EPIGENETIC MODIFICATIONS AND VIRAL INFECTIONS

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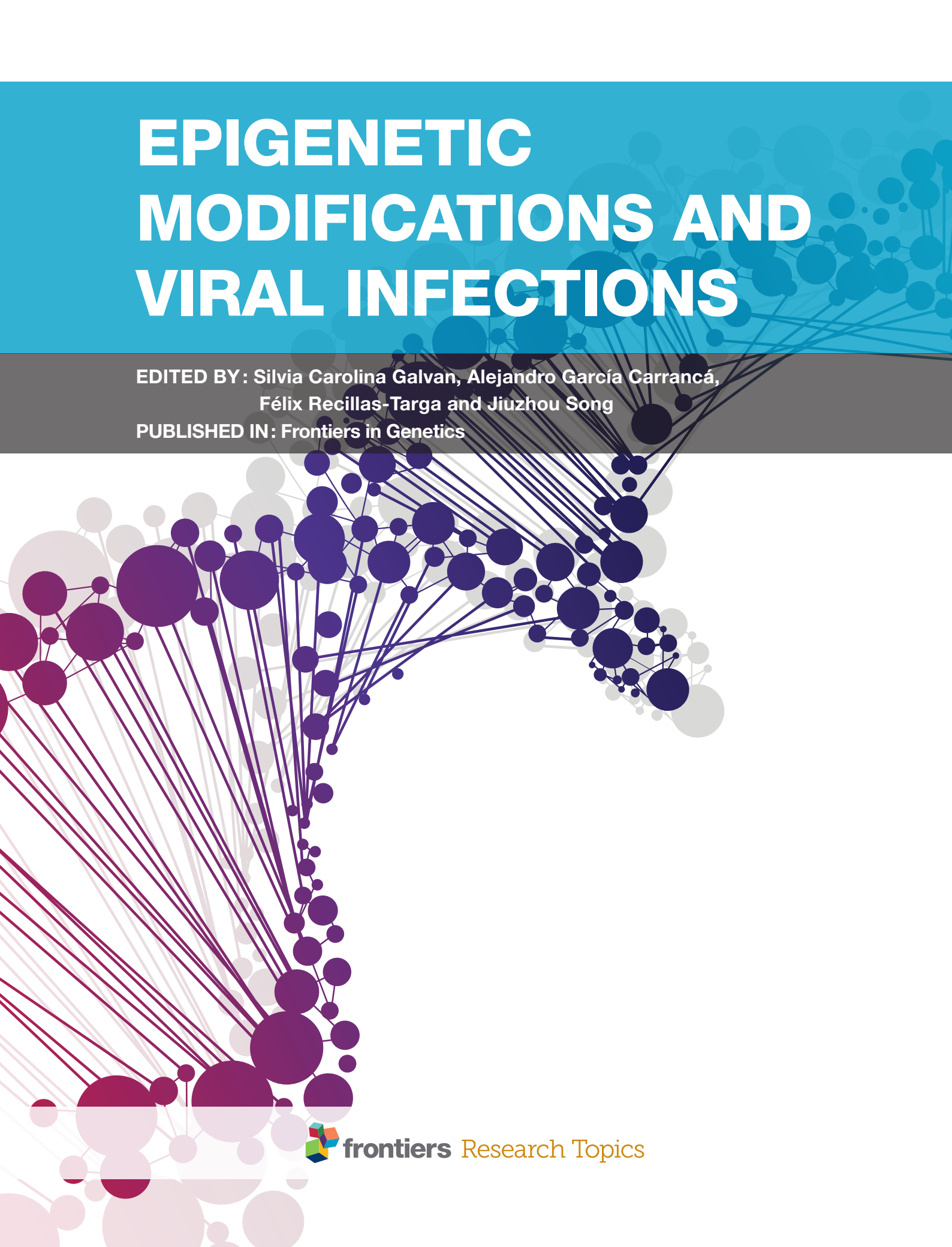

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EPIGENETIC MODIFICATIONS AND VIRAL INFECTIONS

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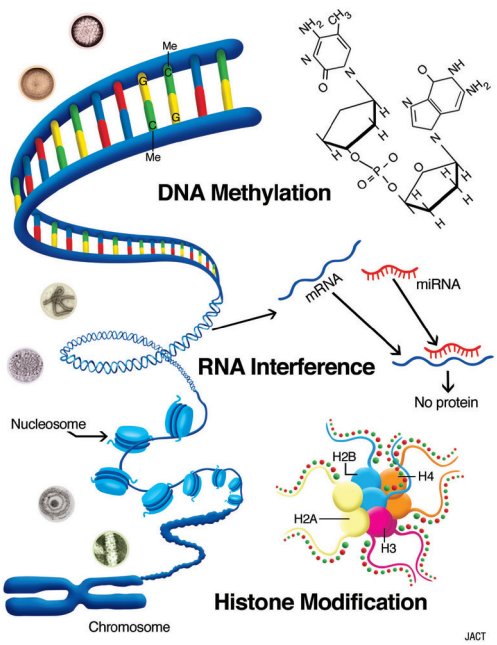

Three epigenetic mechanisms control gene expression: DNA methylation, interference RNA and histone modification. Those are inheritable and may change as a result of environmental stimuli, such as virus infections. The image was designed by Jorge Castro.

Epigenetics is defined as the study of modifications of the genome, heritable during cell division that does not involve changes in DNA sequences. Up to date, epigenetic modifications involve at least three general mechanisms regulating gene expression: histone modifications, DNA methylation, and non-coding RNAs (ncRNAs).

For the past two decades, an explosion in our interest and understanding of epigenetic mechanisms has been seen. This mainly based on the influence that epigenetic alterations have on an amazing number of biological processes, such as gene expression, imprinting, programmed DNA rearrangements, germ line silencing, developmentally cued stem cell division, and overall chromosomal stability and identity.

It has become also evident that the constant exposure of living organisms to environment factors affects their genomes through epigenetic mechanisms. Viruses infecting animal cells are thought to play central roles in shaping the epigenetic scenario of infected cells. In this context it has become obvious that knowing the impact that viral infections have on the epigenetic control of their host cells will certainly lead to a better understanding of the interplay viruses have with animal cells.

In fact, DNA viruses use host transcription factors as well as epigenetic regulators in such a way that they affect epigenetic control of gene expression that extends to host gene expression. At the same time, animal cells employ mechanisms controlling transcription factors and epigenetic processes, in order to eliminate viral infections. In summary, epigenetic mechanisms are involved in most virus-cell interactions.

We now know that some viruses exhibit epigenetic immune evasion mechanisms to survive and propagate in their host; however, there is still much ambiguity over these epigenetic mechanisms of viral immune evasion, and most of the discovered mechanisms are still incomplete. Other animal viruses associated to cancer often deregulate cellular epigenetic mechanisms, silencing cellular tumor-suppressor genes and/or activating either viral or host cell oncogenes. In addition, in several cancers the down-regulation of tumor suppressor protein-coding genes and ncRNAs with growth inhibitory functions, such as miRNAs, have been closely linked to the presence of cell CpG island promoter hypermethylation.

The goal of the aforementioned Research Topic is to bring together the key experimental and theoretical research, linking state-of-the-art knowledge about the epigenetic mechanisms involved in animal virus-cell interactions.

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EDITORIAL
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Epigenetics and animal virus infections

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Epigenetics, modifications of the genome, heritable during cell division, that do not involve changes in DNA sequences include several mechanisms mainly: histone modifications, DNA methylation and related modifications, non-coding RNAs (ncRNAs) and others that regulate gene expression.

The past two decades has seen an explosion of interest for revealing mechanisms that control epigenetic modifications, mainly based on the influence they have on chromatin structure and their impact in biological processes such as programmed DNA rearrangements, imprinting, germ line silencing, developmentally cued stem cell division, and overall chromosomal stability and identity. It has also become obvious that epigenetics changes are fundamental in the interplay between viruses and their host cells. Generally speaking, when retroviruses and DNA viruses integrate their genomes into the host genome, they can stay latent by silencing their genes or can be productive by activating them, and viral gene expression can be regulated just like as the host. In fact, viral DNA uses host transcription factors as well as epigenetic regulators, in such a way that the effect of viral epigenetic control of its own gene expression also extends to regulate host gene expression. At the same time cells use similar mechanisms, transcription factors and epigenetic modifications, in order to try to eliminate viral infections. In summary, epigenetic mechanisms are involved in most of the virus-cell interactions.

The goal of this special issue is to bring together key experimental and theoretical research linking state-of-the-art knowledge of epigenetic mechanisms involved in regulating virus-cell interactions.

This e-book is a compilation of 12 articles. Two of them are methodological, one on the use of new technologies devoted to identify methylated CpG sites on virus genomes and the other on genome-wide mapping of DNase I hypersenitive sites associated with gene expression. Three articles describe original research involving SV40 minichromosomes, DNA methylation fluctuation and the Toll-like receptor pathways. Seven are review articles, including two mini-reviews on Epigenetic Mechanisms associated to Hepatitis B Virus (HBV) and fundamental topics as DNA methylation, histone modifications and viral strategies against the host immune system in Epstein B Virus; cell differentiation of the immune system as a tool for epigenetic studies; epigenetic mechanisms associated to virotherapy, and finally on the recognition of DNA viruses and cell damage by histones.

The article from Sun et al. (2014), is a methodology article designating the relative advantages of the NGS technology compared to pyrosequencing for studying viral DNA methylation. The analytical procedure they used provided further information related to HPV methylation on a single cell basis, showing that there are HPV 16 genomic sequences in cells which are mostly methylated while in others they are unmethylated (methylation mosaic).

Replication of SV40 minichromosomes can serve as an epigenetic switch in which newly replicated chromatin can be epigenetically modified in response to specific signals such as T-antigen binding to site I. This epigenetic switch seems to ensure that newly replicated minichromosomes do not activate early transcription at late times in infection. In addition, this epigenetic switch may control the relative pool sizes of transcribing, replicating, and encapsidating SV40 minichromosomes. In an original research article, Kallestad et al. (2013), shows that in cells containing SV40 minichromosomes, histone modifications associated with chromatin repression can differ significantly depending upon whether the chromatin is being repressed, undergoes transcription or replication.

The review from Russ et al. (2013), describes advantages of studying the immune system for epigenetic regulation of cell differentiation, in particular how T cell identity or plasticity is controlled. The authors focus some of the key findings and general themes emerging from the studies of T cell differentiation, as well as the utility of the immune system as a tool for studying differentiation and development.

Histones are essential components of chromatin structure, and histone modification plays an important role in various cellular functions including transcription, gene silencing, and immunity. In addition, histones also play distinct roles in extrachromosomal settings. Kobiyama et al. (2013), in their review describe the role of histone H2B as a sensor for dsDNA aberrantly present within the cells. According to the results included, extracellular and extrachromosomal histones alert cells to dangerous situations, such as infection, apoptosis, DNA breaks, and cell injury.

Hepatitis B virus (HBV) infection is a global health problem causing a wide spectrum of liver diseases, including acute and chronic infection. Acute HBV infections either resolve or progress to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Because for most patients, available therapies do not lead to the termination of HBV infection, improving our understanding

of HBV–host interactions is necessary for successful antiviral therapeutic strategies development. The epigenetic mechanisms responsible for viral persistence and clearance during Hepatitis B virus (HBV) replication are addressed in the minireview from Zhang et al. (2013). They outline recent information regarding the epigenetic mechanisms regulating HBV replication and transcription.

The mechanism of latent Epstein Bar Virus (EBV) reactivation *in vivo* is not fully understood, however, it is elicited *in vitro* by treatment of latently infected B cells with chemical and biological reagents. Stimulation of the EBV lytic cascade leads to expression of two viral immediate-early genes, BZLF1 (also known as Zta, EB1, ZEBRA, or Z) and BRLF1 (RtaorR). The BZLF1 protein is a transcriptional activator that resembles the cellular AP1 transcription factors and shares structural similarities to the basic leucine zipper (b-Zip) family of transcription factors. This e-book includes two interesting review articles on BZLF1. The minireview from Sinclair (2013) emphasizes that the epigenetic regulation of EBV differs from the current paradigm, indicating that the presence of CpG methylation in a promoter leads to an absence of expression. The review from Murata and Tsurumi (2013), includes studies on the role of BZLF1 in the switch from EBV latency to the lytic cycle, especially the epigenetic mechanisms involved in BZLF1 gene re-programming during that switch.

A third article related to EBV is the review from Allday (2013) on the control that the human tumor-associated Epstein-Barr virus has on the cell intrinsic defense mechanisms that reduce the risk of neoplasia and cancer (named oncogenic stress responses or OSRs). It describes particularly, how the EBV manipulates the host polycomb group of proteins to control key components of the OSR during normal human B cells transformation into permanent cell lines, by mean of epigenetic repression of transcription.

The article from Forbes et al. (2013) is a review on the interplay between epigenetic modifications that regulate anti-viral responses and how they can be manipulated to improve the therapeutic efficacy of Oncolytic Viruses (OV). The authors add a summary of reports on epigenetic modulation affecting permissibility to virus infections, listing genetic target and their cellular functions, the epigenetic modification, and the cell type involved.

Toll-like receptors (TLRs) constitute an evolutionarily conserved signaling system of the innate immune and inflammatory responses against evolutionarily conserved microbial proteins, lipids, and nucleic acids. The avian genome encodes 10 functional TLRs, located either on the cell surface or within endosomes. The original research article from Kogut et al. (2012), evaluate TLR pathway gene expression differences between heterophils from two lines of chickens, one more resistance than the other to infection with *S. enterica*. The authors profiled the expression of all chicken homologous genes identified in a reference TLR pathway, finding differences mainly between heterophils upon infection with SE.

The original research article from Luo et al. (2012), compare epigenetic features between Marek's disease resistant and susceptible chickens. Main findings indicated some genes have higher promoter methylation in MD-susceptible chickens than resistant ones, and that MDV infection induces expression of DNMT1, DNMT3a, and DNMT3b.

In their original article, He et al. (2014), generated a DNase I hypersensitive sites (DNase I HS) map and gene expression profile for functional analysis, in MDV-transformed CD4+ T-cell line (MSB1). The authors found that DNase I HS sites highly correlate with active genes expression in MSB1 cells.

In summary, in our view main contributions from the articles in this eBook include new findings on already explored topics, as well as new scopes in the field of epigenetic modifications and viral infections.

The new findings include the fact that Next Gen sequencing can be used to analyse HPV 16 DNA methylation mosaic pattern with higher throughput, increased resolution and improved efficiency than pyrosequencing (Sun et al., 2014); an epigenetic switch for histone modifications associated with chromatin repression of SV40 minichromosomes depend upon whether chromatin is undergoing either transcription or replication (Kallestad et al., 2013); Toll-like receptor pathways gene expression differs between resistant and sensitive chicken lines to bacterial infections (Kogut et al., 2012); the fact that MDV infection induces expression of all three methyltransferases genes (DNMT1, DNMT3a, and DNMT3b) in both resistant an sensible chickens, as well as higher methylation of their promoters' in MD-susceptible chickens suggest that epigenetic mechanisms may be involved in modulating resistance to MDV infection in chickens (Luo et al., 2012); DNase I HS sites highly correlate with active genes expression in chicken Marek's disease (He et al., 2014).

New scopes deal with the immune system as model for epigenetic regulation of cell differentiation studies (Russ et al., 2013); the role of histone H2B as a sensor for dsDNA aberrantly present within the cells (Kobiyama et al., 2013); the epigenetic mechanisms involved in HBV persistence and clearance, particularly those regulating viral replication and transcription (Zhang et al., 2013); the epigenetic regulation of EBV, where CpG methylation of viral promoter is required for their expression, which differs from the current paradigm (Sinclair, 2013), and how the tumor-associated EBV manipulates the host polycomb group of proteins to control key components of the oncogenic stress responses (OSR), during the transformation of normal B cells (Allday, 2013); the role of BZLF1 in the switch from EBV latency to the lytic cycle, especially the epigenetic mechanisms involved in BZLF1 gene re-programming (Murata and Tsurumi, 2013); and finally the description of epigenetic modifications regulating anti-viral responses and how they can be used to improve the therapeutic efficacy of oncolytic viruses (Forbes et al., 2013).

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Genome-wide mapping of DNase I hypersensitive sites and association analysis with gene expression in MSB1 cells

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DNase I hypersensitive sites (DHSs) mark diverse classes of cis-regulatory regions, such as promoters and enhancers. MSB-1 derived from chicken Marek's disease (MD) lymphomas is an MDV-transformed CD4+ T-cell line for MD study. Previously, DNase I HS sites were studied mainly in human cell types for mammalian. To capture the regulatory elements specific to MSB1 cells and explore the molecular mechanisms of T-cell transformation caused by MDV in MD, we generated high-quality of DHSs map and gene expression profile for functional analysis in MSB1 cell line. The total of 21,724 significant peaks of DHSs was identified from around 40 million short reads. DHSs distribution varied between chromosomes and they preferred to enrich in the gene-rich chromosomes. More interesting, DHSs enrichments appeared to be scarce on regions abundant in CpG islands. Besides, we integrated DHSs into the gene expression data and found that DHSs tended to enrich on high expressed genes throughout whole gene regions while DHSs did not show significant changes for low and silent expressed genes. Furthermore, the correlation of DHSs with lincRNAs expression was also calculated and it implied that enhancer-associated lincRNAs probably originated from enhancer-like regions of DHSs. Together, our results indicated that DNase I HS sites highly correlate with active genes expression in MSB1 cells, suggesting DHSs can be considered as markers to identify the *cis*-regulatory elements associated with chicken Marek's disease.

Keywords: DNase I, DHS, intergenic DHSs, MSB1, CpG islands, gene expressions, long non-coding RNAs, Marek's disease (MD)

INTRODUCTION

The formation of regions of open chromatin or nucleosome loss in eukaryotic genomes is a vital factor revealing potential regulatory activity. In addition, chromatin accessibility, which has been determined traditionally by regions of "open" or "closed" conformation, is governed by accessible *cis*-regulatory elements from DNA sequence, ATP-dependent chromatin remodeling and nucleosome modifications (Bell et al., 2011). However, chromatin accessibility can be examined by DNase I cleavage digestion, and then disclosed by the DNase I cleavage pattern (Wu et al., 1979). The introduction of next generation sequencing technology triggered one of the major breakthroughs in genomic research. The combination of DNase I digestion and deep sequencing (DNase-seq) has been used to reveal chromatin accessibility *in vivo* in a specific tissue or cell-type on a genome-wide scale (Song and Crawford, 2010).

Identification of the causative agent of Marek's disease (MD) had long been the holy grail of MD research and the highly contagious Marek's disease virus type 1 (MDV-1) is an avian herpesvirus that causes T-cell lymphomas and mononuclear infiltration of peripheral nerves (Luo et al., 2012). However, the molecular mechanisms that underlie T-cell transformation caused by MDV are unknown. MSB-1 is an MDV-transformed CD4+ T-cell line derived from a spleen lymphoma induced by the BC-1 strain of MDV-1 (Akiyama and Kato, 1974; Hirai et al., 1990). Therefore, the MSB-1 lymphoblastoid cell line, which shares many properties of Marek's disease (MD) tumors, could be used as a model system for analyzing the molecular pathways and mechanisms of neoplastic transformation in MD tumors. It was known DNase I HS sites are specific for different cell types and tissues (Crawford et al., 2006). In the previous studies, the exploration of chromatin accessibility and recognition of gene regulatory elements by DNase-seq technique were conducted mostly in human or mouse cell types for mammalian. However, genome-wide analysis of DNase I hypersensitive sites in chicken has not been reported yet. Hence, our study is to explore the regulatory pattern of DNase I hypersensitive sites in chicken MSB1 cell line, so as to probe molecular mechanisms of T-cell transformation caused by MDV in MD development.

In the present research, we enriched cleavage fragments of DNA treated with DNase I (200–500 bp) and constructed a DNA sequencing library from chicken MSB1 cell line. From 45,960,000 DHS sequencing reads, 21,724 DHSs were identified with high sensitivity. By combining the genome-wide analysis of DHS and gene expression sequencing, we found a specific correlation between DHS locations and gene expressions in MSB1 cells. Our data suggested DNase I hypersensitive sites provide vital clue to identify *cis*-elements for active genes expressions.

METHODS

PREPARATION OF DNase I TREATED DNA

The MDV-transformed lymphoblastoid MSB-1 cells were obtained from Dr. Mary Delany's lab, University of California, Davis, CA. and grown at 38.5°C in 5% CO_2 in RPMI 1640 medium containing 10% fetal calf serum, 10% tryptose phosphate broth, and 1% sodium pyruvate (Yao et al., 2008). Intact nuclei were prepared and digested with DNase I (He et al., 2012). Briefly, cells were lysed with 0.1% NP40 and nuclei were collected by centrifugation. Intact nuclei were treated with DNase I amounts of 0 units (U), 1 U, 5 U, 40 U, and 80 U per 200 μl reaction at 37°C for 5 min, and reactions were stopped with 0.1 M EDTA. Optimal concentrations of DNase I generated a smear of high-molecular-weight fragments when analyzed by pulsed field gel electrophoresis. The fragments of 200–500 bp were cut from the gel and DNA was extracted using the standard phenol-chloroform technique.

DNA LIBRARY PREPARATION AND HIGH-THROUGHPUT SEQUENCING

The library for sequencing on the Solexa 1G Genome Analyzer (Illumina, USA) was constructed as follows. End repair of the fragmented DNase I treated DNA was performed by NEBNext® End Repair Module (NEB, MA, USA). Then a 3′ polyA was added using DNA polymerase I, Large (Klenow) Fragment (NEB, MA, USA). Also, a pair of Solexa adaptors (Illumina, USA) was ligated to the repaired ends by T4 ligase (Promega, USA). Filtration in a 2% agarose gel was used to select fragments (DNA plus adaptors) from 200 to 500 bp. PCR was conducted to enrich purified DNA fragments by using Phusion® Hot Start High-Fidelity DNA Polymerase (NEB, MA, USA). After purification, DNA quality was examined by using the Qubit assay (Life Technology, USA) and was diluted for sequencing, then we performed sequencing analyses in the Solexa 1G Genome Analyzer (Illumina, USA) following manufacturer protocols.

ALIGNMENT AND PEAK IDENTIFICATION OF DNase I HS SITES

Sequence reads of 50 bp length of DNase-seq were obtained using the Solexa Analysis Pipeline. And then they were mapped to the chicken reference genome by Bowtie and only perfect matches that had a single unique alignment within the genome were retained and used for further analysis. For DNase-seq experiment, peak areas represent *in vivo* locations of DNase I hypersensitive sites. The WaveSeqR package that employs robust method based on the wavelet transformation was applied to identify DNase I peaks (Mitra and Song, 2012). The parameters configuration was window size of 200 bp, minreads of 3, maxscale of 12, the wavelet mother function of "gaussian2," no gap and p.thres of 0.2 to call peaks representing putative DNase I hypersensitive sites. The output result includes the genome coordinates, reads number of each peak, *p*-value, and FDR. Furthermore, for peak related genes, as long as there is 1 bp overlap between regions of a peak and a particular gene (includes regions of up-2 K, exon, intron and down-2 K), we consider that the peak is associated with the correspondent gene. The pathway analysis of genes relevant peaks was conducted by DAVID database. To link the DNA methylation and DNase I HS sites, CpG islands information about chicken was downloaded from the UCSC website (http://hgdownload.cse.ucsc.edu/goldenPath/galGal3/database).

WHOLE GENOME GENE EXPRESSION ANALYSIS

The total RNA extraction was performed by RNeasy Mini Kit (Qiagen, Valencia, CA, USA) from prepared MSB1 cells. Isolation of mRNA from total RNA was achieved using Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. About 300 ng of mRNA was used to synthesize the first strand cDNA by SuperScriptTM II Reverse Transcriptase (Invitrogen, Frederick, MD, USA). The second strand cDNA was synthesized using DNA polymerase I (Invitrogen, Frederick, MD, USA) with addition of Ribonuclease H (Invitrogen, Frederick, MD, USA) to degrade the remaining mRNA. After purification, a Bioruptor Sonicator (Diagenode, NJ, USA) fragmented the double strand cDNA (dscDNA) to approximately 200–500 bp. Then the library was built for sequencing on the Solexa 1G Genome Analyzer (Illumina, USA) following manufacturer protocols.

The total number of tags that uniquely aligned to gene represented its expression level. And the unique mapped tags for each gene were normalized to TPM (number of transcript copies in per million clean tags), equaling to the copy number of clean tags for this gene divided by total number of clean tags and multiplied by one million for multiple samples comparison (Morrissy et al., 2009). Normalized gene expression levels were averaged with two biological duplicates for each gene.

CORRELATION OF DHSs TO GENE EXPRESSION

To study the correlation of DNase I hypersensitive sites with gene expressions, transcriptional levels of genes in chicken MSB1 cells were obtained by RNA-seq analysis. Then, these genes (17,934 genes) were broken up into 170 sets of 100 genes by ranking their expression levels. Four out of the 170 sets shown in **Figure 4** correspond to highly expressed, two degrees of intermediately expressed (medium and low) and silent genes respectively. Tags detected were aligned in each gene set across transcription start sites (TSS) or gene bodies. To calculate the DHSs profiles across the gene bodies, the tag numbers detected in every 5% of the gene-body region and every 1 kb outside of the gene-body region were summed and normalized in the four expressed sets. For DHSs analysis near TSS (**Figure 5**), the tag density (number of tags per base pair) was calculated in the top 1000 high expressed and 1000 low expressed genes relative to the upstream 100 Kb of TSS.

VALIDATION OF DHSs BY REAL-TIME PCR

DNase-qPCR reactions with SYBR green dye were carried out using BIO-RAD MyiQ qPCR machine to confirm the enrichment of selective putative DHSs regions. PCR primer pairs were designed using Primer3 (http://fokker.wi.mit.edu/primer3/input.htm) and confirmed by Oligo 6. Primer sequences were given in Table S3. The DNase-qPCR reactions were triplicated for each site. To determine the relative fold enrichments, the $2^{-\Delta Cp}$ method was used by comparing enrichment values for a positive primer pair (totally 5 pairs) to a negative primer pair between experimental (DNase DNA) and reference (input DNA) samples. For RT-qPCR of gene expression, five candidate genes were selected to validate the association with DHSs and triplicates were

performed for RT-qPCR reactions. Gene expression was normalized against *GAPDH* housekeeping gene in the corresponding samples.

RESULTS

DISTRIBUTION OF DHSs READS

To identify regions of the genome where regulatory factors interact with DNA to modify chromatin structure and gene transcription, DNase-seq has been employed to map regulatory regions in MSB1 cell line. A total of 55.93 and 35.99 million short reads from two biological duplicates were aligned to the chicken reference genome with unique mapping rates of 80.50 and 80.29%, respectively.

To study DHSs distribution regarding genomic region, we divided the chicken genome into five kinds of regions –up-10 K [10 kb upstream of transcription start site (TSS)], exon, intron, down-10 K [10 kb downstream of transcription end site (TES)] and intergenic regions–based on the annotation of "known genes" from UCSC galGa3 database. The reads proportion for each region of the entire genome was indicated (**Figure 1A**). As shown in **Figure 1A**, the majority of reads were assigned to intergenic regions (91.8%) and only a few reads to exonic sections (0.49%). Intronic region constituted 5.34% of the mapped reads and it was approximately ten times higher than for exon region. Further, percentage of reads was 2–3 times higher in upstream and downstream regulatory regions than in exon region. To visualize the distribution trends of DHSs in the gene regions, a composite profile of DHSs for all known genes was generated, spanning their gene bodies and extending it 10 kb upstream and 10 kb downstream (**Figure 1B**). It is notable that the levels of DHS signals were high on gene body regions. Moreover, it appeared that DHSs decreased dramatically at TSS, suggesting that DHSs specifically concentrate in regions proximal to TSS. These results were consistent with previous observations that unique mapped reads of DNase I increased around TSS in HeLa S3 cells (Wang et al., 2012). Besides, our results in **Figure 1B** also showed that more DHSs were enriched in upstream regions of TSS comparing to downstream regions of TES, implying that DHSs could explore some *cis*-regulatory elements, such as enhancers acting on the promoter regions via bounding by activator proteins (Pennacchio et al., 2013).

DISTRIBUTION OF DHSs PEAKS

To determine the DNase I hypersensitive sites within the genome of MSB1 cells, a more robust method, WaveSeqR software, was adopted to accurately identify enriched regions of DNase I HS sites (Mitra and Song, 2012). Statistically, the total of 21,724 DHSs peaks was identified (p-value <0.2). The average and median peak length were 1335 and 1199 bp correspondingly (**Figure 2A**). The reads numbers of peaks and the peaks counts were calculated using cumulative density statistics. Most of peaks can be identified by around 20 reads in MSB1 cells (**Figure 2B**). To study the pattern of DNase I hypersensitive sites in different regions of genes, we also calculated the distribution of peaks in four kinds of genic regions, most peaks (55%) were enriched in intronic region, 10% of them were accounted in upstream-2 kb and downstream-2 kb regions, respectively (**Figure 2C**). The results differ from the patterns of DHSs in HeLa cells, in which more reads were found in upstream 20 kb and downstream 20 kb than in coding region (Wang et al., 2012). In addition, we also found 4465 genes were associated with DHSs peaks, and they actively involved into many biological processes, such as protein amino acid phosphorylation and intracellular signaling cascade, the molecular functions of nucleotide binding and ribonucleotide binding (Table S1). Further pathway analysis demonstrated most of genes related to DHSs involved into ribosome, focal adhesion and Wnt signaling pathway (**Figure 2D**).

DISTRIBUTION OF DHSs ON DIFFERENT CHROMOSOMES

To reveal the difference of DHSs distribution among chromosomes, we mapped the locations of DHSs relative to chromosomes, annotated genes and CpG islands. We found that DHSs

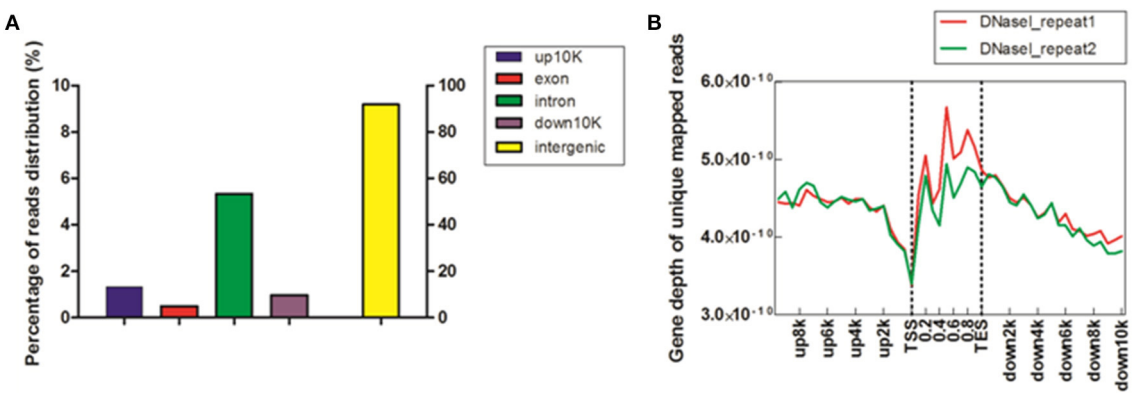

FIGURE 1 | Distribution of DHSs reads in MSB1 cells. (A) Distribution of unique mapped reads among different genomic regions. The chicken genome was divided into five kinds of regions: 10 kb upstream of transcription start site (TSS), exon, intron, 10 kb downstream of transcription end site (TES) and intergenic regions. The histogram described the percentage of unique mapped reads among five genomic regions. **(B)** Coverage depth of unique mapped reads among genic regions. For each gene, the tag numbers detected in every 10% of the gene-body region and every 5000 bp outside of the gene-body region were summed to obtain density levels. These numbers were then normalized by the total number of base pairs in each region (Barski et al., 2007).

