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Gene Silencing Associated with SWI/SNF Complex Loss During NSCLC Development

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Abstract

The SWI/SNF chromatin-remodeling complex regulates gene expression and alters chromatin structures in an ATP-dependent manner. Recent sequencing efforts have shown mutations in BRG1 (SMARCA4), one of two mutually exclusive ATPase subunits in the complex, in a significant number of human lung tumor cell lines and primary non-small cell lung carcinoma (NSCLC) clinical specimens. To determine how BRG1 loss fuels tumor progression in NSCLC, molecular profiling was performed after restoration of BRG1 expression or treatment with an HDAC inhibitor or a DNMT inhibitor in a BRG1-deficient NSCLC cells. Importantly, validation studies from multiple cell lines revealed that BRG1 re-expression led to substantial changes in the expression of CDH1, CDH3, EHF and RRAD that commonly undergo silencing by other epigenetic mechanisms during NSCLC development. Furthermore, treatment with DNMT

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inhibitors did not restore expression of these transcripts indicating that this common mechanism of gene silencing did not account for their loss of expression. Collectively, BRG1 loss is an important mechanism for the epigenetic silencing of target genes during NSCLC development.

Keywords

Chromatin remodeling; lung cancer; DNA methylation; histone acetylation

Implications

Inactivation of the SWI/SNF complex provides a novel mechanism to induce gene silencing during NSCLC development.

Introduction

Lung and bronchus carcinoma remain two of the most lethal cancers in the United States with over 90% of affected individuals succumbing to this disease. Over 85% of these cancers will be classified as non-small cell lung carcinoma (NSCLC) making them the primary clinical focus. For many cancers, treatments for early stage tumors have proven effective while metastatic disease often carries a very poor prognosis (most patients with metastatic lung carcinoma show an overall survival rate of <5% at 5 years). During the last 25 years, investigators have identified many of the genetic changes underlying the appearance of NSCLC including mutations of *BRAF, KRAS, EGFR, FHIT, HER2/NEU, RB,* *p16*^{INK4A}, and *p53* (1, 2). In addition, epigenetic silencing of the *p16*^{INK4A} and *CDH1* also plays a role (3). A study demonstrating the poor survival of patients with 4 epigenetically silenced genes further emphasizes the importance of understanding the contribution of epigenetic mechanisms to NSCLC development (4).

Recent next generation sequencing studies have shown that mutations in components of the SWI/SNF complex occur frequently in NSCLC samples (5). This complex, first discovered in *S. cerevisiae*, shows strong conservation from yeast to *Drosophila* to mammals and contains approximately 10–12 components (6, 7). The complex contains only one of the two mutually exclusive ATPases, BRG1/SMARCA4 or BRM/SMARCA2, to fuel its remodeling activity (8). Perturbation of chromatin remodeling is an emerging theme in cancer progression as evidenced by the discovery of mutations in multiple members of the complex in human cancers including NSCLC, malignant rhabdoid tumors, ovarian carcinomas and renal cell carcinomas (8–14). In NSCLC, mutations often arise in one of the genes coding for the ATPase component that fuels the complex, *BRG1/SMARCA4* (15, 16). However, how mutational inactivation of this gene contributes to NSCLC progression remains an open question.

We have previously shown that re-expression of BRG1 in human cell lines lacking expression of both mutually exclusive ATPases, BRG1 and BRM/SMARCA2, induces expression of genes often associated with epigenetic silencing (17–20). We also observed some overlap between genes activated by BRG1 expression and those activated by treatment with the DNA methyltransferase (DNMT) inhibitor 5dAzaC (17). However, we did not

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assess the effects of histone acetylation in this study, another mechanism for gene silencing (21). Because we only examined a limited number of genes, we could not determine how commonly genes activated by BRG1 expression overlapped with those induced by DNMT inhibition or by HDAC inhibition.

To address the question of how BRG1 inactivation contributes to NSCLC development, we carried out a gene expression array analysis on a BRG1/BRM-deficient cell line treated with a DNMT inhibitor, a HDAC inhibitor or infected with an adenovirus expressing BRG1. An analysis of the results showed that BRG1 re-expression activated a greater number of genes than either chemical reagent. Furthermore, the number of genes activated by both BRG1 and HDAC inhibition was greater than the number induced by both BRG1 and DNMT inhibition. We also did not observe global changes in DNA methylation patterns after BRG1 re-expression. Therefore, it appears that BRG1 loss contributes to gene silencing during NSCLC development via a mechanism independent of changes in DNA methylation. We also identified several important cancer-associated genes that may represent key downstream targets for SWI/SNF complex activity. These findings provide further insight into the role of aberrant SWI/SNF complex activity during NSCLC progression as well as opening new avenues for treatment of the patients.

Material and Methods

Cell culture

The human NSCLC cell lines H460, H522 and A427 and the human adrenal carcinoma cell line SWI3 were obtained from the ATCC and were grown in RPMI1640 with 10% FBS (Gibco, Life Technologies). All experiments were performed with cell lines within 20 passages of receipt (<3 months) to ensure the identity of each cell line. For BRG1 re-expression, we used an adenovirus expressing BRG1 and GFP, kindly provided by Dr. Bremner, Toronto Western Research Institute (22, 23). As a control we used an adenovirus expressing GFP alone provided by the UNC Vector Core Facility, (24). Adenovirus infection followed our previously published protocol (24).

