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## Limited but durable changes to cellular gene expression in a model of latent adenovirus infection are reflected in childhood leukemic cell lines

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### Abstract

Mucosal lymphocytes support latent infections of species C adenoviruses. Because infected lymphocytes resist re-infection with adenovirus, we sought to identify changes in cellular gene expression that could inhibit the infectious process. The expression of over 30,000 genes was evaluated by microarray in persistently infected B- and T-lymphocytic cells. *BBS9*, *BNIP3*, *BTG3*, *CXADR*, *SLFN11* and *SPARCL1* were the only genes differentially expressed between mock and infected B cells. Most of these genes are associated with oncogenesis or cancer progression. Histone deacetylase and DNA methyltransferase inhibitors released the repression of some of these genes. Cellular and viral gene expression was compared among leukemic cell lines following adenovirus infection. Childhood leukemic B-cell lines resist adenovirus infection and also show reduced expression of *CXADR* and *SPARCL1*. Thus adenovirus induces limited changes to infected B-cell lines that are similar to changes observed in childhood leukemic cell lines.

### Keywords

Human adenovirus; Species C; Lymphocytes; Latent; Gene-expression; Leukemia; Oncogenesis; Viral persistence; Adenovirus; Human lymphocytes

## 1. Background

Species C adenoviruses infect most people asymptotically in the first few years of life (Garnett et al., 2002, 2009), and appear to be the most common prenatal viral infection in

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**Appendix A.** Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2016.03.015>.

humans as well (Baschat et al., 2003; Ornelles et al., 2015; Reddy et al., 2005; Wenstrom et al., 1998). These viruses establish long-term, perhaps life-long, latent infections of mucosal lymphocytes as evidenced by the observation of intermittent virus shedding in stool (Fox et al., 1977), and that activation of non-virus-producing tonsil lymphocytes in vitro results in infectious virus production from most individuals (Garnett et al., 2009).

Experimental infections of human T-cell and B-cell leukemia cell lines were used to model the characteristics of adenovirus latency (Zhang et al., 2010). Some of these lymphocyte cells support vigorous adenovirus replication while maintaining nearly normal levels of cell division, in sharp contrast to the rapid cytolytic effect seen in infection of epithelial cells. Following initial infection, some lymphocyte cell lines gradually lose expression of viral genes while maintaining the quiescent viral genome for weeks or months with minimal virus replication detected in the cultures, suggesting a latent state (Zhang et al., 2010). Significantly, expression of the virus receptor (CAR) is lost on the cell surface at late times of infection. Loss of CAR late in infection of lymphocytes is due to absence of CAR mRNA expression (Zhang et al., 2010), suggesting that adenovirus infection elicits long-lasting changes in cellular gene expression by an epigenetic mechanism. Reestablishing CAR expression in persistently infected cells did not restore infectivity, indicating that cellular changes in addition to down-regulation of the viral receptor have occurred. These changes render the cells refractory to adenovirus infection.

At present, alteration in cellular gene expression in virus latency is best understood among the herpesviruses. For example the analysis of gene expression by oligonucleotide or cDNA arrays in models of human cytomegalovirus (HCMV) and Epstein Barr Virus (EBV) latency found dozens and hundreds of cellular genes respectively whose expression is modified compared to uninfected cells (Homa et al., 2013; Slobedman et al., 2004). These studies suggest that many cellular genes are altered by these viruses to produce an intracellular environment conducive to the maintenance of latency. In the present study we assessed alterations in cellular gene expression with the Affymetrix oligonucleotide array technology in models of adenovirus latency in lymphocytes to determine the extent to which cellular gene regulation is occurring in long-term infected cells.

Adenoviruses are the most frequently detected viruses in human cord blood (Ornelles et al., 2015; Reddy et al., 2005; Wenstrom et al., 1998). We, and others, have previously speculated that these viruses are candidates for producing the initiating event leading to the development of childhood acute leukemias (Heath, 2005; Hughes et al., 2007; McNally and Eden, 2004). The absence of adenoviral genetic material in leukemic cells suggests a potential hit-and-run mechanism by which the virus permanently alters some cellular function before being itself ejected from the cell. Using the Affymetrix U133 chip set, we determined that the expression of only six cellular genes is altered in infected B cells. We extended the analysis to include evaluation of the expression of the viral-modulated genes in cell lines of adult and childhood leukemias. Two of these six genes are significantly suppressed in cell lines of childhood leukemia origin but not in those of adult origin. Further, we found that the virus achieved durable changes in the expression of many of these genes by epigenetic mechanisms.

## 2. Materials and methods

### 2.1. Cell lines

BJAB cells are an EBV-negative B cell line that was established in 1975 from an African case of Burkitt's lymphoma. The patient from whose tumor the original BJAB line was established was seropositive for EBV antigens although the tumor was not infected with EBV. BJAB cells were reported to have a normal karyotype at the time of establishment (Clements et al., 1975). KE37 cells are a T cell leukemia cell line established from an acute lymphoblastic leukemia patient in 1979. The cell line used for this study was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig). Cytogenetic analysis indicated a pseudodiploid karyotype with 12% polyploidy. The karyotype was described as:  $46(43-46) < 2n > X,-Y,+ mar, t(8;9)(p11;p24), der(18) t(X;18)(p11;q11)$  (Romano et al., 2009). Additional cell lines used for short term experiments were B cell lines derived from childhood and adult leukemias. The following cell lines were derived from childhood ALL: REH, UoC-B4, RS4;11, SEM, EU-8, KOPN-8, RCH-ACV, and 697 (EU-3). The following cell lines were derived from adult B-cell lymphomas, leukemias or EBV transformed cells: ARH77, Ramos, RPMI-1788, SB, and Raji. ARH77, Raji, Ramos, REH, RPMI-1788, RS4;11 cells were obtained from ATCC. BJAB cells were originally obtained from Ken Takemoto (Klein et al., 1974) and SB cells were obtained from Chas Yang. EU3, EU8 and UoCB4 were obtained from Harry Findley (Zhou et al., 1995). The remaining cell lines, KOPN-8, RSH-ACV and KE37 were obtained from the DSMZ in Germany. RS4;11 CAR-hi were generated by transducing an isolate of RS4;11 cells with a CAR-expression retrovirus, as previously reported (McNees et al., 2004; Zhang et al., 2010). Cell lines were maintained in the recommend growth medium supplemented with fetal bovine serum. Cells were incubated at 37 °C incubator with 5% CO<sub>2</sub> and tested to ensure absence of Mycoplasma.

### 2.2. Infection of lymphocyte cell lines

Ad5 *d*309 is an Ad5 mutant that lacks the genes for E3 RID $\alpha$  and RID  $\beta$  proteins and for the 14,700-molecular-weight protein (14.7 K protein) and was used as the virus for these experiments. Lymphocytes were collected and washed in serum-free medium. Cell density was adjusted to 10<sup>7</sup> cells per ml in serum-free medium. Virus (*d*309) was added to the cell suspension at 100 infectious units (defined on A549 cells) per cell and incubated at 37 °C for 3 h. The infected cells were washed three times with complete growth medium and then resuspended and maintained in complete growth medium at 5 × 10<sup>5</sup> cells per ml.

### 2.3. Chronic virus infection

BJAB and KE37 cells were infected with the phenotypically wild-type virus *d*309 to establish uniformly infected cells. Six independently infected cell lines (three each of BJAB and KE37) were generated. The infected cells were maintained under standard growth conditions ranging between 51 and 78 days. Non-infected cells were maintained as six independent cultures for similar lengths of time. Immediately before isolating RNA from the cell lines, hexon expression was measured by immunofluorescence and flow cytometry (Garnett et al., 2009). The presence of adenovirus DNA in each cell was confirmed by limiting dilution followed by a nested PCR for adenovirus DNA as described in (Garnett et

al., 2009). The average amount of adenovirus DNA per cell was determined by quantitative PCR of the hexon gene.