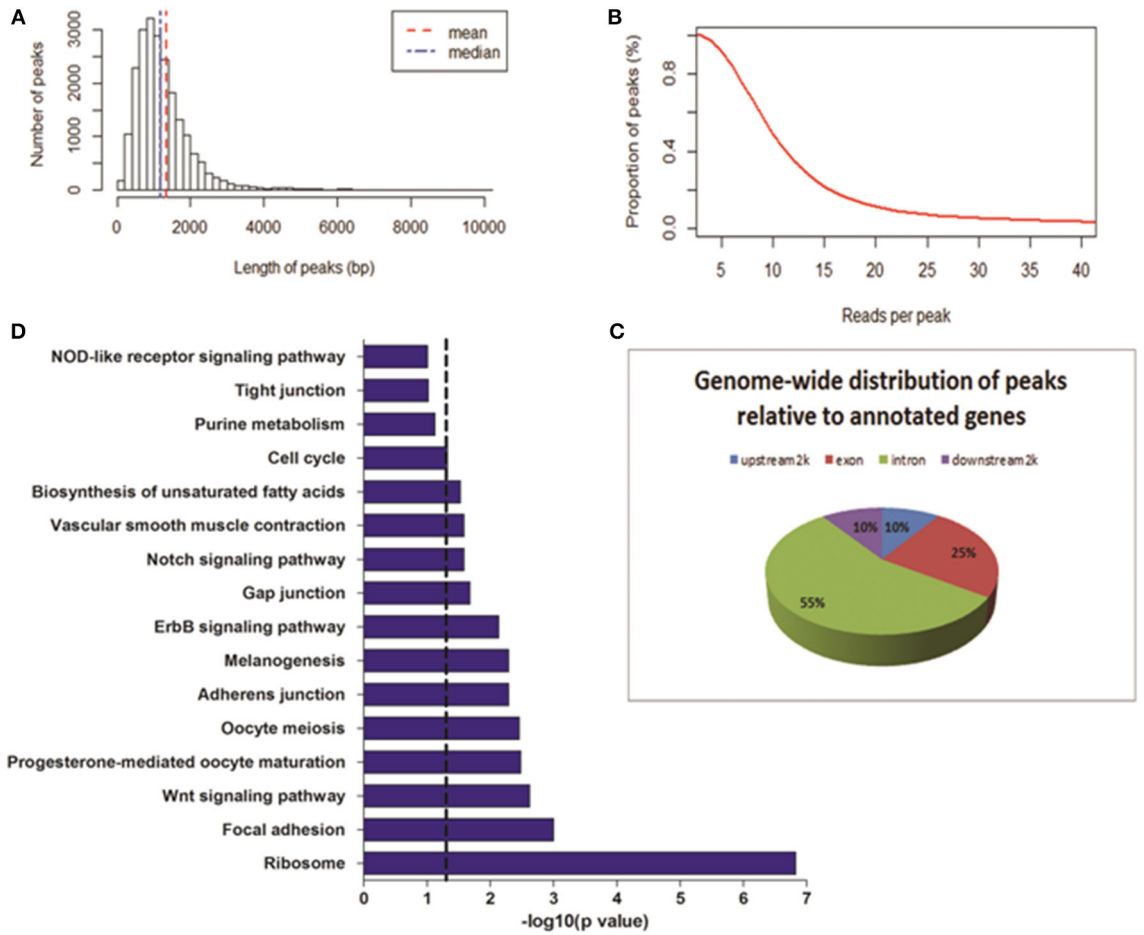

FIGURE 2 | Genome-wide distribution of DNase-seq peaks. (A) Length of peaks. X-axis represents the length of peak; Y-axis represents the number of peaks. **(B)** Proportion of peaks with reads number. Coverage of reads in peak regions was calculated. The reads number of each peak and peak numbers were added with the cumulative density statistics. **(C)** The locations of DHSs relative to annotated genes. Genome-wide distributions of DHS peaks in annotated gene regions were shown. DHS peaks were counted in upstream 2 Kb, exon, intron, and downstream 2 Kb regions. **(D)** Pathways analysis of genes related to DHS peaks. Dashed line: threshold line corresponds to *P*-value of 0.05.

peaks were significantly over-enriched on chromosomes 1, 2, 3, 10, 13, 23, 25, and W, which are known to be especially gene-rich (**Figure 3A**). Besides, the density of DHSs peaks per gene varied among chromosomes and they were highly enriched on these chromosomes. Notable, there were more DHS peaks on chromosome 16 while peaks density per gene was very low, this may be due to smaller chromosome size.

DNA methylation is one of the most prevalent mechanisms to maintain inactive genomic regions in a repressed state, and it is also one of the most stable modifications (Bird, 2002). In order to study the relationship between DNA methylation and DNase I hypersensitive sites, we overlapped DHSs peaks with CpG islands and normalized per Mb in chromosomes except for all random chromosomes. The results showed chromosomes 1, 2, 3, 10, 13, 23, and W highly enriched DHSs peaks per gene appeared to low density of CpG islands (**Figure 3B**), conversely, there was significant high density CpG islands on chromosome 16 when DHSs peaks density per gene was very low on this chromosome, which suggested that DNase I sensitive domain preferred to act within active chromatin domains that present low density CpG islands (Cockerill, 2011).

OVERALL CORRELATION BETWEEN DHS DISTRIBUTION AND GENE EXPRESSION

To reveal the functional consequences of DNase I hypersensitive sites, gene expression profiles were generated by the next-generation sequencing in MSB1 cells. The number of unique mapped reads for each gene was counted and then normalized to TPM (number of transcript copies in per million clean tags) to represent gene expression levels (Morrissy et al., 2009).

To reveal the DNase I regulation pattern in MSB1 cells, the genes whose expression levels were determined by the RNA-seq assay were attributed to multiple groups. Four groups were selected randomly with 100 genes for each group according to their expression levels. The DNase I reads numbers in each region were calculated and normalized throughout the whole transcribed regions and extending 20 kb upstream and 20 kb downstream for four gene sets corresponding to highly expressed, two

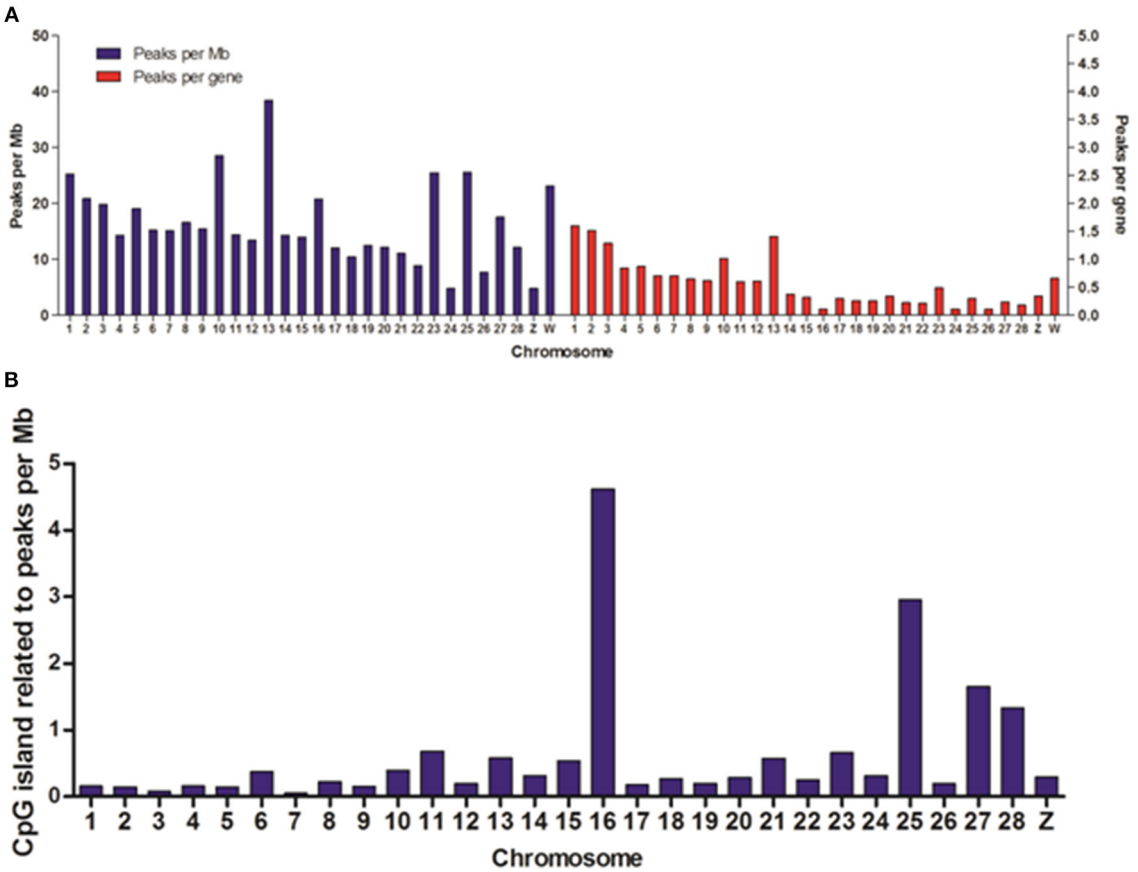

FIGURE 3 | Distribution of DHSs and CpG islands-associated DHSs on different chromosomes. (A) Distribution of DHSs on the annotated genome. DHS peaks were mapped to each chromosome. The peak densities for per Mb (blue bars) and for per gene (red bars) were calculated. **(B)** The densities of CpG island-related peaks for per Mb on different chromosomes. We ignored the characteristics of DHSs on all of random chromosomes.

types of intermediately expressed (medium and low) and silent genes (**Figure 4**). As expected, DNase I hypersensitive sites signals were correlated with gene activation (**Figure 4**). Obviously, DHSs enrichment levels were superior at high expressed genes than at low expressed and silent genes. Intriguingly, DHSs levels were elevated surrounding the TSSs and TES for the highly expressed genes sets (dotted line), though were not significant for the other three sets. To explore DHSs features in extreme high and low expressed genes and reveal the association of DHSs with *cis*-regulatory elements on upstream regions relative to TSS, we analyzed the density levels of DHSs in extending 100 kb upstream for two sets genes with the top 1000 high expressed and 1000 low expressed genes. The result showed that the most pronounced enrichment was observed within 10 kb upstream of promoters of high expressed genes. DHSs enrichment levels appeared to decrease while increasing the distance from TSSs (**Figure 5A**). In contrast, DHS sites enrichment did not change within 100 kb upstream of TSSs in low expressed genes (**Figure 5B**). These observations were consistent with p300 binding sites that a near-ubiquitously expressed component of enhancer-associated protein assemblies drive the expression of adjacent genes in forebrain tissue isolated from mouse embryos at given time point (Visel et al., 2009), suggesting that DNase I hypersensitive sites have a strong relationship with enhancer regulatory element in chicken MSB1 cells.

DHSs AND LONG NON-CODING RNA

It has been reported that some long non-coding RNAs (lncRNAs) originate from intragenic enhancers which behave as alternative promoters producing transcripts when active (Marques et al., 2013). Accordingly, our results showed that abundant DHSs were enriched in intergenic region in MSB1 cells (**Figure 1A**). Therefore, in order to determine whether lncRNAs might originate from active intergenic enhancers examined by DHSs, we also analyzed the distribution of DHSs relative to long intergenic non-coding RNAs (lincRNAs) in MSB1 cell line, based on a stringent lincRNA identification pipeline for RNA-seq data from our lab (unpublished). We found 124 candidate lincRNAs, nonetheless, only 17 of those overlapped with intergenic DHSs (Table S2). These observations indicated that DHSs may possibly be less important as regulatory elements for non-coding RNA genes than for coding genes, which was consistent with information from *C. elegans* (Shi et al., 2009). However, an enhancer examined by DHS sites might affect gene transcription not only in *cis* and it can

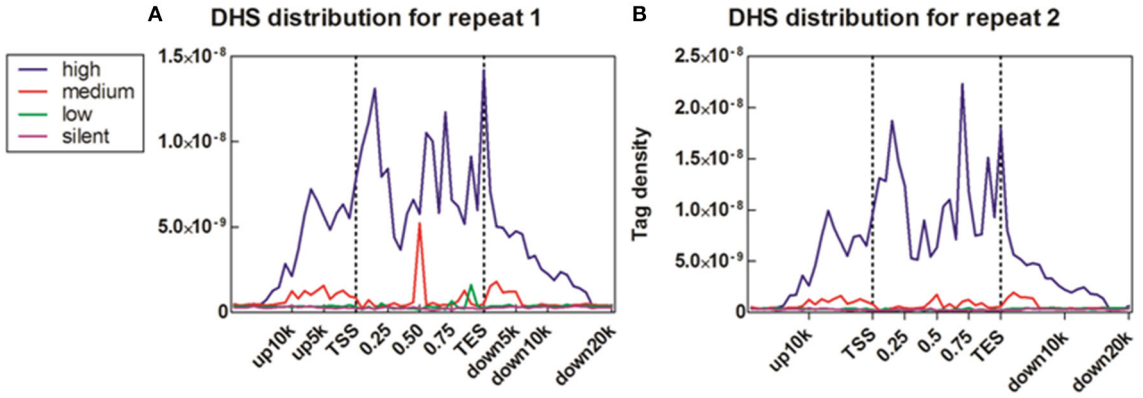

FIGURE 4 | Correlation between DHS distribution and gene expression. **(A)** Profiles of DHSs distribution patterns were shown across the gene bodies for highly active (high), two kinds of intermediately active (medium and low) and silent gene sets. Each gene set included 100 genes according to their expression levels in MSB1 cells line. Here, DHSs reads were aligned extending 20 kb of 5′ and 3′ of the gene bodies of 100 genes in each group (x axis). The y axis shows the detected tag density. For each gene, the tag numbers detected in every 5% of the gene-body region and every 1 kb outside of the gene-body region were summed to obtain DHSs distribution levels. These numbers were then normalized by the total number of base pairs in each region. **(B)** Profiles of DHSs distribution patterns in sample repeat 2 of MSB1 cells.

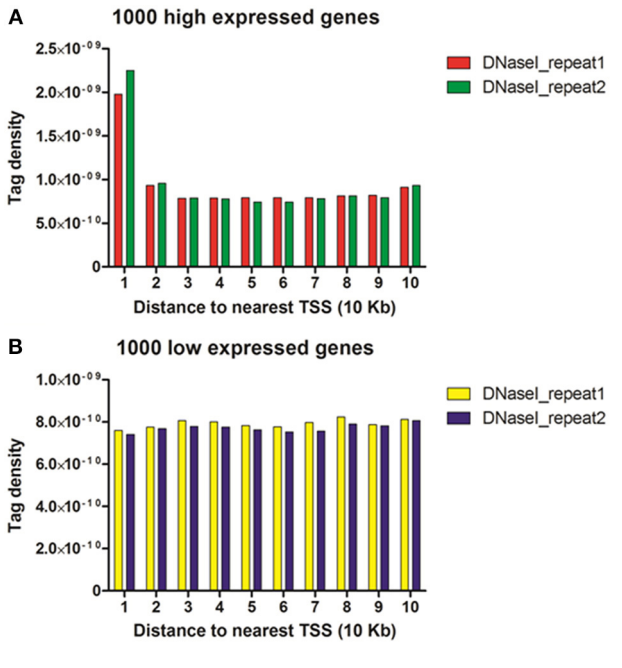

FIGURE 5 | DHSs reads were enriched near genes that were expressed in the MSB1 cells. (A) DHSs density levels in upstream 100 Kb regions relative to TSS for 1000 high expressed genes. **(B)** DHSs density levels in upstream 100 Kb regions for 1000 low expressed genes.

be found within introns or even be excised and inserted elsewhere in the chromosome and still affect gene transcription (Eichenlaub and Ettwiller, 2011). Therefore, we explored whether DHSs as enhancer regulatory elements regulate lincRNAs expressions by calculating the correlation between the enrichments of DHSs and the expressions of overlapping lincRNAs (**Figure 6**). It showed a negative correlation between enrichment of DHS and lincRNA expression. To test whether lincRNAs were co-expressed with protein-coding neighbors, Pearson correlations of expression levels between lincRNAs and neighboring protein-coding genes were also calculated (**Figure 6**).We observed that lincRNAs affect their neighboring protein-coding genes but there is no stable mode, which is similar to previous studies in human and zebrafish (Cabili et al., 2011; Pauli et al., 2012). Therefore, DHSs enhance gene expression and the expression of lincRNAs is associated with the expression of enhancers but act not completely in *cis*.

VALIDATION OF DHSs BY REAL-TIME PCR

To assess the accuracy of the DNase-seq mapping results and confirm the relationship between DNase I HS sites and the expressions of related genes, five DHSs peaks overlapped with neighboring genes, including high expressed and low expressed correspondents, were arbitrarily chosen to confirm their enrichment using DNase I—quantitative PCR (DNase-qPCR) approach. Relative enrichment was quantified for each site with real-time PCR reactions using 0.5 ng DNase I treated DNA or 0.5 ng input DNA and normalized by the negative control without DHSs coverage. For the four candidate peaks of DHSs (**Figure 7A**), the relative enrichments were mostly consistent with DNase-seq profiles. Similarly, the expression levels of genes related to DHSs peaks were also detected with reverse transcription— quantitative PCR (RT-qPCR) and standardized with the *GAPDH* housekeeping gene. The results showed that the expressions of five genes were predominantly consistent with the RNA-seq data (**Figure 7B**) and the enrichment value of G10 gene was significant low. Besides, G2, G3, and G5 are genes overlapping with P2, P3, and P5 peaks, successively (Table S3), and it showed expressions of the neighboring genes to DHSs would decrease with the enrichment levels of DHSs sites declined, which implied DHSs are indeed associated with active genes and they probably represent regulatory elements (e.g., enhancers) to drive adjacent genes expressions. Consequently, DNase-seq can be

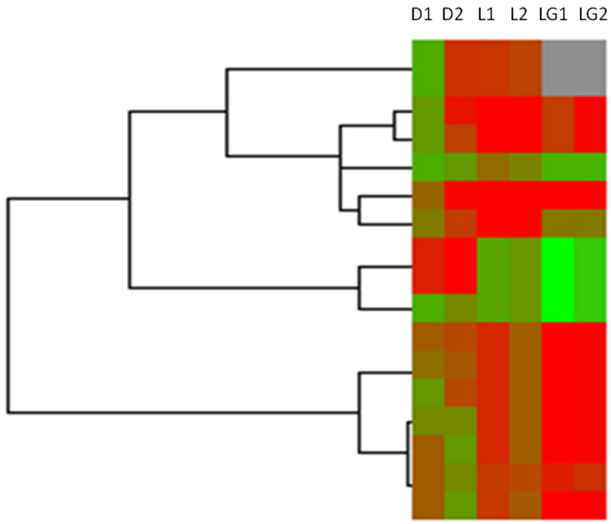

FIGURE 6 | Heatmap of DHSs, overlapped lincRNAs and neighboring genes of lincRNAs in MSB1 cell line. Based on RNA-seq data in MSB1 cell line, 124 candidate lincRNAs were identified and only 17 lincRNAs are overlapped with DNase I hypersensitive sites (DHSs). Normalized intensity values of DHSs (rows) and expressions values of their overlapped lincRNAs and neighboring genes of lincRNAs were ordered using Centroid Spearman Rank Correlation and hierarchical clustering in Cluster3.0 software. The intensity of DHS was represented by reads number in this peak of DNase I hypersensitive site. The expression of lincRNA and its neighboring genes were represented by FPKM (Fragments Per Kilobase of transcript per Million mapped reads). The dendrogram showed the similarity (distance) of DHSs intensity, lincRNAs and neighboring genes expressions and was divided into sub-trees as distinguished from different colors. Arrays (columns) were grouped that *D1* stands for the intensity of DHSs in duplicate 1, *D2* stands for the intensity of DHSs in duplicate 2, *L1* stands for expressions of lincRNAs overlapped DHSs in duplicate 1 and *L2* stands for expressions of lincRNAs overlapped DHSs in duplicate 2; *LG1* and *LG2* are expressions of neighboring genes of lincRNAs in duplicate 1 and duplicate 2, respectively. Red and green colors reflect the high and low intensities, respectively.

reliably and efficiently used for revealing chromatin accessibility and identifying important regulatory elements.

DISCUSSION

DNase-Seq (DNase I hypersensitive sites sequencing) is a method used in molecular biology to identify the location of regulatory regions, based on the genome-wide sequencing of regions super sensitive to cleavage by DNase I (Crawford et al., 2006). Finding peaks from DNase-seq is the main goal to identify the location of candidate regulatory regions. However, the lack of well-established algorithms to handle DNase-seq data and the utilization of a ChIP-seq peak finder which does not completely fit the pattern of the DNase-seq data, inspired us to develope a WaveSeqR method that can be used accurately for both narrow and broad peaks (Mitra and Song, 2012). For the implementation of WaveSeqR, we set gaussian2 as the wavelet mother function that is suitable for diffuse peaks of DNase I hypersensitive sites whereas Morlet mother function is good for sharp and punctate peaks (e.g., TFBS and H3K4me3).

Consequently, 21,724 broad significant peaks of DHSs were identified within the genome of MSB1 cells. To compare the accuracy of different methods for identifying DHSs sites in MSB1 cells, the conventional software MACS (version 1.4.2) was also implemented to find candidate DHSs. The total of 30,834 and 16,669 DNase I HS sites were identified from two replicates, respectively. Of those DHSs, 9911 peaks (p-value $<$ 1e$-$05) were common between two replicates of MSB1 cells. Average and median peak length were 205 and 173 bp respectively (Figure S2). After comparing the WaveSeqR and MACS results, 45% of the peaks (4497) were identified by both methods, which suggested that those DHSs are reliable candidates for DNase I hypersensitive sites. Also, WaveSeqR can be considered an accurate and reliable method for the identification of DNase I hypersensitive sites based on DNase-seq data.

From the **Figure 1A**, we can see that most DHSs reads were allocated to intergenic and intronic regions, however, the percentages of these regions were also greater than others in the whole genome. Therefore, we normalized the DHSs reads distribution to DHSs abundance based on the percentage value of various functional regions (Figure S1). Similarly, reads abundance in intergenic region was still the highest (1.29), followed by exonic region (0.40) and intronic region (0.36), which suggested an orderly preference of DNase I for those genomic sections. Several studies showed that various macrophage-specific DHSs were identified within mouse intron 2. The sequences of those DHSs are highly conserved and some of them can be denoted as intron regulatory elements, such as FIRE, acting as a macrophage-specific enhancer in the *fms* gene expression (Himes et al., 2001). Additionally, it has been reported that 95% of DHSs were observed in intronic and intergenic regions based on 125 different human cell types (Thurman et al., 2012). Therefore, DHSs located in introns and intergenic regions would like to be expected to the vital and conserved regulatory elements without influencing by cell-type and tissue-type specific.

Abundant DHSs were enriched in intergenic region of MSB1. This finding accords with previous studies where approximately half of the DHSs were mapped to intergenic regions in *C. elegans* and were allocated far from annotated genes denoted transcriptional regulatory information (Shi et al., 2009). Moreover, it has been reported that nematode highly conserved non-coding elements (CNEs) were associated with *cis*-regulatory elements (Vavouri et al., 2007). Also it has been reported that DHSs and particularly distal intergenic DHSs, tend to fall in genomic sections that are conserved in two distinct nematode genomes, which implied that conserved DHSs would help to determine what type of functional elements these regions might represent. Our results implied that there was a strong relationship between DHSs enrichments and lincRNAs expressions, and these enhancer-associated lincRNAs probably originated from enhancer-like regions of DHSs (Marques et al., 2013).

The profiles of DNase I hypersensitive sites were determined employing the DNase-seq method on MSB1 chicken cells. Our data showed that most DHSs enriched in intronic, intergenic and upstream regulatory regions. Probably, the intronic and intergenic DHSs are vital and conserved regulatory elements regardless cell or tissue types. By the combination of DNase-seq and

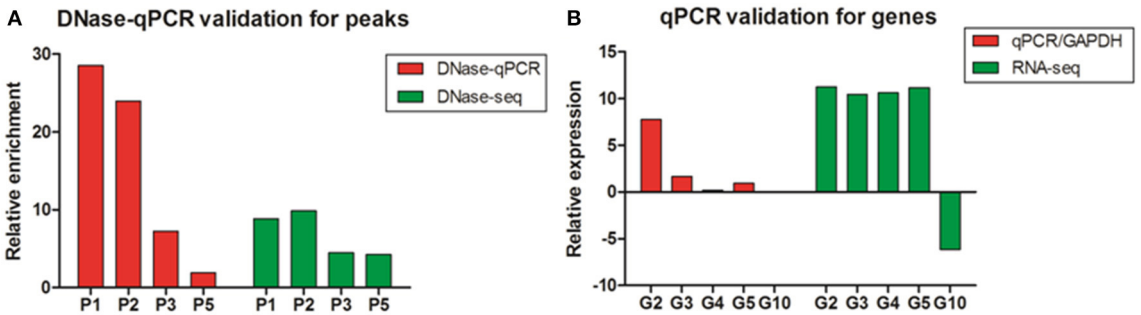

FIGURE 7 | The validation of DNase I hypersensitive sites and gene expression in chicken MSB1 cells. (A) The validation of DHSs peaks by DNase-qPCR. Real-time PCR results showing enrichment of indicated four sites (P1, P2, P3, and P5) in DNase-seq results were carried out in MSB1 cells (red bar). The negative control was selected from regions without DHSs coverage in the whole genome in both of replicates to normalize the relative enrichment levels of DNase I hypersensitive sites. DNase-seq results were also shown by the logarithm base 2 values of average reads number of DNase I in MSB1 cells (green bar). **(B)** Real-time RT-PCR were performed for validation of genes expression and standardized with *GAPDH* housekeeping gene (red bar). RNA-seq result for each selected gene was also shown and the logarithm base 2 values of TPM were used as expression levels (green bar). For G10 gene, it is difficult to show due to very low expression (0.00265).

RNA-seq analyses in MSB1 cells, the function of DNase I HS sites was explored and showed that they were correlated with active genes, especially high expressed genes, implying that DHSs are potential representatives of enhancer regulatory elements. Even though the information of DNase I HS sites in MSB1 cell line provided an important reference for chicken Marek's disease study, it is still necessary to conduct DNase-seq in different cells or tissues, or different states of the same tissue, including normal vs. Marek's disease infected, to identify global changes in regulation. The method of DNase-seq can help to recognize the functional regions of the genome, however, determining the type of regulatory function for each DNase I hypersensitive site still remains a daunting challenge. Clues can be gleaned from correlating DNase I hypersensitive sites with sequence conservation, promoter or enhancer activity, transcription factor binding sites and histone modifications, motif discovery, DNA methylation and more detailed gene expression analysis. Therefore, in the near future, the integrated analysis of genes, regulatory elements and chromatin architecture on a genome-wide scale will be a powerful and well-established method for identifying functional and regulatory elements.

AUTHOR CONTRIBUTIONS

Yanghua He: designed the experiments, analyzed the data and wrote and revised the manuscript; Jose A. Carrillo: analyzed the data; Juan Luo and Fei Tian: implemented the DNase-seq experiment and constructed the sequencing libraries; Yi Ding: validated the work of this study; Irit Davidson and Jiuzhou Song: designed the project, interpreted the data and the results, revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00308/abstract

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Characterization of HPV DNA methylation of contiguous CpG sites by bisulfite treatment and massively parallel sequencing—the FRAGMENT approach

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Invasive cervix cancer (ICC) is the third most common malignant tumor in women and human papillomavirus 16 (HPV16) causes more than 50% of ICC. DNA methylation is a covalent modification predominantly occurring at CpG dinucleotides and increased methylation across the HPV16 genome is strongly associated with ICC development. Next generation (Next Gen) sequencing has been proposed as a novel approach to determine DNA methylation. However, utilization of this method to survey CpG methylation in the HPV16 genome is not well described. Moreover, it provides additional information on methylation "haplotypes." In the current study, we chose 12 random samples, amplified multiple segments in the HPV16 bisulfite treated genome with specific barcodes, inspected the methylation ratio at 31 CpG sites for all samples using Illumina sequencing, and compared the results with quantitative pyrosequencing. Most of the CpG sites were highly consistent between the two approaches (overall correlation, $r = 0.92$), thus verifying that Next Gen sequencing is an accurate and convenient method to survey HPV16 methylation and thus can be used in clinical samples for risk assessment. Moreover, the CpG methylation patterns (methylation haplotypes) in single molecules identified an excess of complete-and non-methylated molecules and a substantial amount of partial-methylated ones, thus indicating a complex dynamic for the mechanisms of HPV16 CpG methylation. In summary, the advantages of Next Gen sequencing compared to pyrosequencing for HPV genome methylation analyses include higher throughput, increased resolution, and improved efficiency of time and resources.

Keywords: human papillomavirus, methylation, next generation sequencing, CpG methylation, methylation haplotypes

INTRODUCTION

Invasive cervical cancer (ICC) is the third most common malignant tumor in women and is caused by persistent infection of oncogenic human papillomavirus (HPV) (Jemal et al., 2011), especially type 16, which accounts for greater than 50% of all ICC (Schiffman et al., 2007; Li et al., 2011; Schiffman and Wentzensen, 2013). Recent data indicates that multiple regions of HPV16 and other oncogenic HPV type genomes show increasing CpG methylation patterns among normal, cervical intraepithelial neoplasia (CIN), and cancer tissues, respectively (Badal et al., 2003; Kalantari et al., 2004, 2009, 2010, 2014; Hong et al., 2008; Brandsma et al., 2009, 2014; Ding et al., 2009; Fernandez et al., 2009; Fernandez and Esteller, 2010; Piyathilake et al., 2011; Sun et al., 2011; Wentzensen et al., 2012; Lorincz et al., 2013; Mirabello et al., 2013). Thus, assays for quantitation of CpG methylation of oncogenic HPV genomes in general and HPV16 in particular, indicate that methylation is a promising biomarker for ICC development (Clarke et al., 2012). Therefore, a fast, accurate, and high-throughput approach to survey DNA methylation in HPV16 should facilitate ICC prevention, diagnosis, and treatment.

So far, the most widely used method for HPV DNA methylation investigation is bisulfite treatment followed by sequencing (Bhattacharjee and Sengupta, 2006), MassArray (Ehrich et al., 2005), SNPshot (Kaminsky and Petronis, 2009), or in particular pyrosequencing (Tost and Gut, 2007a,b; Dejeux et al., 2009) (for review, see Clarke et al., 2012). Despite the accuracy of CpG quantitation by pyrosequencing, it can only provide a relatively short read for each assay per sample and thus is time and labor intensive for testing multiple sites in large numbers of samples, which limits the incorporation of DNA methylation in clinical studies. Moreover, all these approaches constrain the ability to detect the methylation pattern at single-DNA-molecule resolution, which is critical for investigating methyltransferase dynamics. Although the cloning-sequencing approach after bisulfite treatment can provide some insight into this issue, the typically low number of clones analyzed (<10) and the high costs limits this approach.

Next generation sequencing can yield millions of single molecule reads and has been used to determine DNA methylation (Taylor et al., 2007; Bibikova and Fan, 2010; Laird, 2010; Feng et al., 2011; Kim et al., 2011; Komori et al., 2011; Ku et al., 2011; Nejman et al., 2014). Supplemented with DNA barcoding technology, which incorporates a unique index sequence into each PCR segment, this approach can provide a rapid way to simultaneously determine DNA methylation at the single-molecule level in large numbers of samples. However, the accuracy and validity of this approach needs further evaluation, especially in viral genomes such as HPV16.

In the present study, we randomly chose 12 samples with quantitation of CpG methylation within the HPV16 genome by pyrosequencing and performed amplification with primers containing barcodes specific for each sample. After all samples were pooled and purified, the PCR products were deep sequenced, analyzed, and the results were compared with CpG methylation determined by pyrosequencing. The methylation ratio for most CpG sites was highly correlated with those from pyrosequencing, which indicated that Next Gen sequencing of bisulfite treated cervical cells infected with HPV16 was an accurate method of quantitating CpG methylation. Moreover, the single molecule analyses provides a "methylation haplotype" and indicated an excess of full and non-methylated molecules in nearly all samples and a lower proportion of partially methylated molecules in most samples, thus revealing a complex and mosaic methylation pattern in the HPV16 genome.

MATERIALS AND METHODS

CERVICOVAGINAL SAMPLES

Twelve exfoliated cervical samples were randomly chosen from a previously reported nested case-control study of HPV16-positive cervical intraepithelial neoplasia grade 3 (CIN3) and HPV16-positive cervical samples that cleared infection (Mirabello et al., 2012). The lab was blinded to all clinical information. All samples contained HPV16 and the quantitation of CpG methylation of the HPV16 genome had been determined by pyrosequencing, as described (Mirabello et al., 2012). The study was designed to test samples for quantitation of CpG methylation to evaluate Next Gen sequencing compared to pyrosequencing prior to embarking on using this method on precious well-characterized samples from epidemiological studies.

ASSAY DESIGN

Since L1, L2, and E2 ORF regions showed differential methylation among disease groups (Mirabello et al., 2012), these three regions and the most significant CpG sites within them were chosen for the current study. Primers for PCR were designed using MethPrimer (Li and Dahiya, 2002) (http://www.urogene.org/methprimer/index1.html). In total, 3 segments in L1, 4 in L2, and 1 in E2 were included in the current study (**Table 1A**), and in total, 31 CpG sites were surveyed. Each primer was labeled by a unique barcode and 5′ and 3′ padding sequence (**Table 1B**) and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). A map of all 112 CpG sites in the HPV16 7906 bp reference genome is shown in **Figure 1** in the review by Clarke et al. (2012).

Table 1A | Next Gen sequencing assays of bisulfite treated HPV16 DNA in clinical samples.

Assay name	#CpG	CpG position	Length (bp)
L1_1	4	5602, 5608, 5611, 5617	114
L1_2	4	7034, 7091, 7136, 7145	172
L1_7	2	6650, 6581	167
L2_1	5	4240, 4249, 4261, 4270, 4277	130
L2_2	3	4427, 4437, 4441	89
L2_4	3	5128, 5173, 5179	123
L2_5	1	5378	166
E2_1	5	3412, 3415, 3417, 3433, 3436 (*3448, 3462, 3473, 3496*)	169

(), These sites were present in the NGS data but not PSQ.

Table 1B | Description of barcoded primers* for assays shown in this table.