Microarray analyses

Total RNA was extracted from H522 cells either untreated or treated with vehicle, dimethyl sulfoxide (DMSO), 5μM 5-aza-2'-deoxycytidine (5dAzaC), 100nM Trichostatin A (TSA) or infected with adenovirus expressing GFP-tagged BRG1 or GFP alone for 48 hours. RNA was labeled with Cy3 (treated or infected) and Cy5 (untreated) and hybridized to 4×44 whole human genome microarrays (cat#G4112F, Agilent Technologies, Wilmington, Delaware, USA) by the UNC Lineberger Genomics Core for Agilent microarray analysis. After acquiring the raw images from the Agilent Microarray Scanner with SureScan Technology (Agilent Technologies), the raw data from captured image files was extracted using Agilent Extraction Software (Agilent Technologies) and uploaded into UNC Microarray Database (UMD). The normexp background correction and loess normalization procedures were applied to the probe-level data (25). Expression measurements for each gene were calculated by computing the mean of the normalized intensity values for all probes mapping to that gene, as specified in a gene annotation database. This produced

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expression values for 19,749 genes. These data are available at GEO (http://www.ncbi.nlm.nih.gov/geo/), under accession number GSE54033.

The SAMR package was used to detect differentially expressed genes by comparing the expression values in the 5dAzaC-treated and TSA-treated cells to the expression values in the DMSO treated cells, while Ad-BRG1-GFP infected cells were compared to Ad-GFP infected cells in an effort to isolate the effect of BRG1 re-expression. (26). Gene expression values were first standardized within each array. For each of the above comparisons, we then identified differentially expressed genes using a median FDR threshold of .001. R 2.15.1 (27) was used to perform statistical analyses as well as create gene expression heatmaps and Venn diagrams using the gplots and VennDiagram packages, respectively (28, 29).

Gene transfection

For validation studies, we carried out transient transfections using either Fugene 6 (Promega) or Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Either the pBJ5-BRG1 plasmid expressing BRG1 or the empty vector pcDNA was transfected to each plate, and the cells were harvested for both RNA and protein 48 hours post-transfection as previously described (30).

Immunoblotting

Immunoblotting was performed as previously described (31). Protein lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF. The membrane was incubated in blocking buffer and then with a primary antibody overnight at 4°C. After washing, the membrane was incubated with an HRP-conjugated secondary antibody in blocking buffer. The primary antibodies included: BRG1 (A300-813A, Bethyl laboratories), CDH1 (610281, BD Transduction Laboratories), CDH3 (2130, Cell Signaling technology), CD44 (Dr. Larry Sherman, Oregon Health Sciences University), CK18 (DC-10, SCBT), RRAD (Dr. C. Ronald Kahn, Joslin Diabetes Center and Harvard Medical School), and β-ACTIN (A2066, Sigma). Proteins of interest were visualized with ECL Western blotting substrate (GE Healthcare) or a CCD camera imaging system (ChemiDoc™ XRS+, BIO-RAD).

Quantitative RT-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen) according to manufacturer's protocol, and was quantified by nanodrop spectrophotometry. 1 μg was used for cDNA synthesis and reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen) with random primers (Invitrogen) and dNTP mix according to manufacturer's instructions. We determined the amount of cDNA using ABI 7900HT sequence detection system (Applied Biosystems) with TaqMan Universal PCR Master Mix reagents and relative quantification was analyzed by the 2− Ct method with β-actin as an endogenous control. The primers used to detect the expression of CDH1 (Hs00170423_m1), CDH3 (Hs00354998_m1), RRAD (Hs00188163_m1), EHF (Hs00171917_m1), CD44 (Hs01075861_m1), SMARCA4 (Hs00946396_m1) and β-ACTIN (Hs01060665_g1) were purchased from Life Technologies.

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Restriction Genomic Landscape Scanning (RGLS)

We used the RLGS technique as previously described (32, 33). Briefly, 48 hours after transfection with empty vector or pBJ5-BRG1 into H522 or A427, cells were harvested for high molecular weight genomic DNA using Proteinase K digestion followed by phenol chloroform extraction and ethanol precipitation as previously described (34). DNA was digested with NotI and EcoRV for first dimension separation followed by in-gel HinfI digestion prior to second dimension separation, and ~2000 sites visually compared. Differential methylation was detected by either the absence or decrease in signal intensity (>50%). Each gel is internally controlled for successful restriction digests. In an incomplete digest, the strong RLGS spots representing rDNA sequences would show a laddering pattern. Those spots are only present if all restriction digests worked.

Genome-wide DNA methylation data

Dr. David Shames and Dr. Pan Du (Genentech) kindly provided genome-wide DNA methylation data for the H522 and A427 cell lines that was obtained using the Illumina HumanMethylation450 platform (35). Beta values ranging from 0 (lowest) to 1 (highest) measured DNA methylation levels at over 485,000 sites across the genome.

Results

Re-expression of BRG1 and BRM in BRG1/BRM-deficient NSCLC cell lines leads to re-expression of epigenetically silenced genes

We have previously shown that cell lines and genetically engineered mice lacking functional BRG1 or BRM do not express the CD44 transmembrane glycoprotein, a cell adhesion protein which can be epigenetically silenced in some human tumors (18–20, 36). When we transfected these cells with BRG1 or BRM or treated with a DNA demethylating agent, 5-d-Azacytidine (5-dAzaC), we observed induction of endogenous *CD44* transcription (17–20). These initial results suggested that restoration of SWI/SNF complex activity in deficient human tumor cells might reactivate expression of epigenetically silenced genes by affecting DNA methylation. Therefore, we checked whether another gene that commonly undergoes epigenetic silencing in human cancer, *CDH1* or *E-CADHERIN*, behaved in a similar fashion to *CD44*. As shown in Figure 1, either treatment with TSA or 5-dAzaC or re-expression with BRG1 or BRM induced expression of CDH1 protein in 2 BRG1/BRM-negative cell lines, SW13, derived from an adrenal carcinoma and H522, derived from a NSCLC. In contrast, treatment with a dominant-negative form of BRG1 (DNBRG1) that lacks ATPase or with vehicle (DMSO) had no effect.