#### 2.4. Analysis of RNA levels by microarray

Total cellular RNA was isolated from  $10^6$  to  $10^7$  lymphocytes using the Qiagen RNeasy purification kit. The integrity of the purified RNA was verified by the Microarray Core Lab of the Comprehensive Cancer Center of Wake Forest University and used to query the Affymetrix Human Genome U133 Plus 2.0 array which contains 54,675 oligonucleotide probes permitting analysis of 47,000 transcripts from 38,500 human genes. The resulting expression data were analyzed with the open source tools available through the Bioconductor (Gentleman et al., 2004). Expression data were adjusted and normalized by different approaches provided by the Affymetrix and GCRMA packages and by the systematic variation normalization (SVN) method developed by Chou et al. (2007). The limma package was used to perform statistical analysis and assess differential expression of the normalized expression data. The limma package implements an empirical Bayes method to moderate the standard errors of the change in expression level and provides tools to identify significant differences. Raw expression data was adjusted and normalized by the algorithms implemented in the Affymetrix MicroArray Suite 5.0 (MAS5), RMA, GCRMA, and SVN packages. Normalization performed by MAS5 determines a baseline expression level for 100 “housekeeping” genes. The MAS5 algorithm normalizes values for abundantly expressed genes very well but is less efficient for genes that are less abundant. The RMA algorithm computes a robust multi-array average of expression values by background adjustment, quantile normalization among arrays, and summarization. The key property of RMA is the adjustment of the quantile-quantile plot among all arrays to achieve normalization (Irizarry et al., 2003). GCRMA uses a similar algorithm to the RMA method but models probe affinity for perfect match and mismatch oligos in the Affymetrix probe sets in order to perform a more aggressive background correction. The systematic variation normalization (SVN) method of Chou (Chou et al., 2007) performs background subtraction determined by the distribution of pixel intensity values, then linear or non-linear regression, transformation and multi-array normalization to remove systematic variation across multiple array experiments. The SVN algorithm requires fewer regression parameters to normalize the preprocessed data than either the RMA or GCRMA methods. Normalized data were analyzed by a linear modeling approach where the four groups being analyzed were BJAB-mock, BJAB-virus, KE37-mock and KE37-virus. Comparisons between BJAB-mock vs. BJAB-virus, KE37-mock vs. KE37-virus and BJAB-mock vs. KE37-mock were performed by linear modeling. The empirical Bayes method was used to adjust the individual probe variance by augmenting the degrees of freedom for individual variances from the population on the whole. To perform this adjustment and to calculate a moderated t-statistic or false-discovery rate, the proportion of differentially expressed genes (a priori probability) was assumed to be 0.3% or about 150 genes.

#### 2.5. Analysis of RNA levels by real-time quantitative PCR

Purified RNA was treated with Rnase-free DNase (Qiagen Inc. Valencia, CA), quantified and 200 ng were reverse transcribed (RT) into cDNA in 20 uL reaction volumes using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). RT-enzyme

negative controls were included for each reaction. Predesigned primers and probes (PrimeTime) were obtained from Integrated DNA Technologies (Coralville, IA). Primer and probe sequences are reported in Table 1. Probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) reporter molecule and contained dual ZEN and Iowa Black quenchers. The RT-qPCR assay was performed in 25  $\mu$ L using 2 $\times$  TaqMan Gene Expression Master Mix (Applied Biosystems) with 1 nmole of each primer, 0.5 nmole probe, and 2.5  $\mu$ L of cDNA product obtained from the RT reaction. The amplification for each sample was performed in duplicate wells using a standard PCR thermal cycling condition was 50  $^{\circ}$ C for 2 min, 95  $^{\circ}$ C for 10 min, 40 cycles of 95  $^{\circ}$ C for 15 sec and 60  $^{\circ}$ C for 1 min using a 7500 Real Time PCR System (Applied Biosystems). Analysis of relative gene expression in infected BJAB cells was determined using real-time quantitative PCR and the  $C_t$  method (Livak and Schmittgen, 2001).

Relative mRNA levels were determined by a modification of the  $C_t$  method. In some experiments, mRNA for *CXADR* and *SPARCL1* could not be detected in the childhood leukemic cell lines. For these cases, the  $C_t$  was set to a limiting value of 40.  $C_t$  values were first normalized to the housekeeping gene (CD44, EIF1 or GAPDH) within each of nine PCR experiments ( $C_t$ ). Values were further normalized to the mean value of all adult-derived cell lines in the experiment (ARH77, Ramos, Raji and BJAB). These twice-normalized values ( $C_t$ ) were further analyzed by the t-test in order to determine if the mean level for each gene in the childhood leukemic cell lines was significantly different from 0 (log of 1). A  $p$ -value less than 0.05 was considered significant. Because the  $C_t$  measured for *CXADR* and *SPARCL1* was assigned a limiting value in certain cases, the associated  $p$ -values must be considered an estimate.

## 2.6. Analysis of coordinate regulation by EPIG

Patterns of coordinately expressed genes were identified by the method of Chou known as “extracting gene expression patterns and identifying coexpressed genes” or EPIG (Chou et al., 2007). For this analysis, gene expression within each data set was represented as the log<sub>2</sub> ratio of sample to reference or signal. The average signal of each probe from mock-infected cells was set to zero in order to determine the relative level of gene expression in virus-infected cells. An iterative process using Pearson's correlation coefficient, the magnitude of the signal and the signal-to-noise ratio was used to extract groups of coordinately expressed genes as described (Chou et al., 2007).

## 2.7. Functional analysis of coordinately regulated genes

Genes that showed a common pattern by the EPIG analysis were evaluated with the Functional Annotation Clustering tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID), version 6.7 <http://david.abcc.ncifcrf.gov>. Default settings were chosen with the exception of more stringent enrichment threshold (EASE score) of 0.05. The EASE score of each group is derived from the geometric mean of the modified Fisher exact  $p$ -value associated with the enriched annotation terms that belong to the group. The group enrichment score provides a measure of relative importance of the gene groups with higher enrichment scores indicating more significant enrichment. The relative

enrichment for a given group of genes was tabulated against the possible genes represented on the Affymetrix Human 133 Plus 2 Array.

## 2.8. Treatment with epigenetic enzyme inhibitors

Cells were cultured in 24-well plates at a concentration of  $10^6$  cells per mL prior to treatment. TSA (Trichostatin A, Sigma-Aldrich), a histone deacetylase inhibitor, was used at a concentration of 50 nM for UOC-B4 cells, and 300 nM for all other cell lines. 5-Aza (5-Aza-2'-deoxycytidine, Sigma-Aldrich), a DNA methyltransferase inhibitor, was used at a concentration of 10  $\mu$ M. Cells were incubated alone, with TSA, or with 5-Aza for 48 h. Viability of cells was determined by Trypan blue dye-exclusion using a hemocytometer.

## 3. Results and discussion

### 3.1. Persistently infected lymphocytes show limited changes in gene expression

Several human lymphocytic cell lines support long-term adenoviral infections and proliferate at the same rate as non-infected cells (Zhang et al., 2010). Some of these cell lines can maintain the viral genome for more than a year in culture. Within 24 h post-infection the cells lose expression of the coxsackie and adenovirus receptor (CAR) on their surface and this loss is maintained for more than 100 days following infection. The immediate loss of surface CAR on infected cells is facilitated by viral fiber and is followed by the absence of *CXADR* mRNA beginning around 30 days post-infection. Because enforced expression of CAR was not sufficient to revert once-infected cells into becoming susceptible to a secondary infection with the virus (Zhang et al., 2010), we reasoned that other genes are stably altered following infection of lymphocytic cells.

One B-cell line (BJAB) and one T-cell line (KE37) were evaluated for global changes in gene expression approximately two months post-infection. Greater than 90% of the cells were infected at the start of the experiment and a limiting dilution assay determined that the viral genome was maintained in over two-thirds of the BJAB cells and over one-third of the KE37 cells (data not shown). Although viral mRNA could be detected in some of these cultures, expression of the late *hexon* and *L4 100 K* genes was detected in fewer than 1% of the cells (Furuse et al., 2013) thus confirming the persistence of viral DNA with minimal ongoing viral replication.

Six independently established infected cell lines (three infected BJAB and three infected KE37) and six separately maintained mock-infected cells (three BJAB and three KE37) were used to identify genes expressed differentially between infected and non-infected cells using the Affymetrix U133 Plus2.0 microarray. Because the identification of differentially expressed genes is strongly influenced by the normalization method (see Chou et al., 2007; Freyhult et al., 2010), we selected genes concordantly identified by four normalization methods as described in the Materials and Methods. The relative expression and likelihood of differential expression between mock- and virus-infected cells is shown for each method in Fig. 1. As expected, the estimated change in gene expression varied considerably with each algorithm. For the KE37 cell line, a  $\log_2$  cutoff value of 1.5 identified between 4,874 (Fig. 1C, MAS-5) and 6 candidate probes (Fig. 1D, SVN). The number of gene probes



differing in expression by a factor of two or more with odds ratio for differential expression exceeding one was less variable and ranged from 25 to 62 candidates among the four methods. We selected the top 150 differentially expressed genes from each normalization algorithm on the basis of the Bayes-adjusted p-value for differential expression and identified probes common to each algorithm. These results are summarized in the Venn diagrams (Figs. 1E and J). Probes common to three of four normalization algorithms are reported in supplementary data (Supplemental Tables S1 and S2).