Primer name	5′ pad (LP)	3′ pad (RP)	Primer target sequence (5′-3′)
16E2_1F	ACT	GCAG	TTAGGTAGTATTTGGTTAATTATTT
16E2_1R	ACT	GCAG	ATTAAAACACTATCCACTAAATCTCTATAC
16L1_1F	TAC	GTAC	TAATATATAATTATTGTTGATGTAGGTGAT
16L1_1R	TAC	GTAC	AACAACCAAAAAAACATCTAAAAAA
16L1_2F	ACT	GACG	TTTGTAGATTTAGATTAGTTTTTTTTAGGA
16L1_2R	ACT	GACG	TTCAACATACATACAATACTTACAACTTAC
16L1_7F	TAC	GATG	ATGTAGTTTTTGAAGTAGATATGGTAGTA
16L1_7R	TAC	GATG	AATTACCTCTAATACCCAAATATTCAA
16L2_1F	ATC	GACG	TTTTTGTTTGTTTGTTTGTTTTT
16L2_1R	ATC	GACG	ACATATACCTACCTATTTACATATTTTATA
16L2_2F	ATC	GACG	TATGGAAGTATGGGTGTATTTTT
16L2_2R	ATC	GACG	ATTCCCAATAAAATATACCCAATAC
16L2_4F	ATC	GTAC	TTTTGGATATAGTTGTTTTATATAGGTTAG
16L2_4R	ATC	GTAC	CCTTAACACCTATAAATTTTCCACTAC
16L2_5F	ATC	GTCA	TTGTAGAAGAAATAGAATTATAAATTATAA
16L2_5R	ATC	GTCA	AAAAATATAAAAAATACAAATAATACC

**Each primer consisted of 5′ to 3′: 3 bp (LP) – 8 bp Barcode – 4 bp (RP) – Primer Target Sequence.*

BISULFITE TREATMENT, PCR, PURIFICATION, AND DEEP SEQUENCING

DNA samples containing HPV16 DNA were treated with freshly prepared bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. Upon bisulfite treatment, unmethylated C's are converted into U's, which are then converted to T's by Taq polymerase during PCR amplification; methylated C's remain unmodified. Thus, in the CpG sequence a "C" represents a methylated CpG, whereas a "T" represents an unmethyaled CpG. All segments were amplified by HotStart-IT FideliTaq DNA polymerase (United States Biochemicals, Cleveland, OH). After validating size and intensity in a 3% agarose gel, each PCR product was pooled in equal proportions, separated by electrophoresis, and isolated from the gel. After precipitation by isopropanol and washed by 70% ethanol, the PCR products were ligated with adaptor

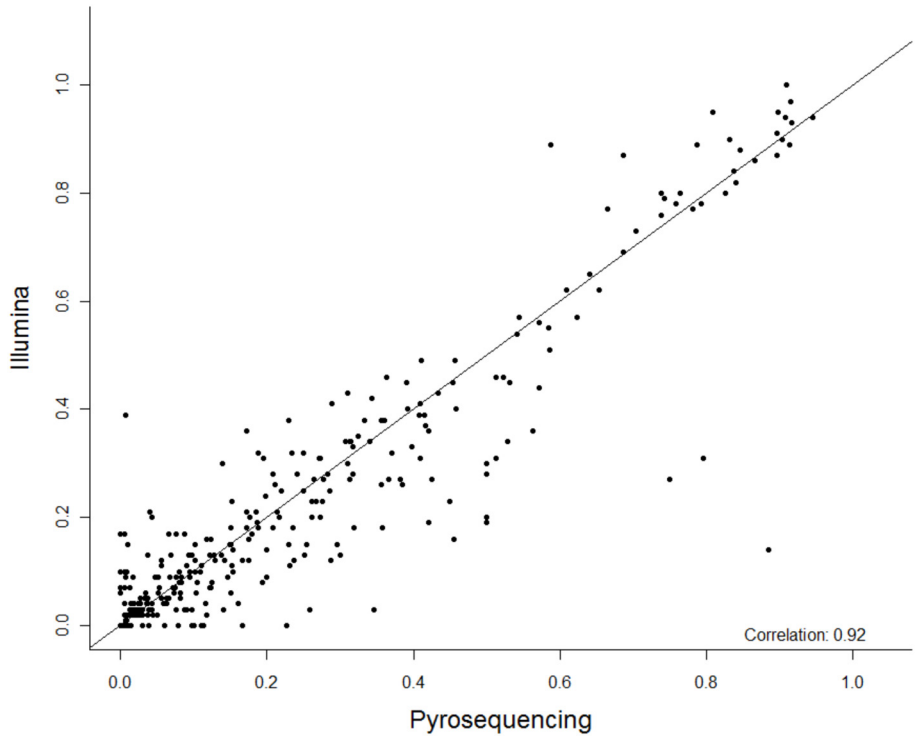

FIGURE 1 | The summary correlation of 27 CpG methylation sites between pyrosequencing and Next Gen sequencing. The x-axis is percent methylation by pyrosequencing and the y-axis is percent methylation by Next Gen sequencing. Each CpG site is plotted for each subject.

(library construction) and sequenced on an Illumina HiSeq 2000 (NG sequencing) within the Albert Einstein College of Medicine, Epigenetics Core Facility (Bronx, NY).

METHYLATION RATIO ANALYSIS

The obtained sequences were filtered by prinseq (Schmieder and Edwards, 2011) with average Phred score (Cock et al., 2010) not less than 20. The barcodes for each sample were split and cut by FastX kit (http://hannonlab.cshl.edu/fastxtoolkit/index.html). After alignment with the reference HPV16 genome by bowtie (Langmead et al., 2009), the methylation status for each molecule was determined by Bismark (Krueger and Andrews, 2011). For each CpG site, the methylation ratio is calculated by the formula: number of C reads divided by the sum of C and T reads at each CpG site. The pyrosequencing result for each site was determined on a PSQ96 ID Pyrosequencer (Qiagen, Valencia, CA) at the Albert Einstein College of Medicine, Genomics Core Facility (Bronx, NY) and the readout was percent methylation, as previously described (Mirabello et al., 2012). The correlation between NG sequencing and pyrosequencing was performed by linear regression in SPSS 16.0 (SPSS Inc., Chicago, IL) and the null hypothesis was rejected when $P < 0.05$. We have deposited the read sequences in the NCBI Sequence Read Archive (SRA) database, accession number SRP040981.

SINGLE MOLECULE ANALYSIS

The methylation pattern for each single molecule and the counts of each pattern were obtained by an in-house script (available on request). Briefly, the Bismark output, which gave the methylation state for each CpG site in each molecule, was parsed, and the methylation pattern of each DNA molecule was then reconstructed based on the unique read name it was assigned by the sequencer. Finally, the prevalence of each unique methylation pattern was counted, for each sample in each assay. The expected probabilities were constructed in two steps. First the singular probabilities of each site being methylated and unmethylated were calculated, by counting the proportions of molecules in each state at each site. Multiplying the appropriate singular probabilities, under the assumption that CpG sites would be methylated independently, produced an expected probability for each methylation pattern. A χ^2 goodness of fit test was then performed to compare observed and expected probabilities for methylation patterns, where the observed probabilities were the proportions of each detected pattern, calculated from their counts.

RESULTS

SEQUENCING DATA STATISTICS

In total, 192.2 million reads 95 bp long were obtained from NG sequencing and 53.4 million (27.8%) possessed an average Phred score above 20 and were used for this analysis (Ewing and Green, 1998). 41.7 million reads (78.1%) were observed to contain one of the incorporated barcodes without mismatch and assigned to a corresponding sample for further analysis. Except one segment in the L2 gene, most segments included ∼4–8 million reads (Figure S1A). For each sample, the read count varied from 2 to 6 million (Figure S1B). Most CpG sites (21/27) were covered by 0.6 to 1.8

million reads (Figure S1C), in total. Although three fragments did not amplify as robustly and had less reads (i.e., L1_2, L2_2, and L2_5 assays containing CpG sites 7034, 7091, 7136, 7145; 4427, 4437, 4441; and 5378, respectively), the correlation between CpG sites within these fragments between the PSQ and NGS assays had reasonable agreement (see **Figure 2**).

METHODOLOGICAL COMPARISON

Among these 31 CpG sites, 27 had been analyzed by pyrosequencng and the two results were compared. Using linear regression, most sites showed significant correlation between the two methods with an overall correlation of 0.92 (**Figure 1**). Only two CpG sites (positions 6650 and 7034) were poorly correlated ($P = 0.069$ and 0.19, respectively, **Figure 2**). These results indicated that next generation sequencing was an appropriate method for determining CpG methylation in the HPV16 genome, yielding results that were highly correlated with a well-established pyrosequencing technique.

SINGLE-MOLECULE RESOLUTION OF CPG METHYLATION

The methylation pattern for each single molecule was determined for each sample across six regions of the HPV16 genome. A substantial proportion (10–80%) of molecules in the six assays were partially methylated (possessing a mixture of methylated and unmethylated sites), and the distributions of patterns were varied. However, the site-wise proportions of methylation for a given sample in a given assay were similar and the distribution of patterns in each molecule did exhibit dependence on that methylation level. To address this issue, we calculated the expected frequencies of all possible methylation patterns in each assay and compared them with the observed patterns (**Figure 3**). In most samples, a relative excess of none- and/or fully methylated molecules was observed (**Figure 3**). In contrast, despite their high prevalences, there was a relative absence of partial methylated molecules (**Figure 3**). As a consequence, most of the samples yielded a significant *P*-value ($p < 0.05$), thus indicating that CpG sites are not likely to be methylated/demethylated in an independent fashion, but that a more complex process determines the methylation state within a region of the HPV16 genome.

DISCUSSION

In the present study, we used Illumina Next Gen single molecule sequencing to survey the methylation of the HPV16 genome in 12 samples and compared the result with pyrosequencing, which provides an average percent methylation for each CpG site. A consequence of using Next Gen sequencing technology was the ability to investigate the methylation pattern at the single-molecule level. Our results demonstrate that the Next Gen bisulfite sequencing protocol was comparable to the well-established pyrosequencing-based method for assaying HPV16 genome methylation (overall correlation = 0.92). The ability to analyze single molecules allowed us to test whether the process of CpG methylation at specific sites was independent. Thus with known percent CpG methylation at each site we compared the observed with the predicted methylation haplotypes. There was a significant difference indicating that CpG methylation of multiple CpG sites on a given fragment of the viral genome is not an independent process. In addition, utilizing DNA barcoding, multiple samples can be pooled together and run in a single sequencing reaction and the result for each single sample can be distinguished without ambiguity. Thus, the high-throughput nature of this technique facilitates large-scale clinical and epidemiological studies.

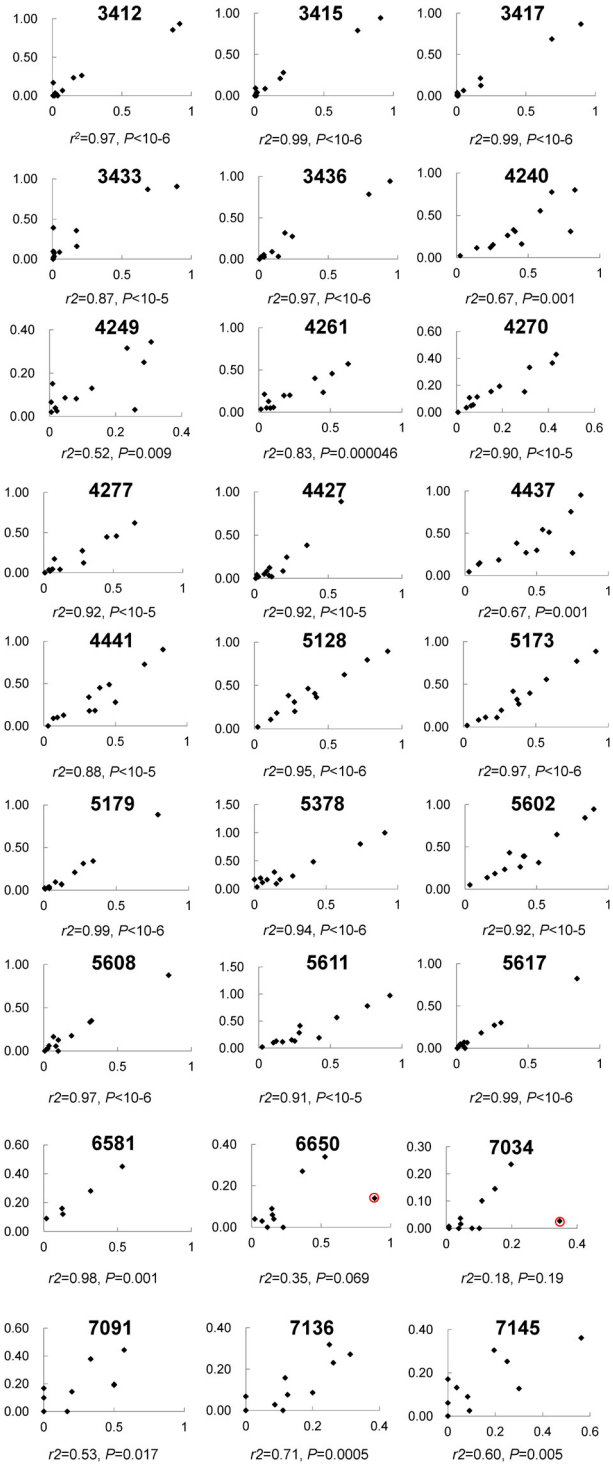

FIGURE 2 | The correlation of 27 individual CpG methylation sites between pyrosequencing and Next Gen sequencing. Each plot displays one CpG site and the location is indicated at the top. The x-axis indicates Illumina sequencing, the y-axis pyrosequencing for percent methylation.

Several CpG sites presented a relatively low read count compared with others. A careful inspection indicated that these were located in the middle of the amplicon and would thus appear at the end of the read in both strand. These end regions usually have a low base call quality, due to constraints of the sequencing technology and are, therefore, often filtered out prior to analysis. The Bismark-based analysis method utilized in this study doesn't exploit the gain in base call quality that can be obtained by comparing overlapping regions of paired-end reads, therefore the latter problem could be surmounted by improvements

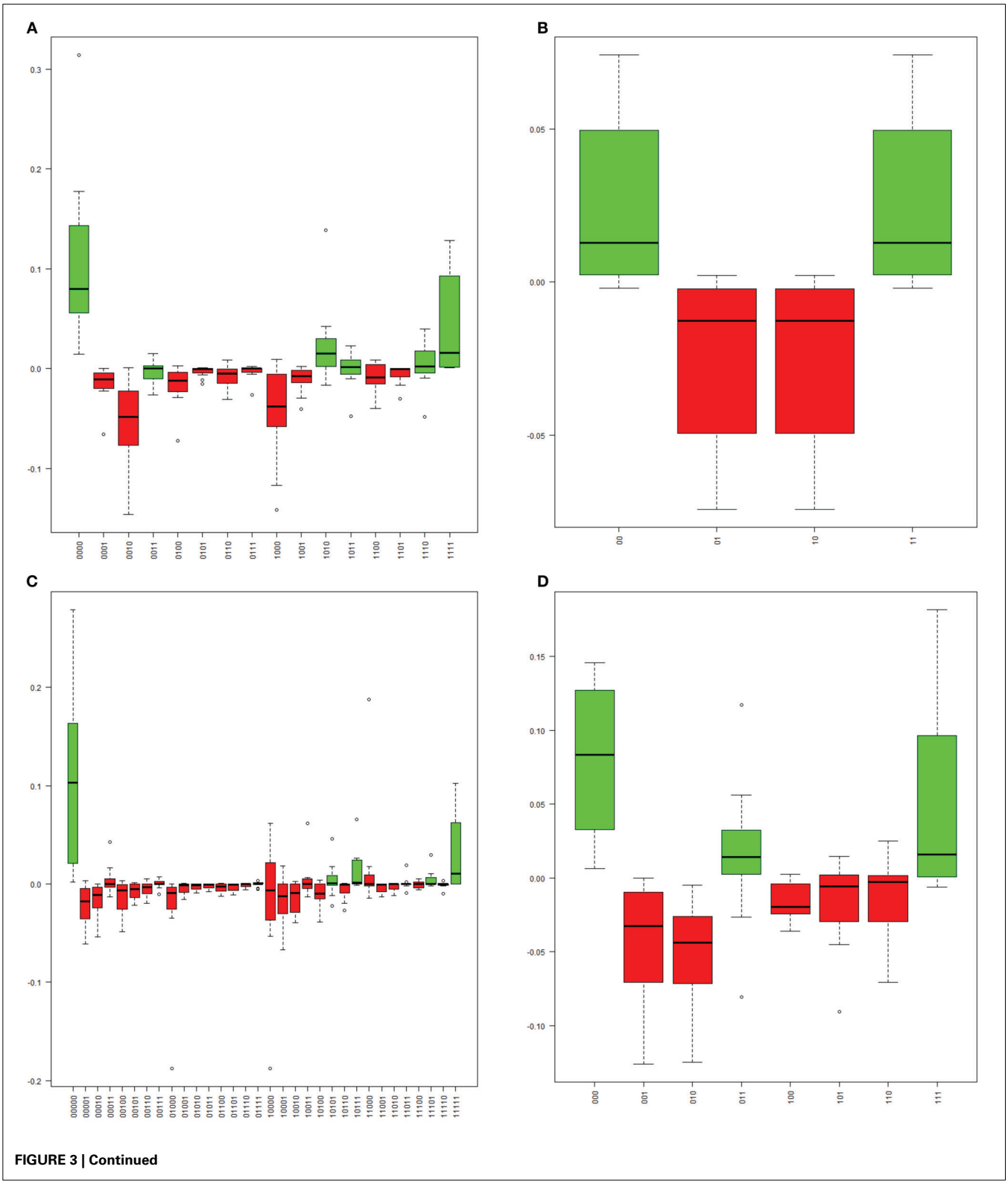

FIGURE 3 | Continued

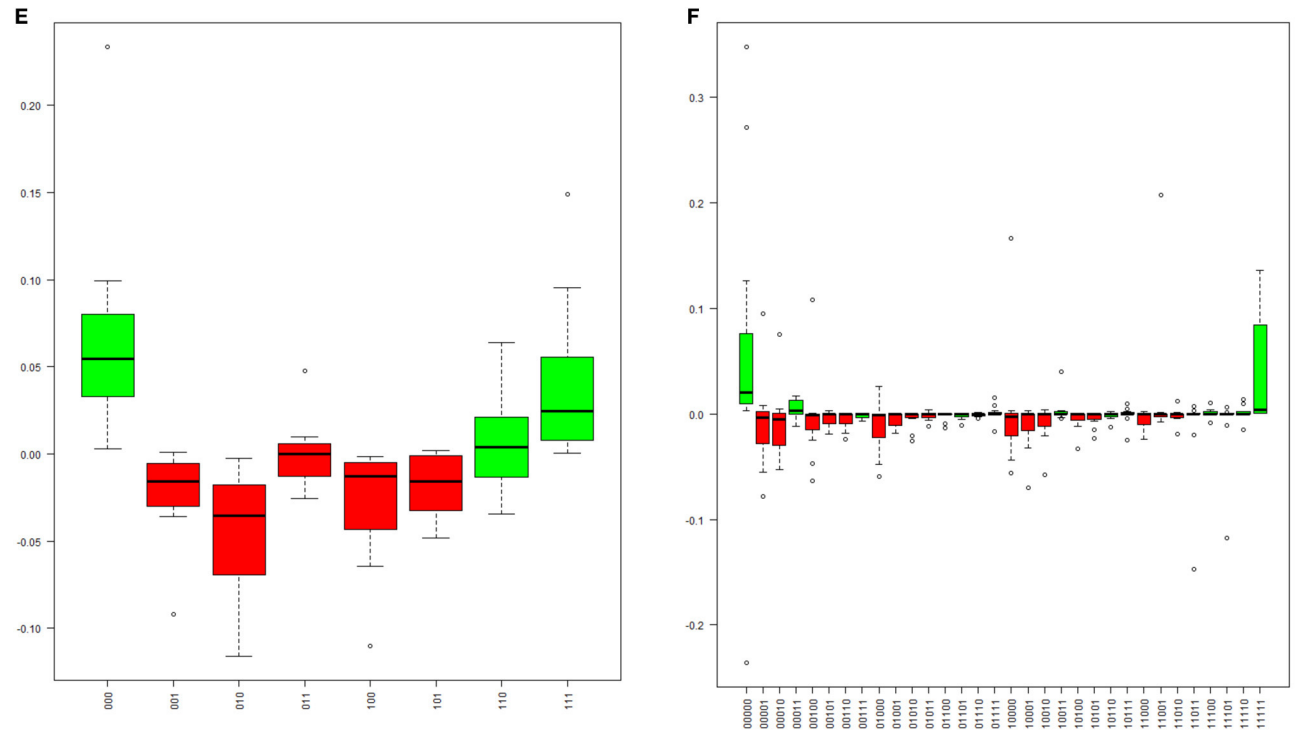

FIGURE 3 | The distribution of the differences between observed and expected frequencies for each "methylation haplotype" in assay L1_1 (A), L1_7 (B), L2_1 (C), L2_2 (D), L2_4 (E), and E2_1 (F). Each boxplot indicate one "methylation haplotype" combination. The green and red bars denote positive and negative value, respectively. **(A)** "Methylation haplotype" frequency differences in assay L1_1. **(B)** "Methylation haplotype" frequency differences in assay L1_7. **(C)** "Methylation haplotype" frequency differences in assay L2_1. **(D)** "Methylation haplotype" frequency differences in assay L2_2. **(E)** "Methylation haplotype" frequency differences in assay L2_4. **(F)** "Methylation haplotype" frequency differences in assay E2_1.

in the bioinformatics pipeline. Alternatively, we could shorten the amplicon, or generate longer reads, to facilitate equal read counts for all sites in an assay. However, there are limitations on shortening or lengthening the amplicons, due to the composition of sequences surrounding CpG sites that are used for the primers. Nevertheless, it is anticipated that with advances in Next Gen sequencing technologies longer fragment reads and improved quality will facilitate the use of the methods described in this report.

Despite the high consistency between next generation sequencing and pyrosequencing results, two CpG sites, 6650 and 7034, failed to yield a significant correlation. Both CpG sites had a relatively high read count (>1.14 and 0.15 million, respectively), thus indicating that the low correlation was not due to stochastic effects. A detailed audit identified one sample showing remarkable discrepancy between the two approaches in both CpG sites (see red circle in **Figure 2**). When this sample was removed from analysis, significant correlations were obtained for both CpG sites ($r^2 = 0.85$, $P = 0.00006$ for 6650 and $r^2 = 0.76$, $P = 0.001$ for 7034), which further verified the strong consistency between the two approaches. However, the reason for the discrepancy in this sample remains unclear. Possibilities include a nucleotide variation at this position, which would skew the results, and/or bisulfite- and PCR-induced artifacts. In addition, potential PCR biases could also result in lower than expected read numbers.

Examining methylation patterns on individual molecules of DNA is essential to understand the methyltransferase dynamics and the mechanism(s) by which methylation interacts with oncogenic HPV viral natural history and progression to cervical cancer. Based on cloning approaches, most previous studies suggested that methylation in promoter regions was more likely to be one (fully methylated) or zero (fully unmethylated) in human genomic DNA (Oates et al., 2006) and HPV genomes (Kalantari et al., 2004, 2009, 2010; Turan et al., 2006, 2007; Ding et al., 2009; Fernandez et al., 2009). However, due to the low number of single molecules analyzed (i.e., clones sequenced), the real composition might be substantially different. Next Gen sequencing, which can survey millions of molecules at the same time, can provide more insight into this issue. In our results, despite the relative excess of fully methylated and fully unmethylated molecules, a substantial proportion of molecules displayed a partial methylation pattern, which verified previous observations (Taylor et al., 2007) and hinted at the complex regulation of the methylation process. Whether there are dynamic changes in CpG methylation patterns remains to be determined.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00150/abstract

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T cell immunity as a tool for studying epigenetic regulation of cellular differentiation

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Cellular differentiation is regulated by the strict spatial and temporal control of gene expression. This is achieved, in part, by regulating changes in histone post-translational modifications (PTMs) and DNA methylation that in turn, impact transcriptional activity. Further, histone PTMs and DNA methylation are often propagated faithfully at cell division (termed *epigenetic* propagation), and thus contribute to maintaining cellular identity in the absence of signals driving differentiation. Cardinal features of adaptive T cell immunity include the ability to differentiate in response to infection, resulting in acquisition of immune functions required for pathogen clearance; and the ability to maintain this functional capacity in the long-term, allowing more rapid and effective pathogen elimination following re-infection. These characteristics underpin vaccination strategies by effectively establishing a long-lived T cell population that contributes to an immunologically protective state (termed *immunological memory*). As we discuss in this review, epigenetic mechanisms provide attractive and powerful explanations for key aspects of T cell-mediated immunity – most obviously and notably, immunological memory, because of the capacity of epigenetic circuits to perpetuate cellular identities in the absence of the initial signals that drive differentiation. Indeed, T cell responses to infection are an ideal model system for studying how epigenetic factors shape cellular differentiation and development generally. This review will examine how epigenetic mechanisms regulate T cell function and differentiation, and how these model systems are providing general insights into the epigenetic regulation of gene transcription during cellular differentiation.

Keywords: epigenetics, T cell Immunity, T cell memory, viral immunity, T cell differentiation

INTRODUCTION

Protection from the myriad of infectious pathogens we are exposed to on a daily basis largely results from the coordinated interaction of the cells and molecules of the mammalian immune system. Key cellular components of the adaptive immune system are white blood cells (*lymphocytes*) of which there are two types: B and T cells. B and T cells share features of adaptive immunity that include the ability to recognize pathogen components via clonal expression of a unique cell surface receptor; the ability to rapidly proliferate upon recognition of a pathogen, coincident with acquisition of cell lineage-specific immune functions; and finally, the ability to persist after the infection is cleared, combined with the capacity to "remember" the pathogen and respond more rapidly and vigorously upon re-infection (termed *immunological memory*).

T cells can be further divided into helper T (T_H) cells and cytotoxic (killer) T cells. T_H cells are distinguished by cell surface expression of CD4 (i.e. $CD4^+$ T cells) and promote effective immunity by secreting molecules that promote effective antibody and cellular responses upon infection. Further, T_H cells can differentiate into at least six subtypes, each characterized by expression of different immune molecules (termed *effector* molecules), which in turn, dictates that each T_H subset can play a different role in immunity to infection. In contrast, killer T cells, distinguished by cell surface expression of CD8 (i.e., $CD8^+$ T cells), are the "hit-men" of the immune system, typically locating and destroying virus-infected host cells, and thus limiting and contributing to the eventual clearance of infection. Killer T cells express a range of effector molecules that equip them to mediate this signature killing capacity.

A cardinal feature of T cell immunity is the ability of naïve T cells to undergo a program of proliferation and functional differentiation upon activation, resulting in a large pool of cells, all capable of recognizing a particular pathogen, and that have acquired the immune functions necessary to control and eventually clear infection (Kaech et al., 2002; van Stipdonk et al., 2003; **Figure 1**). Once an infection is cleared, the majority of the expanded effector T cell population dies, leaving behind a small pool of long-lived cells that can recognize the same pathogen that triggered their initial activation (termed memory T cells; Marshall et al., 2001; Kaech et al., 2002; La Gruta et al., 2004). Importantly, these memory T cells produce a broader array of immune molecules than naïve cells, and in larger quantities, and unlike naïve cells, can respond to infection without the need for further differentiation (Lalvani et al., 1997; Agarwal and Rao, 1998; Oehen and Brduscha-Riem, 1998; Veiga-Fernandes et al., 2000). These features, combined with persistence at a higher frequency, enable memory T cells to respond more rapidly upon secondary infection, enabling earlier control and clearance of infection (**Figure 1**),

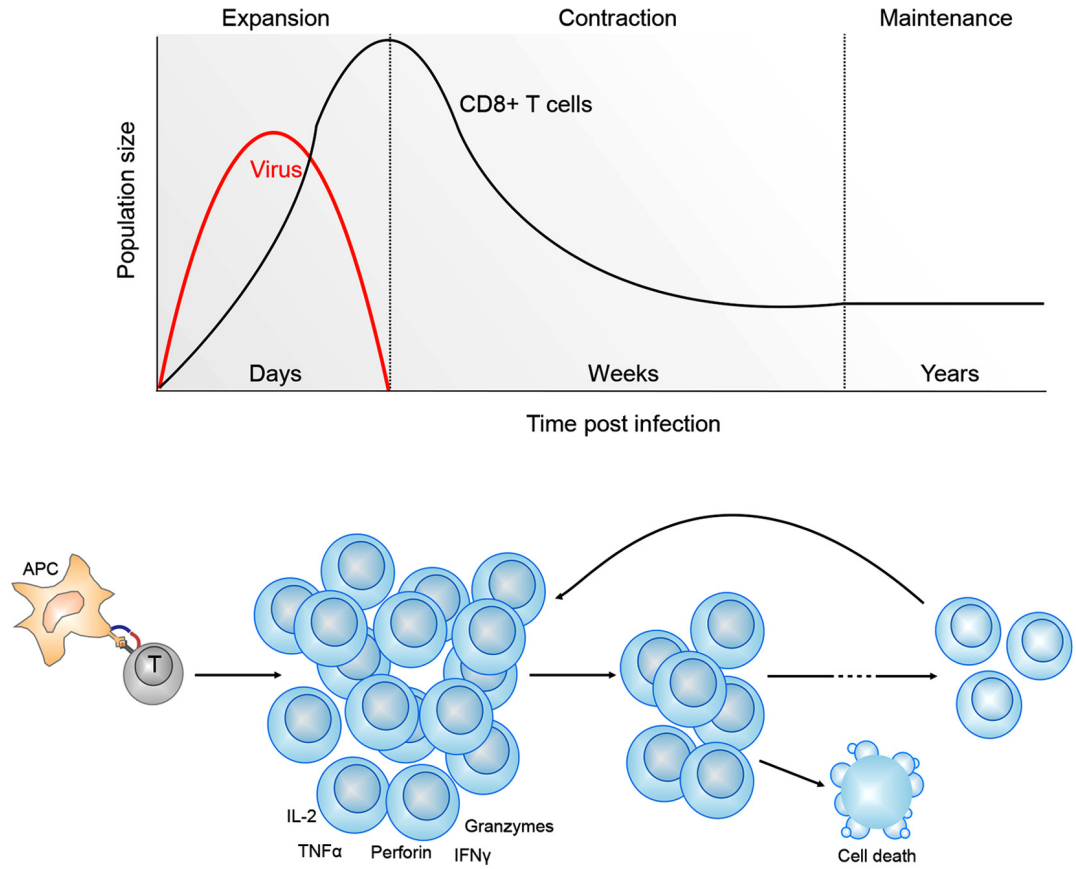

FIGURE 1 | Kinetics of CD8$^+$ T cell differentiation following viral infection. Shown is a typical CD8$^+$ T cell response to a acute viral infection. Antigen presenting cells (APC) present viral antigens to CD8$^+$ T cells. This initiates a program of clonal expansion and differentiation into effector CD8$^+$ T cells capable of lineage-specific effector functions, including the ability to secrete pro-inflammatory (TNF-α, IFN-γ) and cytotoxic (perforin, granzyme) molecules. Following viral clearance, the CD8$^+$ T cells undergo an extensive contraction phase, mediated by programed cell death. The remaining memory CD8$^+$ T cells can persist in the host for years. In the event of a secondary exposure to the same virus, memory CD8$^+$ T cells can rapidly expand and acquire effector functions.

and together, these features of memory T cells provide the basis of T cell-mediated immunity. Importantly, our understanding of the molecular factors that shape cell fate decisions and drive acquisition of T cell effector function is limited, and questions remaining to be determined include how a T cell decides to be a memory versus an effector cell, and what are the molecular mechanisms that enable stable maintenance of rapid effector function within memory T cells in the long-term? In this review we describe what we think are some of the more interesting and important studies addressing these and similar questions, with the aim of demonstrating the utility of the immune system as a tool for studying epigenetics and cellular differentiation. We start by discussing the diversity of T cells phenotypes, before describing our current understanding of how epigenetic regulation influences how these distinct functional T cell populations arise and are maintained.