Analysis of BRG1 re-expression on gene expression in the H522 NSCLC cell line

To further address the relationships among BRG1 re-expression, DNA methylation and histone acetylation, we carried out a gene expression array analysis on the BRG1/BRM-deficient human H522 NSCLC cell line after infection with Ad-BRG1-GFP or Ad-GFP or treatment with vehicle (DMSO), 5μM 5-dAzaC or 100nM TSA. For this analysis, we used 5μM 5-dAzaC to avoid toxicity problems we observed after treatment with 50μM. Similarly, we did not observe toxicity with BRG1 re-expression or 100nM TSA as previously reported

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(18, 37–43). All cells were treated or infected for 48 hours. After hybridization to Agilent whole human genome microarrays, the data was processed as described in the Material and Methods. Expression data for a total of 19,749 genes was available for analysis.

We first analyzed these data by hierarchically clustering both the genes and the arrays in the expression data and then creating a heat map to look for common patterns of gene expression. As shown in Figure 2, the 4 replicates from each treatment group clustered together, which shows consistency of gene expression patterns among the replicates. Of interest, the groups infected with adenovirus showed greater similarity to each other than to either TSA or 5-dAzaC treatments. It also appeared that infection with Ad-GFP alone caused unique changes in gene expression compared to the other treatment groups. Furthermore, the DMSO treatment control showed the least similarity to any of the other treatment groups while TSA and 5-dAzaC treatments showed the most similarity. Therefore, for gene expression altered by TSA or 5-dAzaC treatments, we searched for differential expression between each treatment group and the DMSO treated cells. We identified genes whose expression was induced by BRG1 re-expression by comparing the Ad-BRG1-GFP infection to the Ad-GFP infection.

We first looked for genes that showed changed expression after Ad-BRG1 infection. Our results found expression levels for 5527 genes increased and 6510 decreased after BRG1-GFP re-expression normalized to GFP expression alone (Supplemental Table 1). However, this number represents an over estimation because some genes showed decreased expression under all treatment conditions (Supplemental Table 1). In a similar vein, expression of 2436 genes increased and 2763 genes decreased after 5-dAzaC treatment (Supplemental Table 2). In contrast, we observed fewer changes in gene expression after TSA treatment, where 560 genes went up and 995 genes went down (Supplemental Table 3).

We also identified genes whose expression increased after 2 different treatments. We observed 429 genes that showed increased expression after either 5-dAzaC treatment or BRG1 re-expression (Supplemental Table 4). However, to be conservative in these analyses, we first focused only on those genes whose expression increased >2 fold compared to the parental cell line. Of these 429 genes, Table 1 lists the 145 genes that increased more than 2 fold after BRG1 re-expression including genes such as *CDH3* and *GDF15* that are associated with human tumor development (44, 45). Interestingly, *GDF15* was previously reported to undergo silencing by histone acetylation (46). In a similar analysis for genes whose expression increased after TSA treatment or BRG1 re-expression, we found 186 genes (Supplemental Table 5). Of 140 genes from this group that went up by more than 2-fold after BRG1 re-expression, *CDH1* showed the most robust increase in expression while *CD44, KRT4, KRT8* and *KRT18* also showed strong induction (Table 2). Finally, we looked for genes whose expression went up under all three conditions. This group contained the fewest genes (86), of which 55 genes increased by more than 2 fold after BRG1 re-expression (Table 3, Supplemental Table 6). Interestingly, *GADD45A*, a stress responsive gene, showed strong induction under all 3 conditions (Supplemental Table 6) (47). Finally, we identified genes that showed increased expression after TSA or 5-dAzaC treatment but not BRG1 (Supplemental Table 7). We found 170 genes, of which 148 increased by at least 2-fold after TSA treatment. These results are summarized in a Venn diagram in Figure 2B.

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Validation of gene expression array data

One caveat from studies using adenovirus infection to express proteins is the significant production of protein by the adenovirus infection. To address this issue, we re-expressed BRG1 in the H522 cell line by gene transfection to minimize overexpression. We also included a second BRG1/BRM-deficient NSCLC cell line, A427, where we have previously shown induction of CD44 after BRG1 expression (20). We examined expression of *CDH1, CD44* and *CK18*, as well as potentially novel BRG1 regulated genes, *CDH3, EHF* and *RRAD*. A previous report showed that *RRAD*, a member of the *RAS* superfamily, is epigenetically silenced by DNA methylation in a large percentage of NSCLC while *EHF*, a member of the ETS transcription factor family, is silenced in prostate cancer (48, 49). *CDH3* (P-cadherin) maps next to *CDH1* and can also undergo silencing during human tumor development (45).

As shown in Figure 1B, we observed increased expression of these genes in H522 consistent with microarray data. However, we found increases only in *CD44, EHF* and *RRAD* expression after BRG1 re-expression in the A427 cell line, indicating differences between these 2 NSCLC cell lines. We also assessed protein expression by Western blot analysis for genes with available antibodies (Figure 1C). The Western blot data in Figure 1C recapitulates the qPCR data in panel B i.e. a more robust induction of target protein expression with BRG1 in the H522 cell line versus the A427 cell line (notice the ratio of protein expression to the β-ACTIN control in the 2 cell lines). Thus, the Western blot results appeared consistent with the qPCR findings.

Gene expression changes after BRG1 re-expression in BRG1/BRM-deficient NSCLC cells does not correlate with DNA methylation levels

To further investigate the relationship between BRG1 loss and DNA methylation, we asked whether BRG1 re-expression altered DNA methylation in NSCLC cell lines. We used restriction landmark genomic scanning (RLGS) to detect DNA methylation changes in the 2 BRG1/BRM-deficient NSCLC cell lines after BRG1 re-expression (32, 33). RLGS uses two-dimensional gel electrophoresis system to detect radiolabeled methylation sensitive restriction endonuclease sites and create “landmarks” seen on the resulting autoradiograph. This approach has been successfully used to detect changes in DNA methylation in primary NSCLC samples (50). We first demonstrated that our treatment conditions with 5-dAzaC led to re-expression of methylation-silenced *CDH1* in the H460 NSCLC cell line as previously reported (Figure 3A)(51). In contrast, only treatment with TSA +/− 5-dAzaC or infection with Ad-BRG1 led to *CDH1* and *CD44* expression in H522 cells, in agreement with the gene expression array results (Figure 3A).

