BET Proteins Exhibit Transcriptional and Functional Opposition in the Epithelial-to-Mesenchymal Transition
Guillaume P. Andrieu¹ and Gerald V. Denis¹,2

Abstract
Transcriptional programs in embryogenesis and cancer, such as the epithelial-to-mesenchymal transition (EMT), ensure cellular plasticity, an essential feature of carcinoma progression. As effectors of signal transduction, the bromodomain and extraterminal (BET) proteins are well suited to support plasticity because they function as co-activators or co-repressors of mammalian transcriptomes. Here, using both hormone-sensitive and triple-negative breast cancer (TNBC) model systems, we systematically altered EMT transcriptional profiles by manipulating individual BET proteins and found that BRD2 positively regulates EMT, whereas BRD3 and BRD4 repress this program. Knockdown of individual BET proteins revealed independent transcriptional networks that differed from each other and from the small-molecule pan-BET inhibitor JQ1, which previously had been misleadingly asserted to be BRD4-selective. Available small-molecule pan-BET inhibitors, proposed as antiproliferative agents in cancer clinical trials, obscure these biological differences. Transcriptional profiling reveals that individual BET proteins, inhibited separately, engage in and control EMT through unique processes.

Implications: The distinct and opposing functions of BET proteins in the EMT process suggest the need for more member-selective epigenetic targeting agents.

Visual Overview: http://mcr.aacrjournals.org/content/molcanres/16/4/580/F1.large.jpg.

Introduction
The bromodomain and extraterminal (BET) family of transcriptional regulators includes three somatic members BRD2, BRD3, BRD4, and testis-specific BRDT. The bromodomain, a protein motif first described in *brahma*, binds to e-N-aminocetyl groups of nucleosomal histone lysine and recruits histone

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modifcation enzymes, transcriptional co-activators and corepressors, and chromatin remodeling activities to gene promoters. These epigenetic readers therefore function to upregulate or downregulate gene expression in response to cellular signals (1). Human BET proteins are homologs of female sterile homeotic, a transcriptional regulator important in Drosophila development, a feature of many proteins involved in human cancer. BET proteins share common structural features, with evolutionarily conserved, tandem bromodomains that interact with acetylated histones and an extraterminal domain that recruits specific interactors. The contribution of BET proteins to cancer progression has largely been reported, reinforcing their value as therapeutic targets notably for acute myeloid leukemia, B-cell lymphoma, lung, breast, prostate, pancreatic, and colorectal cancers (1). BET proteins, as crucial transcriptional regulators, control cancer progression. First-generation small molecule inhibitors have been developed that compete with BET bromodomain/acyetyl-peptide binding, displacing both BRD2, BRD3, BRD4, and BRDT from chromatin. These compounds, including IQ1 (2) and I-BET (3), show promising results for in vitro and in vivo cancer models including “nuclear protein in testis” midline carcinoma (2), leukemia (4), lymphoma (5, 6), prostate (7), and breast cancer (8). However, pan-BET inhibition strategies obscure the individual biological functions of each BET protein and can present potential risks for patients (9). Indeed, BET proteins exert specific, protective roles that are ablated by pan-BET inhibition. One illustration is BET protein-controlled active repression of HIV-1 transcription; pan-BET inhibitors reactivate latent HIV-1 and viral outgrowth in infected human T cells (10). Despite their mutual homology, BET proteins have nonidentical genome occupancy patterns, are engaged in different regulatory complexes, and modulate distinct signaling pathways and biological functions (12–15). Thus, full comprehension of the contribution of each BET protein is crucial before envisaging them durably as therapeutic targets for cancer treatment. We have previously reported that depletion of BRD4 inhibits invasion and migration in cellular models of triple negative breast cancer, as a regulator of Jagged1/Notch1 signaling (16). In breast cancer, as a regulator of Jagged1/Notch1 signaling (16), and significantly, only BRD4 regulates this pathway, not BRD2 or BRD3, revealing that some transcription programs are not.