DEFINING THE DIFFERING ROLES OF DISTINCT T CELL SUBSETS IN MEDIATING IMMUNITY

An important feature of T cell immunity is the enormous proliferative potential and functional plasticity of naïve T cells. Acquisition of lineage-specific T cell effector functions is clearly linked to an extended proliferative response, suggesting that T cell activation engages a differentiation program that facilitates effector gene expression (Gett and Hodgkin, 1998; Lawrence and Braciale, 2004; Jenkins et al., 2008). An example of T cell functional plasticity is found after activation of naïve T_H cells that have the potential to differentiate into distinct T cell subsets, largely defined by the soluble effector molecules they secrete (**Figure 2**; Zhu et al., 2010). The best characterized of these are the T_H1 and T_H2 subsets, however, other subsets include T_H17, Tregs (regulatory T cells), T_{FH} (follicular T_H cells) and the more recently described T_H9 cells (**Figure 2**). T_H1 and T_H2 T cells are best characterized by their capacity to secrete interferon-gamma (IFN-γ) and interleukin (IL)-4, respectively. The tailoring of T_H cell responses into distinct functional lineages is a consequence of integration of multiple signals that are present during initial T cell activation (**Figure 2**). For example, naïve T_H cell activation in the presence of the pro-inflammatory molecules, IFN-γ and IL-12, induces T_H1 differentiation while IL-4 is a potent inducer of T_H2 differentiation (Zhu et al., 2010). Importantly, induction of transcription

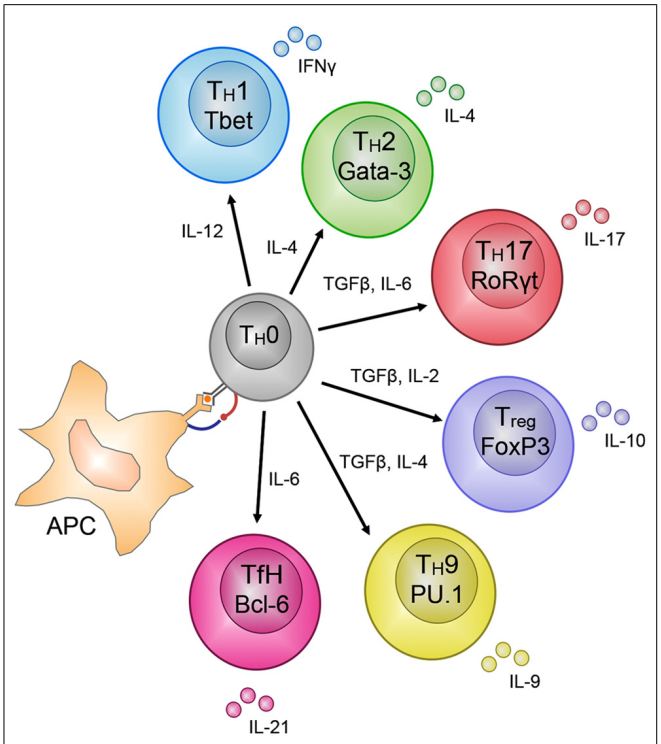

FIGURE 2 | CD4$^+$ T_H – cell subset differentiation. CD4$^+$ T cells show remarkable plasticity and are able to differentiate into many different subsets based on the soluble molecules secreted during priming of the subsets by antigen presenting cells (APC), e.g., IL-12 for T_H1 cells. The different subsets can be distinguished by the transcription factors that regulate and maintain their lineage-specific effector functions, e.g., T-bet for T_H1 cells. The molecules secreted by these subsets, e.g., IFN-γ for T_H1 cells, are finely tuned to control the pathogen that mediated the release of the specific molecules by the APC during activation of the T_H0 cells into the various subsets.

factor (TF) expression by extracellular signals received by activated T_H cells drives T cell differentiation (Kanno et al., 2012); T_H1 differentiation is dependent on STAT1 activation and expression of the T-box TF *Tbx21* (T-bet; Djuretic et al., 2007). Conversely, IL-4 signals activate STAT6 resulting in up-regulation of the TF *Gata3* (Ansel et al., 2003). T_H17 differentiation is associated with IL-6/IL-21 induced expression of the RORγT TF (Dong, 2008) and Treg differentiation with FoxP3 (reviewed in Josefowicz et al., 2012). Such is the importance of these TFs in directing naïve T_H cell commitment to a specific lineage that they are used as definitive markers of T_H subset differentiation (**Figure 2**).

As we learn more about these T_H subsets, it is clear that there is heterogeneity of effector function within a responding T cell population such that no one immune response is uniquely represented by a single T_H subset. Rather, there is tailoring of the total T cell population such that a particular subset may be over-represented. For example, T_H1 type cells dominate the response to extracellular bacterial infections, and in this case, expression of the T_H1 cytokine, IFN-γ, is required to promote immune control of these particular pathogens. In this way, the immune system ensures that the most appropriate immune response is engaged to promote control of infection.

Killer T cells contribute to the control and eventual elimination of intracellular bacteria, viruses and tumor challenges via the coordinated interplay of varied effector mechanisms (Russ et al., 2012). This includes the production of pro-inflammatory cytokines such as IFN-γ and tumor necrosis factor alpha (TNF-α; La Gruta et al., 2004) and the expression of cytolytic effector molecules including perforin (Pfp; Kagi et al., 1994) and the granule enzymes (granzymes, Gzm) A, B, and K (Jenkins et al., 2007; Peixoto et al., 2007; Moffat et al., 2009). Whilst killer T cells are not typically associated with commitment to distinct lineages, it is clear that specific TFs are also important in regulating their differentiation and acquisition of effector function. For instance, two T-box TFs, T-bet (encoded by *Tbx21*) and Eomesodermin (encoded by *Eomes*; Intlekofer et al., 2005) play essential roles in effector CTL differentiation. Analogous to its role in T_H1 T cells, T-bet is rapidly up-regulated upon naïve killer T cell activation and directly regulates the rapid acquisition of IFN-γ production (Cruz-Guilloty et al., 2009). Eomesodermin, a homolog of T-bet, was originally implicated in the regulation of CD8$^+$ T cell granzyme B expression (Pearce et al., 2003), however, recent studies suggest that Eomesodermin is expressed later during CTL differentiation and contributes more to acquisition of perforin expression, while helping sustain the capacity to express IFN-γ (Cruz-Guilloty et al., 2009). IL-2 is a cytokine required for inducing proliferation and survival of activated T cells (Miyazaki et al., 1995). Importantly, high levels of IL-2 signaling at the time of killer T cell activation contribute to granzyme B and perforin expression via STAT5 activation (Janas et al., 2005; Pipkin et al., 2010). In this way, killer T cells integrate signals delivered by extrinsic inflammatory and survival signals during infection that promote effector T cell differentiation.

While the importance of these TFs in lineage determination is clear, exactly how they convey their effects on T cell differentiation is less well understood. As we describe below, at least some of these TFs (i.e., STAT6 and T-bet) exert their effects on T cell differentiation through the recruitment of chromatin modifying enzymes to the sites of TF binding (Lewis et al., 2007; Miller et al., 2010; Onodera et al., 2010). Further, such mechanisms of TF action are known from other systems, suggesting that this mechanism may be common. Thus it appears that TFs and chromatin modifying enzymes cooperate, with the former providing the DNA binding specificity, and the latter the catalytic activity. As described below, once modified, the chromatin can then serve as a substrate for yet other protein complexes that physically rearrange the chromatin, making it more or less permissive for transcription.

EPIGENETIC REGULATION OF CELLULAR DIFFERENTIATION

Cellular differentiation is regulated by the strict spatial and temporal control of gene expression, which at the most fundamental level, is controlled by modulating access of the transcriptional machinery to gene regulatory regions, including promoters and enhancers. In eukaryotic cells, transcription occurs in the context of chromatin – a complex formed between the genome and histone protein octomers (termed nucleosomes), around which the DNA is wound. As the intimate nature of the nucleosome–DNA interaction can occlude binding of the transcriptional machinery,

preventing transcription, this interaction must be tightly regulated to allow appropriate gene expression; this is achieved by controlling the positioning of nucleosomes, and by modulating their affinity for DNA. Histone post-translational modifications (PTMs) are key regulators of changes in chromatin structure that then influence gene expression. Importantly, these modifications are often propagated faithfully at cell division (termed *epigenetic* propagation), maintaining cellular identity in the absence of signals driving cellular differentiation.

Histone PTMs occur primarily at the solvent exposed N-termini, and can take a number of forms, including acetylation, methylation, and ubiquitination (Kouzarides, 2007). The transcriptional consequences of these modifications are then manifested either due to the direct biophysical consequences of the modification, or through the catalytic activities of proteins and protein complexes that recognize and bind modified histones. For instance, acetylation, which reduces the net positive charge on the nucleosome, results in decreased stability of histone associations with the negatively charged DNA, promoting transcription. Therefore, by balancing the expression and genomic localization of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add and remove acetyl groups, respectively, transcription can be activated or repressed (reviewed in Bannister and Kouzarides, 2011). Alternatively, it appears that the effects of histone methylation are conveyed indirectly, with methylated histones serving as a substrate for protein complexes that bind and reconfigure the chromatin. Importantly, histone methylation is associated with both active and repressed transcription, depending on the residue methylated. For example, trimethylation of lysine 4 of histone H3 (H3K4me3) is enriched at promoters of many actively transcribed genes, while trimethylation of lysine 27 of H3 (H3K27me3) is associated with transcriptionally repressed genes (Barski et al., 2007; Wang et al., 2008; Ernst et al., 2011).

Interestingly, activating and repressive modifications can co-localize, even occurring on the same nucleosome, and it appears that the combination and balance of these modifications serves to tune levels of transcription (Wang et al., 2008). Importantly in the context of cellular differentiation, co-localization of opposing PTMs is also employed to poise genes for rapid activation or repression (Bernstein et al., 2006).

As well as controlling access of the transcriptional machinery to the DNA template by modulating nucleosome positioning, transcription is controlled epigenetically by changing the structure of the DNA itself, through the addition and removal of bulky methyl groups. DNA methylation occurs predominantly at cytosine residues occurring in the context of cytosine–guanine di-nucleotides (termed *CpG methylation*), and results in transcriptional repression, both through steric hindrance of transcriptional activator binding (as described below for FoxP3), and through recruitment of methyl-CpG-binding domain proteins (MBD), that in turn, recruit HDACs. For instance, MBD2 has been shown to directly recruit HDAC1, resulting in histone deacetylation, and transcriptional repression (Ng and Bird, 1999). Thus, CpG methylation does not represent a separate system of epigenetic regulation to that described for histone PTMs, but rather is part of the same, inter-connected system.

EPIGENETIC CONTROL OF CD8$^+$ T CELL EFFECTOR FUNCTION

The function of CD8$^+$ killer T cells is defined largely by their capacity to produce effector molecules such as anti-viral cytokines and cytolytic molecules. As with naïve T_H cells, the *Ifng* locus of naïve CD8$^+$ killer T cells is heavily marked by the repressive H3K27me3, with little or none of the permissive H3K9Ac or H3K4me3 PTMs (Denton et al., 2011). Upon differentiation from naïve to effector killer T cells, transcriptional activation of *Ifng* is associated with removal of H3K27me3 and deposition of the permissive H3K9Ac and H3K4me3 PTMs (Denton et al., 2011). Further, in effector CD8$^+$ killer T cells, the *Ifng* locus had reduced levels of total histone H3, indicating nucleosome evacuation from the region, presumably to allow the transcriptional machinery to access the promoter. Taken together, these data suggest that reconfiguration of the chromatin structure within naïve cells is necessary to enable *Ifng* transcription. Moreover, there appears to be conservation of chromatin restructuring and histone PTM modification with a similar pattern observed within other effector gene loci such as granzyme B (*Gzmb*; Juelich et al., 2009) and granzyme A (*Gzma*; Lauren Hatton, Michelle Nguyen, Brendan Russ, and Stephen Turner, data not shown).

As mentioned earlier, memory T cells maintain the capacity for rapid effector gene expression without the need for further differentiation. Strikingly, the permissive signature within the *Ifng* promoter of effector CD8$^+$ killer T cells is maintained into long-term memory. Further, although memory CD8$^+$ killer T cells exhibit little *Ifng* transcriptional activity prior to re-infection, RNA polymerase (RNAp) is docked at the *Ifng* promoter (Denton et al., 2011; Zediak et al., 2011). Taken together, these data suggest that the ability of memory cells to produce IFN-γ rapidly following re-infection is due to the promoter being maintained in a transcriptionally permissive state, and that the rate-limiting step in re-expression of IFN-γ is transcriptional initiation (**Figures 3A,B**). It remains to be determined whether transcriptional poising (as measured by RNAp docking) at other effector gene loci with low transcriptionally activity is evident within memory CD8$^+$ killer T cells. Further, it would be of particular interest to determine the extent of transcriptional poising in memory T cells at a genome-wide level and compare this to naïve and effector cells. In this way, it could determined to what extent transcriptional poising underpins memory T cell characteristics. Moreover, given the direct effect of acetylation on nucleosome density, increased acetylation in memory cells (Araki et al., 2008; Denton et al., 2011) may explain their ability to produce more IFN-γ upon re-infection (La Gruta et al., 2004). In this way, memory T cells are reconfigured at the chromatin level to exhibit more potent effector function and this, in turn, helps ensure more effective and more rapid control of a secondary infection.

Recently, Scharer et al. (2013) applied global approaches to compare CpG methylation in naïve and effector CD8$^+$ T cells. Combining immunoprecipitation of methylated genomic regions with high-throughput sequencing (MeDIP-seq), they identified ∼650,000 regions that were differentially methylated between the two populations, indicating the likely importance of CpG methylation as a means of regulating CD8$^+$ T cell differentiation. As expected, CpG methylation of gene promoters was inversely correlated with gene transcription, but interestingly, ∼40% of

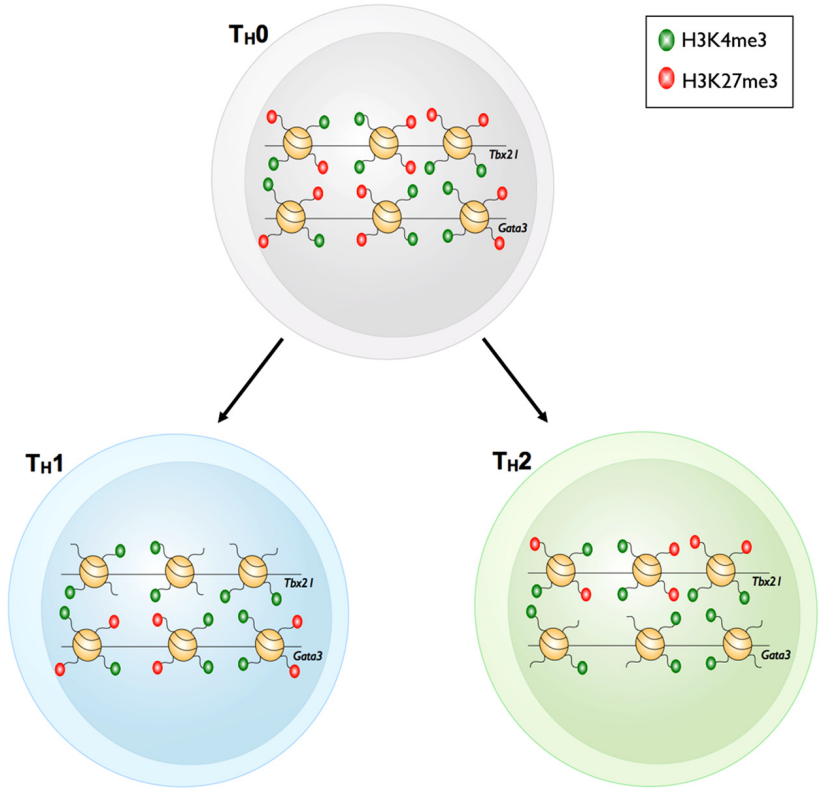

FIGURE 3 | Bivalency of master regulator gene loci in T_H1 and T_H2 cells. Prior to differentiation of T_H0 cells into the T_H1 or T_H2 cell subsets, the master regulator loci of each subset, *Tbx21* and *Gata3* respectively, have both active H3K4me3 and repressive H3K27me3 marks. The bivalent *Tbx21* locus loses the repressive H3K27me3 mark upon differentiation into T_H1 cells. However, the *Gata3* locus remains bivalent. The reverse is true for T_H2 cells whereby *Gata3* loses the repressive H3K27me3 mark yet retains bivalency at the *Tbx21* locus.

genomic regions that differed in methylation state between naïve and effector occurred away from gene promoters. Further analysis showed that these promoter-distal regions largely overlapped candidate transcriptional enhancers identified in developing T cells in the thymus using next-generation sequencing and chromatin immunoprecipitation (ChIP-Seq) for enhancer-enriched histone PTMs (H3K27Ac and H3K4me1). Finally, when these putative enhancers were surveyed for over-represented TF binding sites, known and putative transcriptional regulators of $CD8^+$ T cell differentiation were identified. Therefore, it seems likely that CpG methylation is employed to regulate $CD8^+$ T cell differentiation, both by influencing protein–DNA interactions at gene promoters, and at transcriptional enhancers. Further, this study highlights the utility of such approaches in the identification of regulatory circuits controlling cellular differentiation.

$CD4^+$ T CELL DIFFERENTIATION: A MODEL FOR UNDERSTANDING EPIGENETIC REGULATION

The fact that distinct signals are capable of driving naïve T_H cell differentiation *in vitro* into well-defined subsets makes $CD4^+$ T cell activation a useful model for understanding how epigenetic regulation can influence cellular differentiation and fate determination. Comparison of the epigenetic profiles of signature effector gene loci within T_H1 and T_H2 cells has been particularly informative. In response to T_H1 differentiation signals, the IFN-γ locus of naïve T_H cells is remodeled to a permissive epigenetic signature that reinforces and heritably maintains IFN-γ gene expression in the long-term. At the same time, the IL-4 locus is remodeled to have a repressive epigenetic signature resulting in the shutdown of IL-4 gene expression.

Recent work using ChIP-Seq has been instrumental in providing genome-level insights into how epigenetic processes might regulate T_H cell fate selection. For instance, genome-wide comparison of H3K4me3 and H3K27me3 distribution in naïve, T_H1, T_H2, and T_H17 cells, combined with global transcriptional profiling demonstrated that the distribution of just two histone PTMs (H3K27me3 and H3K4me3) could provide a simple explanation for the differences in phenotypes observed amongst these different T cell subsets.

For example, upon differentiation from a naïve T_H state into the various T_H subsets, H3K4me3 deposition was observed at signature effector gene loci within distinct T_H subsets (e.g., *Ifng* in T_H1, *Il4* in T_H2, and *Il17* in T_H17). Moreover, H3K27me3 deposition was correlated with transcriptional shutdown of effector gene loci that are characteristic of other T_H subsets (Wei et al., 2009; **Table 1**). One might have expected that changes in the epigenetic signatures within gene loci encoding lineage-defining TFs, would simply reflect those observed for lineage-specific effector gene loci.

Table 1 | Major histone methylation patterns at lineage-specific effector gene loci in differentiated CD4$^+$ T$_H$ populations.

	T_H1	T_H2	T_H17
Ifng	H3K4me3$^+$	H3K27me3$^+$	H3K27me3$^+$
Il4	H3K27me3$^+$	H3K4me3$^+$	H3K27me3$^+$
Il17a	H3K27me3$^+$	H3K27me3$^+$	H3K4me3$^+$

For example, the gene locus encoding the T_H17 TF *Rorc* (retinoid-related orphan receptor-γ) was decorated with H3K27me3 in the naïve state, and only acquired H3K4me3, and losing H3K27me3 after T_H17 differentiation. In contrast, the repressive H3K27me3 signature was reinforced under T_H1 and T_H2 differentiation conditions (Araki et al., 2009). However, this was not always the case. The *Tbx21* (T_H1) and *Gata3* (T_H2) gene loci in naïve T_H cells were marked with both H3K4me3 and H3K27me3 (termed bivalent loci), and whilst these loci resolved to a permissive epigenetic signature (H3K4me3$^+$/H3K27me3$^-$) under T_H1 and T_H2 differentiation conditions, respectively, they did not acquire a repressive epigenetic signature when differentiated into opposing lineages, but rather maintained a bivalent state (**Figure 4**). Similarly, the *Tbx21* locus within T_H17 cells was also maintained in a bivalent state. In the case of T_H17 cells, re-stimulation of T_H17 cells in the presence of IL-12 resulted in expression of IFN-γ and conversion to a T_H1 phenotype. This was associated with acquisition of permissive epigenetic signatures at the IFN-γ locus and IL-12-dependent STAT-4 and *Tbx21*-dependent epigenetic silencing of the T_H17 associated *Rorc* locus (Mukasa et al., 2010). Given that epigenetic bivalency is considered a mechanism for poising gene loci for rapid activation or repression, these data suggest that CD4$^+$ T_H subsets can maintain some level of functional plasticity despite lineage commitment. It is tempting to speculate that this provides the immune system with inherent flexibility, allowing the redirection of pathogen-specific T_H responses. In the case of T_H17 cells, it may represent a mechanism that enables switching from a potent inflammatory T_H17 response to a less damaging, more controlled effector response. It also suggests that targeted interventions that drive epigenetic reprograming of T_H responses involved in autoimmune diseases (such as T_H17 in the context of multiple sclerosis) might represent novel immunotherapeutic targets that could lead to decreased pathology.

A number of studies have also defined roles for CpG methylation in the differentiation of CD4$^+$ T cells. For instance, regulated deposition of CpG methylation is important for maintenance of CD4$^+$ T cells that have differentiated to become Tregs. Zheng et al. (2010) showed that mice that had a conserved non-coding sequence (CNS2) within the *Foxp3* locus deleted, had wild-type levels of Tregs in young mice, but greatly reduced numbers in older mice. Further, this was due to a loss of FoxP3 expression in the peripheral Tregs, indicating a role for this TF, not just in Treg differentiation, as described previously (reviewed in Josefowicz et al., 2012), but also in the maintenance of the Treg phenotype. Finally, they were able to show that FoxP3 binds to the CNS2 in Tregs, but not in naïve CD4$^+$ T cells, and that FoxP3 binding was dependent on differentiation-induced demethylation of CpG sites within this region. Thus, FoxP3 binding to CNS2, enabled by differentiation-dependent CpG demethylation, results in a feed-forward signal that enforces Treg fate.

ENZYMES MODULATING HISTONE MODIFICATION DURING T CELL DIFFERENTIATION

Whilst there is a growing understanding of how changes in histone PTMs correlate with dynamic changes in T cell effector functions, it is less clear how the factors that write or erase these histone PTMs are involved in directing T cell differentiation during an immune response. Using the CD4 T_H1 versus T_H2 model system, Allan et al. (2012) examined the role of the histone methyltransferase, Suv39H1, in epigenetic regulation of T_H2 differentiation. Suv39H1 specifically trimethylates H3K9 – a PTM typically associated with transcriptional silencing of gene loci that is in turn recognized by heterochromatin protein 1α (HP1α; Lachner et al., 2001; Peters et al., 2001). Docking of HP1α onto H3K9me3$^+$ gene loci in turn recruits HDAC1 and 2, and the transcriptional repressor MBD1 (Fujita et al., 2003). In this way, H3K9 acetylation, a PTM associated with transcriptional activation, is limited. Thus, Suv39H1-mediated trimethylation of H3K9 is an initial step that triggers histone deacetylation and binding of transcriptional repressor protein complexes that stably silence targeted loci.

While it was possible to skew naïve T_H cells from Suv39H1 gene-deficient mice into the T_H2 lineage *in vitro*, these cells could be reprogrammed to secrete IFN-γ after re-culture in T_H1-inducing conditions. Thus, a lack of Suv39H1 resulted in an inability to stably repress T_H1 effector gene expression. This appeared largely due to an inability of Suv39H1 gene-deficient T_H2 cells to stably silence the transcriptional potential of the key T_H1 TF, T-bet (encoded by *Tbx21*). Consistent with this, Suv39H1-deficient T_H2 cells exhibited increased levels of histone acetylation at the *Tbx21* locus. Of particular interest was the fact that T_H1 cells from Suv39H1 gene-deficient mice stably repressed expression of T_H2 effector genes after re-culture in T_H2-inducing conditions. This suggests that histone PTMs, other than H3K9me3, are used to heritably silence T_H2 effector gene expression during T_H1 differentiation, or alternatively, other H3K9 methyltransferases (such as GP9a, SETDB1/2, or Suv39H2) are utilized by T_H1 cells to establish H3K9me3 repression at T_H2 gene loci. Such a hypothesis would require selective targeting of H3K9 methyltransferases to specific gene loci and this could potentially be facilitated via interactions with specific TFs that bind to specific regulatory regions within target gene loci. Such a precedent has been observed with the demonstration that members of the T-box family of TFs serve to recruit histone methyltransferases to signature effector gene loci within T_H1 cells to promote gene transcription (Lewis et al., 2007). Thus, this mechanism could potentially be a way of ensuring that only certain gene loci are targeted for silencing within either T_H1 or T_H2 cell subsets, thereby ensuring appropriate gene expression, and appropriate immune function.

Taken together, these data demonstrate that Suv39H1 acts to specifically promote T_H2 lineage commitment via epigenetic silencing (via H3K9me3 deposition) of gene loci that drive T_H1 fate commitment (**Figure 5**). One interesting observation was the fact that despite T_H2 cells exhibiting an overall repressive signature

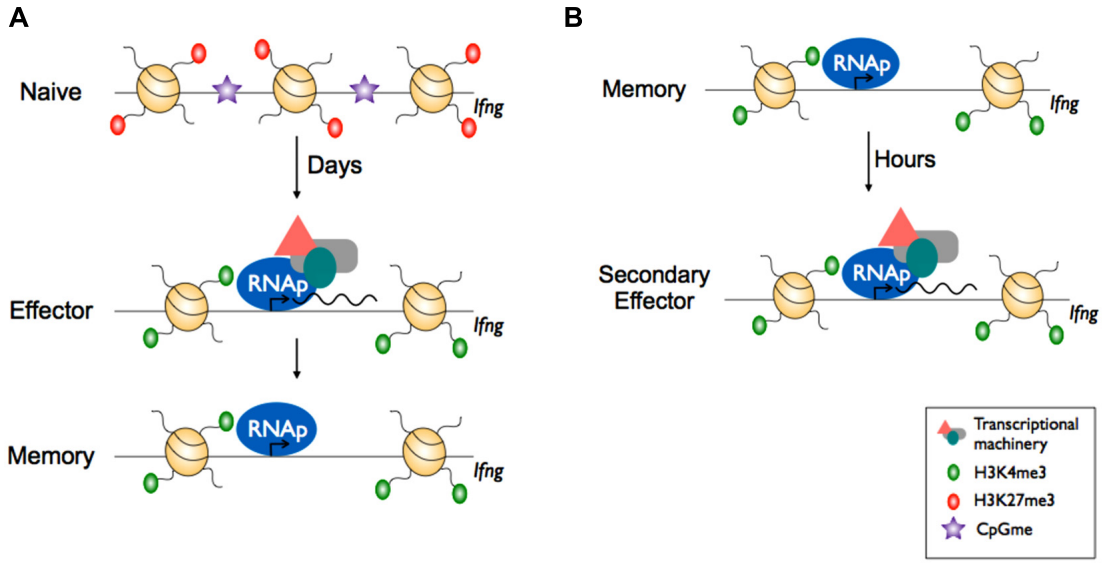

FIGURE 4 | Epigenetic reprogramming within effector gene loci of CD8$^+$ memory T cells enables rapid effector function. **(A)** In naïve CD8$^+$ T cells, effector loci such as *Ifng* display repressive epigenetic marks e.g., H3K27me3 and is inaccessible to transcriptional machinery due to the heterochromatin structure. Upon activation, the chromatin is remodelled whereby it acquires active epigenetic marks e.g., H3K4me3 at key effector loci and nucleosome exit to make the loci accessible by transcriptional machinery and RNA polymerase II (RNAp), allowing transcription. Upon differentiation to memory CD8$^+$ T cells, the chromatin retains the permissive H3K4me3 mark and RNAp remains docked. **(B)** Upon re-infection, the effector loci in memory CD8$^+$ T cells is poised and can undergo rapid transcription.

within the *Tbx21* locus, there is still evidence of H3K4me3 deposition at the promoter. Thus, pharmacological interventions that block Suv39H1 activity could serve to promote *Tbx21* transcription and subsequent T_H1 gene expression. The clinical relevance was made apparent when treatment of mice with a Suv39H1 inhibitor, was able to ameliorate T_H2 cell driven tissue damage in a model of allergic asthma. Treatment of mice resulted in higher numbers of T_H1 T cells, and redirected the immune response toward a less pathogenic state. This study highlights the potential for manipulating epigenetic programing of effector T cell responses using small molecule inhibitors to either promote immunity, in the case of vaccination, or suppress the damage caused by inappropriate immune responses, as is found in autoimmune disease or allergy.

EPIGENETIC CONTROL OF T CELL DEVELOPMENT

Mature, immunologically naïve CD4$^+$ and CD8$^+$ T cells develop in the thymus from multipotent hematopoietic progenitors. Within the thymus, these progenitors progress through at least ten phenotypically distinct stages of development, before exiting the thymus as mature, naïve CD4$^+$ or CD8$^+$ T cells (reviewed in Rothenberg et al., 2010). Hematopoietic progenitor cells enter the thymus expressing neither CD4 nor CD8, and are hence termed double-negative (DN). They then progress through five phenotypically distinct stages of maturation (DN1, DN2a, DN2b, DN3a, and DN3b) before up-regulating both CD4 and CD8 (termed *double-positive*, DP), and following further differentiation, permanently down-regulate either CD4 or CD8 (becoming *single-positive*, SP), before migrating from the thymus. Importantly, events occurring in the thymus not only determine lineage commitment (CD4$^+$ versus CD8$^+$), but also the potential fates of mature T cells; commitment to the CD8$^+$ lineage results in cells with specialized cytotoxic potential, while commitment to the CD4$^+$ lineage results in naïve cells with much broader differentiation potential. Thus an interesting question is when is fate potential programed, and what is the contribution of epigenetic mechanisms?

Rothenberg's group recently studied the molecular signatures that underpin lineage commitment and differentiation occurring in the early phases (DN1–DP) of thymic development in mice (Zhang et al., 2012). Combining ChIP-Seq and RNA-Seq, they determined the global distribution and dynamics of three histone PTMs, and the transcriptional signatures of immature thymocytes, at each stage of differentiation. Specifically, they studied the distribution of H3K9/14Ac (Gett and Hodgkin, 1998; Djuretic et al., 2007) and H3K27me3, which is enriched within the promoters and enhancers of actively transcribed and repressed genes, respectively, and H3K4me2, which defines active enhancer elements, and is often associated with transcriptionally poised gene promoters.

Aside from highlighting the extraordinary complexity of the mechanisms regulating T cell differentiation, this study provided novel insights into the mechanisms controlling cellular differentiation. A key finding of the paper was that the repressive H3K27me3 PTM is often deposited at genes after transcription has already been shutdown, indicating that the likely role of this modification is not to directly regulate transcription, as is generally accepted, but rather to stabilize repression. Further, there appeared to be multiple mechanisms of transcriptional repression, since only approximately a third of genes that were

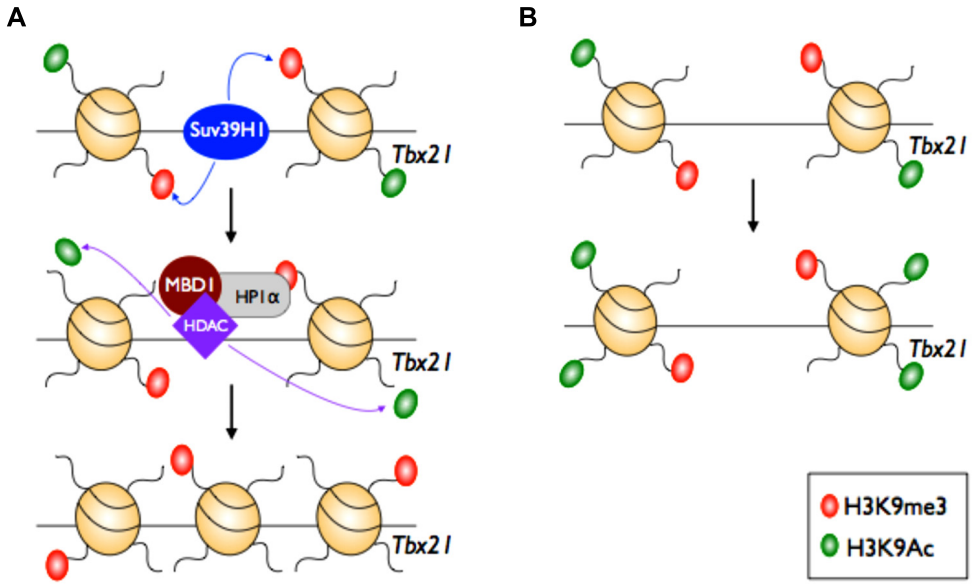

FIGURE 5 | Epigenetic maintenance of T_H2 lineage commitment. In the T_H2 cell subset, the master regulator of T_H1 cells (*Tbx21*) is silenced. The histone methylase Suv39H1 adds the repressive H3K9me3 mark at the *Tbx21* locus. This initiates recruitment and docking of heterochromatin protein 1 alpha (HP1α), histone deacetylase (HDAC) 1 and 2, and methyl-binding domain protein (MBD1). HDACs then remove the active H3K9ac mark to maintain silencing, mediated by H3K9me3, at the *Tbx21* locus.

developmentally repressed during thymic differentiation were associated with H3K27me3.

In contrast, histone acetylation was strongly and temporally correlated with mRNA levels, indicating that this modification may be added just prior to transcription, and as such, likely represents a rate-limiting step in the activation of gene transcription. Further histone deacetylation may be a key means of gene repression during T cell differentiation since this observation also implies that acetylation is either rapidly removed from promoters following transcriptional repression, or is a direct cause of transcriptional repression. Finally, H3K4me2 deposition often preceded transcription. This finding is consistent with a previous study showing that H3K4me2 marks lineage-specific hematopoietic genes in multipotent progenitor cells, in the absence of transcription (Orford et al., 2008). As many H3K4me2-marked genes lost this modification as differentiation preceded (toward an erythroid fate), it appears that H3K4me2 poises genes for a rapid response to differentiation signals, whereby, following the receipt of signals, non-lineage-specific genes lose H3K4me2 and are not expressed, while at lineage-specific genes, H3K4me2 is converted to H3K4me3 – a positive correlate of transcription.