RLGS analysis of H522 cells transfected with vector or BRG1 revealed only three prominent landmarks that appeared after BRG1 re-expression (Figure 3B). However, sequencing of these DNAs revealed that they originated from the BRG1 transgene and not from changes in methylation of the H522 cellular DNA. Similar results were observed for the A427 cell line (Supplementary Figure 1). We could not detect any additional changes after BRG1 re-expression. Therefore, this “snapshot” of global DNA methylation did not

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show major changes after BRG1 re-expression. We cannot, off course, exclude that DNA methylation changes below the detection limit of about 5% change occurred.

Finally, we examined whether the DNA methylation status of these genes would predict their response to BRG1 re-expression i.e. do high levels of DNA methylation inhibit the effects of BRG1 re-expression? To address this issue, we took advantage of a recent study that used DNA methylation arrays to compare the patterns among 69 human NSCLC cell lines including H522 and A427 (35). We compared the methylation patterns on 4 genes that showed increased expression in one or both cell lines, *CD44, CDH1, RRAD* and *CDH3*. As shown in Figure 4, A427 cells displayed significantly more DNA methylation along the length of the *CD44* gene than H522 cells. However, BRG1 re-expression induced *CD44* expression only in the A427 cell line (Figure 1B). In contrast, H522 cells showed significantly less methylation in the promoter region of *CDH3* than A427 cells, consistent with BRG1 re-expression inducing its expression in H522 alone. Furthermore, *RRAD* basal expression was higher in A427 cells despite the presence of significantly more DNA methylation along the entire promoter and coding region. Similarly, we did not observe an association between *CDH1* methylation and expression. Taken together, our results support the notion that DNA methylation correlates poorly with altered gene expression after BRG1 re-expression.

Discussion

Epigenetic alterations are generally accepted as critical components of neoplastic transformation (52, 53). Most studies have focused upon the mechanisms of DNA methylation and histone modifications as inducers of gene silencing. In this study, we provide evidence that loss of SWI/SNF complex activity via mutations in the ATPase components may provide another mechanism for gene silencing during lung tumor development. This role for aberrant SWI/SNF complex activity may account for the significant number of mutations found in most of the subunits across a large number of human tumors (5, 14)

How could decreased expression or loss of SWI/SNF complex activity lead to changes in gene expression? One potential mechanism could involve altered activity of transcription factors associated with the SWI/SNF complex. Previous studies have shown interactions between various SWI/SNF complex members and transcription factors including c-MYC, NRF2, p53 and NFkB (54–57). These studies also established that loss of BRG1 or SNF5 altered downstream signaling of these transcription factors. Several recent reports have also demonstrated global changes in nucleosome positioning after loss of BRG1 expression (55, 58, 59). Therefore, alterations in SWI/SNF complex activity could affect gene expression by either causing nucleosome positioning changes at gene promoters or at transcription factor binding sites.

Our studies also showed that treatment with a HDAC inhibitor or re-expression of BRG1 could increase expression of several genes frequently silenced in NSCLC, such as *CDH1* and *CD44*. This brings up the possibility that loss of BRG1 and BRM proteins, the critical ATPase subunits of the SWI/SNF complex, could influence gene silencing by affecting the

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activity of other histone or chromatin modifying complexes. Multiple examples exist in the literature illustrating the interdependence between components of the SWI/SNF complex and other chromatin remodeling complexes (60, 61). Thus, loss of SWI/SNF complex activity could lead to gene silencing through the repressive effects of other complexes such as the ISWI or polycomb complexes.

The SWI/SNF complex can also associate with different histone-modifying enzymes. The SWI/SNF complex cooperates with histone acetyl transferases to promote epigenetic marks at histones (62, 63). Therefore, loss of SWI/SNF complex activity could also lead to loss of histone acetylation marks in tumor cells. In the NUMAC complex (nucleosomal methylation activation complex), members of the SWI/SNF complex including BRG1 interact with the co-activator-associated arginine methyltransferase-1 (CARM1) to regulate activity for histone methylation (64). This again provides another mechanism where loss of SWI/SNF complex function could potentially lead to loss of activating marks on histones. The SWI/SNF complex also cooperates with other histone-modifying enzymes such as histone deacetylases-3 (HDAC3) and the transcriptional co-repressors KAP-1 (Krab associated protein 1) within the NCoR-1 (Nuclear receptor corepressors-1) complex (65). Therefore, loss of BRG1 and BRM could alter their activities by potentially increasing their repression of gene transcription.

Although existing next generation sequencing studies have found a significant number of BRG1 mutations and deletion in squamous cell and adenocarcinomas of the lung, their effects on SWI/SNF complex activities remain unknown. Many of the mutations result in truncated forms of BRG1 that appear to be degraded (11, 18). However, in these cohorts, five of eight mutations in squamous cell and ten out of twenty mutations in adenocarcinomas are missense. Therefore, determining the effects of these single amino acids changes on protein function becomes imperative. Importantly, several recent reports have shown that missense mutations in BRG1 can dramatically alter its biological activity (30, 66). Whether these BRG1 mutant proteins can also result in gene silencing remains an important unanswered question.

Our current study implicates BRG1 loss as another route for epigenetic silencing during NSCLC development. The large number of changes in gene expression associated with BRG1 re-expression compared to treatment with a DNMT or HDAC inhibitor appears consistent with this notion. While the mechanism for this effect requires further study, experiments identifying changes in BRG1 binding sites by ChIP-seq and nucleosome positioning by MNase-seq should help resolve this issue. Furthermore, the validation of additional BRG1 target genes should lead to the generation of new treatment approaches for individuals with BRG1-deficient NSCLC. Our results also strongly suggest that treatment with DNMT or HDAC inhibitors may not prove efficacious in patients with BRG1-deficit NSCLC. The cell lines developed in this study as well as genetically-engineered mouse models for BRG1-induced tumor development will accelerate the translation of future treatment options into clinical practice (67, 68).

