-1 for 12 h or 24 h in independent triplicates and subjected to whole genome transcriptomic analysis by RNA Sequencing (RNA-seq). Unsupervised hierarchical clustering clearly segregated the samples by treatment (+)-JQ-1 vs. (−)-JQ-1 (Fig. S3).

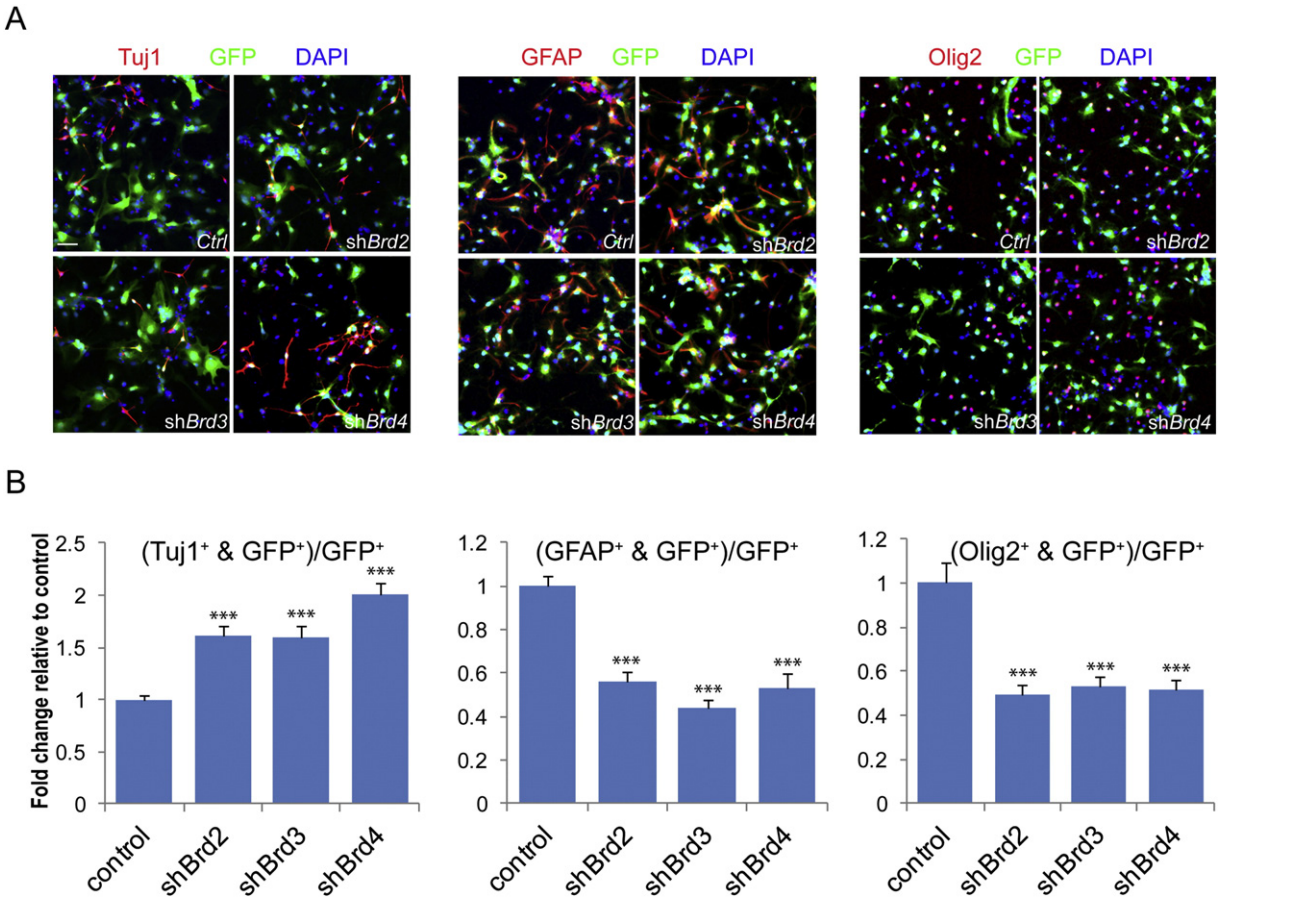

Fig. 3. shRNA knockdown of Brd2, Brd3, and Br4 enhances neuronal differentiation while inhibiting the generation of astrocytes and oligodendrocytes. A) Representative immunostaining images of differentiated NPC following lentiviral shRNA transduction (labeled by GFP). The differentiation of neuron, astrocyte and oligodendrocyte lineage was labeled by Tuj1, GFAP, and Olig2 respectively. Scale bar, 50 μm. B) Quantification of differentiated neuron, astrocyte and oligodendrocyte populations for NPC transduced with lentiviral shRNA targeting Brd2, Brd3, and Brd4 respectively relative to control shRNA. Data were mean ± SEM from 3 independent replicates with 9 fields per replicate for each shRNA construct. Each field had 400–600 GFP^+ cells. *P < 0.001 versus control shRNA were determined by unpaired *t*-test.

Global analysis of gene expression profiling showed that in contrast to a number of upregulated genes, (+)-JQ-1 treatment produced suppression of significant number of genes (Fig. 5A, a and a', 82 upregulated vs. 871 downregulated genes in 0.5 μM, 24 h-treatment group by 1.5-fold or greater, FDR < 0.05). With increased (+)-JQ-1 concentration or time in treatment, the number of differential gene expression increased (Fig. 5A, a', 531 vs. 871 downregulated genes in 0.2 μM or 0.5 μM dose group at 24 h treatment; b', 398 vs. 475 downregulated genes that are common in both 0.2 μM and 0.5 μM dose group at 12 h or 24 h treatment), with a large degree of overlap between the two concentrations or time points (Fig. 5A). To understand