Taken together, these studies suggest that different histone PTMs play distinct roles in transcriptional regulation; acetylated histones appear to rate-limit transcription, probably by directly regulating promoter accessibility, while H3K27me3 appears to operate "after the fact" – stabilizing transcriptional repression rather than directly repressing transcription. Finally, H3K4me2 apparently functions as an intermediate between unmethylated H3K4, and the activating trimethylated state at gene promoters, thus allowing rapid transcriptional change following differentiation signals.

In the context of T cell development, CpG methylation plays important roles both during thymic development, and in later (peripheral) fate decisions (described above). For instance, by deleting DNA methyltransferase 1 (DNMT1) at the DN stage of thymic development, Lee et al. (2001) showed an ∼90% reduction in the numbers of DP T cells, as well as large decreases in mature peripheral T cells of both $CD4^+$ and $CD8^+$ lineages. Further, the T cells that did develop had greatly reduced survival relative to the wild-type. However, when DNMT1 was deleted at the (later) DP stage, peripheral T cell numbers and composition were normal, but when either ($Dnmt1^{-/-}$) $CD4^+$ or $CD8^+$ T cells where stimulated *in vitro*, they had aberrant cytokine production profiles, in that they produced IL-2, IL-3, and IFN-γ more rapidly than wild-type cells. This latter observation is consistent with the demonstration that demethylation of regions controlling the transcription of *Ifng* and *Il2* in effector $CD8^+$ T cells (Kersh et al., 2006; Northrop et al., 2006) and *Il2* in effector $CD4^+$ T cells (Thomas et al., 2005) coincides with their demethylation. Further, it suggests that methylation might be a safeguard against inappropriate expression of these genes, which might otherwise lead to immune pathology. Taken together, these results indicated a central role for DNMT1, and CpG methylation, both during thymic and post-thymic development and differentiation of T cells of both $CD4^+$ and $CD8^+$ lineages.

SUMMARY

Both current effective vaccine strategies, and the design of novel vaccine strategies that specifically target adaptive T cell immunity, rely on acquisition and maintenance of T cell functional potential to establish protective immunity. Conversely, these same characteristics of adaptive T cell immunity are also at play during adverse

immune reactions where priming of T cells to either environmental or self-antigens, can manifest as T cell hypersensitivities or T cell-mediated autoimmune diseases, respectively. Thus, a greater understanding of the molecular mechanisms, and specifically epigenetic mechanisms, that shape acquisition and maintenance of lineage-specific T cell function, will be key if we are to make advances in novel therapeutic strategies for a variety of disease contexts. We have tried to highlight what we think are some of the key findings and general themes emerging from the studies of T cell differentiation, as well as the utility of the immune system as a tool for studying differentiation and development. By comparison with studies performed on stem cells, it appears that conclusions made from studies of T cells are broadly relevant to differentiation in other cell types and tissues. In particular, the concepts of transcriptional poising and promoter bivalency as mechanisms that regulate fate decisions are pertinent during the differentiation of stem cells and less primitive tissues. The studies of Rothenberg et al. (2010), in particular, highlight the value of the immune system as a tool for studying differentiation – because of the detailed ontogenies and the ability to resolve different stages of T cell development based on characteristic and defined cell surface phenotypes.

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EBV finds a polycomb-mediated, epigenetic solution to the problem of oncogenic stress responses triggered by infection

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Viruses that establish a persistent infection, involving intracellular latency, commonly stimulate cellular DNA synthesis and sometimes cell division early after infection. However, most cells of metazoans have evolved "fail-safe" responses that normally monitor unscheduled DNA synthesis and prevent cell proliferation when, for instance, cell proto-oncogenes are "activated" by mutation, amplification, or chromosomal rearrangements. These cell intrinsic defense mechanisms that reduce the risk of neoplasia and cancer are collectively called oncogenic stress responses (OSRs). Mechanisms include the activation of tumor suppressor genes and the so-called DNA damage response that together trigger pathways leading to cell cycle arrest (e.g., cell senescence) or complete elimination of cells (e.g., apoptosis). It is not surprising that viruses that can induce cellular DNA synthesis and cell division have the capacity to trigger OSR, nor is it surprising that these viruses have evolved countermeasures for inactivating or bypassing OSR. The main focus of this review is how the human tumor-associated Epstein–Barr virus manipulates the host polycomb group protein system to control – by epigenetic repression of transcription – key components of the OSR during the transformation of normal human B cells into permanent cell lines.

Keywords: Epstein–Barr virus, PcG, epigenetic, oncogenic stress response, oncogene-induced senescence, $p16^{INK4a}$, BIM, B cell transformation

INTRODUCTION – THE BIOLOGY OF EPSTEIN–BARR VIRUS (EBV)

Epstein–Barr virus (EBV) is a human gamma-herpesvirus (HHV4) and as such is characterized by a tropism for lymphocytes and an ability to persist life-long in the infected host. Data on persistent EBV infection in humans are consistent with the viral genome residing in a population of long-lived, largely non-dividing memory B cells. To establish persistence, EBV first infects resting (naïve) B cells – probably in tissues of the oropharynx – and transiently drives these to proliferate as activated B-blasts. The expanding B-blast population is thought to then either migrate into, or nucleate the formation of, a germinal center in local lymphoid tissue and therein the cells differentiate to become centroblasts, centrocytes, and finally resting memory B cells that enter the peripheral circulation (reviewed in Thorley-Lawson and Gross, 2004; Roughan and Thorley-Lawson, 2009). While the precise series of events that the EBV-positive B cells undergo to reach the memory compartment is not yet known, it is generally agreed that it involves regulated shut-down of latent EBV gene expression from an initial state called latency III, via latency II, until in quiescent memory B cells no EBV proteins can be detected in a state called latency 0. However, there is still some controversy as to whether or not the differentiation of EBV-infected B-blasts to resting memory B cells can occur anywhere outside the microenvironment of a germinal center (Rowe et al., 2009; Heath et al., 2012; Thorley-Lawson et al., 2013).

In more than 90% of the global population, following primary infection in infancy, EBV establishes an asymptomatic, stable, life-long, persistent infection in this long-lived pool of circulating memory cells. Periodic activation of an infected memory B cell by exposure to cognate antigen or aberrant T cell activity is thought to trigger plasma cell differentiation and concomitant "lytic" viral replication with the production of infectious virus that is released in the oropharynx and shed in saliva (reviewed in Thorley-Lawson and Gross, 2004; Laichalk and Thorley-Lawson, 2005; Roughan and Thorley-Lawson, 2009).

Primary EBV infection can cause the benign self-limiting disease infectious mononucleosis (IM) in some adolescents who were not infected in childhood. Uncontrolled proliferation of infected B cells in the immunocompromised of any age may result in a fatal form of IM, a chronic B lymphoproliferative disease or rarely the development of malignant immunoblastic lymphoma (Williams and Crawford, 2006). In normal individuals EBV-infected B-blasts are targets for EBV-specific cytotoxic T lymphocytes (CTLs) that recognize and destroy the EBV-infected proliferating B-blasts – so an equilibrium is established between B-blast proliferation on the one hand, and their immune-mediated elimination or differentiation to resting memory B cells on the other (Babcock et al., 1999; Thorley-Lawson and Gross, 2004; Hislop et al., 2007). Individuals who are co-infected with malaria or HIV are at increased risk of developing EBV-associated lymphomas, including Burkitt's lymphoma (BL). EBV is also etiologically linked to subgroups of Hodgkin's lymphoma (HL) and diffuse large

B cell lymphoma (DLBCL), in addition to various non-B cell malignancies (reviewed in Young and Rickinson, 2004).

Infection of resting naïve B cells *ex vivo* with EBV can also induce the proliferation of the B-blast-like cells that *in vivo* would differentiate to become memory cells. *In vitro* these B cells do not differentiate, but are transformed to continuously proliferating permanent lymphoblastoid cell lines (LCLs) that retain the activated B cell phenotype and carry the viral genome as extra-chromosomal episomes. Only the nine latency III-associated proteins six nuclear (EBNAs 1, 2, 3A, 3B, 3C, and LP) and three membrane-associated (LMP1, LMP2A, and 2B) together with several RNA species are expressed from the viral genome (reviewed in Bornkamm and Hammerschmidt, 2001; Young and Rickinson, 2004; Forte and Luftig, 2011). These latency-associated gene products are responsible for activating the quiescent primary cells into the cell cycle, inducing and sustaining their proliferation and maintaining the extrachromosomal episome in these blast-like cells. There is general agreement – that at least in the initial stages after infection – LCL outgrowth recapitulates the early events of establishing latency prior to differentiation and long-term persistence *in vivo*. EBV may therefore be considered one of the few viruses that initiate and sustain the proliferation of infected cells as a necessary step in their life cycle, in the natural host. Some of the molecular details of how EBV does this in the face of intrinsic barriers to aberrant proliferation are the focus of this review. Specific attention will be paid to the polycomb group (PcG) protein-mediated epigenetic repression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ and the pro-apoptotic BH3-only inducer of apoptosis BIM.

ONCOGENIC STRESS RESPONSES (OSRs) AND ONCOGENE-INDUCED SENESCENCE (OIS)

The seminal discovery in 1992 that the Myc proto-oncoprotein can trigger rapid apoptosis as well as cell growth and proliferation, led to the compelling hypothesis that apoptotic pathways must be disabled for oncogenes to promote neoplastic transformation of cells and the development of cancer (Askew et al., 1991; Evan et al., 1992). About 5 years later an equally influential discovery was that oncogenic mutant Ras protein – in addition to activating proliferative signaling pathways – also provokes in normal fibroblasts a cell cycle arrest resembling premature cell senescence. This was associated with the accumulation of tumor suppressors (ts) p53 and $p16^{INK4a}$ (Serrano et al., 1997), and further endorsed the hypothesis that normal mammalian cells possess intrinsic defenses against oncogenic transformation. These observations inspired the concepts of "OSR," "intrinsic tumor suppression," and "OIS" and produced many detailed descriptions of mechanisms involving the p53 and retinoblastoma (Rb) tumor suppressor pathways that prevent deregulated oncogenes causing cancer (**Figure 1**; reviewed in Sherr, 1998, 2012; Lowe et al., 2004; Braig and Schmitt, 2006).

RELATIONSHIP BETWEEN OSR/OIS AND DNA DAMAGE RESPONSES (DDRs)

Since cell proto-oncogenes generally control signaling pathways and/or gene networks that link proliferative signals to the cell cycle machinery, when they are deregulated this can result in unscheduled entry into S phase and aberrant DNA synthesis (sometimes referred to as "replicative stress"). As a consequence, oncogene activation can produce the stalling of DNA replication forks that results in damaged DNA – particularly double strand breaks. Such lesions can also be caused by the action of multiple physical and chemical agents and they can trigger, primarily via the ATM/ATR-kinase signaling pathway, the stabilization and activation of p53 and also the induction of 16^{INK4a}. Depending on the physiological and cellular context this leads to DNA repair, cell death, or senescence. This complex response is known as the DDR. It has been proposed that the induction of apoptosis or cell cycle arrest/senescence by oncogenic stress is a general downstream manifestation of the DDR acting as a barrier to cell transformation *in vitro* and tumor progression *in vivo* (Di Micco et al., 2006; Bartek et al., 2007; Halazonetis et al., 2008). However, it remains unclear whether all oncogene-mediated stress responses act via the DDR, or whether alternative signaling pathways directly regulate downstream effectors (see for example induction of $p16^{INK4a}$ in response to oncogenic RAS/RAF signaling (Agger et al., 2009; Barradas et al., 2009) and the relationship between MYC and BIM in B cell lymphomas described below). The links between DDR, OSR, and OIS have been extensively reviewed (Braig and Schmitt, 2006; Gil and Peters, 2006; Kim and Sharpless, 2006; Wade and Wahl, 2006; Bartek et al., 2007; Halazonetis et al., 2008).

A common feature of herpesviruses is their capacity to activate DDRs in infected cells (Shirata et al., 2005; Gaspar and Shenk, 2006; Koopal et al., 2007; Tarakanova et al., 2007; Nikitin et al., 2010). Although in some cases this is associated with lytic or productive infection, when the virus has a requirement for rapid replication of its genome prior to virion assembly, at least two gamma-herpesviruses (Kaposi's Sarcoma associated herpes virus (KSHV, aka HHV8) and EBV) trigger DDRs during the establishment of a latent infection. This is largely because

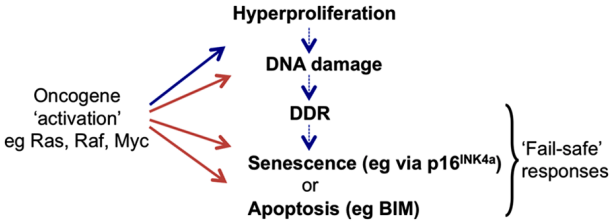

FIGURE 1 | Activation of cell proto-oncogenes can lead to oncogenic stress responses (OSR). Oncogene "activation" by mutation or constitutive expression at supra-physiological levels can induce aberrant cell division that may become manifest as rapid cell proliferation (hyperproliferation). However higher vertebrates have evolved cell intrinsic "fail-safe" responses to recognize such cells and so block their proliferation or eliminate them completely. These can include the induction of tumor suppressors (ts) such as $p16^{INK4a}$ that halt the cell cycle and can cause cells to enter a prolonged state of arrest called senescence, or pro-apoptotic proteins such as BIM that can induce programmed cell death (apoptosis). Responses may involve direct activation of the ts genes by oncoproteins or they can result from secondary signaling pathways (DDR) linked to the detection of damaged DNA produced during periods of aberrant DNA replication and cell division.

latency-associated viral proteins drive cells into the cell cycle and can induce hyperproliferation, replication errors, and associated DNA damage (Koopal et al., 2007; Nikitin et al., 2010). Moreover, it has been suggested that EBV infection of B cells *in vitro* may also induce reactive oxygen species (ROS) that can damage DNA (reviewed in Allday, 2009; Gruhne et al., 2009). EBV and KSHV appear to have evolved mechanisms for the attenuation of the DDR to ensure latent infection is maintained. Virus-associated responses involving the DDR have recently been comprehensively reviewed elsewhere (Leidal et al., 2012; Nikitin and Luftig, 2012) and for EBV will be reconsidered below.

THE *INK4b-ARF-INK4a* LOCUS, $p16^{INK4a}$, OSR/OIS, AGING, AND CANCER

Within the *INK4b-ARF-INK4a* locus at human chromosome 9p21, *CDKN2A* encodes two potent tumor suppressors, $p16^{INK4a}$, and $p14^{ARF}$ ($p19^{ARF}$ in mice); these proteins are critical negative regulators of cell proliferation. Although exons 2 and 3 are shared by *INK4a* and *ARF*, the proteins result from differential splicing and are encoded in alternative reading frames (reviewed in Gil and Peters, 2006; Kim and Sharpless, 2006; Sherr, 2012). Adjacent to *CDKN2A* is a second related gene *CDKN2B* that encodes a protein closely related to $p16^{INK4a}$ called $p15^{INK4b}$ (**Figure 2**). The cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$ acts on the cyclin D-dependent kinases (CDK4 and CDK6) abrogating their binding to D-type cyclins and so inhibiting CDK4/6-mediated phosphorylation of the Rb protein. By binding CDKs and blocking Rb hyperphosphorylation, increased $p16^{INK4a}$ expression causes a G1 cell cycle arrest and senescence (Gil and Peters, 2006; Kim and Sharpless, 2006; Sherr, 2012). Although the CDK inhibitor $p15^{INK4b}$ has about 85% amino acid similarity to $p16^{INK4a}$ and biochemically behaves in much the same way, in most mammalian cells – for unknown reasons – it has distinct functions. In contrast to the CDK inhibitors, the p14 and p19 ARF proteins regulate the stability of p53 by inactivating MDM2 – a p53-specific ubiquitin ligase that facilitates p53 degradation. The concomitant stabilization and activation of p53 leads to G1 and G2 cell cycle arrest by inducing the CDK regulator $p21^{WAF1}$ or apoptosis by inducing pro-apoptotic factors such as NOXA and PUMA (Vousden and Prives, 2009; Sherr, 2012).

The products of *CDKN2A* can therefore be key mediators of OSR and potent barriers to the "immortalization" of cells in culture and the development of cancers *in vivo*. Both $p16^{INK4a}$ and ARF are also progressively up-regulated with tissue aging, when they probably contribute to the aging process by reducing reservoirs of stem cells capable of self-renewal (Kim and Sharpless, 2006; Collado et al., 2007). There is general agreement that $p19^{ARF}$ plays the more important role in all these processes in mice, whereas in human cells $p16^{INK4a}$ is the dominant player. It is therefore not surprising that in a wide variety of human cancers *INK4a* is inactivated by gene deletion, mutation, or promoter DNA methylation (Gil and Peters, 2006; Kim and Sharpless, 2006; Popov and Gil, 2010). The whole *INK4b-ARF-INK4a* locus appears to be coordinately regulated epigenetically by polycomb protein complexes generating repressive histone modifications (Gil and Peters, 2006; Popov and Gil, 2010). Although induction of $p16^{INK4A}$ in fibroblasts and epithelial cells is generally associated with cell cycle arrest and senescence, in B cells – which exhibit no obvious characteristics of senescence – there may be some crosstalk between $p16^{INK4a}$ and the apoptotic machinery, since in lymphocytes the default pathway triggered by $p16^{INK4a}$ can be death rather than prolonged cell cycle arrest (Lagresle et al., 2002; Bianchi et al., 2006).

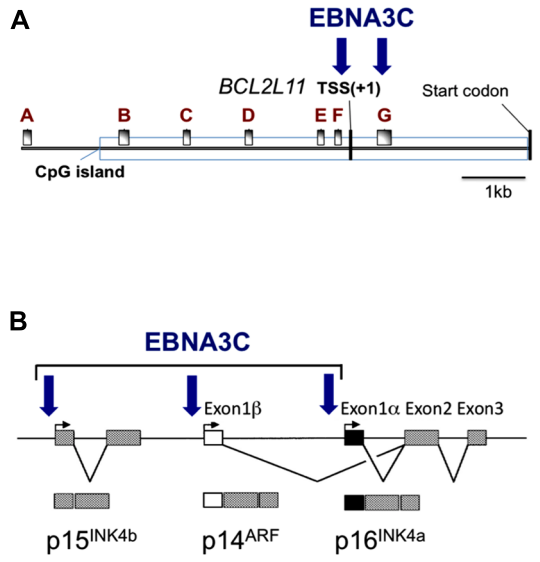

FIGURE 2 | Epitope-tagged EBNA3C associates with the promoter for BIM and genes in the *INK4b-ARF-INK4a* locus. Schematic representations of **(A)** approximately 9kb of the *BCL2L11* (BIM) promoter and **(B)** approximately 40 kb including the *INK4b-ARF-INK4a* locus. Vertical arrows indicate the positions where EBNA3C has been detected by chromatin immunoprecipitation (ChIP) analyses using lymphoblastoid cell lines (LCL) established using EBV expressing an epitope-tagged EBNA3C. These same regions of chromatin are marked by the polycomb (PRC2)-mediated modification H3K27me3 when EBNA3A and EBNA3C are present (adapted from Paschos et al., 2012; Skalska et al., 2013). The *BCL2L11* gene transcriptional start site (TSS) and the protein products of *INK4b-ARF-INK4a* are indicated. A–G in **(A)** mark the positions of RT-PCR primer sets used in (Paschos et al., 2012).

BIM, B CELLS, AND MYC

BIM (*B*cl2-*i*nteracting *m*ediator) is a pro-apoptotic member of the BH3-only family of BCL2-like proteins and is encoded by the *BCL2L11* gene at human chromosome 2q13. BIM acts as a potent, direct initiator of apoptosis because it binds with high affinity to BCL2 and all the other pro-survival family members to inactivate them. BIM also binds and activates pro-apoptotic BAX to initiate cytochrome-c release from mitochondria (Strasser, 2005; Gavathiotis et al., 2008). BIM is particularly important in the immune system, acting as a major regulator of life-and-death decisions during lymphocyte development including the negative selection of auto-reactive B cells and programmed death of low-affinity antibody-expressing germinal center-derived B cells (Enders et al., 2003; Strasser, 2005; Fischer et al., 2007). *Bim*-null mice accumulate excess lymphoid and myeloid cells and loss of *Bim* accelerates B cell lymphomagenesis induced by an *Eμ-Myc* transgene. Even loss of a single allele accelerates lymphomagenesis significantly,

indicating *Bim* is a haploinsufficient tumor suppressor and that the level of Bim protein is rate-limiting in murine B cell survival (Egle et al., 2004).

Extending this *Eμ-Myc*-lymphoma model to human B cell lymphomagenesis, the relationship between *MYC* and BIM in EBV-negative BL was investigated (Dang et al., 2005; Hemann et al., 2005). This study brought into sharp focus the activation of *BCL2L11/BIM* by MYC, and led to the proposal that *MYC*-induced apoptosis can be overridden by inactivation of any one of several *MYC* effectors – including p53, $p14^{ARF}$, or BIM – causing apoptosis-firing to drop below a critical threshold to allow cell proliferation. It also established that *BCL2L11/BIM* is a $p14^{ARF}$/p53-independent target of MYC and that its activation does not require MYC-induced hyperproliferation (Dang et al., 2005; Hemann et al., 2005). Thus BIM is a uniquely important tumor suppressor in cells of the hematopoietic lineage and operationally its activation by MYC is a component of the OSR in B cells. Since MYC is induced and becomes constitutively expressed early after EBV infection of primary human B cells, modulation of BIM expression by EBV is likely to be a contributory factor in B cell transformation and the development of any EBV-associated B cell lymphomas (discussed in more detail below).

POLYCOMB GROUP PROTEINS AND EPIGENETIC REPRESSION

Epigenetic gene regulation is heritable and results from changes in a chromosome without alterations to DNA sequence (Berger et al., 2009). Such changes can be mediated by chemical modifications to chromatin on either DNA or DNA-associated histones and may involve non-coding RNAs. PcG proteins were first identified in *Drosophila* and are best known as repressors of the homeotic (Hox) transcription factor genes during embryonic development. They are very highly conserved from flies to humans and homologues regulating developmental transitions are found in plants. PcG proteins form multi-protein complexes called polycomb repressive complexes (PRCs) that bind and epigenetically regulate hundreds of genes, predominantly associated with cell-fate decisions and development (reviewed in Bracken and Helin, 2009; Margueron and Reinberg, 2011; Bemer and Grossniklaus, 2012; Simon and Kingston, 2013). They can repress transcription by introducing post-translational covalent modifications on histones in chromatin located in the regulatory regions of target genes. This repression/silencing is stable and heritable so can be described as epigenetic (Berger et al., 2009).

PRC2 is a multi-component complex that mediates trimethylation at lysine 27 of histone H3 (H3K27me3). In humans the core complex is comprised of three polycomb proteins: suppressor of zeste (SUZ)12, embryonic ectoderm development (EED), and enhancer of zeste (EZH)2. EZH2 contains the catalytic SET domain responsible for lysine methyltransferase activity (Bracken and Helin, 2009; Margueron and Reinberg, 2011; Simon and Kingston, 2013). Other components of PRC2 are histone chaperone RbAp46/48 and recently an ancillary factor, JARID2, has been identified as being essential for recruitment of PRC2 to some polycomb-target genes (Murzina et al., 2008; Landeira et al., 2010; Margueron and Reinberg, 2011; Simon and Kingston, 2013). It remains unclear how in most cases the polycomb proteins are recruited to specific promoters in mammalian cells, although sequence context is probably important and a preference for regions rich in CpG dinucleotides (CpG-islands) has been reported (Ku et al., 2008). However, for most target genes, it remains to be determined whether specificity comes from sequence-specific transcription factors, PRC2-interacting non-coding RNA species, or yet to be identified mechanisms (Bracken and Helin, 2009; Khalil et al., 2009; Gupta et al., 2010; Kanhere et al., 2010; Simon and Kingston, 2013).

H3K27me3 on chromatin attracts the binding of a second complex, PRC1 that mediates the repressive ubiquitinylation at lysine 119 of histone H2A (H2AK119Ub). PRC1 core proteins include chromobox (CBX) proteins, whose chromodomains are thought to recruit the complex to the H3K27me3 mark, and RING finger proteins, such as RING1B, MEL18, and BMI1 that are responsible for the E3 ubiquitin ligase activity that produces H2AK119Ub. PRC1 mediates chromatin compaction and the local formation of heterochromatin (Grau et al., 2011) and together with PRC2, increases the chances of the more stable CpG DNA methylation mark being deposited (reviewed in Cedar and Bergman, 2009). Although recent evidence suggests H3K27me3 is stable and heritable (Simon and Kingston, 2013) this histone modification can be rapidly removed by demethylase enzymes such as JMJD3 (aka KDM6B; Agger et al., 2009; Barradas et al., 2009). Moreover, if a promoter carries H3K27me3 and simultaneously has the activation-associated modification H3K4me3 at the same locus, it is repressed but is described as "bivalent" and thought to be poised for rapid reactivation by removal of H3K27me3; genes with such bivalent domains are common in stem cells (Bernstein et al., 2006; Voigt et al., 2013). Cancer cells and stem cells often share gene expression patterns and multiple reports suggest that polycomb complexes contribute to the aberrant CpG DNA methylation profiles that are critical in the genesis and progression of many diverse cancers (Cedar and Bergman, 2009). The mechanism for this is suggested by the capacity of various polycomb proteins to physically interact with DNA methyl transferases (DNMTs) and recruit them to chromatin. It has been estimated that PcG-target genes are up to 12 times more likely to be aberrantly methylated in cancer than non-targets (Widschwendter et al., 2007).

EPIGENETIC REGULATION OF BIM AND $p16^{INK4a}$ EXPRESSION BY EBV

EBNA3A AND EBNA3C COOPERATE AS ONCOGENIC REPRESSORS OF TRANSCRIPTION

The EBV EBNA3 proteins (EBNA3A, EBNA3B, and EBNA3C) are large (>900 aa) latency-associated nuclear proteins that show no significant similarity to known cell or viral factors. Although none of them appears to bind DNA directly, they all bind the cellular DNA-binding factor CBF-1 (aka RBP-JK; reviewed in Bornkamm and Hammerschmidt, 2001; Young and Rickinson, 2004). All three EBNA3s can also interact with cellular factors associated with the covalent modification of histones, the repression of transcription, and gene silencing; for example, EBNA3A and EBNA3C associate with histone deacetylases (HDACs) and the conserved co-repressor CtBP (Radkov et al., 1999;

Bornkamm and Hammerschmidt, 2001; Touitou et al., 2001; Hickabottom et al., 2002; Young and Rickinson, 2004). EBNA3A, EBNA3B, and EBNA3C are all robust repressors of transcription when targeted directly to DNA in transient assays (Bain et al., 1996; Cludts and Farrell, 1998 and our unpublished data), and EBNA3A and EBNA3C – but not EBNA3B – are necessary to establish LCLs from purified B cells (Tomkinson and Kieff, 1992; Tomkinson et al., 1993). EBNA3A and EBNA3C also cooperate with oncogenic Ha-Ras in the transformation/immortalization of primary rodent fibroblasts and require the interaction with CtBP to do this (Parker et al., 1996; Touitou et al., 2001; Hickabottom et al., 2002). All the data are therefore consistent with EBNA3A and EBNA3C acting as oncoproteins in the transformation of B cells and in EBV-associated lymphomagenesis. However EBNA3B is unnecessary in these processes, and can even act as a tumor suppressor (White et al., 2012).

Recent microarray gene-expression analyses using LCLs or lymphoma cells infected with recombinant B95.8 strain EBVs that express defined EBNA3 mutants, suggest that together the EBNA3s can regulate >1000 host genes in B cells – often repressing transcription. The regulation of many of these genes seems to require the functional interaction of at least two EBNA3s and in several cases that have been subjected to further analysis, gene repression appears to utilize the host PcG system to inhibit transcription via the H3K27me3 chromatin modification (Hertle et al., 2009; Skalska et al., 2010; White et al., 2010, 2012; Maruo et al., 2011; Zhao et al., 2011; McClellan et al., 2012; Paschos et al., 2012). Two genes repressed by the combined action of EBNA3C, EBNA3A, and PcG proteins – and of particular interest in the context of OSR – encode BIM and $p16^{INK4a}$.

REPRESSION OF BIM TRANSCRIPTION

The first indication that EBNA3A and EBNA3C can cooperate to repress specific host cell genes came using a panel of EBNA3-knockout recombinant B95.8-derived EBVs to infect EBV-negative BL cells. This revealed that expression of both EBNA3A and EBNA3C are necessary to repress transcription of *BCL2L11/BIM* (Anderton et al., 2008). Subsequently it was found that DNA in a large CpG island located at the 5′ end of *BCL2L11/BIM* becomes methylated on CpG dinucleotides in EBV-positive BLs (Paschos et al., 2009). However a reduction in BIM expression occurred soon after EBV infection of B cells in culture and did not initially involve detectable CpG methylation, but correlated with the deposition of the polycomb signature H3K27me3 on chromatin proximal to the transcription start site (TSS; Paschos et al., 2009, 2012). Detailed chromatin immunoprecipitation (ChIP) analyses of the chromatin around the *BCL2L11/BIM* promoter revealed that latent EBV triggers the recruitment of polycomb repressive complex 2 (PRC2) core subunits and the trimethylation of histone H3 lysine 27 (H3K27me3) at this locus. It appears that in uninfected BL cells, RbAp48, and JARID2 already associate with the chromatin proximal to the TSS and that EBV infection is necessary to recruit SUZ12 and EZH2 to establish functional PRC2. Assembly of PRC2 at the locus was absolutely dependent on both EBNA3A and EBNA3C being expressed, and using a recombinant EBV expressing an epitope-tagged EBNA3C, it was shown by ChIP that EBNA3C associates with chromatin near the TSS – it is therefore likely to physically interact with PRC2 (Paschos et al., 2012; **Figure 2** and model in **Figure 3**). Since the activation mark H3K4me3 is largely unaltered at this locus irrespective of H3K27me3- or EBNA3-status the establishment of a "bivalent" chromatin domain is suggested. Consistent with the "poised" nature of these domains, RNA polymerase II (RNA Pol II) occupancy at the *BCL2L11/BIM* TSS was not altered by EBV. However, further analysis of phospho-serine 5 on RNA Pol II indicated that when EBNA3A and EBNA3C are both expressed they inhibit this phosphorylation step and block the initiation of the *BIM* transcripts. It was not determined whether this involves the direct action of an EBV protein on the kinase CDK7 or is a consequence of the recruitment of PRC2 and/or PRC1 to this particular locus. B cell lines carrying EBV encoding a conditional EBNA3C-modified estrogen receptor-fusion revealed that this epigenetic repression of *BIM* was reversible, but took more

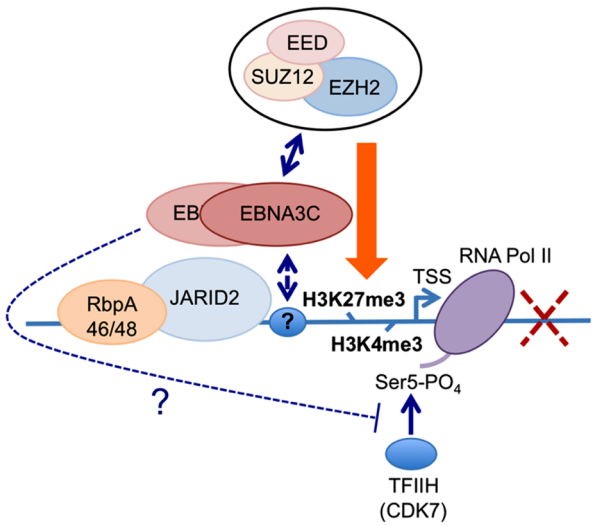

FIGURE 3 | Working hypothesis for the role(s) of EBNA3C and EBNA3A in the PRC2-mediated repression of the BIM promoter. The available data indicate that EBNA3C (and EBNA3A) are recruited to regions proximal to the *BCL2L11/BIM* transcriptional start site (TSS) in EBV-infected B cells (Paschos et al., 2012; our unpublished data and **Figure 2**). Irrespective of whether EBNA3C or EBNA3A are expressed in these cells, the PRC2-associated factors RbpA46/48 and JARID2 are present at the locus. Similarly the activation mark H3K4me3 and RNA polymerase II (RNA Pol II) occupy the TSS irrespective of which EBNA3s are expressed. Only when both EBNA3C and EBNA3A are present are core components of the PRC2 complex found at this site and the repressive chromatin mark H3K27me3 is detected across the TSS; concomitantly the level of transcription and BIM expression are reduced. The simultaneous presence of both H3K4me3 and H3K27me3 at the locus define it as a "bivalent" or "poised" domain and is consistent with RNA Pol II always being detected. However only in the absence of either EBNA3C or EBNA3A is RNA Pol II phosphorylated on serine residue 5 (RNA Pol II Ser 5), suggesting that in addition to playing a key role in the recruitment of PRC2 core complex, the presence of EBNA3C and EBNA3A might interfere with serine 5 phosphorylation of RNA Pol II and therefore block the initiation of transcription. Since EBNA3A and EBNA3C can be co-immunoprecipitated from infected B cells and both are necessary for repression of BIM (and $p16^{INK4a}$) expression, in this model we assume they are co-localized at these loci. The identity of the factor(s) responsible for targeting EBNA3C and/or EBNA3A to this particular stretch of chromatin is still unknown, as is the mechanism of interaction with PRC2.

than 30 days from when EBNA3C was inactivated, emphasizing the stability of these chromatin modifications through rounds of cell division. Lentivirus delivery of shRNAs against PRC2 and PRC1 subunits disrupted EBV repression of *BCL2L11/BIM*, thus confirming the requirement for PcG complexes (Paschos et al., 2012).