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Supplementary Material

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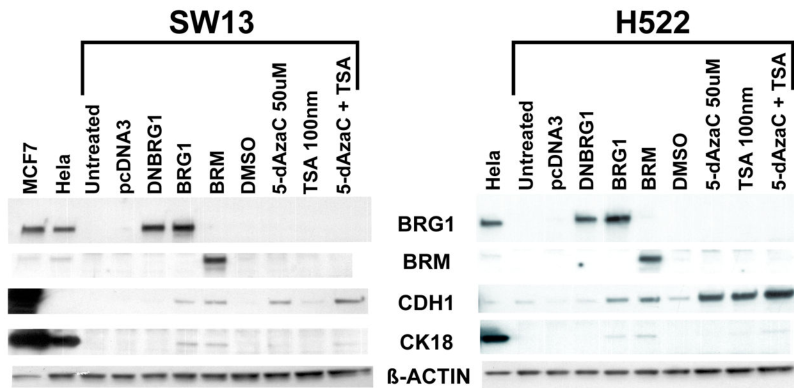

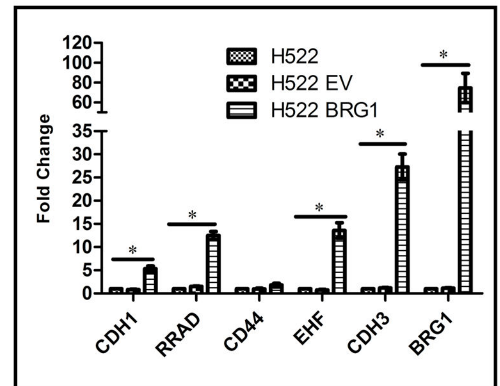

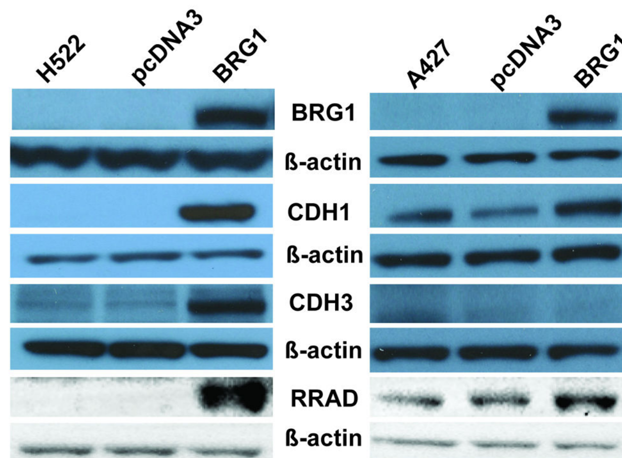

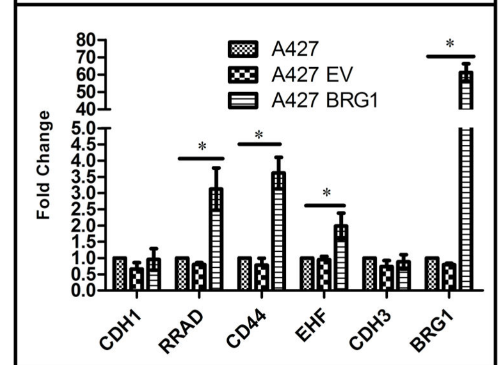

Figure 1. Re-expression of silenced genes in BRG1/BRM-deficient cell lines

(A) SW13 or H522 cells were either treated with 5-dAzaC and/or TSA or transfected with the empty expression vector pcDNA3 or expression vectors for BRG1, DNBRG1 or BRM. After 72 hours, protein was extracted, separated and immunoblotted for the indicated proteins as described in the Material and Methods. β-actin served as the loading control. (B) & (C) H522 and A427 cells were transfected with BRG1 (pBJ5-BRG1) or empty vector (pcDNA3). After 48 hours, cells were harvested for either total RNA or protein. Gene expression was then evaluated by qPCR using ABI primers (B) or by western blotting (C). For qPCR, expression for each gene was normalized to β-actin levels; for western blotting, β-actin served as a loading control. * means P-value< 0.05, and error bars represent +S.E.M.