Only four genes were identified as differentially expressed between infected and non-infected T-cells (Fig. 1E). Of these four genes, one was upregulated (*TAF1D*) and three (*BCL2A1*, *CXXC5*, and *HEIH*) were downregulated. Among the four algorithms, the change in expression for *TAF1D* varied between 1.6- and 2.4-fold. Relative expression among the downregulated genes ranged from a factor of 1.7- to 4.1-fold lower than mock-infected cells. By contrast, the decrease in relative gene expression for the six genes identified in B-cells (*CXADR*, *SPARCL1*, *BNIP3*, *BTG3*, *SLFN11*, and *BBS9*) varied among the four algorithms from four to 6,470-fold (Table 2). Importantly, the reduction in *CXADR* (*CAR*) expression previously observed in these cells (Zhang et al., 2010) was confirmed by this method.

We focused on evaluating the changes in expression of the six B-cell associated genes by quantitative PCR after reverse transcription because these changes were uni-directional and of much larger magnitude than the T-cell associated gene changes. Among infected KE37 T-cells, levels of expression for all but *BNIP3* were no different from mock-infected cells (Fig. 2A). The level of *BNIP3* mRNA was slightly higher than in mock-infected T cells ( $p < 0.05$ ). In the infected BJAB B-cells, the level of mRNA for each of the candidate genes was reduced and this change was significant for all but *BBS9*, and *CD44*, which served as a control (Fig. 2B). *SPARCL1* expression decreased the most substantially at over 1000-fold. *BTG3*, *SLFN11* and *CXADR* (*CAR*) were the next most decreased, with 11.6-, 8.7- and 6.4-fold reductions, respectively. *BBS9* and *BNIP3* were reduced by approximately 2-fold. Although the fold-change in gene expression determined by microarray typically overestimated that determined by qPCR, the SVN, RMA and MAS-5 methods reported a similar pattern among the candidate genes with a comparable overall deviation compared to qPCR. By this comparison, these methods were better than the GC-RMA method (Table 2). Overall, these results reveal that infected lymphocytes are characterized by surprisingly limited changes in gene expression as measured by mRNA levels and that a greater number and more substantial changes in gene expression can be detected in the infected B-cell line than the infected T-cell line used in this study.

### 3.2. Patterns of coordinately regulated genes in chronically infected B- and T-cell lines

Strikingly few genes exhibited large differences in expression in the chronically infected lymphocytic cells. Less dramatic changes in individual gene expression, however, can occur in a coordinated manner reflecting a molecular signature (see Wang et al., 2008). Six patterns of genes whose expression differed from non-infected cells in a coordinated manner were identified (Fig. 3). The largest group of coordinately expressed genes (Pattern 1) included 372 gene probes whose levels were modestly increased in infected KE37 T-cells

(red closed circles), but were unchanged in BJAB B-cells (blue symbols, Fig. 3A). Conversely, Pattern 2 contained 285 gene probes whose levels were modestly decreased in infected KE37 cells and largely unchanged in BJAB cells. However, it appears that for this group, the corresponding genes were more highly expressed in the infected cells than non-infected cells (Fig. 3B). Patterns 3 and 4 represent genes that were unchanged in KE37 cells, but were either modestly increased (Pattern 3, 194 gene probes) or decreased (Pattern 4, 114 gene probes) in infected BJAB cells (blue closed squares). Only 43 gene probes were found to be coordinately altered across both infected B-cell and T-cells, with 22 probes found to increase modestly in infected lymphocytes (Pattern 5) and 21 probes found to decrease modestly in infected lymphocytes (Pattern 6). Overall, more than twice as many probes were coordinately regulated in the chronically infected T-cell line (657) than in the B-cell line (308).

To determine if these groups of coordinately expressed genes were biologically related, the Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to identify functionally related groups based on gene ontology terms and published gene annotations (Huang da et al., 2009a, 2009b). The Affymetrix probes clustered into the six patterns described above were submitted to the online resource (<http://david.abcc.ncifcrf.gov>) using a background of all genes represented on the U133Plus2 microarray.

Seven clusters were identified among 309 of the 372 probes in pattern 1, which is elevated in the infected T cell line (Supplemental Table S3). None of these genes showed an obvious association with infectious processes. Nine clusters were identified from 221 of the 285 probes in pattern 2, which were decreased in the infected T cell line. Unlike the upregulated genes, two clusters among these genes suggested a relationship to the biology of an infected lymphocyte. One cluster was involved in programmed cell death and negative regulation of apoptosis. The other cluster was associated with the adaptive immune response and lymphocyte activation.

Among the 194 gene probes that increased in the infected B-cell line (pattern 3), 148 were mapped to six clusters that included genes associated with lymphocyte activation, adhesion and differentiation. Only two clusters were identified among the 114 probes in pattern 4 that were downregulated in the B-cell line. The coxsackie and adenovirus receptor (*CXADR*) was among these genes although clustered in a group of three genes associated with muscle development: *CXADR*, *AFG3L2*, and *DNER*. The stringency was relaxed in the DAVID algorithm to search for potential clusters associated with the remaining patterns of 22 and 21 probes; however, no significant clusters were identified as signified by enrichment scores less than 1. In total, these results suggest that infection with adenovirus results in changes in seemingly unrelated biological clusters.

To see if the coordinately regulated genes share common transcriptional regulators, genes identified in patterns 1 through 4 were analyzed with the Distant Regulatory Elements (Gotea and Ovcharenko, 2008) or DiRE tool (<http://dire.dcode.org/>). The number of genes in patterns 5 and 6 were below the recommended number for analysis. DiRE generates a weighted list of known regulatory elements based on their abundance in the promoter,



introns and untranslated regions of the target genes compared to their abundance in a set of 7500 background genes. Using a relaxed importance cutoff value of 0.275, common regulatory elements were identified only for genes downregulated in KE37 T-cells (pattern 2; *VBP*, *CIZ*, *FREAC7*, and *S8*) and upregulated in BJAB B-cells (pattern 3; *POLY*, *S8*, *HFH3*, *ICSBP* and *IRF*) (data not shown). It may be significant that these were the only two patterns to have clusters of potential biological significance to the infected cell. These findings could suggest that coordination of gene expression in the other patterns of gene expression may occur through less direct means such as epigenetic modification.

### 3.3. Several Ad-modulated genes appear to be repressed by epigenetic mechanisms in infected BJAB cells

Although the changes in gene expression following infection were quite limited, we successfully detected the expected reduction in *CXADR* (CAR) expression in infected B-cells with the Affymetrix microarray. This change, as well as those of five other genes downregulated in infected B-cells, was detected 50–70 days post-infection suggesting that the change persists long after the initial infection. The long-term loss of CAR expression following infection was reported to be maintained by epigenetic mechanisms because treatment with a histone deacetylase inhibitor (HDACi) and a DNA methyltransferase inhibitor (DNMTi) restored expression of the gene (Zhang et al., 2010). To determine if expression of the other genes was down modulated following infection by similar mechanisms, infected BJAB cells were treated with Trichostatin A (TSA), an HDACi, or 5-Aza-2'-deoxycytidine (5-Aza), a DNMTi. Expression of the genes of interest was compared between infected B-cells untreated or treated with TSA alone or 5-Aza alone. As expected, treatment with the inhibitor of DNMT, 5-Aza, was able to significantly increase expression of CAR. Treatment with TSA alone for 48 h induced very little change in the expression of any of the genes (Fig. 4). By contrast, treatment with 5-Aza for 48 h was able to induce expression of *SPARCL1*, *SLFN11* and *BNIP3*, but not *BTG3* or *BBS9*. The expression of *4-IBBL* has been reported to be repressed in human colorectal carcinoma cells by histone deacetylation (Kumari et al., 2013), and *4-IBBL* expression was robustly increased (18-fold) in SW620 cells following treatment with TSA as positive control in our experiments (data not shown). Interestingly, expression of this gene was not influenced greatly by HDACi in our human B-cell line demonstrating that the four genes identified are specifically repressed in our infected cells by these epigenetic mechanisms.