Interestingly, only BRD4 regulates this pathway, not BRD2 or BRD3, revealing that some transcription programs are not regulated by all BET proteins (16). Because the dependence of target genes on individual BET proteins in each cancer cell type is impossible to predict a priori, it is necessary to map the signaling pathways with an unbiased approach.

The epithelial-to-mesenchymal transition (EMT) is a developmental program that cancer cells often activate to acquire a highly plastic phenotype that promotes invasion, metastasis, as well as chemoresistance and cancer stem cell generation (17). Several transcription programs triggered by key transcription factors induce drastic changes in epithelial cells to confer mesenchymal phenotypes and properties. Here, we investigated patterns of transcriptional activation and repression of genes important for EMT that are controlled by BET proteins in breast cancer models to resolve the unique and independent functions of these transcription regulators.

Materials and Methods

Cell culture

Human breast cancer cell lines maintained at the NCI Office of Physical Sciences-Oncology Centers (PS-OC) Network Bioresource Core Facility (PBCF) were contractually obtained through the ATCC, under a Material Transfer Agreement. The cell lines have been authenticated by the NIH Physical Sciences Oncology Consortium. Mycoplasma contamination was prevented by treating the cells with Plasmocin (25 mg/mL for 2 weeks, Invivogen) following thawing prior the experiments. MDA-MB-231 and MCF-7 were cultured in DMEM. SUM149PT cells were cultured in DMEM/F12 + 5 μg/μL insulin and 0.5 μg/μL hydrocortisone (Sigma). T47D cells were cultured in RPMI. All culture media were supplemented with 10% FBS. Cells were cultured at 37°C in a humid 5% CO2 atmosphere.

Antibodies and reagents

The following antibodies were used: anti-BRD2, anti-BRD3, and anti-BRD4 (Bethyl Laboratories), anti-E-cadherin (24E10), anti-N-cadherin (13A9), anti-Snail (C15D3), anti-Slug (C19G7), anti-vimentin (D21H3), anti-ZEB1 (D80D3) (Cell Signaling Technology), anti-Sna3 (Abcam), anti α-tubulin (DM1A), Twist (H-81), and anti-ZEB2 (E-11) (Santa Cruz Biotechnology). Fluorochrome-conjugated secondary antibodies were obtained from The Jackson Laboratory. IQ1 was purchased from Tocris Bioscience.

Plasmids, siRNAs and transfection

Plasmids coding for His-tagged BET proteins or control vector pReceiver-M01 were purchased from GeneCopoeia. ON-TARGETplus BET proteins siRNAs were obtained from Dharmacon. Cells were transfected with plasmids and siRNAs by Lipofectamine 2000 reagent (Thermo Fisher Scientific) as previously validated (16). Efficient depletions or overexpressions were obtained 3 days posttransfection.

qRT-PCR

Total RNA was extracted using the RNeasy Kit (Qiagen). Reverse transcription reactions were performed on 1 μg of RNA with the QuantiTect Reverse Transcription Kit (Qiagen). The primer sequences used for this study are listed in Supplementary Table S1. PCR amplifications were performed with the MESA GREEN qPCR MasterMix (Eurogentec) on an ABI Prism 7500 Fast Block thermal cycler.

The gene screening was conducted with the RT2 Profiler PCR EMT Array (Qiagen). Z scores were calculated and heatmaps were generated using MATLAB software (MathWorks).

Immunocytochemistry staining, confocal imaging, and analysis

Cells were fixed in absolute methanol for 5 min at –20°C then permeabilized with 0.2% Triton X-100 in PBS buffer for 10 minutes. After saturation in blocking buffer (0.02% Triton X-100, 2% BSA in PBS) for 30 minutes, cells were incubated with primary antibodies, then fluorochrome-conjugated secondary antibodies, both diluted in blocking buffer for 1 hour. Finally, coverslips were mounted with ProLong Gold with DAPI (Thermo Fisher Scientific). Image acquisition was conducted using a Leica SP5 confocal microscope. For z-stack acquisition, a step of 0.3 μm was set. Fluorescence intensities were determined using ImageJ software (NIH). Intensities were corrected for background then expressed as a ratio of mean intensities per cell area before normalization.
Statistical analyses

Statistical analyses were either performed with Student t test or ANOVA according to the datasets by using GraphPad Prism 7 software. The following symbols were used to indicate significant differences: ns, \( P > 0.05; \) \( * * , P < 0.01; \) \( ** *, P < 0.001. \)

All the experiments executed for this study have been conducted in accordance with the NIH guidelines under the review of the Boston University Institutional Biosafety Committee.

