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Generation of a Cellular Reporter for Functional BRD4 Inhibition

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[Abstract] The ubiquitously expressed bromodomain-containing protein 4 (BRD4) is an epigenetic reader, which recruits transcriptional regulatory complexes to acetylated chromatin. Because of its role in enhancing proliferation, BRD4 has become a therapeutic target in oncology, as the inhibition of this protein leads to the reduction of the growth of many tumours. Even though BRD4 is more and more studied, its mechanism of action has not been fully described yet. Therefore, we aimed at generating a cellular reporter system to monitor BRD4 inhibition. Such reporter can be potentially used in high throughput chemical and genetic screenings, in order to uncover new possible BRD4 functional pathways. The deeper understanding of the mechanism of action of BRD4 activity will certainly help in developing new therapy strategies for those cancers so called BRD4-dependent.
Keywords: Cellular chromatin reporters, Epigenetic, Chromatin reorganization, Heterochromatinization, BRD4

[Background] Research in the epigenetic field has recently highlighted the central role of BRD4 in cancer progression. BRD4 is an acetyl-lysine reader of the BET (bromodomain and extraterminal domain) family (Dey *et al*., 2003; Filippakopoulos *et al*., 2012; Wang *et al*., 2012) able to bind to acetylated histones at promoter and enhancer regions (Dey *et al*., 2003; Filippakopoulos *et al*., 2012; Nagarajan *et al*., 2014). The mechanism of action of this epigenetic reader consists in the activation of gene promoters and enhancers by recruiting several transcription factors, cofactors and RNA polymerase II (RNApol II), which results in modulating, mostly enhancing, the transcription of certain target genes. The BRD4-histone module has been described to play a key role regulating cell cycle progression (Dey *et al*., 2003; Wu and Chiang, 2007; Yang *et al*., 2008; Devaiah and Singer, 2013) and genomic structure and stability (Wu and Chiang, 2007; Floyd *et al*., 2013); for those reasons, BRD4 has frequently been associated with cancer development and progression (Yang *et al*., 2008; Zuber *et al*., 2011; Nagarajan *et al*., 2014; Wu *et al*., 2015).

Chromatin reporter cell lines have been already developed in order to identify modulators of position effect variegation (Tchasovnikarova *et al*., 2015) or to discover new chromatin-targeting compound (Johnson *et al*., 2008; Best *et al*., 2011; Wang *et al*., 2013). In contrast to previous approaches, we wanted to develop a protocol for the generation of a reporter cell line able to monitor the BRD4-dependent heterochromatization of a generic reporter. To achieve that, we used a common retroviral vector carrying an RFP (Red Fluorescent Protein) gene, and selected clones that integrated it in fully

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repressed genomic regions specifically reactivated by BRD4 inhibition. The haploid nature of the cell line used (KBM7 [Andersson *et al*., 1995]), makes the reporter easily amenable not only to chemical screens, but also to genetic screens. Both methods can be used for the identification of new BRD4 direct and functional partners, and results from these approaches will provide further insights into BRD4 biology.

Materials and Reagents

1. Pipette tips
2. 6-well plates, tissue culture treated (Corning, Costar®, catalog number: 3506)
3. 15 ml Falcon® conical centrifuge tube (Corning, Falcon®, catalog number: 352196)
4. 0.45 µm syringe filters (VWR, catalog number: 514-8021)
5. 24-well plates, tissue culture treated (STARLAB INTERNATIONAL, catalog number: CC7682-7524)
6. 96-well plates, tissue culture treated (Corning, catalog number: 3598)
7. Viewplate-96 black, optically clear bottom, tissue culture treated, sterile, 96-Well with lid (PerkinElmer, catalog number: 6005182)
8. 10 cm plates, tissue culture treated (Corning, catalog number: 430167)
9. 293T cell line (ATCC, catalog number: CRL-3216)
10. KBM7 cell line (Chronic Myeloid Leukaemia) (Horizon Discovery, catalog number: C628)
11. Fluorescent reporter vector (LZRS-RFP-ires-ZEO retroviral vector, a gift from S. Nijman Lab, Ludwig Cancer Research, Oxford)
12. Packaging vector (*e.g*., pCMV-Gag-Pol retroviral vector, Addgene, catalog number: 14887; pCMV-VSV-G envelope vector, Addgene, catalog number: 8454)
13. DMEM media (Thermo Fisher Scientific, Gibco™, catalog number: 41965039)
14. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10500064)
15. Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen™, catalog number: 11668019)
16. Opti-MEM
17. IMDM media (Thermo Fisher Scientific, Gibco™, catalog number: 21980032)
18. Phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 14190094)
19. (*S*)-JQ1 (MedChemExpress, catalog number: HY-13030)
20. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)