### 3.4. Similar to infected lymphocytes, childhood leukemic B-cell lines are refractory to Ad infection

Adenovirus infection inhibits the cell's normal DNA-damage response (Hart et al., 2005; Karen and Hearing, 2011), potentially leading to alterations of cellular DNA such as translocations. In a prior study, cord blood lymphocytes from one donor contained both high level of adenovirus DNA and a genetic translocation commonly associated with acute lymphoblastic leukemia (ALL) (Ornelles et al., 2015). We, and others, have previously speculated that these viruses are candidates for producing the initiating event leading to the development of childhood acute leukemias (McNally and Eden, 2004; Ornelles et al., 2015). The absence of adenoviral genetic material in leukemic cells would suggest a potential hit-and-run mechanism by which the virus permanently alters some cellular function before

being eliminated from the cell. Though we saw limited changes in gene expression in infected cells we observed a large effect on subsequent virus infection and infected cells are refractory to re-infection (Zhang et al., 2010). To determine if cells derived from ALL shared any characteristics with cells once infected with adenovirus we evaluated the ability of several diverse leukemic B-cell lines to be infected with adenovirus. A total of 14 human leukemia cell lines were infected and monitored as described previously (McNees et al., 2004) for support of the adenovirus replication cycle (Table 3). Many cell lines (8 of 14, 57%) were productively infected with adenovirus as measured by hexon expression (> 10% positive) or viral DNA replication. DNA replication ranged from 43-to 5800-fold (maximum increase above that found at the time of infection). Productively infected cells contained from 1300 to 176,000 viral genome copies per cell at the peak. The cell lines in Table 3 have been organized according to their identified gene rearrangements and translocation-generated fusion proteins. Most cell lines (6 of 8; 75%) containing the common translocation-generated fusion proteins of childhood ALL (TEL-RUNX1, E2A-PBX1, MLL-AF4, and ENL-MLL) failed to replicate adenovirus. Only two of the eight cell lines containing common translocations of childhood acute lymphoblastic leukemia permit adenovirus replication (EU3 and SEM cells). Curiously, almost all of the childhood leukemic cell lines also did not express surface CAR (7 of 8, 87%). Adenovirus, however, can infect CAR-negative cells through other receptors such as the integrins or MHC-I (Cupelli and Stehle, 2011). Accordingly, productive infections were observed in RPMI-1788 and SB cells, which are both CAR-negative. Thus the lack of CAR is not the only barrier to infection (McNees et al., 2004; Zhang et al., 2010). Collectively, these observations suggesting that CAR expression is not required, nor is it sufficient, to support virus replication and gene expression in human leukemia cell lines. Interestingly, all of the adult derived leukemic cell lines were able to support DNA replication and late gene expression, regardless of surface CAR expression. Overall, we found that while adult derived cell lines could support viral infection, very few of the childhood derived cell lines could support infection. These findings lead us to hypothesize that certain proteins associated with childhood ALL, such as ETV6-RUNX1, limit the outcome of an adenovirus infection in lymphocytic cells.

### 3.5. CAR expression is repressed in childhood leukemic cell lines as compared to adult leukemic cell lines

To determine if other changes associated with adenovirus-infected B-lymphocytes were present in childhood leukemic cells we evaluated expression of the six identified “fingerprint” genes between childhood and adult derived leukemic cells. Two of the six genes repressed in Ad-infected B cells, *CXADR* (CAR) and *SPARCL1*, also were significantly repressed in childhood leukemic cell lines as compared to adult leukemic cell lines (Fig. 5). No discernable pattern of expression that could be segregated by childhood versus adult origin was observed for the other four genes (*SLFN11*, *BBS9*, *BNIP3* or *BTG3*).

Because the expression of several of these genes appears to be reduced by epigenetic mechanisms following infection of B-cells with adenovirus we also sought to determine if these genes were epigenetically repressed in leukemic cell lines. Both adult (ARH77 and

BJAB) and child leukemic cell lines (UOC-B4, RS4;11, and KOPN-8) were treated with TSA or 5-Aza for 48 h. For all cell lines except UOC-B4, viability was above 90% during 48-h treatment with 300 nM TSA. UOC-B4 cells were more sensitive to the drug and were instead treated with 50 nM TSA (Busbee et al., 2014) to maintain viability above 90% during treatment (data not shown). Treatment of childhood cell lines with DNMTi caused a robust increase in *CXADR* expression (200-fold; RS4;11 and 137-fold; UoC-B4 and 183-fold; KOPN-8) in the childhood cell lines (Table 4) as compared to changes occurring in both adult cell lines (1.5-fold). In fact, *CXADR* expression, which was undetectable in untreated RS4;11 cells, was only detectable after 5-Aza treatment. *SPARCL1* shows similar upregulation after DNMTi treatment of the childhood derived cell line RS4;11 only (36-fold) but not as robustly in the other childhood lines (UoCB4; 2.6-fold change and KOPN; 2.8-fold) (Table 4). *SPARCL1* was, however, moderately upregulated following DNMTi in the adult cell line BJAB (7-fold). There was some response to HDACi among the childhood cell lines. UoC-B4 and KOPN-8 cells responded to TSA-treatment by increased expression of *CXADR*, and RS4;11 cells upregulated *SPARCL1* expression. However, expression of these genes appeared to be much more sensitive to treatment with the DNMTi 5-Aza than to treatment with the HDACi TSA. By contrast, *BTG3* showed no evidence of epigenetic repression similar to infected B cells (Fig. 4). The other three target genes (*SLFN11*, *BNIP3*, and *BBS9*) showed evidence of epigenetic regulation, but no divisible pattern of regulation between adult versus childhood cell lines was observed (data not shown). These data suggest that CAR expression is robustly repressed by epigenetic means in childhood leukemic cell lines as compared to adult leukemic cell lines.

#### 4. Conclusions

Adenovirus is a DNA tumor virus with the ability to transform and immortalize rodent cells (Nevels et al., 2001) and immortalize primary human cells of neural origin (Arnhold et al., 2008; Fallaux et al., 1996; Shaw et al., 2002) in culture. However, unlike other human DNA tumor viruses, adenovirus has not been shown to be the cause of any human cancer. However, adenovirus is similar to another DNA tumor virus EBV by its ability to persist in lymphocytic cells. EBV establishes a latent infection in memory B cells and can transform peripheral blood B cells into immortalized lymphoblastoid cells (Speck and Ganem, 2010). Changes in cellular gene expression provoked by EBV have been well-characterized during type I and III latent infections, which precedes transformation into lymphoblastic cells (Speck and Ganem, 2010). These changes include decreased expression of tumor suppressor genes and increased expression of growth promoting genes (Fukayama et al., 2008; Klein et al., 2010; Niller et al., 2009). Several adenovirus genes are well known for their ability to alter the expression or activity of these classes of cellular genes (Vink et al., 2015; Zhao et al., 2007). Consequently, one focus of this study was to evaluate gene expression in lymphocytic cells infected persistently with species C adenovirus and in order to identify changes that could contribute to oncogenesis.

Lymphocytes infected with species C adenoviruses survive and continue to proliferate at rates similar to uninfected cells (Zhang et al., 2010). Lymphocytic cells display little of the cytopathic effect that characterizes infected epithelial cells despite carrying as many as 100,000 copies of the viral genome while growing in culture. Despite such a heavy viral

burden, the breadth of change in cellular gene expression in these cells is surprisingly limited. By microarray, we found six genes in the B-cell line (BJAB) and four genes in the T-cell line (KE37) that were differentially expressed in the persistently infected cells (Fig. 1). We consider these to be durable changes because they were detected in cells 50–80 days after initial infection and at a time when viral gene expression was virtually absent in most cells. Curiously, the genes modulated in the B cell line all exhibited decreased expression. Moreover, downregulation of CAR was confirmed as has been previously reported (Zhang et al., 2010).