the biological relevance of these expression changes, we performed functional process and pathway analyses using Metabase. Gene Ontology Biological Process (GO) analysis displayed an enrichment of key processes involved in neurogenesis, cytoskeleton rearrangement, cell adhesion, Notch and Wnt signaling, transcriptional and translational regulation, and cell cycle, which are known processes for NPC proliferation, fate specification and differentiation (Fig. 5B) (Faigle and Song, 2013; Gotz and Huttner, 2005). Further functional pathway analysis using Metabase identified a number of statistically significant signaling pathway categories, including neural stem cell lineage commitment, the differentiation of oligodendrocyte and astrocyte, EGFR, and PTEN signaling pathways (Fig. 5C). Among these processes and pathways, we identified representative highly characterized genes for neurogenesis, astrogenesis, gliogenesis, cell cycle and key regulators in NPC development and fate specification. Consistent with the phenotype of NPC in response to BET inhibition, proneurogenic genes including DLX2, BCL6, PROX were upregulated by (+)-JQ-1, while genes related to cell cycle progression (CDKN1C, CCND1, MYC, MYCN), glial fate specification (NKX2, SOX10, BMP4 and receptor), differentiation of astrocytes (MAPK3, ID1, ID3, S100B, ALDH1A1) and oligodendrocytes (PDGFα and receptors, Cspg4/NG2, PLP1, MBP, MAG) were significantly downregulated (Fig. S4). Quantitative real-time PCR analysis of NPC treated with 0.5 μM of (+)-JQ-1 for 12 h or 24 h further validated the similar pattern on significant changes of gene expression (Fig. 5D).

Collectively, these data demonstrated BET bromodomain inhibition induced broad but specific transcriptional programs which inhibit cell cycle progression and gliogenesis but promote neurogenesis.