Results

We sought to determine how individual BET proteins transcriptionally control EMT in breast cancer cells. We performed a PCR array analysis of 84 genes involved in EMT regulation in different breast cancer cell lines, specifically depleted for each BET protein (Fig. 1). Our analysis revealed that individual depletion of each BET protein produced a unique transcription profile, indicating that BRD2, BRD3, and BRD4 exert independent control over EMT (Fig. 1A). Under BRD2 depletion, we found that 34 genes were significantly downregulated and two others upregulated in triple-negative MDA-MB-231 cells (Fig. 1B, Z score \( \geq 2 \) or \( \leq -2 \), \( P < 0.05 \)). Conversely, BRD3 depletion (16 genes upregulated, 3 downregulated) and BRD4 depletion (7 genes upregulated, 2 downregulated) are mostly associated with gene upregulation, suggesting these BET proteins principally act as repressors. Remarkably, only a few genes vary in the different BET-depleted signatures, revealing that BET proteins regulate EMT sometimes in opposition to each other (Fig. 1A and B). Similar results were obtained in triple-negative breast cancer SUM149PT cells or luminal A breast cancer MCF-7 cells (Supplementary Fig. 1A and S1B). Functional analysis of the genes deregulated under BET depletion, suggests that BRD2 positively regulates EMT, whereas BRD3 and BRD4 repress this program.

We also compared the transcriptional consequences of single BET silencing with pan-BET inhibition by JQ1 treatment. A panel of downregulated genes was common to BRD2 depletion or JQ1 treatment, whereas only few co-occurrences were shared with JQ1 in BRD3 or BRD4 depletion (Fig. 1B). Most of the commonly regulated genes are EMT transcription factors (Fig. 1B and C and Supplementary Table S2), suggesting that BET proteins exert a transcriptional control on EMT. This result strongly suggests that pan-BET inhibition is most similar to BRD2 depletion, and clearly opposes single BRD3 or BRD4 depletion, indicating that any result obtained with pan-BET inhibitors like JQ1 should not be interpreted as a specific targeting of any BET protein, as misleadingly asserted in several reports (9).

EMT is driven by multiple transcription programs induced by several major transcription factors, including the Snail family (Snail, Slug, Sna1a3), Twist and the ZEB family (ZEB1, ZEB2; ref. 17). To confirm that BET proteins regulate EMT through different transcription programs, we modulated single BET protein expression by either specific depletion or overexpression of the main EMT transcription factors in multiple breast cancer cell lines. Triple-negative breast cancer MDA-MB-231 and SUM149PT cell lines exhibit a mesenchymal phenotype. In these cell lines, BRD2 depletion induced a significant down-regulation of Snail, Slug, Sna1a3, but also Twist and ZEB1, ZEB2 (Fig. 2A and B and Supplementary Fig. S2A). Conversely, BRD3 and BRD4 depletions significantly increased the expression of all these major EMT transcription factors. The luminal A breast cancer line MCF-7 presents an epithelial phenotype and barely expresses EMT transcription factors under normal conditions (Fig. 2B). Upon BRD2 depletion, we noted a moderate down-regulation of Snail and Twist. However, BRD4 silencing led to a strong up-regulation of the Snail and ZEB family members along with Twist. Interestingly, BRD3 depletion phenocopies BRD4 silencing but fails to upregulate Sna1a3 or ZEB2 in our models, suggesting independent control of these transcription factors. We then overexpressed each BET protein in these cell lines and monitored the expression of the major EMT transcription factors (Fig. 2C). We found that BRD2 overexpression induced expression of each of these factors, assayed in both cell lines (Fig. 2D and Supplementary Fig. S2B). However, BRD3 and BRD4 overexpression led to downregulation of several EMT factors, including Twist, ZEB1, and ZEB2. These data support the idea that BRD2 positively regulates EMT, whereas BRD3 and BRD4 repress this program. Importantly, the overexpression of single BET proteins also led to individual and distinct transcriptional signatures relevant to EMT (Supplementary Fig. 1C).