REPRESSION OF TRANSCRIPTION FROM THE CDKN2A LOCUS

Direct evidence that EBNA3C modulates the cell cycle during EBV-mediated transformation of B cells came from Maruo et al. (2006). Using a recombinant Akata strain EBV made conditional for EBNA3C function by fusing EBNA3C with a modified estrogen receptor, they revealed that EBNA3C represses expression of the CDK inhibitor $p16^{INK4A}$ in LCLs. Removing the inducer of EBNA3C activity (4-hydroxytamoxifen, 4HT) from the culture medium resulted in an accumulation of both $p16^{INK4A}$ mRNA and protein, de-phosphorylation of Rb, and concomitant cell cycle arrest (Maruo et al., 2006). Using a similar recombinant virus expressing an EBNA3A-fusion, the same authors showed that inactivation of EBNA3A also resulted in reduced proliferation, although the mechanism was not determined (Maruo et al., 2003). Since EBNA3A and EBNA3C are necessary for the H3K27me3-mediated chromatin manipulation and epigenetic repression of *BCL2L11/BIM*, and since the *CDKN2A* locus that encodes $p16^{INK4a}$ had been identified as a target of polycomb-mediated repression in proliferating cells, it was not surprising to discover that the combined action of EBNA3C and EBNA3A repressed *CDKN2A* in cycling B cells by facilitating the deposition of H3K27me3 across the locus – primarily around the $p16^{INK4a}$ TSS (Skalska et al., 2010). Furthermore, establishing LCLs with recombinant viruses encoding CtBP-binding mutants of EBNA3C and EBNA3A revealed that their interaction with this highly conserved cellular co-repressor was necessary for the efficient deposition of H3K27me3 and repression of $p16^{INK4a}$ expression. ChIP analysis for the epitope-tagged EBNA3C expressed in an LCL revealed EBNA3C at the TSS of $p16^{INK4A}$ and ARF, and also the *CDKN2B* gene encoding $p15^{INK4b}$ (**Figure 2**; Skalska et al., 2013). Although it was initially unclear whether the EBNA3C-associated H3K27me3 deposition at *CDKN2A* was a cause or a consequence of cells exiting from the cell cycle, regulation of the locus by EBNA3C in an Rb-null LCL (Skalska et al., 2010) and in several $p16^{INK4a}$-null LCLs (Skalska et al., 2013 and see below) unequivocally established that EBNA3C regulation of the locus is independent of the degree of cell proliferation. As with *BLC2L11/BIM*, B cell lines carrying EBV encoding the conditional EBNA3C-modified estrogen receptor-fusion revealed that this epigenetic repression of *CDKN2A* was reversible by adding or removing 4HT from the medium. Taken together all these data suggest that EBNA3C (cooperating with EBNA3A) coordinately regulates the whole *INK4b-ARF-INK4a* locus by directing the recruitment of PRC2 to the three transcriptional start sites. Consistent with this we have recently found that the level of $p15^{INK4b}$ mRNA is coordinately regulated with that of $p16^{INK4a}$ in EBNA3C-conditional LCLs (our unpublished data).

The specific role of $p16^{INK4a}$ as a target for EBNA3C and a major barrier to B cell transformation was further explored making use of an "experiment of nature" in the form of "Leiden" B cells carrying a homozygous genomic deletion that specifically ablates production of functional $p16^{INK4a}$ (Brookes et al., 2002; Hayes et al., 2004). These cells were infected with recombinant B95.8-derived EBVs that express either the conditional EBNA3C or no EBNA3C (Skalska et al., 2013). A comparison of p16-null LCLs with LCLs established from normal B cells showed unequivocally that, if $p16^{INK4a}$ is not functional, then EBNA3C is unnecessary to sustain cell proliferation. Consistent with this – and providing formal proof that $p16^{INK4a}$ is the main target of EBNA3C – it was possible to transform p16-null B cells into stable LCLs with EBV, but without any functional EBNA3C ever having been expressed.

INHIBITING OSR/OIS IS NECESSARY FOR LCL OUTGROWTH

A reasonable but speculative explanation for why EBV has evolved a mechanism for suppressing $p16^{INK4a}$ (and BIM) expression became apparent from examining the outcome of attempted transformations of normal B cells with EBNA3C-deficient EBV (**Figure 4**; Skalska et al., 2013). These experiments revealed that infection with a "wild type" EBV modestly induced $p16^{INK4a}$ transcription in the first few days after infection – when EBNA2 transactivates inducers of cell cycle progression (e.g., MYC and cyclin D2) and a period of hyperproliferation has been described (Sinclair et al., 1994; Spender et al., 1999; Nikitin et al., 2010). It is likely that unscheduled entry into S-phase, is interpreted by the cell as oncogenic stress and activation of $p16^{INK4a}$ transcription is a consequence. When the infecting virus expressed functional EBNA3C (and EBNA3A) there was a halt to the increase of $p16^{INK4a}$ expression from about day 7 onwards. However, if EBNA3C was not expressed or was non-functional (i.e., no 4HT in the medium), transcription from *INK4a* continued unrestrained and the level of mRNA progressively increased over the next 2–3 weeks, until most of the cells stopped proliferating. Early after infection BIM expression is down-regulated, and very soon (<5 days) reaches a steady state, but if EBNA3C is deleted or functionally inactivated in the infecting EBV – beginning about 4 days post infection – the level of mRNA corresponding to BIM also increases, in parallel with that of $p16^{INK4a}$. This increase continues for the next week or two until cells arrest or die (Skalska et al., 2013). Largely similar results were obtained with EBNA3A-negative virus (our unpublished data).

The EBNA3C/3A-mediated epigenetic inhibition of *INK4a* and *BCL2L11/BIM* transcription is therefore critical for EBV to bypass an intrinsic host cell defense against oncogenic transformation probably triggered by EBNA2 acting through MYC (summarized in **Figure 4**; see also Nikitin et al., 2010). Thus expression of both EBNA3C and EBNA3A ensures expansion of the infected B cell population and LCL outgrowth *in vitro* and *in vivo* the initiation of latency. Strictly speaking, in this context, EBNA3C and EBNA3A do not actually repress *INK4a* and *BCL2L11/BIM* transcription, but rather prevent their activation. This most likely involves the recruitment of PcG protein complexes to the loci, leading to H3K27me3 modifications on chromatin around the TSSs, as is seen in established LCLs; however this has not yet been formally demonstrated in newly infected cells.

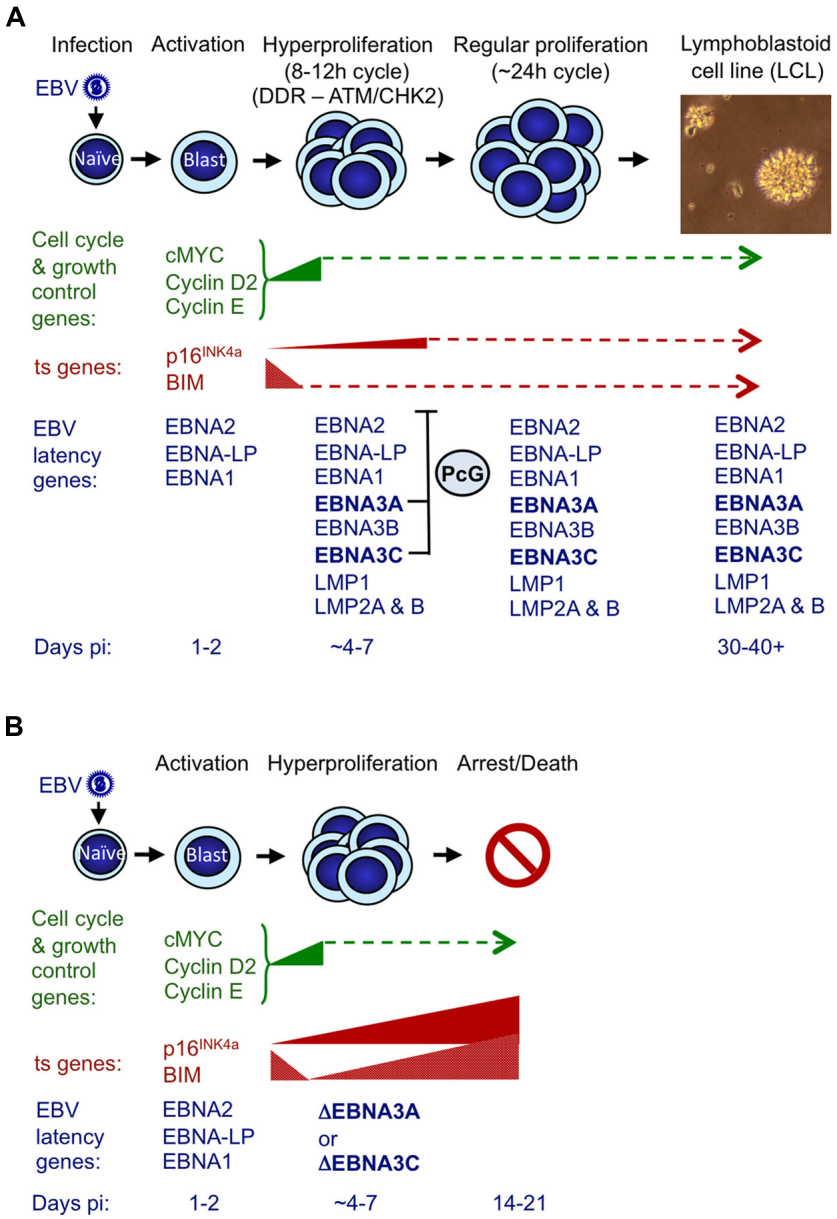

FIGURE 4 | Events following infection of primary resting B cells by EBV that initiate transformation into continuously proliferating LCLs. **(A)** During the first 24–48 h post-infection (pi) with a B95.8-derived EBV, cell genes associated with growth and cell cycle are transactivated and their products (e.g., MYC, cyclin D2, cyclin E) drive cells from G0 to G1, to become enlarged, activated and start proliferating. The whole process is driven by the EBV transactivator protein EBNA2, probably assisted by the co-factor EBNA-LP (Sinclair et al., 1994; Spender et al., 1999; Nikitin et al., 2010). During the next 3–4 days cells undergo rounds of rapid cell division (hyperproliferation) and in some cells this results in damage to DNA that can activate the DNA damage response (DDR) and initiate a signaling cascade involving the kinases ATM and CHK2 (Nikitin et al., 2010). If the full complement of nine EBV latency-associated proteins is expressed, the DDR becomes attenuated (in part by EBNA3C) and cells continue to proliferate to produce polyclonal LCLs that have a population doubling time of about 24 h. Early after infection BIM expression is down-regulated, and although the level of $p16^{INK4a}$ expression increases slightly, this soon reaches a steady state. In both cases we assume that EBNA3A and EBNA3C cooperate by harnessing the polycomb group (PcG) protein system to epigenetically repress (or restrain the transcription of) these ts genes via H3K27me3 (Anderton et al., 2008; Paschos et al., 2012; Skalska et al., 2013). **(B)** If EBNA3C or EBNA3A are deleted (ΔEBNA3C and ΔEBNA3A) or functionally inactivated in the infecting EBV, beginning about 4–7 days pi, the levels of mRNAs corresponding to $p16^{INK4a}$ and BIM progressively increase and continue to do so for the next week or two until finally most of the cells arrest and/or die (Skalska et al., 2013 and our unpublished data). The PcG-mediated repression of these two ts genes – in particular $p16^{INK4a}$ (see text) – is part of a critical countermeasure evolved by EBV to bypass an intrinsic host defense against oncogenic transformation. If primary B cells are $p16^{INK4a}$-null, functional EBNA3C is dispensable for the outgrowth of LCLs. This is consistent with $p16^{INK4a}$ being the dominant barrier to outgrowth and subsequent proliferation of LCLs, and the principal requirement of EBNA3C appears to be to restraining transcription of $p16^{INK4a}$ (see text for details and Skalska et al., 2013). The precise relationships between DDR, $p16^{INK4a}$ and EBNA3C/EBNA3A have yet to be defined.

CONCLUDING REMARKS

Through the combined action of EBNA3C and EBNA3A and their interaction with the cellular PcG protein system, EBV has evolved a very effective countermeasure to OSR/OIS that appears to be critical in its normal life cycle to establish a latent infection and therefore initiate long-term persistence in B cells. *In vitro* this mechanism neatly overcomes a major early obstacle to cellular "immortalization," making EBV one of the most potent transforming/immortalizing biological agents to have been identified. By utilizing an epigenetic mode of gene regulation to tackle the problem of OSR/OIS, key target ts genes including *INK4a* and *BCL2L11/BIM* are repressed not only in the infected cells, but also in their progeny; furthermore the genes become particularly predisposed to complete silencing by DNA modification. It is self-evident – since EBV stably ablates at least two major barriers to oncogenic transformation – that this will substantially increase the likelihood of EBV-infected B cells undergoing additional genetic and/or epigenetic changes leading to cancer (discussed further in Thorley-Lawson and Allday, 2008; Allday, 2009; Skalska et al., 2010; Paschos et al., 2012). This manipulation of the PcG system to specifically regulate key tumor suppressor genes in B cells makes EBV – to our knowledge – unique among tumor viruses. Now the challenges are to provide complete biochemical descriptions of how the EBNA3 proteins interact with PcG complexes and – employing genome-wide screens such as ChIP-seq – determine the extent of polycomb-mediated epigenetic reprogramming of B cells by EBV.

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Regulation of hepatitis B virus replication by epigenetic mechanisms and microRNAs

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The hepatitis B virus (HBV) genome forms a covalently closed circular DNA (cccDNA) minichromosome that persists in the nucleus of virus-infected hepatocytes. HBV cccDNA serves as the template for viral mRNA synthesis and is subject to epigenetic regulation by several mechanisms, including DNA methylation and histone acetylation. Recently, microRNAs (miRNAs), a class of small non-coding RNAs, were also directly connected to the epigenetic machinery through a regulatory loop. Epigenetic modifications have been shown to affect miRNA expression, and a sub-group of miRNAs (defined as epi-miRNAs) can directly target effectors of the epigenetic machinery. In this review, we will summarize recent findings on the epigenetic mechanisms controlling HBV cccDNA function, primarily focusing on the epi-miRNA functions operating in HBV replication. Investigation of the epigenetic regulation of HBV replication may help to discover novel potential therapeutic targets for drug development with the goal to eradicate the HBV cccDNA pool in hepatocytes.

Keywords: hepatitis B virus, microRNA, epigenetic regulation, histone deacetylases, DNA methyltransferase

INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem that causes a wide spectrum of liver diseases, including acute or chronic HBV infection. Acute HBV infections either resolve or progress to chronicity. Chronic hepatitis B (CHB) is associated with chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC; McMahon, 2009). It is estimated that more than 350 million patients worldwide are chronically infected with HBV, with the majority of these patients living in the Asia-Pacific region. More than one million deaths occur each year as a direct consequence of CHB (Dienstag, 2008). Medical intervention using antiviral nucleoside/nucleotide analogs and interferon (IFN) was established to treat chronically infected patients (Pardo et al., 2007). However, currently available therapies do not lead to the termination of HBV infection in the majority of patients (Mailliard and Gollan, 2006). There is a consensus that the improved understanding of the HBV–host interaction is a prerequisite for new antiviral therapeutic strategies. Recently, many aspects pertaining to the epigenetic mechanisms responsible for viral persistence and clearance during HBV replication have been addressed, including methylation of viral DNA, acetylation of histone complexes, and microRNA (miRNA) regulation. These topics are described in this review.

HBV cccDNA STRUCTURE AND ITS ROLE IN HBV INFECTION

Hepatitis B virus is the prototype member of the family Hepadnaviridae and has a partially double-stranded DNA genome of approximately 3.2 kb in length. The viral genome harbors seven open reading frames, coding for the viral polymerase, HBV core, and e antigens (HBcAg and HBeAg); the regulatory HBx protein; and the preS/S gene encoding the three surface antigens (LHBsAg, MHBsAg, and SHBsAg). The genome also contains a number of regulatory elements (Seeger and Mason, 2000). The entry of HBV virions is likely initiated through a non-specific interaction with negatively charged glycans at the surface of hepatocytes (Schulze et al., 2007; Bremer et al., 2009) followed by specific binding to the sodium-taurocholate cotransporting polypeptide (NTCP) receptor by a specific sequence (2-48aa) located in the preS1 domain of the LHBsAg protein (Yan et al., 2012). After uncoating, the HBV capsid is transported by the cellular machinery to the nuclear pore. The open circular form of HBV genomic DNA is then converted to a covalently closed circular DNA (cccDNA) molecule in the nucleus. This process requires that the covalently attached viral polymerase is removed from the negative DNA strand by a proteinase and that the positive strand DNA is completed by the cellular replicative machinery so that it matches the negative strand to covalently join the two ends to form a circular, supercoiled molecule (Gao and Hu, 2007).

In the nucleus, HBV cccDNA is incorporated into the host chromatin and exists as an individual minichromosome with a "beads-on-a-string" structure, which is revealed by electron microscopy (Bock et al., 1994; Newbold et al., 1995). This minichromosome has been shown to consist of both histone and non-histone proteins. By immunoblotting with HBcAg, the histone proteins H3 and H2B were the most prominent species, while lower levels of H4, H2A, and H1 were also detectable (Bock et al., 2001). Using the cccDNA-ChIP assay, the group of Massimo Levrero has confirmed the recruitment of the H3 and H4 histones along with the

Abbreviations: 5-AzaC, 5-azacytidine; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; DNMT, DNA methyltransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; IFN, interferon; miRNA, microRNA; SIRT1, sirtuin 1; TSA, trichostatin A.

HBcAg and HBx proteins to the cccDNA minichromosome. Using the same approach, several cellular transcription factors (CREB, ATF, YY1, STAT1, and STAT2) and chromatin modifying enzymes (PCAF, p300/CBP, HDAC1, SIRT1, and EZH2) have been shown to bind to the cccDNA in human hepatoma cells containing replicating HBV (Pollicino et al., 2006; Belloni et al., 2009, 2012). The histone acetyltransferases (HATs) p300/CBP and PCAF and the histone deacetylases (HDACs) HDAC1 and SIRTl were shown to be recruited with different kinetics onto HBV cccDNA, implying that HBV cccDNA-bound histones may be subjected to regulatory post-translational modifications (Levrero et al., 2009).

Because cccDNA is the transcriptional template of the virus (Quasdorff and Protzer, 2010), it is required for the maintenance of HBV infection. Unlike HBV transcripts and replicative intermediates, cccDNA is very stable in quiescent hepatocytes and is responsible for the persistence of infection during the natural course of chronic HBV infection and during prolonged antiviral therapy (Werle-Lapostolle et al., 2004). The cccDNA may persist for many years in the liver of patients, even after successful antiviral treatment and reinforcement of immunologic control (Zoulim, 2005). Currently, little is known about the mechanism of HBV cccDNA maintenance in the nuclei of hepatocytes. However, it has been shown that the cccDNA can be eliminated when infected hepatocytes are removed by immune cell-mediated killing or other non-cytopathic mechanisms (Murray et al., 2005) and replaced by cell turnover (Lutgehetmann et al., 2010).

REGULATION OF HBV cccDNA TRANSCRIPTION BY EPIGENETIC MODIFICATION

HISTONE ACETYLATION AND METHYLATION

Recently, it was proposed that the functionality of HBV cccDNA might be controlled by epigenetic mechanisms, regulating its transcriptional activity and HBV replication. Histones and non-histone proteins either bind directly to the cccDNA or are indirectly recruited to viral minichromosomes through protein–protein interactions. Thereby, the acetylation and deacetylation of cccDNA-bound histones may regulate HBV transcription. Exploring a ChIP assay using anti-acetylated-H3 or -H4 antibodies, Pollicino et al. (2006) found that HBV replication is indeed regulated by the acetylation status of H3/H4 histones bound to the viral cccDNA, both in cell-based replication systems and in the liver of chronically HBV infected patients. The co-recruitment of PCAF and p300/CBP parallels viral replication *in vitro*, whereas HDAC1 recruitment onto the HBV cccDNA correlates with low HBV replication *in vitro* and with low viremia *in vivo*. The importance of epigenetic modifications of cccDNA-bound histones in the regulation of HBV replication is further confirmed by experiments exploring the class I and class III HDAC inhibitors trichostatin A (TSA), valproate, and nicotinamide (NAM). These HDAC inhibitors induce an evident increase of both cccDNA-bound acetylated H4 and HBV replication. Another study demonstrated a similar role for the acetylation of cccDNA-bound histones, as well as a role for methylation and phosphorylation of these proteins (Gong et al., 2011).

A recent study demonstrated that in cultured hepatoma cells with HBV replication and in mouse models with repopulated human hepatocytes, administration of IFN-α resulted in the active recruitment of the transcriptional corepressors HDAC1, SIRT1, and polycomb repressor complexes 2 (EZH2 and YY1) to HBV cccDNA as well as the hypoacetylation/hypermethylation of cccDNA-bound histones. IFN-α treatment also reduced the binding of the transcription factors STAT1 and STAT2 to the IFN-sensitive response element on active cccDNA (Belloni et al., 2012). These observations suggested that IFN-α could epigenetically regulate HBV replication, and the hypoacetylation/hypermethylation of histones was associated with decreased replication of HBV. Furthermore, it was shown that small molecules that inhibit p300 and PCAF or activation of SIRT1/2 and EZH2 could induce an "active epigenetic suppression" of the HBV cccDNA minichromosome to suppress HBV replication (Palumbo et al., 2013).

HBV DNA METHYLATION

In addition to post-translational modification of histones, methylation of the CpG islands on HBV genomic DNA also contributes to the regulation of HBV gene expression (Mogul et al., 2011; Rivenbark et al., 2012). It has been shown that early integrated HBV DNA is methylated in HCC cells (Miller and Robinson, 1983; Chen et al., 1988). The non-integrated HBV DNA (Vivekanandan et al., 2008b) and cccDNA (Guo et al., 2009) could also be methylated in liver tissues from patients. Currently, at least six CpG islands have been identified in the HBV genome, including three conventional regions overlapping the start site of the HBV S gene (island 1), the region encompassing enhancer I and the X gene promoter (island 2), and the Sp1 promoter and start codon of the P gene (island 3; Zhang et al., 2013b). Methylation of CpG islands 1 and 2 was found in HBV DNA extracted from liver biopsies from CHB patients, suggesting that increased methylation of HBV DNA may decrease the production of viral proteins (Vivekanandan et al., 2008b). The hypermethylation of island 2 was correlated with low levels or absence of HBsAg production (Vivekanandan et al., 2008a), as well as reduced HBeAg expression (Guo et al., 2009). It was shown that individuals with occult HBV infection, which is characterized by the persistence of HBV DNA in the liver of individuals who test negative for the HBsAg, had a higher degree of methylation in island 2 compared to non-occult CHB patients (Vivekanandan et al., 2008a). Another study with a cohort of cirrhosis patients did not find an association between the methylation status of HBV cccDNA and HBsAg expression in liver tissues, but confirmed that a higher methylation density was associated with lower viral load, lower RNA copies per cccDNA, and lower virion productivity (Kim et al., 2011).

Consistent with these findings, transfection of methylated HBV DNA in HepG2 cells resulted in reduced HBV mRNA levels, decreased intracellular HBsAg and core HBcAg expression, and decreased secretion of HBV viral proteins into cell supernatants. Furthermore, an *in vitro* equivalent of cccDNA showed decreased viral protein production in HepG2 cells after DNA methylation (Vivekanandan et al., 2009). After transfection of HBV DNA into HepG2 cells, an inverse relationship between methylated HBV DNA and viral mRNA levels was observed in dependence on the upregulation of host DNA methyltransferase (DNMT). Cotransfection with DNMT3a and HBV DNA was associated with decreased production of HBsAg and HBeAg, as well as host proteins implicated in carcinogenesis (Vivekanandan et al., 2010).

These data from cell culture experiments suggest that HBV DNA methylation is associated with down regulation of viral protein production.

INTERPLAY BETWEEN HBV, miRNAs, AND THE EPIGENETIC MACHINERY

miRNAs PLAY A PIVOTAL ROLE IN THE EPIGENETIC REGULATION NETWORK

MicroRNAs are approximately 22 nucleotide-long non-coding RNAs that are emerging as key players in regulating gene expression in eukaryotes, influencing various biological processes such as development, infection, immunity, and carcinogenesis (Ambros, 2004). The biogenesis and mechanisms of action of these tiny but potent molecules have been described in detail (Bartel, 2004). Briefly, miRNAs are transcribed from the host genome and generated by Drosha- and Dicer-mediated enzymatic cleavage. Mature miRNAs are engaged in either translational arrest or degradation of targeted transcripts through imperfect base pairing with the 3′-untranslated region (UTR) or the coding region of the target transcripts. Currently, more than 2000 miRNAs have been identified in human organs (Griffiths-Jones et al., 2008). The expression profiles of these miRNAs in different cells or tissues may exhibit temporal or tissue-specific patterns (Skalsky and Cullen, 2010).

Many studies have shown that a set of miRNAs play a pivotal role in the epigenetic regulation network (Chuang and Jones, 2007; Iorio et al., 2010). Epigenetic modifications, such as promoter methylation or histone acetylation, have been demonstrated to affect miRNA expression and are potentially responsible for the aberrant miRNA regulation observed in cancer (Baer et al., 2013). Along with the epigenetic regulation of miRNA expression, many miRNAs themselves can regulate the expression of components of the epigenetic machinery, creating a highly controlled feedback mechanism. A number of the miRNAs related to epigenetic regulation were defined as so-called "epi-miRNAs." For example, DNMT1 overexpression was responsible for the hypermethylation of the miR-148a and miR-152 promoters. As a direct target of miR-148a and miR-152, DNMT1 was inversely related to the expression levels of miR-148a and miR-152 (Chen et al., 2013). Similarly, miR-1 and miR-449a, which could be induced by 5-AzaC/TSA treatment (Datta et al., 2008) or by HDAC1-3 knock down (Buurman et al., 2012) in HCC cells, directly targeted HDAC4 (Chen et al., 2006) and HDAC1 (Noonan et al., 2009), respectively.

HBV INFECTION AFFECTS miRNA EXPRESSION

Although the viral miRNAs encoded by HBV have not been verified, the products of HBV were shown to alter miRNA expression profiles. In chronic HBV infection or HBV-related HCC, the miRNA profiles in liver tissue or serum levels from numerous studies are controversial and complicated (Ura et al., 2009; Hou et al., 2011; Liu et al., 2011; Wang et al., 2012b). For instance, it was reported that subviral HBsAg circulating in the blood of HBV carriers could carry liver-specific miRNAs (miR-27a, miR-30b, miR-122, miR-126, and miR-145) as well as immune regulatory miRNAs (miR-106b and miR-223) that were involved in hepatocarcinogenesis and HBV persistence (Novellino et al., 2012). In another study, three miRNAs (miR-122, miR-22, and miR-99a) were upregulated at least 1.5-fold in the sera of HBV-infected patients (Hayes et al., 2012). The highly liver-enriched, abundantly expressed miR-122 was consistently upregulated in HBV infected patients, and miR-145 could be used as a candidate tumor suppressive miRNA in the early steps of HBV-related hepatocarcinogenesis (Gao et al., 2011).

Recently, molecular studies have revealed that the HBx protein, which is essential for virus replication *in vivo*, induced epigenetic changes, including aberrations in DNA methylation, histone modifications, and miRNA expression. HBx expression has been found to be associated with alterations in the host miRNA profile through different epigenetic mechanisms (Tian et al., 2013). MiRNAs upregulated by HBx include miRNA-29a and miR-143 (Zhang et al., 2009; Kong et al., 2011). HBx-downregulated miRNAs include miR-101, miR-122, miR-132, miR-148a, miR-152, let-7, and the miR-16 family (Huang et al., 2010; Wang et al., 2010; Wu et al., 2011; Song et al., 2013; Wei et al., 2013a,b; Xu et al., 2013). In addition, HBx was shown to activate HBV transcription through opposition to the protein phosphatase 1 and HDAC1 complex on the HBV cccDNA (Cougot et al., 2012), or down-regulate DNMT3A expression through miR-101 induction (Wei et al., 2013b). Loss of HBx reduced recruitment of p300, caused rapid hypoacetylation of the cccDNA-bound histones and increased early recruitment of SIRT1 and HDAC1, accompanied by lower HBV replication (Belloni et al., 2009).

CELLULAR miRNAs INHIBIT HBV REPLICATION BY DIRECT BINDING

As HBV produces different transcripts during its life cycle, the transcripts are proposed to be targeted by cellular miRNAs. In a screen for cellular miRNAs affecting HBV replication, Zhang et al. (2010) employed a loss-of-function approach by transfecting antagomirs targeting 328 human miRNAs into HepG2 cells. Two miRNAs, miR-199a-3p and miR-210, were shown to suppress HBsAg expression. The direct effect of these two miRNAs on HBV RNA transcripts was validated by GFP reporter assay (Zhang et al., 2010). In addition, Russo's group found that miR-125a-5p is able to interfere with HBsAg expression, thus reducing the amount of secreted HBsAg (Potenza et al., 2011). Recently, many cancer-related miRNAs, including miR-15a/miR-16-1 (Wang et al., 2013a), the miR-17-92 cluster (Jung et al., 2013), and miR-224 (Scisciani et al., 2011), were shown to target HBV mRNAs directly by luciferase reporter assay and inhibit HBV replication (summarized in **Figure 1**). Notably, the expression of these miRNAs was also linked to epigenetic regulation, as well as to promoter methylation (Dakhlallah et al., 2013) and histone acetylation (Zhang et al., 2013a; Wang et al., 2013b).

CELLULAR miRNAs REGULATE HBV REPLICATION INDIRECTLY

In addition to direct targeting, some cellular miRNAs, including epi-miRNAs, were found to be capable of inhibiting or stimulating HBV replication by indirectly regulating cellular transcription factors. It was shown that the transcription of HBV cccDNA was tightly regulated by a number of liver-enriched transcription factors and nuclear receptors through the recognition of HBV promoter/enhancer elements (Quasdorff and

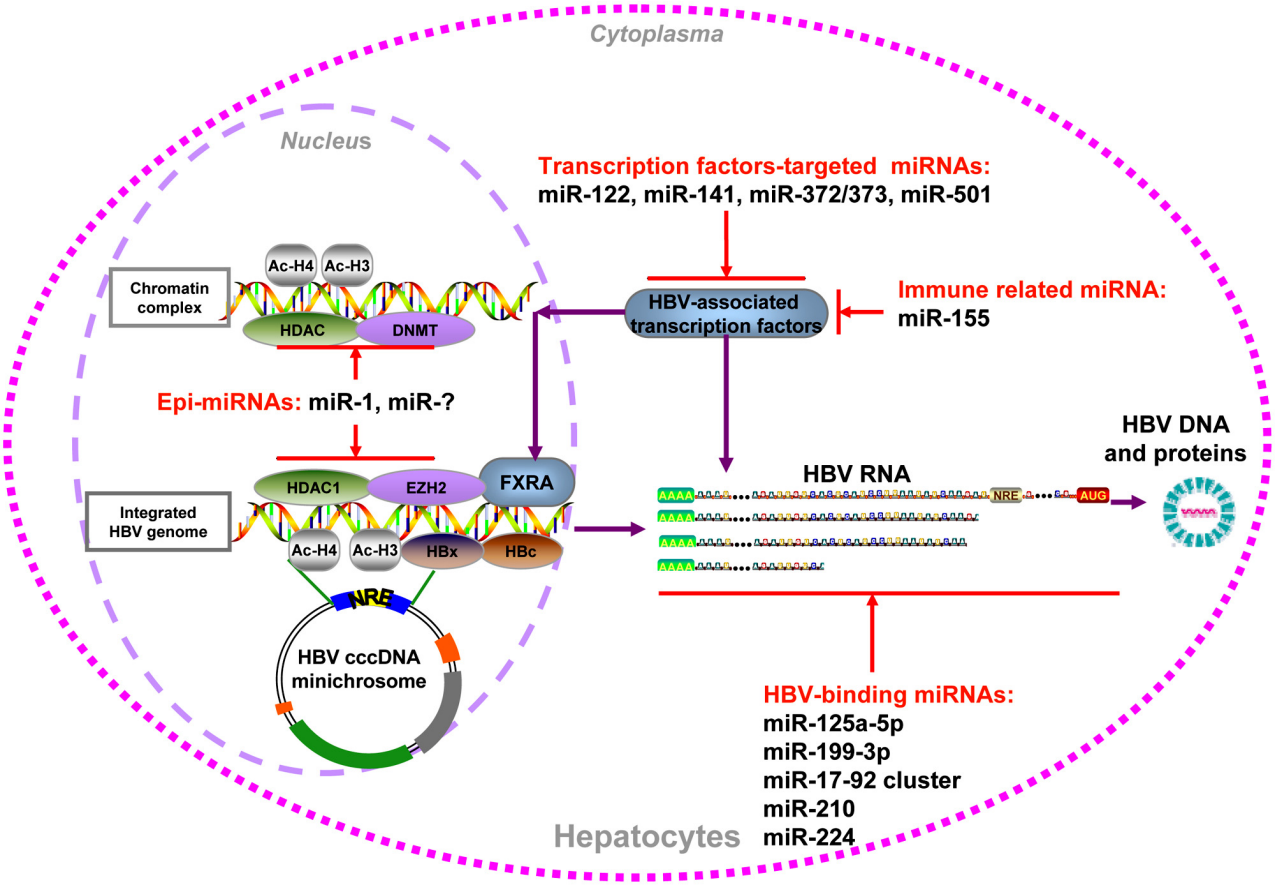

FIGURE 1 | Summary of cellular miRNAs effect on HBV replication. HBV DNA could be integrated into host genome or form cccDNA minichromosome in the hepatocytes nucleus. It serves as the temple for viral transcription, viral DNA, and antigens production. The miRNAs which regulate HBV replication through epigenetic mechanism, transcription factors regulation, immune regulation, and direct targeting viral transcripts were indicated.