Equipment

1. Pipettes (Gilson)
2. Cell culture centrifuge (Sigma Laborzentrifugen, model: 3-18K, catalog number: 10290)
3. FACS (BD, BD Bioscience, model: BD FACSCALIBUR)

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4. FACS sorter (BD, BD Biosciences, model: BD FACSAria)
5. Fluorescence microscope (PerkinElmer, model: HH12000000)
6. Cell culture hood (Thermo Fisher Scientific, model: Herasafe™ KS, catalog number: 51022515)
7. Cell culture incubator (Eppendorf, model: Galaxy® 170 R, catalog number: CO170R-230-1000)

Procedure

1. Virus preparation
   a. 293T cells are seeded at 200,000 cells/ml and grown until 60% confluence in DMEM plus 10% FBS in 6-well plates. When they reach such confluence rate (usually the day after), they are transfected using Lipofectamine 2000, according to manufacturer's instructions, in order to produce the virus needed for the reporter generation (see details below). In order to get optimal virus production, it is important to use 293T cells at low passage number, and which have never been overconfluent.
   b. For the LZRS-RFP-ires-ZEO retrovirus production, 1.5 µg of LZRS-RFP-ires-ZEO retroviral vector were used in combination with 1.5 µg of packaging vectors (pCMV-Gag-Pol retroviral vector 8:1 pCMV-VSV-G envelope vector). Briefly (for 1 well), vectors are diluted and gently mixed in 250 µl of Opti-MEM. 5 µl of Lipofectamine 2000 are diluted in 250 µl of Opti-MEM and incubate for 5 min at RT (room temperature). After the 5-min incubation, the vector dilution and the Lipofectamine 2000 dilution are combined and gently mixed. This suspension is incubated for 20 min at RT and then added to the cell media drop by drop. Finally, the plate is mixed gently by rocking back and forth in order to distribute homogenously the transfection solution.
   c. Virus harvest is done at 30 and 48 h post transfection by collecting the media, centrifuging it in 15 ml Falcon conical centrifuge tubes at 1,200 *x g* for 5 min at RT and then filtering it through 0.45 µm syringe filters into new 15 ml Falcon conical centrifuge tubes (RT).
   Note: At this point the virus can be used for titration and cell infection (steps 2 and 3) or stored at -80 °C.
2. Virus titration
   a. KBM7 cells are seeded in a 6-well plate in IMDM plus 10% FBS at 2 x 10^6 cells/ml (1 ml/well), and then infected using different ratios of media/virus. The virus is resuspended in 1 ml of IMDM plus 10% FBS. In this way, the final cell concentration is of 10^6 cells/ml.
   b. The ratio typically tested were: 1/100, 1/40, 1/20, 1/10, 1/4 and 1/2 (virus volume vs. cell volume).
   c. 5 µg/ml (final concentration) of Polybrene is added to each well by pipetting it in the cell media, once the virus has been added (Polybrene stock solution is 5 mg/ml).
   d. 24 and 48 h post infection cells are washed in PBS and the media is replaced with fresh aliquots (2 ml/well). Cells of each well are resuspended once in 5 ml of PBS (RT) and then centrifuged at 1,200 *x g* for 5 min at RT.