To compare single BET depletion versus pan-BET targeting on EMT transcription factor expression, we then treated breast cancer cell lines with JQ1 and repeated the aforementioned experiments. We found that JQ1 treatment led to a significant downregulation of SNA1a, SNA1a3, TWIST1, ZEB1, and ZEB2 in MDA-MB-231 and MCF-7 cells (Supplementary Fig. S2C and Supplementary Fig. S2B), as also observed under BRD2 targeting (Fig. 2B and Supplementary Fig. S2B). Collectively, our results demonstrate that BRD2 opposes BRD3 and BRD4 to transcriptionally regulate EMT. This duality may be explained by divergent regulation of the key EMT transcription factors. Interestingly, BRD3 seems to exert only moderate control of EMT transcription programs compared to BRD4; BRD3 modulation does not affect all the EMT transcription factors depicted here. Critically, we confirmed that, in a model of transcription control of EMT, pan-BET inhibition with JQ1 most closely parallels BRD2 silencing and opposes BRD3 or BRD4 targeting.

We then confirmed that BET proteins regulate morphological and phenotypical changes relevant to EMT in breast cancer cells. We immunostained breast cancer cell lines, to detect epithelial and mesenchymal markers upon BET modulation. Luminal A breast cancer cells, such as MCF-7 or T47D, present a cuboidal morphology characterized by strong expression of the epithelial marker E-cadherin at tight junctions, and lack the mesenchymal markers N-cadherin or vimentin, as illustrated in control cells treated with scrambled siRNA (Fig. 3A). Upon BRD2 depletion, we observed increased expression of E-cadherin, consistent with repression of EMT. Conversely, BRD3 or BRD4 depletion induced a significant decrease in E-cadherin expression, and an increase in either N-cadherin or vimentin expression. Under BRD3 or BRD4 depletion, we observed disruption of cell morphology, exemplified by cell flattening and increased cell area. Significantly, opposite results were obtained by overexpressing BET proteins (Fig. 3B). BRD2 overexpression induced downregulation of E-cadherin, increased N-cadherin, and provoked similar morphological modifications as observed in BRD3- or BRD4-depleted cells, consistent with initiation of EMT. Taken together, the results indicate that BET proteins control EMT transcription factors and either engage

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Individual BET proteins control independent EMT transcriptomes. 

A, Heatmap presenting Z scores of a PCR array of 84 EMT genes expressed in MDA-MB-231 cells upon BET protein depletion by siRNA (50 nmol/L for 3 days; \( n = 3 \)). Independent transcriptional signatures relevant to EMT regulation were obtained for each BET protein. Pan-BET inhibition using small molecule JQ1 (400 nmol/L for 3 days) obscured these individual profiles. A color code is used to illustrate Z score variations. Normalization is set to scramble.

B, Systemic analysis of the EMT signatures. BRD2 depletion exhibit a strong association with JQ1 treatment with 25 common genes downregulated. Conversely, BRD3 and BRD4 depletions are mostly associated with a small number of upregulated genes that do not overlap. Most of the BET-regulated genes are EMT transcription factors. Graphs plot Z scores from BET depletion or JQ1 datasets. Significantly altered genes are indicated (\( Z \) score \( / \leq 2 \) or \( \geq 2 \), \( P \) -value < 0.05).

C, Functional analysis of the EMT signatures. Most of the genes modulated by BET depletion are transcription factors, indicating that BET proteins transcriptionally regulate EMT.
or repress EMT programs, leading to changes in epithelial/ mesenchymal marker expression and cell architecture.