Protzer, 2010). miR-122 may exert its effect on HBV indirectly via downregulation of its target cyclin G1, thus interrupting the interaction between cyclin G1 and p53 and abrogating p53-mediated inhibition of HBV replication (Wang et al., 2012a). miR-372 and -373 are upregulated in HBV-infected liver tissues and promote HBV gene expression through a pathway involving the transcription factor nuclear factor I/B (Guo et al., 2011). The higher expression of miR-501 in HCC tissues could enhance HBV replication partially by targeting HBXIP (Jin et al., 2013). In contrast, miR-141 significantly suppresses HBV expression and replication in HepG2 cells. Bioinformatic analysis and experimental assays indicate that peroxisome proliferator-activated receptor alpha is a relevant target of miR-141 during this process (Hu et al., 2013). For immune-related miRNAs, miR-155 enhances innate antiviral immunity by promoting the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway through the targeting of SOCS1, mildly inhibiting HBV infection in human hepatoma cells (Su et al., 2011).

By screening a set of cellular miRNAs, our group found that epigenetically regulated miR-1 over-expression resulted in a marked increase in HBV replication, accompanied with upregulated HBV transcription, antigen expression, and progeny secretion. HDAC4, the cellular target of miR-1, was able to suppress HBV replication. The expression of nuclear receptor farnesoid X receptor alpha (FXRA) was increased by miR-1, leading to the enhanced transcriptional activity of the HBV core promoter (Zhang et al., 2011). Furthermore, another epi-miRNA that targets HDAC1, miR-449a, had an even higher capacity for enhancing HBV replication but a lower level of induction of FXRA (Zhang et al., unpublished data). Additionally, both of these two defined epi-miRNAs could inhibit the G1/S cell cycle transition and promote cell differentiation by increasing the expression of hepatocyte-specific factors, which may be beneficial for HBV replication (Zhang et al., 2011). Collectively, host epi-miRNAs can modulate HBV replication by regulating cellular epigenetic factors or specific transcription factors that directly bind to the HBV cccDNA minichromosome (summarized in **Figure 1**).

CONCLUSION AND PERSPECTIVE

In this review, we summarize the available information about the epigenetic mechanisms involved in the regulation of HBV cccDNA function. Notably, miRNAs could be considered part of a multilevel regulatory mechanism aimed to precisely modulate

HBV replication and gene expression, likely in the response to the changing hepatic microenvironment. Considerably, many cellular miRNAs indirectly influence the HBV life cycle by regulating the expression of relevant cellular proteins and may play important roles in hepatitis B pathogenesis. Future studies need to be performed to elucidate the regulatory loop involving miRNAs and the cccDNA epigenetic machinery and certainly to investigate how to translate these findings into clinical applications.

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Exploiting tumor epigenetics to improve oncolytic virotherapy

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Oncolytic viruses (OVs) comprise a versatile and multi-mechanistic therapeutic platform in the growing arsenal of anticancer biologics. These replicating therapeutics find favorable conditions in the tumor niche, characterized among others by increased metabolism, reduced anti-tumor/antiviral immunity, and disorganized vasculature. Through a self-amplification that is dependent on multiple cancer-specific defects, these agents exhibit remarkable tumor selectivity. With several OVs completing or entering Phase III clinical evaluation, their therapeutic potential as well as the challenges ahead are increasingly clear. One key hurdle is tumor heterogeneity, which results in variations in the ability of tumors to support productive infection by OVs and to induce adaptive anti-tumor immunity. To this end, mounting evidence suggests tumor epigenetics may play a key role. This review will focus on the epigenetic landscape of tumors and how it relates to OV infection. Therapeutic strategies aiming to exploit the epigenetic identity of tumors in order to improve OV therapy are also discussed.

Keywords: oncolytic virotherapy, epigenetic modulation, cancer, tumor heterogeneity, anti-viral response, antigen presentation

INTRODUCTION

While genetic information establishes the primary blueprint for cellular identity, multiple regulatory layers responsive to extra and intra-cellular signals ultimately control the manifestation of this blueprint. Changes in cellular state, including initiation of DNA synthesis, activation of apoptotic programs, or triggering of antiviral defense mechanisms, result from an integrated response to stimuli received by the cell. These are controlled in large part by gene/protein expression profiles unique to each cell. It is now well understood that activation of transcription factors that bind in a DNA sequence-specific manner at promoter and enhancer elements is responsible for many of the changes in gene expression that occur in response to environmental or developmental cues. However transcription factors and their associated gene targets are themselves further regulated by the accessibility of DNA sequences. Since the genome resides in the finite space provided by the nucleus, it interacts with proteins known as histones to form chromatin and facilitate its compaction. The configuration of chromatin compaction is modulated by epigenetic modification and is a key determinant for transcription factor-mediated activation of gene transcription (Magnani et al., 2011).

Epigenetic modifications create a reversible imprint that may be inherited through cell division. For example, DNA methylated at promoter CpG islands is associated with gene silencing and can be reversed by treatment with DNA methyltransferase inhibitors such as 5-AZA (5-aza-2′-deoxycytidine) leading to the reactivation of silenced genes (Baylin and Jones, 2011; Krecmerova and Otmar, 2012). Similarly, chromatin structure can alter accessibility to the DNA template and can be readily remodeled by histone post-translational modifications (PTMs). PTMs including acetylation, methylation, phosphorylation, ubiquitination, and many others can be added to numerous residues of histone proteins (Bannister and Kouzarides, 2011). Different PTMs will favor chromatin compaction while others will increase its accessibility to DNA binding proteins. Histone modifications and DNA methylation are highly interdependent processes and define the epigenetic code (Cedar and Bergman, 2009). The epigenetic code is regulated by a complex interplay of enzymatic erasers, readers, and writers that exhibit specificities toward different histones and residues (Rice and Allis, 2001). For example, the level of histone acetylation is regulated by the relative activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs), proteins with opposing

enzymatic activities that are often found in the same protein complexes (Johnsson et al., 2009; Peserico and Simone, 2010). This also applies to histone lysine methyltransferases (KMTs) and lysine demethylases (KDMs). Consequently, modulating the activity of histone-modifying enzymes can profoundly alter the epigenetic profile of a cell (Egger et al., 2004; Yoo and Jones, 2006).

Given their critical role in the regulation of normal cellular physiology, it is not surprising that aberrations in epigenetic modifications can contribute to the manifestations of human disease. For example, a cell's epigenetic profile can impact the progression of acute microbial diseases (discussed in more detail below) as well as the development and treatment of chronic diseases such as cancer. DNA hypermethylation is often observed in cancer cells (Patel et al., 2012). The genome-wide distribution of histone modifications can also be altered in the course of cancer development (Akhtar-Zaidi et al., 2012; Magnani et al., 2013). As well, the activity of various histone-modifying enzymes can be altered through mutations (Taylor et al., 2011), aberrant expression (Schildhaus et al., 2011; Bennani-Baiti et al., 2012) and/or recruitment to target histone residues via oncogenic fusion proteins (Lubieniecka et al., 2008). Consequently, many cancers are sensitive to epigenetic modulators such as 5-AZA, HDAC, or KDM inhibitors (Hurtubise et al., 2008; Taylor et al., 2011; Schenk et al., 2012) and epigenetic modifications have been shown to influence the response to chemotherapy (Glasspool et al., 2006; Magnani et al., 2013).

ONCOLYTIC VIROTHERAPY

While epigenetic modulators hold promise as anticancer agents, it is clear that like for many other cancer therapies, tumor-specificity is of paramount importance. Tremendous efforts have been made over the past decades to tackle the difficult task of developing more selective cancer therapies, aiming to exploit the sometimes-subtle differences between normal tissues and tumors. One promising new class of therapeutics comes to us from the field of virology. Since the early 1900s it has been observed that cancers can be uniquely susceptible to virus infection (Dock, 1904). While the first clinical trials using replication-competent viruses to treat cancer began in the seventies (Asada, 1974; Kelly and Russell, 2007; Pol et al., 2013), approval of the first oncolytic virus (OV) is only now in the foreseeable future in North America (Carroll, 2011; Galanis et al., 2012; Heo et al., 2013). The more recent clinical success of OVs is in large part due to our more complete understanding of the molecular biology of both cancer cells and viruses that allowed us to create virus strains with improved selectivity and anti-tumor activity, and clinical safety profile (Breitbach et al., 2011). Rapid proliferation and deregulated metabolism (Fritz and Fajas, 2010), disorganized vasculature (Jain, 2005), and defective antiviral innate immune responses (Dunn et al., 2006) in malignant tumors are hallmarks that not only define cancer, but also favor viral growth. Building on these observations, several OVs have been engineered or selected to take advantage of one or more of these features (Russell et al., 2012). A variety of OV platforms are currently under clinical evaluation including those based on herpes simplex virus (HSV), Reovirus, vaccinia virus (VV), Adenovirus, Measles virus, and vesicular stomatitis virus (VSV; U.S. National Library of Medicine, 2013).

ONCOLYTIC VIROTHERAPY AND THE CELLULAR INNATE ANTIVIRAL RESPONSE

It is now well established that cancer cells that evolve to frank malignancies often acquire defects in their ability to mount a successful antiviral response and this attribute/deficit contributes to the selectivity of many if not all OVs (Norman and Lee, 2000; Stojdl et al., 2000, 2003). This is often a consequence of the observation that approximately 65–70% of tumors are unable to produce or respond to type I interferon (IFN), a key mediator of the cellular antiviral response (Stojdl et al., 2003; Dunn et al., 2006). IFNs are antiviral cytokines induced following recognition of viral proteins and nucleic acids by cellular pattern recognition receptors such as Toll-like receptors (TLRs) that signal through to transcription factors such as interferon regulatory factors (IRFs). There are many isoforms of IFN, which can be functionally sub-divided in at least three types (types I/II/III). While type I/III IFNs (e.g., IFN-α, IFN-β/IFN-λ) stimulate cellular antimicrobial immunity; type II IFNs (e.g., IFN-γ) coordinate the host immune response. IFNs elicit their transcriptional effects through autocrine and paracrine activation of IFN receptors and signaling through the Jak/STAT signaling pathway (Borden et al., 2007). This induces the transcriptional up-regulation of interferon-stimulated genes (ISGs), many of which have direct antiviral/pro-apoptotic activities (e.g., RNAseL, TNF-α, TRAIL) and/or immune-stimulatory properties (e.g., components of major histocompatibility complex).

ONCOLYTIC VIRUSES AND THE GENERATION OF AN ANTI-TUMOR IMMUNE RESPONSE

In addition to taking advantage of a niche provided by aberrations unique to cancer and the tumor microenvironment, OVs have been used as platforms to express a range of therapeutic transgenes, from suicide genes to immune-stimulatory cytokines (Merrick et al., 2009; Maldonado et al., 2010; Chai et al., 2012; Stephenson et al., 2012; Lange et al., 2013). In this regard, it is now well recognized that beyond simply lysing infected tumor cells, OVs effectively "de-cloak" tumors by stimulating immune cells to recognize cancer antigens, ultimately leading to tumor destruction and in some cases, long-term cures (Sobol et al., 2011; Huang et al., 2012). Many tumors evade immune recognition due to a dysfunctional antigen presentation pathway, which is under tight multilayered transcriptional control ultimately dictated by type I/II IFNs and the class II transactivator (CIITA). This transcription factor controls the expression of numerous genes involved in antigen presentation, including class I and II MHC molecules, which display tumor or pathogen derived peptides to killer T cells ($CD4^+$/$CD8^+$; LeibundGut-Landmann et al., 2004).

The antigen presentation pathway is influenced by both tumorigenesis and OV therapy. Many tumor cells including leukemias, lymphomas, and carcinomas, avoid immune recognition due to a dysfunctional antigen presentation pathway, largely caused by epigenetic silencing (e.g., histone deacetylation or DNA methylation) of *MHC2TA*, the gene encoding CIITA (LeibundGut-Landmann et al., 2004). OV therapies can enhance tumor-associated antigen presentation through various mechanisms. In response to OV infection, type I and II IFN secretion by infected cells within the tumor environment (which also includes normal tumor infiltrating cells) leads to the up-regulation of hundreds of ISGs including

IRF-1, which up-regulates CIITA expression (Muhlethaler-Mottet et al., 1998). Notably, this response is dependent upon the ability to respond to IFN, which can be limited in many cancer cells (Stojdl et al., 2003; Dunn et al., 2006).

Oncolytic virotherapy can have a positive influence on antigen presentation and the anti-tumor response. Some OVs including HSV, reovirus, and measles virus, induce syncytia formation in infected and neighboring cells. These large multinucleated tumor cells secrete an abundance of "syncytiosomes," which are exosome-like vesicles that present tumor-associated antigens via MHC molecules (Bateman et al., 2000, 2002). Finally, destruction of cancer cells following infection by OVs provides an additional source of tumor antigens available for capture by antigen-presenting immune cells. The immunostimulatory nature of the virus itself, through activation of TLRs and subsequent cellular production of pro-inflammatory cytokines stimulates the recruitment of antigen-presenting cells that sample tumor-derived and virus-expressed antigens. Presentation of tumor antigens to killer T cells ($CD4^+/CD8^+$) through MHC molecules in the presence of inflammatory cytokines can thus lead to generation of a robust and long-lasting immune responses directed against the tumor.

To capitalize on these beneficial immunological effects, some groups have developed OV/vaccine hybrid strategies. These strategies are designed specifically to re-educate the adaptive immune system to recognize and respond to tumor antigens. Thus, OVs can be engineered to express not only immune-stimulatory cytokines but also tumor-specific antigens to further stimulate an anti-tumor immune response following OV infection of cancer cells (Diaz et al., 2007; Pulido et al., 2012). Indeed, several studies have shown that this "tumor antigen vaccination" effect can be further amplified using a prime-boost strategy, by priming with an antigen then boosting the response using an OV expressing the same antigen (Bridle et al., 2010, 2013). As discussed below, it is possible to use epigenetic modifiers to further fine-tune this oncolytic vaccine approach. It is also possible to take advantage of this vaccine effect by infecting cancer cells *ex vivo* and re-injecting the inactivated "oncolysate" to generate prophylactic and even therapeutic anti-cancer immune responses. The resulting up-regulation of MHCs and co-regulatory factors and presentation of tumor antigens at the surface of OV infected cells as well as the presence of immune-stimulating virus is thought to be at the root of this effect (Lemay et al., 2012). Overall, these studies emphasize the important role of antigen expression/presentation in OV-stimulated anti-tumoral responses.

TUMOR HETEROGENEITY: INHERENT BARRIER TO OV THERAPY

Despite promising clinical data, it is clear that there is considerable inter- (and likely intra-) tumor heterogeneity in the responsiveness to OV therapy *in vitro* as well as *in vivo* in both pre-clinical and clinical settings (Breitbach et al., 2011; Sobol et al., 2011). Because overcoming the innate cellular antiviral response and generating a robust anti-tumor response are critical to observe meaningful therapeutic benefits from oncolytic virotherapy, it is important to understand what tumorigenic processes influence these closely linked pathways in order to manipulate them to improve therapeutic outcomes.

Given the profound epigenetic divergence that prevails in tumor cells (Akhtar-Zaidi et al., 2012; De Carvalho et al., 2012), it is foreseeable that tumor-specific gene expression response profiles induced by virus infection may be altered by epigenetic modifications and that this could contribute to the heterogeneity of tumor responsiveness to OVs. As discussed previously, epigenetic reprogramming is well known to play an important role in oncogenic transformation and numerous reviews extensively cover the role of epigenetics in cancer (Muntean and Hess, 2009; Baylin and Jones, 2011; Hatziapostolou and Iliopoulos, 2011; Suva et al., 2013). Thus, the remainder of this review aims to highlight current knowledge of genes epigenetically regulated in cancer that are also involved in pathways critical for OV therapy, namely the IFN-mediated antiviral response and antigen presentation (**Table 1**), and how this contributes to tumor heterogeneity (**Figure 1**).

THE ROLE OF EPIGENETICS IN HOST SUSCEPTIBILITY TO VIRAL INFECTION

Epigenetic regulation of innate and adaptive immune processes is emerging as a key determinant of susceptibility to viral infection. Several reports suggest that cell type-specific epigenetic regulation of antiviral ISGs leads to differences in permissibility to virus infections in both normal and tumor cells (Naka et al., 2006; Nguyen et al., 2008; Fang et al., 2012; Chen et al., 2013; Cho et al., 2013). Recently, histone H3K9 di-methylation, a repressive heterochromatin mark, was found to be elevated within IFN genes and ISGs in non-professional IFN-producing cells (e.g., fibroblasts) as compared to professional IFN-producing plasmacytoid dendritic cells (pDCs). Interestingly, inhibiting the KMT G9a by both genetic and pharmacological means led to increased IFN production and responsiveness in fibroblasts. In line with this, G9a-ablated fibroblasts were also rendered more resistant to infection by viruses (Fang et al., 2012; **Figure 1**).

Another recent study in mice harboring the murine viral susceptibility locus *Tmevp3* revealed the intriguing role of *NeST*, a long non-coding RNA (lncRNA) adjacent to the IFN-γ locus in both mice and humans (Vigneau et al., 2001). NeST was found to function as an epigenetically driven enhancer element (Gomez et al., 2013) leading to increased IFN-γ production in mouse $CD8^+$ T cells by directly interacting with the H3K4 histone methyltransferase complex and increasing H3K4 trimethylation, an activating mark. This novel epigenetic modification culminated in heightened susceptibility to persistent viral infection in mice (Gomez et al., 2013; **Figure 1**). Although the role of *NeST* in human epigenetic regulation is currently unknown, it is likely lncRNAs contribute to epigenetic regulation and manifestation of cell phenotypes including permissiveness to virus infection and cancer.

CANCER EPIGENETICS IMPACT THE REGULATION OF ANTIVIRAL RESPONSE GENES

As previously discussed, the majority (but not all) of cancer cells are dysfunctional in their ability to produce and/or respond to IFN (Dunn et al., 2006). While crosstalk between oncogenic signals and the antiviral response pathways have been shown to play a role (Farassati et al., 2001; Shmulevitz et al., 2005); epigenetic events are also likely contributors to this phenotype. One indication of this comes from a series of studies on cells derived from

Table 1 | Epigenetic control: implications in cancer and OV therapy.

Genetic target	Cellular function	Epigenetic modification	Cell type	Reference
ISGs (*IFI27*, *9–27*, *LMP2*, *LMP7*, *Viperin*, *IFI44*, *IFIT2*, *ISG56*)	Antiviral response	DNA hypermethylation	Huh-7 cells (Human hepatoma)	Naka et al. (2006)
STAT1, *ISGs* (*IFI27, IRG1*, *Viperin, Cxcl10, ISG15, IFI44)*	Antiviral response, anti-tumor response, antigen presentation	Histone deacetylation	Human cortical neurons	Cho et al. (2013)
CREB3LI, *MX1*	Antiviral response	DNA hypermethylation	Human hepatoma Huh-7 cells	Chen et al. (2013)
IFN-β, ISGs (*MX1*, *IFIT1*, among many)	Antiviral response	H3K9 dimethylation	Mouse embryonic fibroblasts, mouse splenic dendritic cells	Fang et al. (2012)
IFN-γ	Antiviral response, anti-tumor response	H3K4 trimethylation	Mouse $CD4^+/CD8^+$ T cells	Gomez et al. (2013)
IRF7, IFN regulated genes	IFN-β induction, antiviral response	DNA hypermethylation	Li-Fraumeni immortalized cells	Fridman et al. (2006)
IRF7, IFITM1, OAS1, OAS2, STAT1, MX1, TIP30, IL-8, TRAIL, HLA-F, HLA class I locus C heavy chain, among others	IFN-α/β induction, antiviral response, anti-tumor response, antigen presentation	DNA hypermethylation	Li-Fraumeni immortalized cells	Kulaeva et al. (2003)
IRF7	IFN-α/β induction	DNA hypermethylation	Li-Fraumeni immortalized cells	Li et al. (2008)
IRF8	IFN signaling, differentiation, apoptosis, tumor suppression	DNA hypermethylation	Nasopharyngeal, esophageal, breast, and cervical primary carcinomas	Lee et al. (2008)
IRF4, IRF5, IRF8	IFN signaling, differentiation, apoptosis signaling, tumor suppression	DNA hypermethylation	Gastric carcinoma	Yamashita et al. (2010)
STAT1, STAT2, and *STAT3*	Antiviral response, antigen presentation, anti-tumor response	DNA hypermethylation	Colon carcinoma	Karpf et al. (1999)
JAK1 kinase	Antiviral response, antigen presentation, anti-tumor response	DNA hypermethylation, histone deacetylation	Prostate adenocarcinoma	Dunn et al. (2005)
Apo2L/TRAIL receptor 1 (DR4), RASSFIA, XAF1, TRAIL	TRAIL-mediated apoptosis	DNA hypermethylation	Melanoma cell lines, renal carcinoma, experimentally transformed human cell lines	Reu et al. (2006a,b), Bae et al. (2008), Lund et al. (2011)
unknown	TRAIL-mediated apoptosis	Histone deacetylation	Medulloblastoma	Hacker et al. (2009)

(Continued)

Table 1 | Continued

Genetic target	Cellular function	Epigenetic modification	Cell type	Reference
IFITM1	Antiviral response	DNA hypermethylation	Gastric carcinoma	Lee et al. (2012)
ISGs (Global regulation)	Antiviral response, Anti-tumor response	Histone deacetylation	U2OS (osteosarcoma), HeLa (cervical carcinoma)	Chang et al. (2004)
ISGs under ISRE control	Antiviral response, Anti-tumor response	Histone deacetylation	Human foreskin fibroblasts	Sakamoto et al. (2004)
IFN-β, FGF2, VEGFC, CASP1, CASP9, ISGs (OAS2, MyD88, IFIT1, ISG15, TGFB1, IRF7, IL-8, among others)	Antiviral response, Angiogenesis, Apoptosis	Histone deacetylation	Human fetal microglia, astrocytes	Suh et al. (2010)
STAT-1 dependent genes, ISGs	Antiviral response, apoptosis, anti-tumor response	Histone deacetylation	Colorectal carcinoma cells; L929 cells (mouse fibroblasts)	Génin et al. (2003), Klampfer et al. (2004)
2′–5′ OAS, ISG54, IFITM3, IP-10	Antiviral response	Histone deacetylation	2fTGH (sarcoma) cells	Nusinzon and Horvath (2003)
CIITA	Antigen presentation	Histone deacetylation	Mouse plasmacytoma cells; squamous cell carcinoma; rhabdomyosarcomas	Kanaseki et al. (2003), Chou (2005), Londhe et al. (2012)
unknown	Antigen presentation	Histone deacetylation	Mouse plasmacytomas	Khan et al. (2004)
CIITA	Antigen presentation	H3K27 trimethylation	Uveal melanoma cells, breast cancer cells	Holling et al. (2007), Truax et al. (2012)
CIITA	Antigen presentation	DNA hypermethylation	Head and neck cancer cells, choriocarcinoma cells, uveal melanoma, colorectal and gastric carcinomas	Morris et al. (2000), Satoh et al. (2004), Radosevich et al. (2007), Meissner et al. (2008)
CIITA	Antigen presentation	Histone deacetylation, DNA hypermethylation	Myeloid leukemia	Morimoto et al. (2004)
TAP-1	Antigen presentation	Histone H3 acetylation	Carcinomas	Setiadi et al. (2007)

Numerous reports have cited instances of epigenetic modulation affecting permissibility to virus infection, many of which occur in tumor cells. Here we present a summary of these reports, listing the genetic target and its cellular function, the epigenetic modification, and the cell type involved. IFN, interferon; ISG, interferon stimulated gene; IFI, IFN alpha inducible protein; LMP, low molecular weight polypeptide; STAT, signal transducer and activator of transcription; CXCL1, C-X-C motif ligand 1; CREB3L1, cAMP responsive element binding protein 3 like-1; MX1, myxovirus resistance 1; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IRF, IFN regulatory factor; OAS, 2′–5′ oligoadenylate synthetase; TIP30, TAT-interacting protein 30; IL, interleukin; TRAIL, tumor necrosis factor- related apoptosis-inducing ligand; HLA, human leukocyte antigen; JAK1, janus kinase 1; DR4, Apo2/TRAIL receptor 4; XAF1, x-linked inhibitor of apoptosis-associated factor 1; ISRE, IFN sensitive response element; FGF2, fibroblast growth factor 2; VEGFC, vascular endothelial growth factor C; CASP, caspase; TGFB1, transforming growth factor beta 1; CIITA, Class II MHC transactivator; TAP-1, transporter 1, ATP-binding cassette, sub-family B.

cancer-prone Li-Fraumeni syndrome patients. Cells from these patients spontaneously immortalize when serially passaged in tissue culture due to mutations in the tumor suppressor p53, however transformation is inhibited upon treatment with 5-AZA (Kulaeva et al., 2003; Fridman et al., 2006). DNA methylation profiling of these immortalized cells revealed hypermethylation at the promoters of numerous genes involved in the type I IFN pathway, including IRF7 (Kulaeva et al., 2003; Fridman et al., 2006; Li et al., 2008). Interestingly, these immortalized Li-Fraumeni patient-derived cells were inherently more sensitive to VSV infection (Fridman et al., 2006; **Figure 1**).

Indeed, epigenetic repression of IFN and associated genes correlates with IFN insensitivity in many cancers. IRFs 4, 5, 7, and 8 are the target of DNA methylation, leading to dysfunctional responsiveness to type I and II IFNs in gastric cancer (Yamashita et al., 2010), while IRF8 is silenced by the same mechanism in

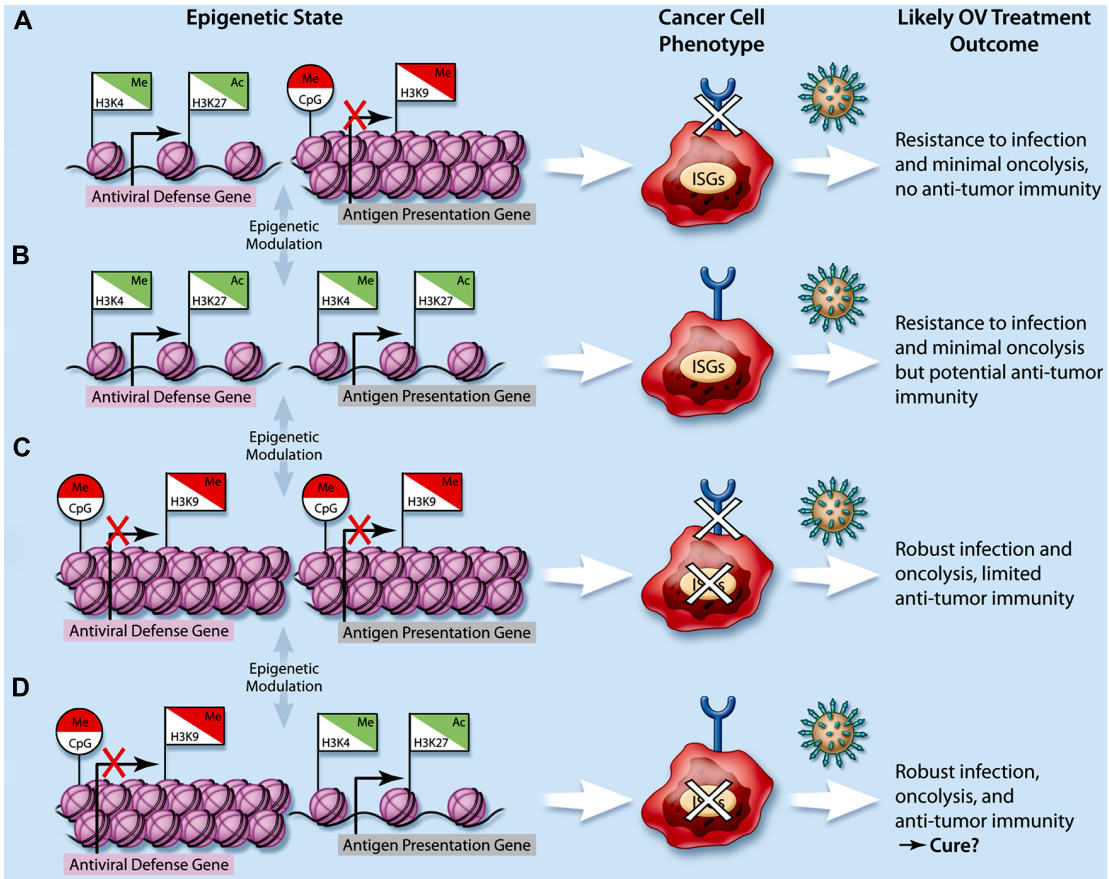

FIGURE 1 | Impact of cancer epigenetics on oncolytic virotherapy. The integration of repressive epigenetic marks such as DNA CpG methylation (Me, circle flags) and histone H3K9 methylation (Me, square flags), and activating epigenetic marks such as histone H3K4 methylation and histone H3K27 acetylation (Ac, square flags) lead to higher-order nucleosome packaging and repression (red flags) or open chromatin and gene expression (green flags). In cancer cells, dysregulation of epigenetic processes leads to various possible epigenetic states with respect to genes involved in the antiviral response (e.g., type I IFN, interferon stimulated genes or ISGs) as well as those involved in antigen presentation (e.g., MHC I/II expression, represented by a semi-circle at the end of a stick). This ultimately leads to a variety of cancer cell phenotypes **(A–D)** and subsequently, a variety of potential therapeutic responses to oncolytic viruses (OVs, represented by spiked green circles).

several carcinomas (Lee et al., 2008). Similarly, IFN responsiveness was found to be suppressed in colon carcinoma cells due to DNA methylation at STAT1, STAT2, and STAT3, which can be restored following 5-AZA treatment (Karpf et al., 1999; **Figure 1**). Along the same signaling axis, epigenetic silencing of JAK1 in prostate adenocarcinoma cells was associated with unresponsiveness to both type I and type II IFNs (Dunn et al., 2005).

IFN-induced apoptosis is mediated by ISGs including Apo2L/TRAIL, which are also often dysfunctional in cancers (Reu et al., 2006b; Borden, 2007; Bae et al., 2008; Burton et al., 2013). Genes involved in Apo2L/TRAIL signaling, including TRAIL, the TRAIL receptor DR4, RASSF1A, and XAF1 are epigenetically silenced in melanomas (Reu et al., 2006a,b; Bae et al., 2008), leukemia (Soncini et al., 2013), renal carcinoma (Reu et al., 2006a) and experimentally transformed cells (Lund et al., 2011). Interestingly, 5-AZA treatment can restore TRAIL-mediated apoptosis induced by type I and II IFN (Reu et al., 2006a,b; Bae et al., 2008; Lund et al., 2011; Soncini et al., 2013; **Figure 1**). However, this cell death pathway is likely also epigenetically silenced through histone PTMs given that in medulloblastoma, IFN-γ could induce apoptosis via TRAIL only following treatment with the HDAC inhibitor valproic acid (Hacker et al., 2009).

Overall, these studies highlight multiple epigenetic mechanisms that transcriptionally repress IFN-associated genes, culminating in dysfunctional and non-responsive IFN signaling across various cancer subtypes. However, in some instances alterations to epigenetic modifications in cancer lead to the up-regulation of antiviral factors. In both gastric tumors and gliomas, overexpression of the ISG IFITM1 promotes cancer cell migration and invasion, and its elevated expression is linked to reduced CpG methylation levels (Yu et al., 2011; Lee et al., 2012). Alongside its oncogenic properties, IFITM1 has antiviral properties, through its ability to inhibit viral membrane fusion (Li et al., 2013; **Figure 1**).