The four different normalization methods used here identified a range of 33 and 35 candidate genes differing in expression by a factor of two for BJAB and KE37 cells, respectively (Supplemental Table S1 and S2). The range of the changes observed in lymphocytes harboring an apparently latent infection with adenovirus is comparable to that reported for other DNA viruses (Homa et al., 2013; Slobedman et al., 2004). For example, Slobedman et al., reported that 35 host genes changed expression in cells latently infected with CMV (Slobedman et al., 2004) and a study by Hoever et al., found 11 host genes changed by latent CMV infection (Hoever et al., 2005). Carter et al., (2002) reported 38 host RNAs were consistently twofold more abundant in the EBV-positive lymphoblastic cell lines when compared to EBV-negative Burkitt's lymphoma by microarray analysis. In contrast to other studies that typically use a single algorithm to identify differentially expressed genes, we chose to increase the rigor of our analysis by applying four different algorithms. By this approach, we identified only six differentially expressed genes (Table 2). Nonetheless, each algorithm identified additional and different genes (Supplemental Table S1 and S2). The RMA algorithm identified 20 and 16 differentially expressed genes in infected BJAB cells and KE37 cells, respectively. The MAS approach identified 19 genes in infected BJAB cells and 12 in infected KE37 cells. In addition to determining the changes in gene expression defined by the magnitude of the gene, we also searched for patterns of coordinately regulated genes in chronically infected B- and T-cell lines (Fig. 3). The more biologically suggestive clusters in the KE37 T-cell line were identified among the downregulated genes. These genes seem to be related to the infectious process in lymphocytes and were related to programmed cell death, the adaptive immune response and lymphocyte activation. By contrast, the more biologically suggestive clusters in the infected BJAB B-cell line were found among the upregulated genes, which were loosely associated with lymphocyte activation. Very few coordinately regulated genes were identified for both types of lymphocytes (Fig. 3, pattern 5 and 6) and no significant functional clusters were found among these few genes (data not shown). The apparently unrelated nature of coordinately regulated genes also has been observed for many of the differentially expressed genes identified in herpesvirus-infected cells (Carter et al., 2002; Price et al., 2012).

The changes in cellular gene expression reported here persist for many cell divisions. EBV also elicits epigenetic changes in the host cell genome (Leonard et al., 2011; Niller et al., 2014) that are retained for several cell generations. We found that of the six genes down regulated in infected B cells, four were under epigenetic control by methylation because treatment with an inhibitor of DNA methyltransferase restored expression (Fig. 4). Further methylation appeared to be the dominant mechanism of gene repression when compared to acetylation mechanisms. Adenoviral proteins such as E1A (Ferrari et al., 2012; Horwitz et

al., 2008) and E4-ORF3 (Soria et al., 2010) have both been reported to induce epigenetic changes in infected cells. Further, the E1A promoter itself is subject to methylation (Hsu et al., 2010). *CXADR* has been reported to be epigenetically repressed in virally infected lymphocytes (Zhang et al., 2010) and epigenetically regulated in other cells (Pong et al., 2003). *BNIP3*, which is silenced by epigenetic mechanisms in several malignant epithelial cell lines (Bacon et al., 2007), was the most robustly de-repressed gene following treatment with DNMTi. Interestingly, although not much is known about the function of the other two epigenetically regulated genes they both are associated with cancer. Secreted protein acidic and rich in cysteines-like protein 1 (*SPARCL1*) is an extracellular matrix glycoprotein that has been implicated in the pathogenesis of several disorders including cancer. The downregulation of *SPARCL1* was correlated with lymphatic metastasis and poor tumor grade in breast cancer (Cao et al., 2013) as well as increased metastatic potential in prostate cancer (Xiang et al., 2013). Schlafen-11 (*SLFN11*) expression in cancer specimens from ovarian cystoadenocarcinoma and colon adenocarcinoma was reduced compared to that in normal tissues (Zoppoli et al., 2012).

It is estimated that viruses contribute to approximately 15% of human cancers (Butel, 2000) and an infectious etiology for ALL has been widely supported (Einav et al., 2005; Heath, 2005; McNally and Eden, 2004). Because several of the genes downregulated in infected B cells have been associated with other cancers, we sought to determine how similar adenovirus-infected B cells were to leukemic B cells from ALL. We found that leukemic cells derived from childhood leukemia were refractory to adenovirus infection in a manner similar to once-infected BJAB cells (Table 3). B cells derived from adult leukemias were much more susceptible to infection. Because the six genes we identified as downregulated in infected B cells were repressed long after infection with adenovirus, we evaluated the expression of these genes in leukemic B cells. Two of the six genes, *CXADR* and *SPARCL1*, were indeed downregulated in childhood leukemic cells (Fig. 5). In addition, *CXADR* expression was epigenetically repressed across the childhood leukemic cell lines (Table 4). Similar to infected B cells, gene expression was most robustly increased following treatment with an inhibitor of DNMT. CAR is a cellular adhesion protein that is used by adenovirus to gain entrance to the cell (Cupelli and Stehle, 2011). Downregulation and reduced expression of this gene understandably prevents adenovirus from entering cells. However, forced re-expression of this gene does not allow re-infection (Zhang et al., 2010), suggesting that other changes must restrict virus replication. In addition to being a negative regulator of cell growth and proliferation that is downregulated in a number of cancers (Claeskens et al., 2000; Sullivan and Sage, 2004), *SPARCL1* (also known as HEVIN and SC1/ECM1) encodes an extra-cellular matrix glycoprotein that is highly expressed during embryogenesis (Sullivan and Sage, 2004). We have not yet determined if enforced expression of *SPARCL1* is sufficient to restore susceptibility to infection with adenovirus. Because *SPARCL1* has been shown to augment the proliferation of B cells in response to mitogens (Oritani et al., 1997), downregulation of this gene may contribute to the persistence of adenovirus in these B cells, but may also be involved in the malignancy of the childhood leukemic cell lines.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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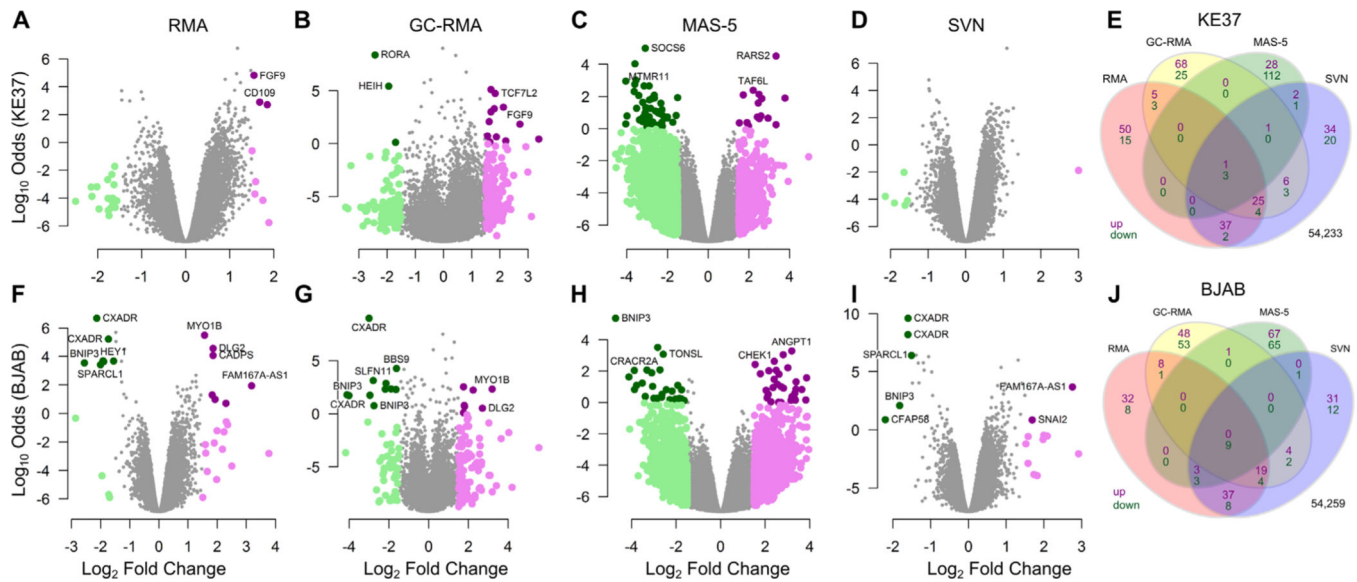
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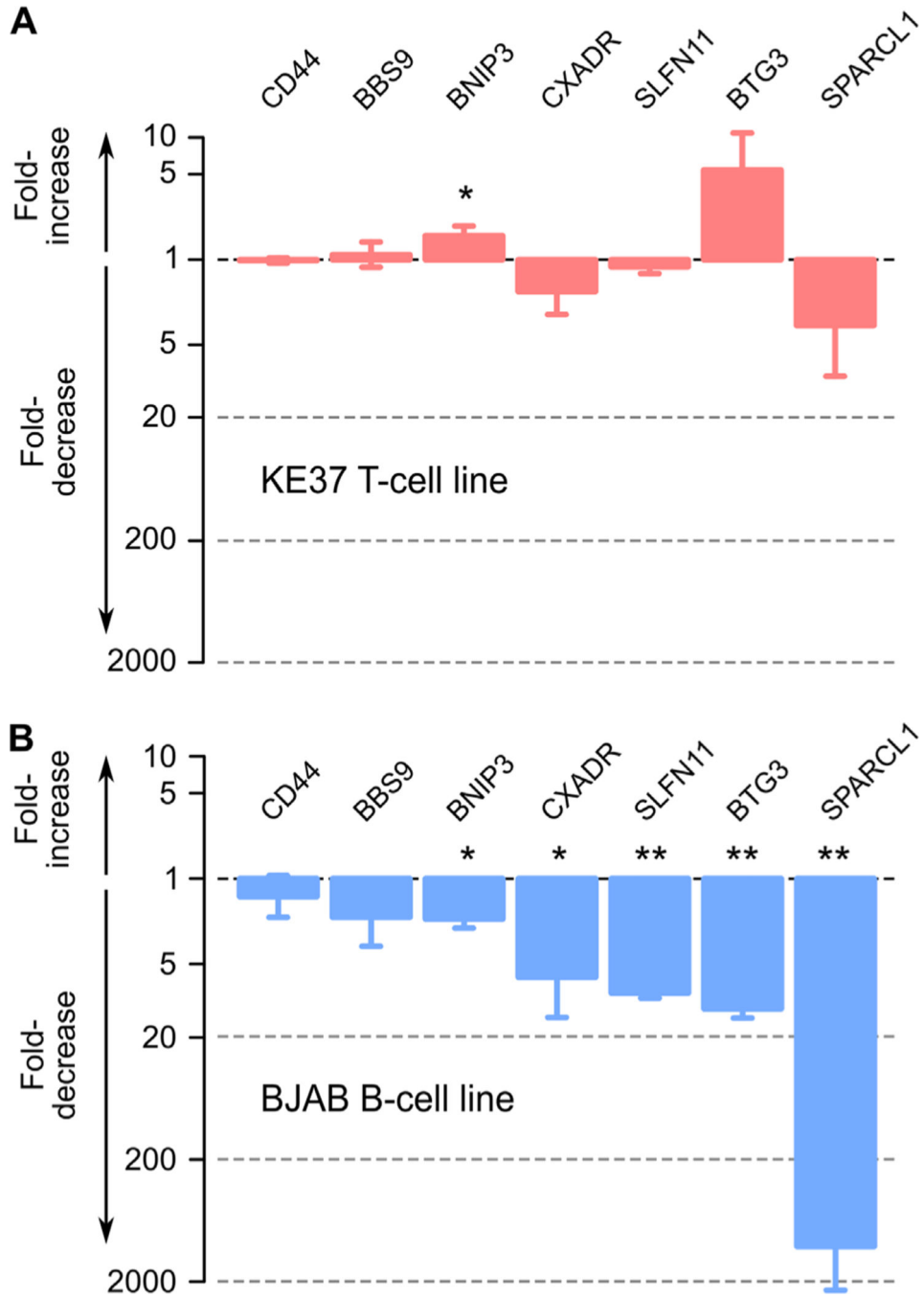
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**Fig. 1.**