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3. 48 h post infection, FACS analysis is performed in order to check the percentage of infection of each well/ratio. A concentration of virus giving between 25% and 40% of cell infection (25-40% of RFP positive cells) is chosen to be used for the reporter generation (therefore ensuring that likely no more than one infection event could happen per cell). LZRS-RFP-ires-ZEO infection.
   a. KBM7 cells are seeded in 6-well plates in IMDM plus 10% FBS at 10^6 cells/ml (30 x 10^6 cells are used in total).
   b. KBM7 cells are treated with 0.5 µM (*S*)-JQ1 for 18 h and then infected with the LZRS-RFP-ires-ZEO retrovirus (using the virus concentration determined in step 2).
   c. Media is changed after 24 and 48 h post infection; (*S*)-JQ1 is kept in the culture media at 0.5 µM.
4. First sorting (pool)
   RFP-positive cells are sorted in presence of 0.5 µM (*S*)-JQ1 (the whole population is sorted: cells number is at this stage approximately 120 x 10^6).
5. Second sorting (single cells)
   (*S*)-JQ1 is removed from the media and after 30 h the RFP negative population is sorted into single cell clones. Approximately 10 96-well plates are filled; the rest of cell is frozen in IMDM media plus 10% FBS and 10% DMSO.
   Note: Usually for the sorting procedure cells must be washed at least once in 5 ml of PBS (RT), centrifuged at 1,200 x g for 5 min at RT and resuspended in 0.5 ml of PBS (maximum cell concentration allowed is 30 x 10^6 cell/ml); we recommend to add 10% of cell media to the resuspension in order to avoid cell-cluster formation, especially during the first sorting.
6. Single clone amplification and reporter selection
   a. Outgrowing clones (about 10 days after step 5) are amplified and frozen in aliquots of 10 x 10^6 cells in IMDM media plus 10% FBS and 10% DMSO. 20,000 cells of each clone are seeded in 96-well plates (Viewplate-96 black, optically clear bottom) and treated with (*S*)-JQ1 for 24 h.
   b. Cells are imaged using the Operetta high-content imaging system (20x objective and non-confocal mode) (Figure 1). A clone is selected as reporter if after (*S*)-JQ1 treatment is able to express RFP.

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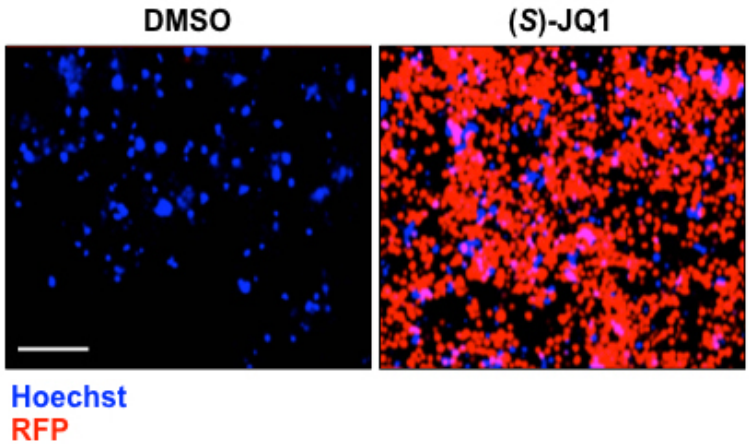

Figure 1. (*S*)-JQ1 treatment activates RFP expression in KBM7 reporter cells. Examples of live cell imaging pictures of KBM7 reporter cells treated with 0.5 μM of (*S*)-JQ1 for 24 h; equal amount of DMSO was added as control (scale bar = 100 μm).

Notes

1. All the cells used in this protocol were grown in a cell culture incubator at 37 °C and 5% CO_2.
2. KBM7 freezing media: IMDM, 20% FCS, 10% DMSO. Cells are gradually frozen at -80 °C and then passed to liquid nitrogen.
3. LZRS-RFP-ires-ZEO vector can be replaced with any other fluorescent reporter vector, either retro or lentiviral.
4. (*S*)-JQ1 treatment can be replaced with any other epigenetic or not epigenetic drug treatment in order to generate reporters for the specific enzyme of interest.
5. KBM7 cells can be replaced with any other cell line of interest. We have chosen this cell line because they are haploid and therefore allows unambiguous monoallelic genetic configurations.

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