Discussion

Our results reveal functional opposition between BRD2, BRD3, and BRD4. BRD2 is a positive regulator of EMT, whereas BRD3 and BRD4 are repressors of this program. Similarly to previous studies, we conclude that each BET protein carries its own functions, sometimes overlapping with another family member’s, sometimes distinct or opposite (12–15). Therefore, investigators in the BET protein field risk over-interpreting one particular member’s role, like BRD4, based solely on experiments with pan-BET inhibitors. Rather, studies should be conducted by selectively targeting each member. We have commented on how misleading interpretations are problematic for our mechanistic understanding of how small molecule BET inhibitors work or should best be combined with other modalities (9). Family member-selective small molecules are urgently needed to advance the clinical translational impact of these recent discoveries. Among the newly reported small molecules targeting the BET proteins, MZ1 shows promise (18). This Proteolysis Targeted Chimera (PROTAC), which combines a BET binder motif based on JQ1 structure and a ligand for the E3 ligase VHL, induces BET protein degradation in a family member-specific manner, depending on the dose.

By accomplishing EMT, cancer cells acquire numerous properties relevant for migration, invasion, and survival in response to chemotherapy or in a stressful microenvironment, or cell identity and differentiation (17). Furthermore, reports have shown that EMT can generate cancer stem-like cells (CSC; refs. 19, 20). Depending on the activated EMT transcription factors, several programs can be triggered, leading to different EMT-related outcomes. For instance, EMT is not always associated with increased metastatic potential. A recent publication reported that EMT is dispensable for metastasis but elicits development of chemoresistance in a pancreatic cancer model (21). In a previous report, we showed that BRD4 silencing ablates breast cancer cell migration and invasion (16). Therefore, we can speculate that EMT triggered by BRD4 loss in breast cancer may not generate highly invasive cells but rather might elicit subpopulations with higher survival or CSCa properties. BRD4 has been reported to control pluripotency and therefore embryonic stem cell (ESC) identity (22). In ESCs, BRD4 targeting induces EMT markers, indicating that the regulation we discovered may not be limited to cancer but can also occur during development and physiologic EMT. The role of BET proteins in normal and cancer stem cell functions demands further investigation, considering that BET proteins likely

Figure 2.

BRD2 opposes BRD3 and BRD4 to control key EMT transcription factors. A, Validation of BET depletion by siRNA in MDA-MB-231 and MCF-7 cells (50 nmol/L for 3 days). B, Protein expression of key EMT transcription factors upon BET protein depletion in MDA-MB-231 or MCF-7 cells. C, Validation of BET overexpression in MDA-MB-231 and MCF-7 cells. D, Protein expression of key EMT transcription factors upon BET protein overexpression in MDA-MB-231 or MCF-7 cells. Blots are representative of three independent experiments. Molecular weights are indicated (kDa).
Figure 3.
BET protein manipulation triggers EMT in epithelial breast cancer cells. A, Representative images of MCF-7 depleted for BET proteins (50 nmol/L for 3 days) and stained for E-cadherin (green), N-cadherin (red), and vimentin (gray). B, Representative images of MCF-7 overexpressing BET proteins and stained for E-cadherin (green), N-cadherin (red), and DNA (DAPI, blue). For measurement of relative fluorescence intensities, lines represent means ± SEM of three independent experiments. Each dot represents a single cell value. For cell area measurement, histograms represent means ± SEM of three independent experiments. Blots depict protein expression of key EMT markers upon BET protein depletion (A) or overexpression (B) in MCF-7 cells. Molecular weights are indicated (kDa). Statistical analyses were conducted by one-way ANOVA. The following symbols were used to indicate significant differences: ns, P > 0.05; *, P < 0.01; ***, P < 0.001. Images and blots are representative of three independent experiments. Bar scale, 10 μm.
activate trithorax (23), which balances Polycomb group proteins to regulate proliferation and self-renewal in stem cells. Previously, we reported that BRD2 deficiency in murine ESCs induces insulin transcription, reinforcing the concept that these factors play important roles during the earliest stages of mammalian development. Understanding the biological functions of each individual BET proteins is critical knowledge preliminary to improved design and development of targeted epigenetic therapeutics for cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: G.P. Andrieu
Development of methodology: G.P. Andrieu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.P. Andrieu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.P. Andrieu
Writing, review, and/or revision of the manuscript: G.P. Andrieu, G.V. Denis
Study supervision: G.V. Denis
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