Relative levels of gene expression in infected and non-infected KE37 and BJAB cells. Gene expression levels were determined after normalization by the four methods indicated in Fig. 1 and described in the Materials and Methods. An empirical Bayes method was used to calculate a moderated t-statistic for differentially expressed genes where the proportion of differentially expressed genes (a priori probability) was assumed to be 0.4% or about 150 genes. Volcano plots (panels A–D and F–I) display the log of the odds ratio for differentially expressed genes in virus-infected cells versus the  $\text{log}_2$  fold change in gene expression. Green symbols indicate probes with a  $\text{log}_2$  fold-decrease of at least 1.5. Magenta symbols indicate probes associated with a  $\text{log}_2$  fold-increase of at least 1.5. Saturated colors highlight probes with an odds ratio greater than one. The top 150 differentially expressed Affymetrix probes from each normalization algorithm are compared in the Venn diagrams in panels E and J. The number of probes found to increase in the virus-infected cells are shown in magenta. The number of probes found to decrease are shown in green. The four probes common for the KE37 cell line represent four genes (*BCL2A1*, *CXXC5*, *HEIH*, *TAF1D*). The nine probes common for the BJAB cell line represents six genes (*BBS9*, *BNIP3*, *BTG3*, *CXADR*, *SLFN11*, and *SPARCL1*).



**Fig. 2.** Confirmation of differentially expressed candidate genes from chronically infected BJAB cells. RNA was isolated from mock-infected and chronically infected KE37 or BJAB cells and analyzed by qPCR after reverse transcription. The relative level of expression for the indicated genes compared to mock-infected cells was determined by the  $-Ct$  method using EIF1 as an endogenous control. The average change in expression determined in three independent infections is plotted on a log scale with error bars representing the SD. Levels

in virus-infected cells that were significantly different compared to mock-infected cells were identified with the t-test applied to the Ct. (\* $p$ -value  $\leq 0.05$ ; \*\* $p$ -value  $\leq 0.005$ ).

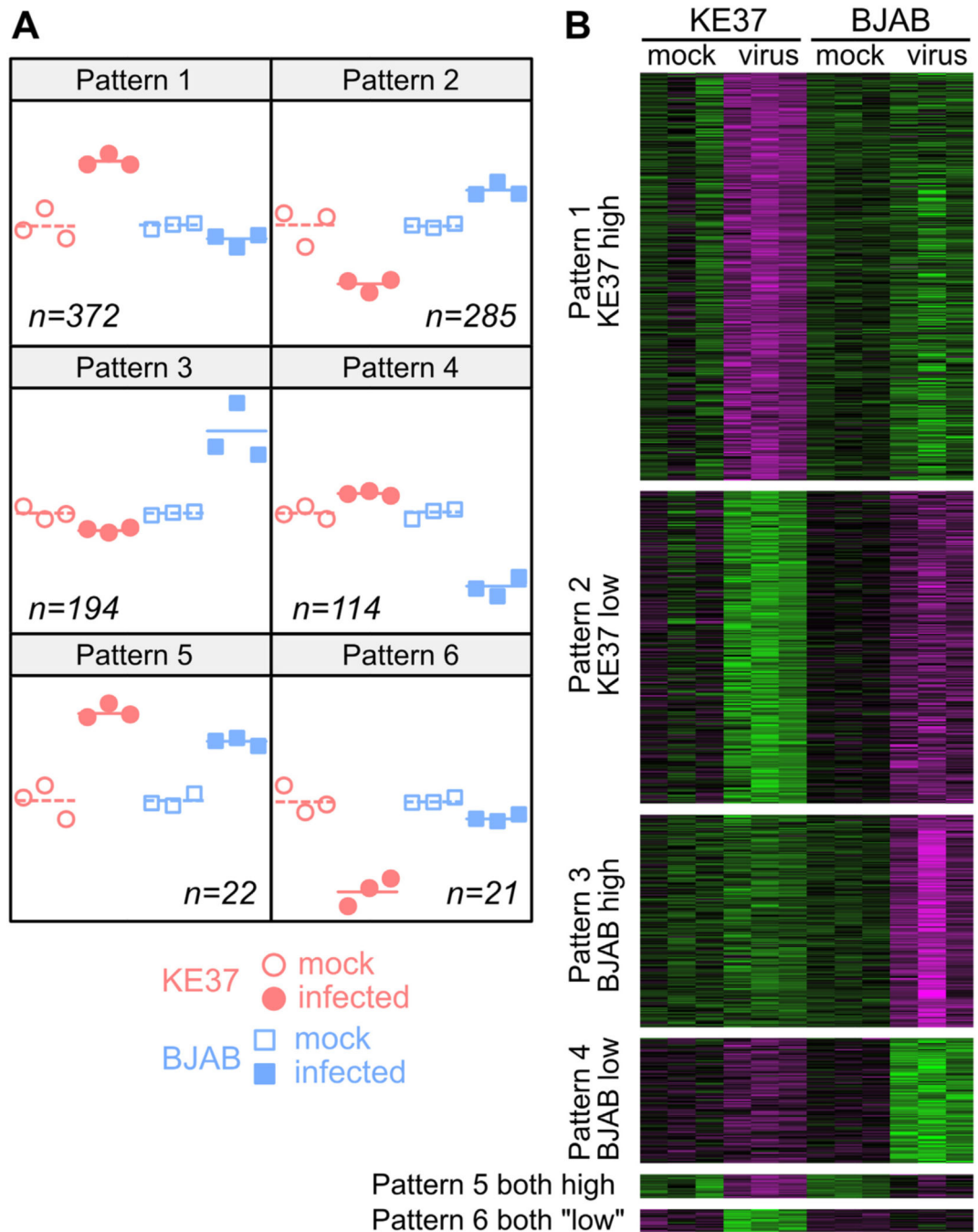
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**Fig. 3.**