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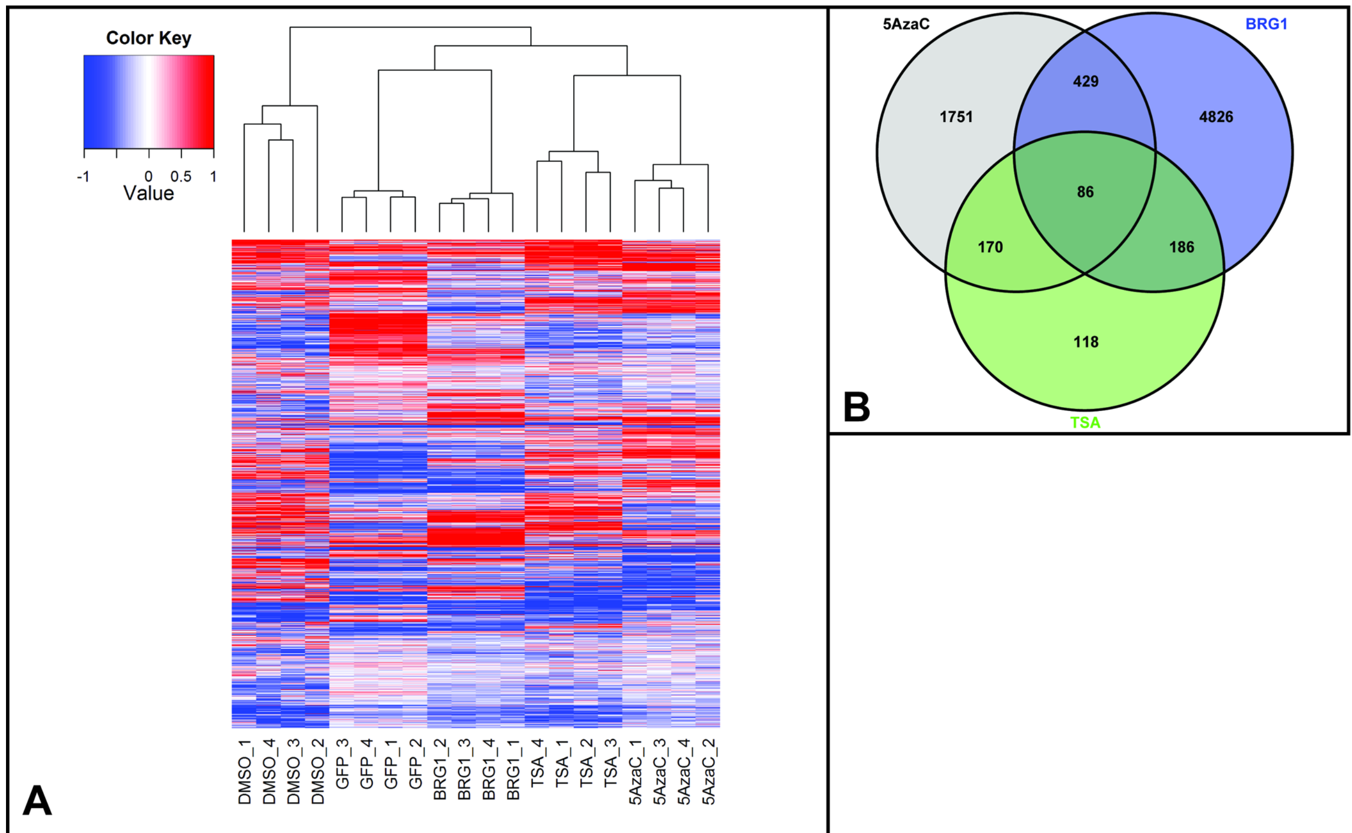

Figure 2. Characterization of gene expression changes in H522 NSCLC cells

Gene expression analysis using array technology was carried out as described in the Material and Methods. (A) Heatmap of gene expression data in H522 NSCLC cells after standardizing expression values within arrays, median centering within each gene and hierarchically clustering both genes and arrays (gene dendrogram not displayed). Expression values for 19,749 genes are shown. (B) Venn diagram displaying counts of differentially up-regulated genes according to the comparison groups described in the Material and Methods.

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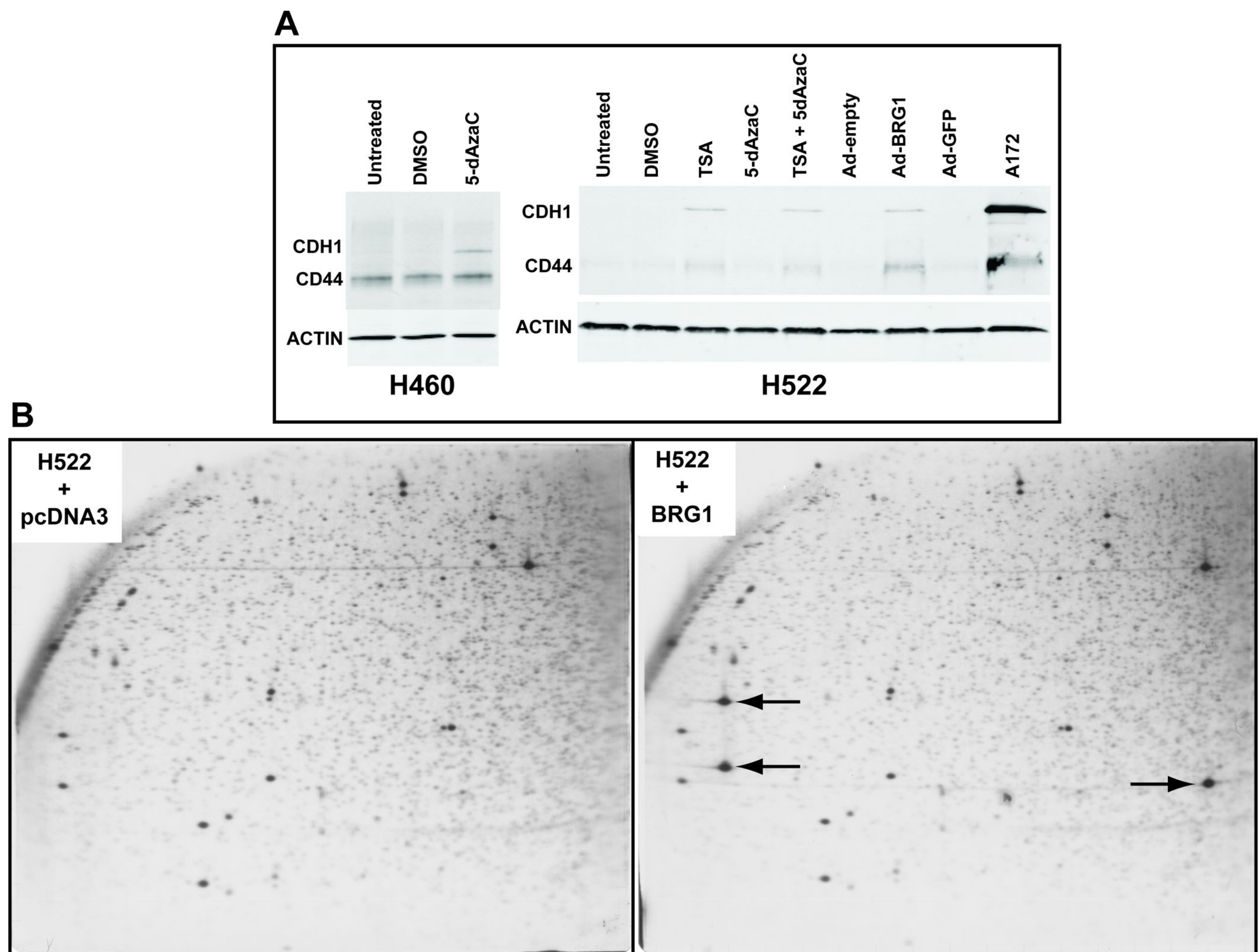