4. Discussion

Phenotypic screens have led to the discovery of many first-in-class drugs (Swinney and Anthony, 2011). However, a significant challenge with phenotypic screenings is to determine the molecular target of the compounds. Target deconvolution could be a time-consuming and complicated process with limited success rate (Schenone et al., 2013). Among small molecules identified from previous neurogenesis phenotypic screens, the P7C3 neuroprotective chemical class was identified from an impressive in vivo chemical screen for hippocampal neurogenesis in mice (Pieper et al., 2010). With photocrosslinking and various biochemical methods, nicotinamide phosphoribosyltransferase was recently identified as the intracelluar target of P7C3 (Wang et al., 2014). Through a cell-based phenotypic screen, Isoxazole stem cell modulators were identified to trigger neuronal differentiation through a neurotranmitter-evoked Ca^{2+} signal (Schneider et al., 2008). However, the precise target of isoxazole in NPC remains unknown. To identify

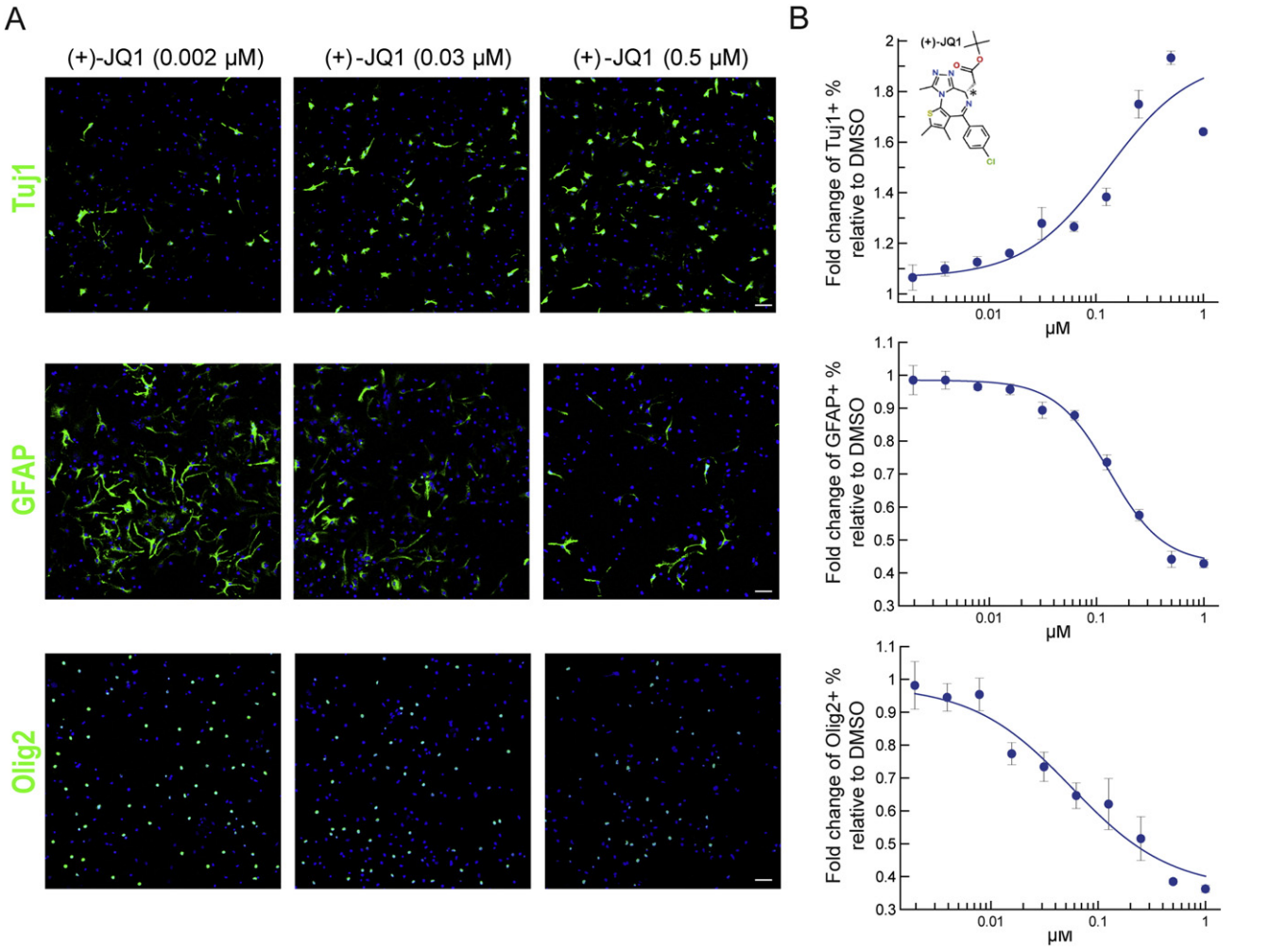

Fig. 4. BET-bromodomain inhibitor (+)-JQ-1 induces neuronal differentiation of NPC while blocking glial differentiation towards astrocytes and oligodendrocytes. A) Representative immunostaining images of differentiated NPC treated with (+)-JQ-1 at indicated concentration. The differentiation of neuron, astrocyte and oligodendrocyte lineage was labeled by Tuj1, GFAP, and Olig2 respectively. Scale bar, 50 μm. B) Full dose-response curves of (+)-JQ-1 on NPC differentiation into neuron, astrocyte and oligodendrocyte compared to vehicle control. EC_{50} values were indicated. Data were mean ± SEM from 3–5 replicates.

drug candidates with tractable targets for neurogenesis, and to circumvent any potential issues associated with target deconvolution, we used GSK high-quality proprietary compound libraries that not only have broad target coverage but the compounds for each annotated target is highly selective. Through this primary NPC based phenotypic screen, we discovered that BET bromodomain-containing proteins are the novel target for neurogenesis. Using highly potent and selective chemical probes and shRNA knockdown, our