Six patterns of coordinately expressed genes are identified by the EPIG algorithm. (A) Relative mRNA levels determined by the SVN algorithm were used to identify coordinately expressed genes. Genes from infected cells (closed symbols) are compared with those of mock-infected cells (open symbols) on a log scale. Horizontal lines indicate the geometric mean for each group of three independent cultures. KE37 T-cells are shown in red, BJAB B-cells in blue. Mean values range from a 1.6-fold increase to a 1.6-fold decrease. (B) The relative changes in all 1008 coordinately expressed genes from each sample are represented

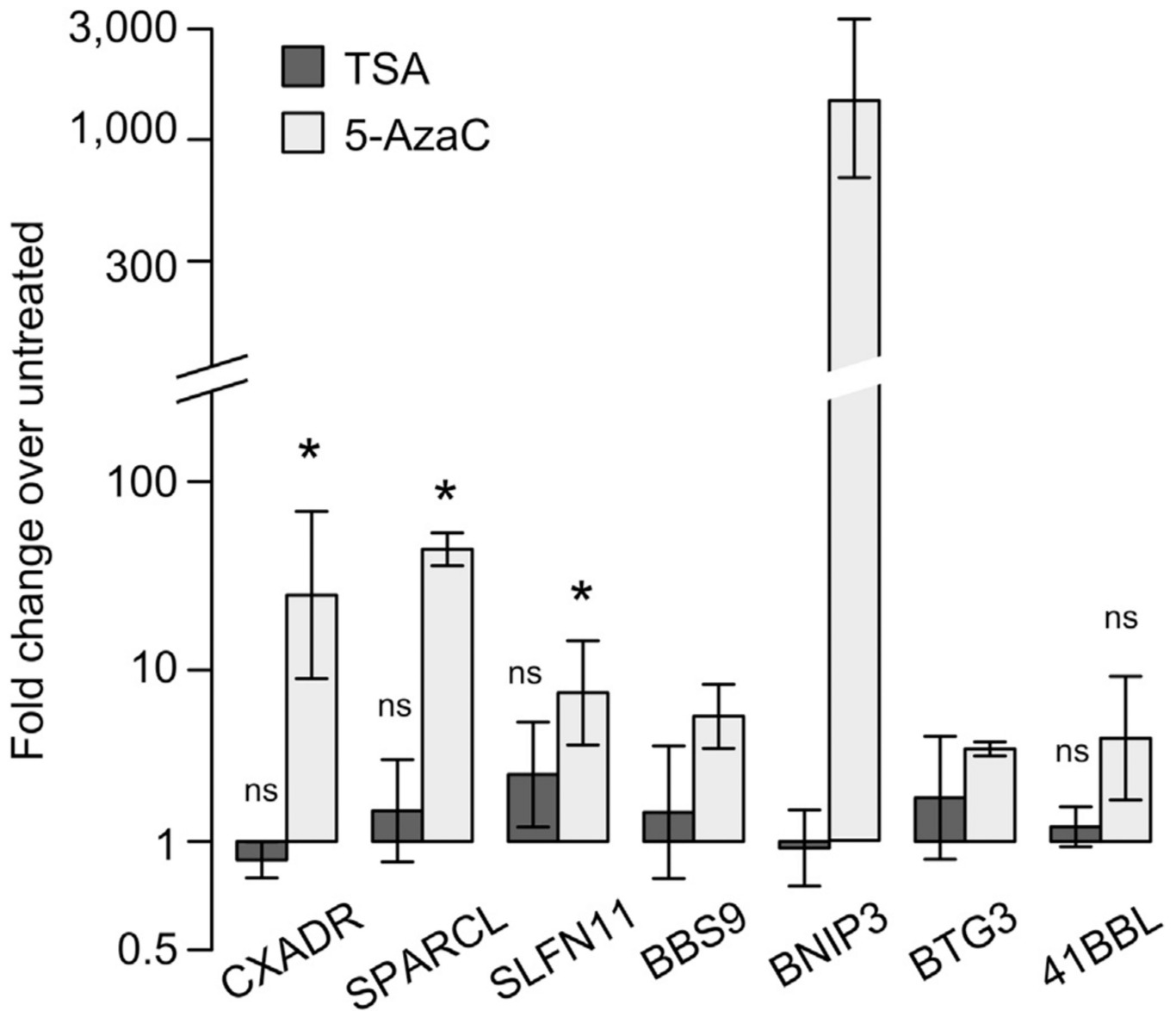
by green and magenta for a relative decrease or increase in virus-infected cells, respectively. Darker colors indicate the lack of differential expression.

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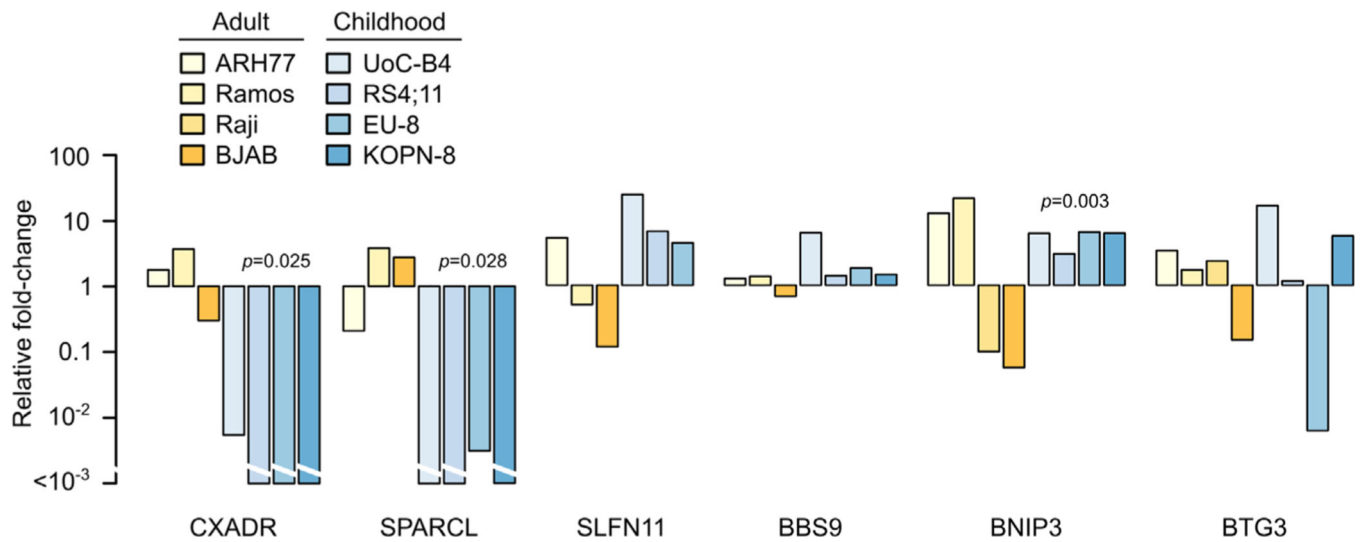
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**Fig. 4.** Epigenetic repression of *CXADR*, *SPARCL1*, *SLFN11*, and *BNIP3* in AdV-infected BJAB cells is released through inhibition of DNA methyltransferase. Persistently infected BJAB cells were treated with 300 nM TSA (HDAC Class I & Class II inhibitor), 10  $\mu$ M 5-AzaC (DNA methyltransferase inhibitor) for 48 h. Relative quantity of gene message was analyzed by RT-qPCR. *4-1BBL* is shown as a control gene whose expression exhibits little change in these cell lines and was not significantly modulated following infection of lymphocytic cell lines. Fold-change in gene expression is normalized to a housekeeping gene and compared to untreated cells (expression set to 1). Error bars represent SD. Results show the average of two or three independent experiments. Statistical significance is indicated for values with three independent measurements (\* $p < 0.05$ ; ns,  $p > 0.05$ ).