Figure 3. Changes in DNA methylation does not account for gene expression changes after BRG1 re-expression in H522 and A427 cells

(A) H522 cells were infected with adenovirus expressing human BRG1 and/or GFP as described in the Material and Methods. H522 and H460 cells were treated with DMSO, 5μM 5-dAzaC or 100nM TSA as described in the Material and Methods. After 48 hours, dishes were harvested for total protein. Expression of CDH1, CD44 and CK18 was then measured by Western blotting. β-actin served as a loading control and the H460 cell line served as a positive control for 5-dAzaC treatment (51). (B) H522 cells were transfected with BRG1 (pBJ5-BRG1) or empty vector (pcDNA3). After 48 hours, cells were harvested for DNA and used for RLGS as described in the Material and Methods.

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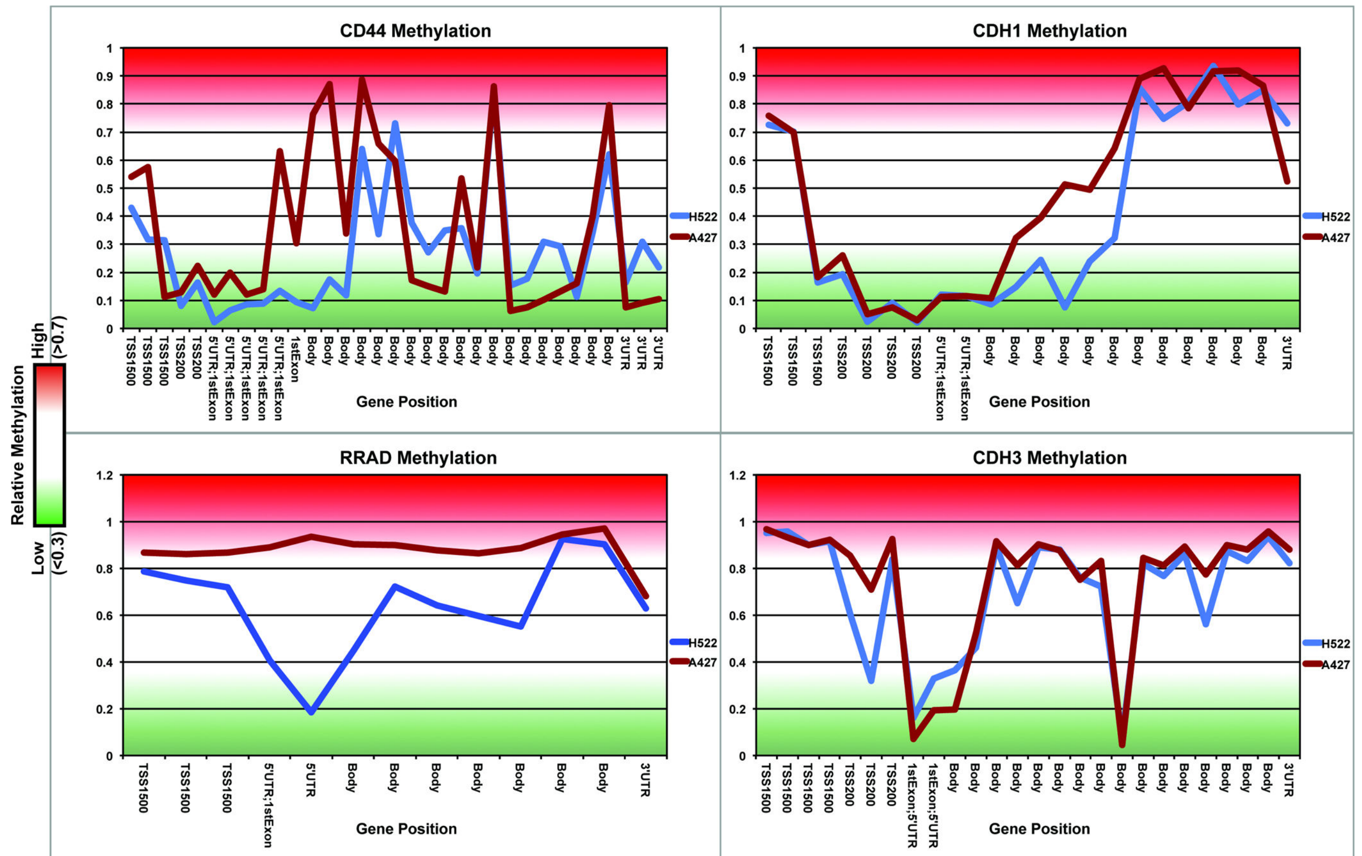

Figure 4. DNA methylation does not correlate with gene expression changes after BRG1 re-expression in H522 and A427 cells

Beta values ranging from 0 (lowest) to 1 (highest) show DNA methylation levels at sites across four representative BRG1 target genes, *CD44, CDH1, RRAD*, and *CDH3*, for both H522 and A427 cell lines. Methylation levels are coded as follows: low (green, beta < 0.3), intermediate (white, 0.3 < beta < 0.7), high (red, beta > 0.7).

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Table 1
Genes up-regulated by both 5-dAzaC treatment and BRG1 re-expression

Differentially expressed genes were identified, as described in Material and Methods. Of the 429 genes up-regulated in both 5-dAzaC treatment vs. DMSO and BRG1-GFP re-expression vs. GFP, the 145 genes that increased more than two fold after BRG1 re-expression are shown.