study provided pharmacologic and genetic validation of BET bromodomains as a target for neurogenesis. Importantly, our genome wide transcriptome analysis on changes induced by selective bromodomain inhibitor (+)-JQ-1 revealed that BET inhibition is sufficient for initiating transcriptional programs that promote neurogenesis while repress gliogenesis and cell cycle progression, shedding light on the mechanistic basis of BET proteins as epigenetic readers in NPC development and neurogenesis.

Among four BET proteins described in vertebrates, Brd2, Brd3 and Brd4 are expressed in the CNS (Shang et al., 2004). The exact role of BET family in the development and proper functioning of the brain and how their dysfunction might contribute to neurological disorders remain elusive. Previous genetic studies on bromodomain-containing proteins suggested a role of BET proteins in neural development (Gyuris et al., 2009; Shang et al., 2009). However, their widespread expression during development and early embryonic lethality hindered a thorough study of BET proteins in NPC development and neuronal differentiation (Gyuris et al., 2009; Houzelstein et al., 2002; Shang et al., 2009). Pleiotrophin (Ptn), an intracelluar antagonist of Brd2 that can destabilize the association of Brd2 with chromatin has recently been identified to play a role in the transition from neural progenitor cell proliferation to differentiation (Garcia-Gutierrez et al., 2014). Endogenous regulators like Ptn that mediate BET-directed epigenetic program are yet to be discovered to have a better understanding the role of BET proteins in health and various diseases. On the other hand, chemical biology approach as highlighted in the discovery of small molecule inhibitors of BET bromodomains could be a powerful tool to advance our biological understanding and to discover potentially novel therapeutics (Bunnage et al., 2013). Using chemical biology techniques we identified BET bromodomains as a novel target for neurogenesis through a phenotypic screen of small-molecule libraries, but also revealed the role of BET proteins in NPC development and potential applications of BET inhibitors as an adjunct in regenerative medicine.