**Fig. 5.**

Two of the six genes repressed in AdV-infected cells show significantly reduced expression in childhood leukemic cell lines compared to adult leukemic cell lines. Relative mRNA levels comparing adult and childhood leukemic lines were determined and are plotted as a fold-change ( $2^{-Ct}$ ). In some experiments, mRNA for *CXADR* and *SPARCL1* could not be detected in the childhood leukemic cell lines. For these cases, the mRNA level was set to a limiting value determined by a Ct of 40. The *t*-test was used to determine if the mean level for each gene in the childhood leukemic cell lines (blue) was significantly different the mean level for the adult cell lines (yellow/orange). A *p*-value of less than 0.05 was considered significant. Because the Ct was assigned a limiting value in certain cases, *p*-values for *CXADR* and *SPARCL1* must be considered an estimate. The fold-change shown for these values (indicated with a break in the bar) represents an upper limit.

**Table 1**

Primer-probe sequences.

Gene symbol	Gene name	Oligo	Sequence
BTG3	BTG family, member 3	Primer 1	GCTTTCAGGACATCAGGATCA
BTG3	BTG family, member 3	Primer 2	CAGTTGAGAGGTTTGTCTGAGA
BTG3	BTG family, member 3	Probe	TCTGAAATTTATTGACACGAATACATCTGTAGGCC
CXADR	coxsackie virus and adenovirus receptor	Primer 1	GGCTTTTTCAATCATCTCTTCAGG
CXADR	coxsackie virus and adenovirus receptor	Primer 2	TGCTGTGCTTCGTGCTC
CXADR	coxsackie virus and adenovirus receptor	Probe	AACTTCTGGCGAAATCCACTACTCCG
SPARCL1	SPARC-like 1 (hevin)	primer1	GATCCTTCAATCCCAGCTCTT
SPARCL1	SPARC-like 1 (hevin)	primer2	CAGCAGTATCCACAGAAGACG
SPARCL1	SPARC-like 1 (hevin)	probe	AGCCATGAACAGTCAGCAGAACAGG
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	primer1	TGTTGGTATCTTGTGGTGTCTG
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	primer2	GCATGAGTCTGGACGGAGTA
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	probe	CTCACTGTGACAGCCCACTCG
SLFN11	schlafen family member 11	primer1	TGTTCCAGGCCTTTCTTGG
SLFN11	schlafen family member 11	primer2	GATGACAGACACAGATCCAGAT
SLFN11	schlafen family member 11	probe	CCTCCCCTTAGCAGACCAGTGT
BBS9	Bardet-Biedl syndrome 9	primer1	GCACGTAGACTGATAATTTGGC
BBS9	Bardet-Biedl syndrome 9	primer2	ACTGACCTTGTCCCTTCTGT
BBS9	Bardet-Biedl syndrome 9	probe	ACTCTGTTCTGCAGTGTGACCTTCAC
CD44	CD44 molecule (Indian blood group)	primer1	TCCAAATCTTCCACCAAACCT
CD44	CD44 molecule (Indian blood group)	primer2	TCTTCAACCCAATCTCACACC
CD44	CD44 molecule (Indian blood group)	probe	ACGCTTCAGCCTACTGCAAATCCA
EIF1	eukaryotic translation initiation factor 1	primer1	GATATAATCCTCAGTGCCAGCA
EIF1	eukaryotic translation initiation factor 1	primer2	GTATCGTATGTCCGCTATCCAG
EIF1	eukaryotic translation initiation factor 1	probe	CTCCACTCTTTCGACCCCTTGGCT
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	primer1	TGTAGTTGAGGTCAATGAAGGG
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	primer2	ACATCGCTCAGACACCATG
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	probe	AAGGTCGGAGTCAACGGATTTGGTC

**Table 2**

Fold-decrease in infected BJAB cells relative to mock-infected cells.

Gene	Method <sup>a</sup>					
	qPCR	SVN	RMA	MAS-5	GC-RMA	
<i>BBS9</i>	2.1	3.9	4.9	6.8	8.4	
<i>BNIP3</i>	2.1	9.0	28	488	2810	
<i>BTG3</i>	11.7	5.9	6.9	5.6	17	
<i>CXADR</i>	6.4	8.2	14.5	119	6470	
<i>SLENI1</i>	8.7	5.6	13.7	10.4	120	
<i>SPARCL1</i>	1030	7.2	16.2	320	34	
deviation <sup>b</sup>	0	2.0	2.3	2.8	5.3	

<sup>a</sup>The method used to determine the relative change in gene expression is indicated.

<sup>b</sup>The relative deviation from values determined by qPCR is the average magnitude of the difference of the log<sub>2</sub>-transformed values for all six genes.



Table 3

Leukemic B cell lines from children are refractory to adenovirus infection compared to adult B cell lines.

Cell Line	Translocation <sup>a</sup>	Fusion Gene <sup>b</sup>	CAR <sup>c</sup>	Viral Products	
				Early <sup>d</sup>	Late <sup>e</sup> DNA <sup>f</sup>
B cell (childhood)					
REH	t(12;21)(q13;q22)	<i>ETV6-RUYX1</i>	-	-	-
UoC-B4	t(12;21)(q13;q22)	<i>ETV6-RUNX1</i>	-	-	-
RS4;11	t(4;11)(q21;q23)	<i>MLL-AF4</i>	-	+	-
SEM	t(4;11)(q21;q23)	<i>MLL-AF4</i>	-	+	+
EU-8	t(4;11)(q21;q23)	<i>MLL-AF4</i>	-	-	-
KOPN-8	t(11;19)(q23;p13)	<i>ENL-MLL</i>	-	+	-
RCH-ACV	t(1;19)(q23;p13)	<i>E2A-PBX1</i>	-	+	-
697 (EU-3)	t(1;19)(q23;p13)	<i>E2A-PBX1</i>	Yes	+	+
B cell (adult)					
ARH77	Unknown	None	Yes	+	+
Ramos	Unknown	None	Yes	+	+
RPMI-1788	Unknown	None	-	-	+
SB	Unknown	None	-	-	+
Raji	t(8;14)(q24;q32)	<i>MYC-IGH</i>	Yes	+	+
BJAB	t(2;8)(p12;q24)	<i>IGK-MYC</i>	Yes	+	+

<sup>a</sup>Known chromosomal translocation associated with leukemia.

<sup>b</sup>Leukemic fusion product arising from the chromosomal translocation.

<sup>c</sup>Cell lines with at least 5% of cells displaying levels of CAR above background as measured by flow cytometry were considered positive for CAR (yes).

<sup>d</sup>The ability to detect early gene expression was assessed with an EGFP-expressing adenovirus vector. Cell lines that showed at least 25% GFP-positive after infection with the viral vector within 3 dpi were considered permissive for early gene expression (+).

<sup>e</sup>The ability to detect late gene expression was assessed by infection with a wild-type virus. Cell lines with at least 30% hexon-positive cells measured within 3 dpi by flow cytometry were considered permissive for late gene expression (+).

<sup>f</sup>Cell lines that amplified viral DNA by at least 4-fold over input levels following infection with the wild-type virus were considered to support viral DNA replication (+).

*CXADR* and *SPARCL* expression is epigenetically regulated in childhood leukemic cell lines<sup>a</sup>.

**Table 4**

Gene	Fold-change relative to untreated cells <sup>b</sup>					
	Treatment <sup>c</sup>	Cell line				
<i>CXADR</i>	TSA	1.2				
	AzaC	1.5				
<i>SPARCL</i>	TSA	1.4				
	AzaC	2.3				
<i>BTG3</i>	TSA	0.9				
	AzaC	1.8				
	ARH77	UoC-B4	24.1	1.2	3.2	
		RS4;11	137.3	199	183	
	KOPN-8	TSA	1.1	1.7	6.8	1.4
		AzaC	6.9	2.6	36.3	2.8
	BJAB	TSA	1.1	1	1.2	0.97
		AzaC	1.5	1.1	6.1	0.94

<sup>a</sup>Total cellular RNA was isolated from cells that were untreated, treated with TSA or 5-AzaC for 48 h.

<sup>b</sup>Relative levels of mRNA for the indicated gene were measured by RT-qPCR and are expressed relative to the level measured in untreated cells for each cell line.

<sup>c</sup>TSA was used at 50 nM for UoC-B4 cells and 300 nM for all other cell lines. 5-AzaC was used at a 10 uM.