ABCG4	GALNAC4S-6ST	MAPKAPK3	SESN2
ABHD11	GALNTL4	MCFD2	SHC2
AIM1L	GCC1	METRNL	SLC1A4
AKAP8	GDF15	MGLL	SMOX
APLN	GJB2	MMP1	SPEG
ARHGAP22	GOT1	MMP19	STC2
ARHGEF3	GPIHBP1	MORC4	STX4
ASB2	GPN1	MPPED1	TCF7
ATP6V1D	GPRIN2	MRPL18	TICAM1
BLCAP	GPT2	MSI2	TMEM130
BMP5	GRB10	MT1E	TMEM198
C10orf49	GUCA1B	MYLK2	TNFRSF10B
C15orf52	HIVEP3	MYO5B	TNFRSF8
C18orf25	HMGCS1	NEFM	TNNC2
C21orf34	HSPA4	NPPB	TPD52L1
C6orf145	HSPH1	NR3C1	TPRXL
C7orf29	IDH1	NUPR1	TRIP6
CABLES1	IFFO2	OGDHL	TSSC4
CAMK2N1	IFRD1	OR3A3	UPP1
CARD10	IL11	P2RY2	VDR
CBS	IL17D	PCBP4	WARS
CCDC130	INHBB	PDZD2	YKT6
CCDC3	KCNA7	PHLDB3	ZFAND2A
CDH3	KCNQ1	PINK1	ZNF354A
CEBPB	KIAA1199	PMAIP1	ZNF592
CKMT1A	KIAA1683	PREX1	
CXorf40B	KRT19	PRR17	
DKFZp547K054	LEPREL1	RAB3B	
DNAJA4	LGALS8	RAGE	
DUSP2	LHFP	RBKS	
DYNC1H1	LHX6	RBP4	
DYSF	LIMK2	REEP1	
EIF4G2	LOC387763	RGS16	
ELF4	LOC392335	RHBDL3	
ENO3	LOC732215	ROM1	
ETS2	LONRF2	RRP12	
FAM101A	LYG1	S100A13	

FCGBP	LZTS1	S100A16
FGFBP3	MAL	SAT1
FHL2	MALT1	SCEL

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Table 2

Genes up-regulated by both TSA treatment and BRG1 re-expression

Differentially expressed genes were identified, as described in Material and Methods. Of the 186 genes up-regulated in both TSA treatment vs. DMSO and BRG1-GFP re-expression vs. GFP, the 140 genes that increased more than two fold after BRG1 re-expression are shown.

A4GALT	ETV7	LOC149501	TBC1D9
AFAP1	F3	LOC441376	TGFA
AGPS	FAM127A	LOC442249	TKT
AK2P2	FAM151A	LOC54492	TMEM37
AKR1C3	FAM174B	LOC647954	TNNC1
ANKRD13A	FAM65B	MAP1B	TRIM2
ARL4D	FLJ40504	MAP4K4	TSPAN5
ASMTL	GATA6	MYLIP	TTYH1
BAMBI	GDPD5	MYO10	TUBA4A
BTG2	GLDC	MYO1E	TUBB2A
C11orf67	GSN	MYOF	TUFT1
C19orf33	GUK1	NACAD	TXNIP
C1orf167	HABP4	NCRNA00087	TXNRD1
C1orf226	hCG_1988300	NPPC	VSTM2L
CALB2	HDAC5	NPTX2	WNT10A
CAPN2	HEATR5A	OBFC2A	WNT4
CCL2	HIST1H2AD	OSGIN1	WNT6
CCND1	HRCT1	P4HA3	ZCCHC12
CD44	HS3ST2	PEG10	ZCCHC17
CDH1	HTRA1	PGD	ZNF365
CDKN1A	IGSF3	PGM2L1	
CITED4	IL18	PLCD3	
CLU	JAKMIP1	PMEPA1	
CNN1	JUN	PPL	
COL16A1	KLK10	PRAGMIN	
CPE	KRT18	PRSS23	
CPM	KRT18P19	PTRF	
CRMP1	KRT18P28	RAB11FIP1	
CRTAC1	KRT18P30	RELL1	
CTSL1	KRT18P33	RGL1	
CXCR4	KRT18P40	RNF144B	
CYP2U1	KRT18P42	S100A4	
DAPL1	KRT18P49	S100P	
DDIT4	KRT4	SH3BP5	
DHRS3	KRT8	SIK1	
DLK1	LBH	SLN	
DUSP5	LCN2	SNTA1	

EFHD1	LGI2	SUSD2
ENC1	LIPH	SYT17
ERRFI1	LOC100128116	TAGLN2

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Table 3

Genes up-regulated by 5-dAzaC treatment, TSA treatment, and BRG1 re-expression

Differentially expressed genes were identified, as described in Material and Methods. Of the 86 genes up-regulated in 5-dAzaC treatment vs. DMSO, TSA treatment vs. DMSO, and BRG1-GFP re-expression vs. GFP, the 55 genes that increased more than two fold after BRG1 re-expression are shown.

ANKRD6	GFOD1	GLIPR2	NDRG1	RAB31
ANXA2	GFPT2	ID3	NGEF	RASSF2
ATF3	DDX58	ID4	NMNAT2	RHOU
BCL6	EGR2	KIF5C	OCC-1	RIPK4
BIK	EMILIN2	KRT18P34	ODC1	S100A2
C16orf45	FAM89A	LAMB3	PAQR9	SLC30A3
C6orf114	FEZ1	LGALS3	PEA15	SMAD3
CCK	FTL	LMCD1	PLCE1	SRXN1
CD83	FYN	LOC26010	PODXL	SULF2
CGNL1	GABARAPL1	MEG3	PPP1R14C	TPM1
CHST2	GADD45A	MTM1	RAB11FIP5	WWC3