Once committed to a certain cell fate, NPC undergo cell cycle arrest and terminal differentiation leading to the exhibition of cell-type-specific features. The onset and processes of neuronal and astroglial differentiation involve extensive chromatin remodeling and precise regulation of transcription (Hirabayashi and Gotoh, 2010). Epigenetic enzymes including epigenetic writers and erasers, and small molecules that can target some of the enzymes have been demonstrated to induce the alternations of a large array of gene expression and the modulation of multiple neural development pathways during these processes

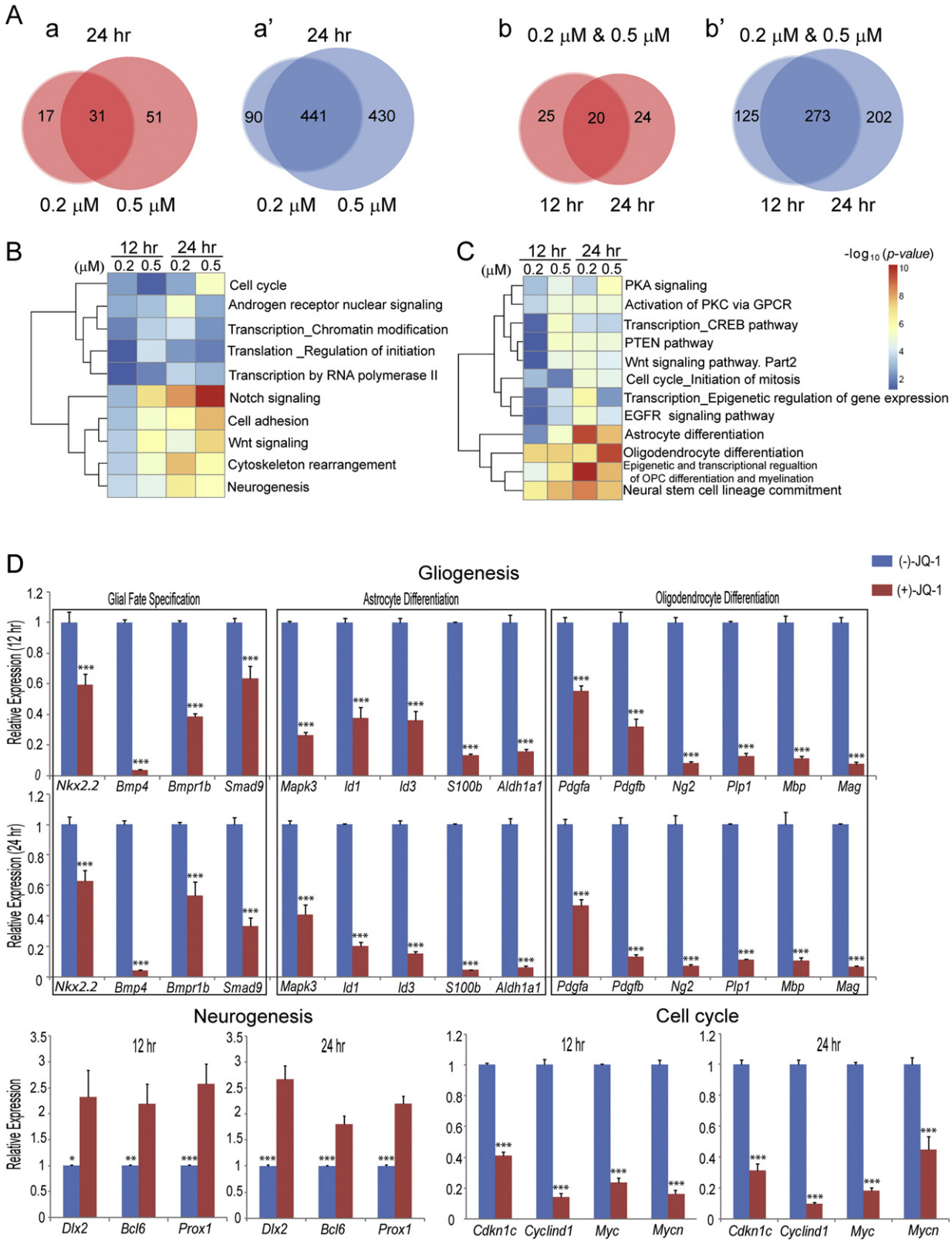

Fig. 5. BET inhibition induces broad but specific transcriptional programs in NPC during differentiation. A) Venn analyses showing up-regulated, down-regulated and overlapped differentially expressed genes (DEGs) following treatment of NPC with 0.2 μM and 0.5 μM of (+)-JQ-1 for 12 h, 24 h compared to inactive enantiomer (−)-JQ-1. a and a', up-regulated (a), down-regulated (a') DEGs in 24 h treatment of 0.2 μM, 0.5 μM of (+)-JQ-1 and the overlapped subset between 2 concentrations; b and b', up-regulated (b), down-regulated (b') DEGs that are common in both 0.2 μM and 0.5 μM treatment group at 12 h, 24 h and the overlapped subset between 12 h and 24 h treatment. B) Global biological process (GO) analysis of DEGs enriched in NPCs treated with (+)-JQ-1. C) Global functional pathway (MetaBase) analysis of DEGs enriched in NPCs treated with (+)-JQ-1. The color grade of the heat maps in both B and C indicates the statistical significance, which describes the enrichment of DEGs in a defined subset of genes associated with a particular biological process term or functional pathway annotation. D) Quantitative RT-PCR analyses of key regulatory genes involved in gliogenesis, neurogenesis and cell cycle progression in NPC, which were treated with 0.5 μM of (+)-JQ-1 for 12 h, 24 h. Relative fold change compared to (−)-JQ-1 were shown. Data shown as mean ± SEM were from 3 independent studies with 3 replicates each study. *P < 0.05, **P < 0.01, ***P < 0.001 versus control (−)-JQ-1 were determined by unpaired t-test.

(Swaminathan et al., 2014). HDAC inhibitors such as valproic acid (VPA) were found to induce neuronal differentiation of NPC, whereas it inhibited astrocyte and oligodendrocyte differentiation. VPA treatment upregulated proneurogenic genes NeuroD and Neurogenins (Ngn) through promoting recruiting Actylated-H4 to their promoters for transcriptional activation (Hsieh et al., 2004; Yu et al., 2009). BET proteins as epigenetic readers bind acetylated histones and are a crucial regulator of transcription in many cell types. Brd4, a well-studied member of the BET family, was found to recruit transcriptional regulatory complexes including P-TEFb and Mediators to acetylated chromatin thereby coupling the acetylation state of chromatin with Pol II elongation. Interestingly, global transcriptomic analyses on the effects of BET bromodomain inhibitors revealed that the transcriptional effects of BET inhibitors are often highly specific to the cell type and/or the physiology and pathophysiology process being examined (Shi and Vakoc, 2014). For example, treating macrophage cells with I-BET prevented the activation of a specific subset of LPS-inducible genes that encode cytokines, chemokines and various transcription factors involved in the inflammatory response. In the absence of LPS stimulation, I-BET treatment led to minimal changes to global gene expression in macrophages, indicating selective effects of BET inhibitors on inflammatory genes in this cell type (Nicodeme et al., 2010). In NPC among the genes regulated by (+)-JQ-1, biological process and pathway analyses revealed that bromodomain inhibition leads to the most significant changes in NSC lineage commitment, the differentiation of oligodendrocytes and astrocytes, neurogenesis, and the processes and signaling pathways associated with NPC development. A significantly large number of genes were downregulated, which are likely to be the direct and secondary targets of BETs as a result of their function as transcriptional activators (Shi and Vakoc, 2014). Both astroglial and oligodendroglial differentiation pathways were significantly perturbed in response to (+)-JQ-1 treatment, and the expression of their downstream target genes were markedly suppressed. Bone morphogenetic proteins (BMPs) are known to promote glial differentiation and inhibit neuronal fate specification (Bonaguidi et al., 2005; Lim et al., 2000). BMPs mainly potentiate JAK/STAT signaling through the formation of a STAT-SMAD co-activating complex (Faigle and Song, 2013). Platelet-derived growth factor (PDGF) is not only a mitogen for the proliferation of oligodendrocyte progenitor cells, but also an instructional signal for the differentiation of neural stem cells into oligodendrocyte lineage (Fruttiger et al., 1999; Hu et al., 2008). During the neurogenic phase of NPC development, suppression of alternative fates while promotion of neurogenesis are known mechanisms for neuronal fate specification (Sun et al., 2001). (+)-JQ-1 induced suppression of transcriptions on these lineage specification signals (for example BMP and PDGF) and downstream genes towards the differentiation and maturation of astrocyte and oligodendrocyte might directly contribute to the inhibition of gliogenesis and promotion of neuronal differentiation. Moreover, (+)-JQ1 treatment induced increased gene expression involved in chromatin assembly, epigenetic regulation and neurogenesis. Distal-less homeobox 2 (Dlx2) is a helix-loop-helix transcription factor that is heavily controlled by epigenetic modifications (Lim et al., 2009). Over-expression of Dlx2 is required for GABAergic neuron production. It negatively regulates Oligo2-dependent oligodendrocyte formation (Brill et al., 2008; Petryniak et al., 2007; Suh et al., 2009). Bcl6, an oncogene in B lymphocytes encoding a BTB/POZ zinc finger transcription repressor, was recently identified as a proneurogenic gene necessary for proper cortical neurogenesis and pyramidal neuron differentiation through selective regulation of Notch-dependent transcription (Tiberi et al., 2012). The "cell cycle length hypothesis", in vitro and in vivo studies suggested that inhibition of the cell cycle favors neurogenesis and prevents proliferation of NPCs (Arai et al., 2011; Gotz and Huttner, 2005; Knoepfler et al., 2002). Consistent with this hypothesis, accompanied with increased neuronal differentiation, the transcriptions of Cdkn1C, CyclinD1, Myc and MycN genes that are involved in cell cycle progression were markedly suppressed by (+)-JQ-1 treatment. The genome-wide analysis of Brd4 chromatin occupancy using chromatin immunoprecipitation coupled with DNA sequencing (CHIP-Seq) showed that Brd4 is associated with essentially all active promoters and a significant fraction of active enhancers in the genome of various normal and transformed cell types (Anand et al., 2013; Loven et al., 2013). Examination of Brd4 occupancy at genes whose transcription is particularly sensitive to (+)-JQ-1 has led to the observation that such genes often exhibit high levels of Brd4 occupancy at nearby super enhancer regions. The association of high-level of Brd4 occupancy with these lineage-specific enhancers has been linked to the lineage-specific gene regulation by BET inhibition (Shi and Vakoc, 2014). Future study on CHIP-seq analysis of (+)-JQ-1 treated differentiating NPC might be able to identify these enhancer elements that are selectively targeted by BET inhibition and underlie the effects of I-BET in NPC development.

Neural stem cell transplantation is currently investigated as a treatment for different CNS injuries and disorders. One key challenge for a successful cell therapy is that an inflammatory environment in the diseased CNS might trigger glial differentiation of transplanted NSCs or NPCs (Aboody et al., 2011; Reekmans et al., 2012; Robel et al., 2011). Additionally, there might be a safety concern on the oncogenic potential of human iPSC-derived NSCs. Previous studies on BET bromodomain inhibition have demonstrated profound anti-proliferative and anti-inflammatory effects (Belkina and Denis, 2012). Our study provided new evidence on a dual role of BET inhibition in promoting neurogenesis and suppressing gliogenesis. Together it suggests a potential clinical application of I-BET in neural stem cell therapy for enhanced efficacy and improved safety in cell-based transplantation.

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