It is also notable that while most cancers display IFN pathway defects, approximately a third of cancer cells are fully functional in their ability to produce and respond to IFN (Stojdl et al., 2003; Norman and Lee, 2000). Importantly, several studies have shown that HDAC inhibition using a variety of chemical inhibitors

modulate IFN-induced expression of ISGs, type I IFN, and TLR3/4 (Génin et al., 2003; Nusinzon and Horvath, 2003; Chang et al., 2004; Klampfer et al., 2004; Sakamoto et al., 2004; Suh et al., 2010), which leads to increased OV activity in resistant cells (Nguyen et al., 2008). This further highlights the key role of epigenetic regulation in the generation of an antiviral response and suggests that it may be possible to improve OV efficacy in resistant tumors by manipulating the cancer epigenome as will be discussed shortly.

CANCER CELLS EPIGENETICALLY REGULATE GENES INVOLVED IN ANTIGEN PRESENTATION

In addition to inactivating the antiviral response to escape anti-proliferative/pro-death signals, tumors must also evade immune recognition and clearance. To this end, many tumor types epigenetically suppress CIITA expression by mechanisms including histone deacetylation/methylation and DNA promoter methylation, resulting in suppressed IFN-γ mediated MHC-I and MHC-II gene expression and dysfunctional antigen presentation (Morris et al., 2000; Kanaseki et al., 2003; Morimoto et al., 2004; Satoh et al., 2004; Chou, 2005; Holling et al., 2007; Radosevich et al., 2007; Meissner et al., 2008; Londhe et al., 2012; Truax et al., 2012; **Figure 1**). Interestingly, treatment of cancer cells with HDAC inhibitors can promote antigen presentation and ultimately help to induce anti-tumor immunity (Khan et al., 2004; Chou, 2005). For example, trichostatin A (TSA)-treated irradiated B16 melanoma cells administered prophylactically as a cancer vaccine are significantly more effective then control irradiated B16 cells at protecting from a subsequent challenge with live B16 tumor cells (Khan et al., 2007). Cancer immune evasion can also be mediated by dampened expression of the transporter associated with antigen processing 1 (TAP1), a key factor for antigen presentation by MHC molecules (Johnsen et al., 1999). In carcinoma cells, decreased TAP1 expression was attributed to reduced levels of histone H3 acetylation at the TAP-1 promoter (Setiadi et al., 2007; **Figure 1**).

In addition to these direct epigenetic effects on components of the antigenic response within cancer cells, the tumor microenvironment has also been shown to epigenetically drive tumor infiltrating $CD4^+$ T cells to tolerance. In colon cancer, infiltrating $CD4^+$ lymphocytes displayed high levels of DNA methylation at the IFN-γ promoter, and consequently required treatment with 5-AZA to enable tumor antigen-stimulated IFN-γ production (Janson et al., 2008; **Figure 1**). Overall, these studies highlight the role of epigenetic control in conferring "stealth" status to tumor cells such that they may evade the immune surveillance.

HDAC INHIBITORS CAN ALTER SUSCEPTIBILITY TO ONCOLYTIC VIRUSES

As alluded to earlier, defects in the IFN pathway are common in many malignancies but a significant proportion of tumors retain an active antiviral response (Stojdl et al., 2003; Dunn et al., 2006). Overcoming this antiviral response has been identified as a key barrier to the success of OV therapy and is the focus of many research groups including our own (Parato et al., 2005; Chiocca, 2008; Diallo et al., 2010; Liikanen et al., 2011; Russell et al., 2012). To overcome this barrier, many groups have looked at the possibility of using HDAC inhibitors in combination with OV therapy due to their repressive effects on the IFN-mediated antiviral response.

In one of the earliest reports, the anti-tumor effect of oncolytic adenovirus (OBP-301) in human lung cancer cells was found to synergize with FR901228 (Romidepsin), a class I HDAC inhibitor (Watanabe et al., 2006). However, in this report, increased activity was attributed to the upregulation of coxsackie adenovirus receptor (CAR) expression in cancer cells as opposed to direct effects on the antiviral response. Intriguingly, valproic acid, a class I/II HDAC inhibitor was found by another group in parallel to inhibit oncolytic adenovirus through the up-regulation of p21 (WAF1/CIP1; Hoti et al., 2006). Subsequently, TSA and valproic acid, two pan-HDAC inhibitors were found to enhance HSV oncolysis in squamous cell carcinoma and glioma cells (Otsuki et al., 2008; Katsura et al., 2009). Around the same time, Nguyen et al. (2008) showed that several HDIs could synergize with the oncolytic VSV-Δ51, an attenuated oncolytic VSV-mutant that is incapable of blocking IFN production (Stojdl et al., 2003). Combination treatment with HDIs resulted in synergistic cell killing, due to both enhanced induction of cell death and increased viral output (typically over 100-fold). Enhanced viral spreading of VV and semliki forest virus (SFV) was also observed in this study. Subsequent to this, TSA was shown to be particularly effective for improving VV-based OVs in several resistant cancer cell lines *in vitro* and in subcutaneous xenograft and syngeneic lung metastasis mouse models (MacTavish et al., 2011). Importantly, the impacts of HDAC inhibitors on OV spread and efficacy remain restricted to tumors and not normal cells, presumably because cancer cells exhibit a number of additional aberrations, such as increased metabolism, that promote viral growth independent of the status of the antiviral response.

HDAC INHIBITORS AS MODULATORS OF ONCOLYTIC VIRUS-ASSOCIATED ANTI-TUMOR IMMUNITY

While initial experiences with HDAC inhibitors in combination with OVs exploited mainly the ability of these epigenetic modifiers to improve the infectivity of resistant tumors, at least in part by dampening the innate cellular antiviral response, more recent studies have further exploited the broader immunological effects of HDAC inhibitors. For example, one report showed that valproic acid suppresses NK cell activity by blocking STAT5/T-BET signaling leading to enhanced oncolytic HSV activity (Alvarez-Breckenridge et al., 2012). Also of note, a recent report by Bridle et al. (2013) demonstrated significant improvements in the generation of an anti-tumor immune response elicited against aggressive melanoma following a heterologous prime-boost vaccination strategy. After the establishment of intracranial melanomas, immune-competent mice were primed with a non-replicating adenovirus expressing the dopachrome tautomerase (hDCT) melanoma antigen, and then boosted with oncolytic VSV expressing hDCT. While this prolonged survival, mice were fully cured (64%) only when VSV-hDCT was administered in combination with the class I HDAC inhibitor MS-275. Remarkably, MS-275 reduced VSV-specific neutralizing antibodies and memory $CD8^+$ T cells while maintaining prime-induced levels of humoral and cellular immunity against the tumor antigen. Interestingly, MS-275

also ablated autoimmune vitiligo typically observed following immunization against the melanocyte-expressed antigen (Bridle et al., 2013).

USE OF OTHER EPIGENETIC MODULATORS TO IMPROVE ONCOLYTIC VIROTHERAPY?

Given the epigenetic regulation of the antiviral response and antigen presentation pathways, it is tempting to speculate that other epigenetic modulators, in addition to HDAC inhibitors, may also be used to amplify therapeutic responses in combination with OVs. To this end, a recent study by Okemoto et al. (2012) showed that 5-AZA treatment could enhance HSV replication when co-administered with IL-6 (**Figure 1**). However, given numerous reports of cancers epigenetically silencing antiviral genes by DNA methylation (**Table 1**), we would expect that in general 5-AZA and other DNA methyltransferase inhibitors should be ineffective at overcoming the cellular antiviral response. On the other hand, the advent of new pharmacological inhibitors of KMTs and KDMs brings forth new possibilities for improving OV efficacy. For example, given the finding that histone H3K9 dimethylation observed at ISGs correlates with repression and reduced IFN response/expression, investigating the potential utility of H3K9-demetylase inhibitors for enhancing OV spread in resistant tumors seems warranted. However, it is of critical importance that, as is observed for HDAC inhibitors, OV-enhancing effects remain tumor-selective.

CONCLUSION

While genetic mutations are believed to be essential initiators of carcinogenesis, it is clear that epigenetic deregulation plays a key role in augmenting and/or maintaining the tumor phenotype. OVs are promising biotherapeutics that among others take advantage of the epigenetic silencing of cellular antiviral response genes and in many ways unmask cancer antigens as they destroy cancer cells and promote an inflammatory response. While additional studies on the impact of epigenetic regulation on the antiviral and immunological responses are needed, it is already recognized from studies using HDAC inhibitors that epigenetic modulators can positively impact OV efficacy. Additional *in vitro* and *in vivo* studies evaluating the effect of other epigenetic modulators are needed to determine whether these could be used in combination with promising OV platforms anticipated to reach the clinic in the near future, to further improve their therapeutic impact.

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Epigenetic control of Epstein–Barr virus transcription – relevance to viral life cycle?

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DNA methylation normally leads to silencing of gene expression but Epstein–Barr virus (EBV) provides an exception to the epigenetic paradigm. DNA methylation is absolutely required for the expression of many viral genes. Although the viral genome is initially un-methylated in newly infected cells, it becomes extensively methylated during the establishment of viral latency. One of the major regulators of EBV gene expression is a viral transcription factor called Zta (BZLF1, ZEBRA, Z) that resembles the cellular AP1 transcription factor. Zta recognizes at least 32 variants of a 7-nucleotide DNA sequence element, the Zta-response element (ZRE), some of which contain a CpG motif. Zta only binds to the latter class of ZREs in their DNA-methylated form, whether they occur in viral or cellular promoters and is functionally relevant for the activity of these promoters. The ability of Zta to interpret the differential DNA methylation of the viral genome is paramount for both the establishment of viral latency and the release from latency to initiate viral replication.

Keywords: Epstein–Barr virus, CpG-DNA methylation, DNA binding, transcription factor, replication cycle, cancer

In cellular genomes, the methylation of 5′ cytosines in CpG-dinucleotides leads to recruitment of methyl-DNA binding proteins that co-operate with other epigenetic events to promote the repression of transcriptional activity (reviewed in Wade, 2001; Klose and Bird, 2006; Jones, 2012; Muers, 2013). Although the double-stranded DNA genome of Epstein–Barr virus (EBV) γ herpesvirus resides in the nucleus of human cells and carries the hallmarks of cellular chromatin, the viral genome provides an exception to this rule during the replication phase of its life cycle.

EPSTEIN–BARR VIRUS ASSOCIATION WITH MAN

Epstein–Barr virus is an almost ubiquitous human virus, which is transferred from person to person in saliva. Infection results in virus entry into both B-lymphocytes and epithelial cells. EBV promotes the proliferation of infected B-lymphocytes and readily generates immortalized cell lines when infection is undertaken in an *in vitro* culture system. The majority of these immortalized cells are recognized by the host immune system and destroyed but some enter the memory B-cell pool, down regulate EBV gene expression and persist in a latent state. Viral latency can be a long-term event and the association of EBV with an infected individual is considered to be for life. EBV is associated with the development of several types of cancer associated with lymphocytes or epithelial cells, principally Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma. Primary infection with EBV can also result in infectious mononucleosis (Rickinson and Kieff, 2007).

EPIGENETIC CHANGES DURING THE EBV LIFE CYCLE

Epstein–Barr virus interacts with cells in a complex manner: the virus is either in a latent state in which only a small sub-set of the viral genes are expressed or it undergoes a lytic replication cycle in which the entire repertoire of EBV genes is expressed and viral progeny are generated (Rickinson and Kieff, 2007). Crucially, the switch from latency to the lytic replication cycle is triggered by physiological stimuli, which can be reproduced in *in vitro* culture systems. It is at this point that the normal epigenetic paradigm is broken.

Following infection, the viral double strand DNA genome is established in the nucleus of the cell where it circularizes to form an episome and then replicates once per cell cycle in synchrony with the host genome. During this time, the majority of the viral promoters are silent, with just a few directing the expression of the latency-associated genes. Many studies of individual viral promoters have demonstrated an inverse correlation between promoter activity and the presence of DNA methylation at CpG-dinucleotides within the promoter (reviewed in Minarovits, 2006; Niller et al., 2009). Indeed, recent genome-wide analyses support the contention that the EBV genome is extensively methylated during latency, with only the few active promoter regions spared (Fernandez et al., 2009; Kalla et al., 2010; Woellmer et al., 2012). In contrast, following the onset of the lytic replication cycle, the viral genome becomes largely un-methylated at CpG-dinucleotides (Fernandez et al., 2009). Thus, the majority of the viral genome cycles between an un-methylated and a heavily methylated state (**Figure 1**).

This biphasic methylation state poses an intriguing question. If the promoters of the genes required for lytic replication are silenced by DNA methylation during latency, how is the silencing overturned? There are no reasons to suspect that the mechanisms involved in gene repression are specific to EBV. First, repressive histone modifications, such as the heterochromatin-associated tri-methylation of lysine 9 (H3K9me3) and polycomb-associated tri-methylation of histone 3 at lysine 27 (H3K27me3) marks have been identified on the EBV genome (Murata et al., 2012;

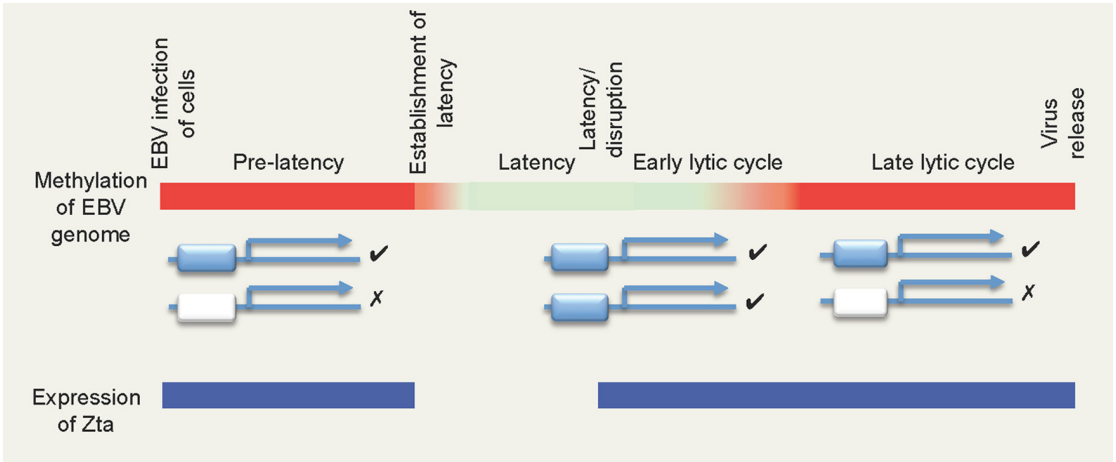

FIGURE 1 | The biphasic DNA methylation cycle of the EBV genome and its impact of ZREs. The colored bar depicts the methylation state of EBV genome during different phases of the viral life cycle, with non-methylated DNA in red and methylated DNA represented in green. Two types of Zta-responsive gene are shown below: those containing ZREs that are independent of DNA methylation (blue) and those that are dependent on methylation (white). Note that non-methylated CpG-ZREs cannot be bound by Zta. Periods where Zta is expressed are indicated in blue.

Ramasubramanyan et al., 2012b; Woellmer et al., 2012; reviewed in Murata and Tsurumi, 2013). Second, histone remodeling and the appearance of activating marks such as tri-methylation of lysine 4 on histone 3 (H3K4me3) occurs during the latency/lytic cycle transition (Woellmer et al., 2012). Third, and most importantly, sensitive methylation mapping suggests that no change in DNA methylation status occurs prior to the activation of lytic cycle gene expression (Woellmer et al., 2012).

The surprising finding was that the EBV genome requires DNA methylation to reactivate it from latency (Kalla et al., 2010, 2012). This has been fine-mapped to several EBV lytic cycle gene promoters. In comparison with the control of host gene expression, a requirement for DNA methylation at viral promoters presents a paradox. The key to resolving this paradox rests with the unique properties of the EBV-encoded transcription factor, Zta (BZLF1, ZEBRA, Z, EB1).

THE Zta TRANSCRIPTION FACTOR

Zta is a member of the bZIP family of transcription factors, but it has an unusual dimerization domain, driving the exclusive formation of homodimers (Petosa et al., 2006). Zta contains a classical transactivation domain, which interacts with RNA polymerase II (RNA pol II) associated proteins presumably stabilizing RNA pol II at Zta associated promoters (Lieberman and Berk, 1991). Zta interacts with sequence specific motifs (Zta-response elements, ZREs), resembling AP1 sites, within the promoters of responsive genes. Seminal studies from the Kenney lab revealed that at some promoters, the association of Zta with DNA is dependent on CpG methylation (Bhende et al., 2004, 2005; Dickerson et al., 2009). This key observation led to the recognition of different categories of ZRE, depending on the presence of a CpG-dinucleotide in the sequence. The class I (Karlsson et al., 2008) or simple ZREs (Bergbauer et al., 2010), do not contain a CpG and the binding of Zta is independent of methylation. Class III (Karlsson et al., 2008) or Me-ZREs (Bergbauer et al., 2010) do contain a CpG and the binding of Zta is strictly dependent on methylation. At a minority of ZREs, referred to as class II (Karlsson et al., 2008), DNA methylation has an intermediate impact. Importantly, this classification scheme also applies to ZREs in the host cell genome. For example, *Egr1*, which is activated by Zta (Kim et al., 2007) contains a CpG-ZRE that is methylation dependent (Heather et al., 2009). It is not known whether additional mechanisms are in place to aid Zta activation of DNA-methylated compared to non-methylated promoters.

Zta expression is restricted to two phases of the EBV life cycle; immediately after infection and during the EBV lytic replication cycle. Zta is not expressed during viral latency, indeed enforced expression of Zta promotes cells to initiate the lytic replication cycle. Following physiological stimulation of cells harboring latent EBV, Zta is the first viral lytic replication cycle gene to be expressed and then activates the expression of many viral genes. Zta is expressed initially when the viral genome is heavily methylated and remains expressed when the genome is largely non-methylated. Zta interacts with several hundred sites on the viral genome and at about half of these site binding is dependent on the DNA methylation status (Bergbauer et al., 2010; Flower et al., 2011; Ramasubramanyan et al., 2012a). Many of them occur within important promoters that control the expression of genes essential for the EBV lytic replication cycle (Bergbauer et al., 2010; Flower et al., 2011; Ramasubramanyan et al., 2012a,b). Thus, a sub-set of viral lytic replication cycle promoters is dependent on DNA methylation for activation by Zta (**Figure 1**). This could explain the requirement for genome methylation during the EBV life cycle.

It is puzzling to understand how these methylation-dependent promoters evolved. Why is it advantageous to encode a transcription factor with both methylation-dependent and -independent recognition sites if both classes of ZRE should be equally "visible" to Zta in the methylated state? To understand the driving force

behind the differential binding of Zta at ZREs, we need to consider the situation where the EBV genome is non-methylated and the CpG-ZREs become "invisible" (**Figure 1**).

RELEVANCE OF THE NON-METHYATED EBV GENOME

There are two stages in the life cycle of EBV when the differential recognition of methylation sensitive and insensitive ZREs in promoters could occur; in both the viral genome is non-methylated and Zta is expressed (**Figure 1**).

(i) During the late stage of the EBV lytic replication cycle, large numbers of non-methylated viral genomes and Zta protein accumulate within the nucleus. Whether the demethylation occurs via an active or passive process has not been determined. However, it is clear that Zta interacts with the non-methylated EBV genomes that are present during late lytic cycle (Ramasubramanyan et al., 2012a). Indeed, genome-wide comparisons of Zta binding sites revealed that methylation-independent ZREs are preferentially recognized at this stage (Ramasubramanyan et al., 2012a). This suggests that there could be a switch in Zta-orchestrated gene expression between the early and late stages of lytic replication cycle but this will require further investigation.

(ii) Immediately following infection of cells, the non-methylated EBV genome enters the nucleus, accompanied by a transient burst of Zta expression (Wen et al., 2007; Halder et al., 2009; Kalla et al., 2010). The short-lived nature of this event has thus far precluded a biochemical analysis of Zta binding patterns, but it is clear that only a sub-set of the lytic cycle genes are expressed at this stage and there is no associated generation of infectious virions (Halder et al., 2009; Shannon-Lowe et al., 2009; Kalla et al., 2012). This phase has been termed an abortive lytic cycle or pre-latency step (Woellmer and Hammerschmidt, 2013) and it is postulated that the lack of DNA methylation on the viral genome prevents Zta from activating the full set of lytic replication cycle genes. The advantage to the virus might be that the expression of a limited set of genes provides a boost to the growth or survival of infected cells prior to latency becoming fully established. Indeed, Zta is known to activate the expression of host cytokine genes (Murata and Tsurumi, 2013; Woellmer and Hammerschmidt, 2013) and has a role in the development of lymphomas in a model system (Ma et al., 2011).

CONCLUSION

The EBV genome provides an exception to the epigenetic paradigm of DNA methylation correlating with a silencing of gene expression. The virus also exploits a unique transcription factor to activate genes embedded in methylated DNA. The ability of Zta to differentially recognize methylated sequence elements together with the biphasic methylation cycle of the viral genome suggest that the selection of these properties was driven by the need to differentially regulate binding to different sub-sets of ZREs. Indeed Zta expression during the pre-latency stage and the lytic cycle results in the expression of different sub-sets of target genes, these are related to the location of methylation-dependent or -independent ZREs in their promoters and the methylation status of the viral genome.

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Transcription and replication result in distinct epigenetic marks following repression of early gene expression

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Simian virus 40 (SV40) early transcription is repressed when the product of early transcription, T-antigen, binds to its cognate regulatory sequence, Site I, in the promoter of the SV40 minichromosome. Because SV40 minichromosomes undergo replication and transcription potentially repression could occur during active transcription or during DNA replication. Since repression is frequently epigenetically marked by the introduction of specific forms of methylated histone H3, we characterized the methylation of H3 tails during transcription and replication in wild-type SV40 minichromosomes and mutant minichromosomes which did not repress T-antigen expression. While repressed minichromosomes following replication were clearly marked with H3K9me1 and H3K4me1, minichromosomes repressed during early transcription were not similarly marked. Instead repression of early transcription was marked by a significant reduction in the level of H3K9me2. The replication dependent introduction of H3K9me1 and H3K4me1 into wild-type SV40 minichromosomes was also observed when replication was inhibited with aphidicolin. The results indicate that the histone modifications associated with repression can differ significantly depending upon whether the chromatin being repressed is undergoing transcription or replication.

Keywords: simian virus 40, viral epigenetics, H3K9, H3K4, transcription, replication

INTRODUCTION

The selective methylation of the amino terminal tails of histone H3 and H4, a well-known form of epigenetic regulation, has been associated with a number of important biological regulatory processes including the control of transcription and cellular differentiation (Atkinson et al., 2008; Corry et al., 2009; Bonasio et al., 2010; Gibney and Nolan, 2010; Lister et al., 2011; Shafa et al., 2011; Skinner, 2011). Functionally, epigenetic regulation of transcription can occur either to control a particular gene's expression during a cell's life, or to pass along transcriptional information following cell division. The former would be an example of intra-generational epigenetic regulation while the latter would be an example of trans-generational regulation. While both forms of regulation might occur in association with a particular gene, it has not yet been established whether the same forms of histone methylation invariably mark the chromatin of the regulated gene during intra-generational and trans-generational regulation, nor how these two forms of epigenetic regulation might be related.

Since the passing of epigenetic information from a parental cell to daughter cells during cell division is critical to trans-generational epigenetic regulation, the mechanism of this inheritance has been the subject of much interest (Abmayr and Workman, 2012). A model for the inheritance of cellular trans-generational epigenetic information has emerged in which nucleosomes containing parental epigenetic information are randomly passed to daughter DNA during replication. These nucleosomes then act to direct the modification of histones present in the newly replicated nucleosomes added to the DNA in order to conserve the parental epigenetic modifications in the daughter chromatin (Corpet and Almouzni, 2009; Zhu and Reinberg, 2011).

Simian virus 40 (SV40), a member of the polyomavirus family, has been extensively studied as a model for eukaryotic molecular biology since its initial identification in 1960 because of its small size, organization into typical chromatin structure, and almost complete use of cellular enzymes and factors to complete its life cycle. A time course of SV40 transcription, replication, and encapsidation is shown in **Figure 1**. Upon infection the SV40 is rapidly transported to the nucleus with removal of the virus coat proteins and within 2 h early transcription begins. As the level of the major product of early transcription, T-antigen, increases it serves to repress its own expression through a feedback mechanism in which it binds to a site in the transcriptional regulatory region known as Site I. By 8 h post-infection repression of early transcription is extensive. Between 12 and 24 h post-infection late transcription and DNA replication begin with late transcription slightly preceding replication. At approximately 48 h post-infection replication is maximal. Beginning at approximately 48 h, newly replicated SV40 is bound by the products of late transcription, VP1, VP2, and VP3, to encapsidate new virus particles in a process which continues until the infected cell lyses and the newly synthesized virus is released (Acheson, 1981).

We have recently shown using a SV40 mutant which does not repress early SV40 transcription, that repression is strongly associated at late times in infection with mono-methylation of H3K9 and weakly associated at this time with mono-methylation of H3K4 (Milavetz et al., 2012). Specifically, we compared the

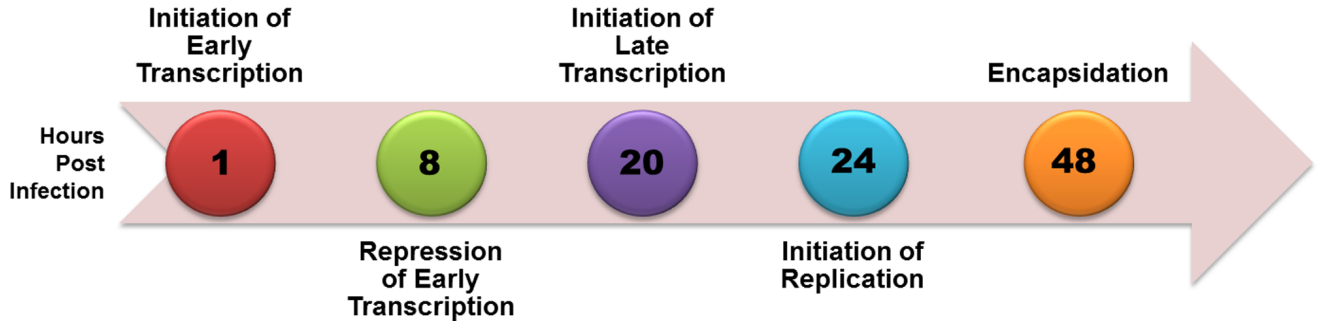

FIGURE 1 | Time course of biological processes during SV40 lytic cycle.

levels of methylated H3K4 and H3K9 at 48 h post-infection in wild-type SV40 which represses early transcription and the mutant cs1085 which contains a 30-bp deletion in the regulatory region encompassing T-antigen binding Site I and does not repress early transcription (DiMaio and Nathans, 1982). We found that the percentage of SV40 minichromosomes containing H3K9me1 was reduced from 22 ± 10% in the wild-type minichromosomes to 0.66 ± 0.06% in the mutant which fails to repress. Similarly, we observed a reduction in H3K4me1 from 0.1 ± 0.07% in wild-type minichromosomes to 0.005 ± 0.007% in the mutant. In contrast, H3K4me2 went from 0.4 ± 0.3 to 0.02 ± 0.02%, H3K4me3 went from 0.08 ± 0.06 to 0.02 ± 0.02%, H3K9me2 went from 0.04 ± 0.03 to 0.17 ± 0.2%, and H3K9me3 went from 12 ± 6 to 8.2 ± 5% comparing the wild-type to the mutant. Moreover, we also showed that the changes in methylation patterns which occurred in SV40 minichromosomes during infection in mutants or following other changes in environment could also be represented in the SV40 chromatin present in virions and transferred to a subsequent infection in the viral equivalent of trans-generational epigenetic regulation (Milavetz et al., 2012). However, we do not know whether transcriptional repression occurring prior to DNA replication also results in the same effects on histone methylation. For this reason, we have extended our studies on early repression to early times in infection and characterized the changes which occur to the methylation patterns of SV40 minichromosomes. In addition, we have also investigated the role of DNA replication in introducing each of the methylated forms of H3K4 and H3K9.

MATERIALS AND METHODS

CELLS AND VIRUSES

Wild-type and mutant SV40 minichromosomes were prepared in the monkey kidney BSC-1 cell line (ATCC) using either wild-type 776 virus, cs1085 virus (from Dr. Daniel Nathans) or SM virus (from Dr. Chris Sullivan). The recombinants pBM129-1 and pBM131-1 were prepared in our laboratory and previously described (Hermansen et al., 1996).

CELL CULTURE AND INFECTION

BSC-1 cells were maintained and infected as previously described with the exception of incubating cs1085 virus with the cells for 1 h, in order to increase the minichromosome yield, instead of the typical 30 min (Balakrishnan and Milavetz, 2006; Milavetz et al., 2012). SV40 minichromosomes were isolated at the indicated times post-infection as described for each of the analyses. DNA replication was inhibited with aphidicolin (final concentration 6 μM). Aphidicolin in ethanol (4 μl) was added at 24 h post-infection and minichromosomes were prepared from treated cells at 48 h post-infection.

PREPARATION OF SV40 MINICHROMOSOMES

SV40 minichromosomes were harvested at the desired time as previously described (Balakrishnan and Milavetz, 2006; Milavetz et al., 2012) with one minor modification. After transferring the lysed cells to the 15 ml centrifuge tube, an additional 1 ml of nuclei preparation buffer was used to rinse the flask and was subsequently added to the centrifuge tube in order to maximize the yield of minichromosomes from each infection.

CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) kits were obtained from Millipore and the protocol was followed as previously described (Milavetz et al., 2012). The antibodies used included: H3K4me1 (07-436, Millipore), H3K4me2 (39141, Active Motif), H3K4me3 (04-745, Millipore), H3K9me1 (ab9045, Abcam), H3K9me2 (ab1220, Abcam), H3K9me3 (ab8898, Abcam), and RNA polymerase II (RNAPII; 05-623, Millipore). All antibodies were ChIP validated by the respective vendors. Hundred microliters of protein A agarose, 800 μl of ChIP dilution buffer, and 7.5 μl of each antibody was used in a protein low-bind tube. The mixture was rotated for 5 h at 4°C on an end to end rotator in a refrigerator to bind the antibody to protein A agarose. Following binding of the antibody, the protein A agarose was spun down at 2,000 × *g* for 2 min and the supernatant discarded. Eight hundred microliters of fresh ChIP dilution buffer was added and either 100 or 200 μl of the chromatin to be analyzed was added. The samples containing antibody bound to protein A agarose and chromatin were incubated with end to end rotation for a further 7 h at 4°C. The chromatin bound to protein A agarose was washed according to the manufacturer's protocol and eluted as previously described (Milavetz et al., 2012).

PREPARATION OF DNA

Samples were prepared for PCR using an MP Bioscience Geneclean Spin Kit (#111101-200) with the following modifications. The

glassmilk reagent (100 µl) was mixed with 100 of sample in a 1.5-ml centrifuge tube. The tube was mixed by repeated inversion at 2 min and again at 4 min of incubation. Following 5 min of room temperature incubation, the samples were centrifuged at 6,000 rpm for 30 s in a Micro One (Tomy) to pellet the glass. The supernatant was discarded and 200 µl of the wash buffer was added to the tube. While adding the wash the pipette tip was used to break up the pellet by both physically rubbing and vigorously pipetting up and down. The samples were inverted twice and centrifuged at 6,000 × *g* for 30 s to again pellet the glass. The supernatant was discarded and the pellets where dried in a vacuum for 5 min. The glass pellet with bound DNA was resuspended in 25 µl of Tris EDTA (TE) buffer.

PCR AMPLIFICATION

DNA was amplified from the promoter region of the SV40 genome using the primers 5′-TTG CAA AAG CCT AGG CCT CCA AA-3′ and 5′-TGA CCT ACG AAC CTT AAC GGA GGC-3′ in a CFX Connect Real Time System thermal cycler (Bio-Rad) using "SSO Advanced DNA polymerase" (Bio-Rad). Immediately before use, the primers and DNase free water were added and 28 µl of the mix was used per sample. Two microliters of the resuspended glass milk in TE buffer was added per sample. Samples were amplified by PCR in triplicate with a melt curve applied afterward to ensure specific amplification. All sample preparation for PCR was done in either a Nuaire biological safety cabinet Model NU_425-400 or an AirClean 600 PCR Workstation (ISC BioExpress).

RESULTS

In order to test whether the repression of early transcription which occurs prior to replication was also associated with the same forms of histone methylation observed when replication was occurring, we used two distinct strategies. First, we determined whether there were changes in histone methylation during the first 8 h post-infection in a wild-type infection consistent with what we previously reported for repression of early gene expression late in infection during DNA replication (Milavetz et al., 2012). We hypothesized that if transcriptional repression occurring at early times was associated with mono-methylation (me1) of H3K9 as observed during DNA replication, we would observe an increase in H3K9me1 over the first hours of an infection perhaps approaching the 20% value seen at late times when transcriptional repression was occurring. In contrast if early transcriptional repression was not associated with mono-methylation of H3K9 we would expect no effect on the levels of H3K9me1. Since we previously reported that the fraction of SV40 minichromosomes containing RNAPII decreased during the first hours of infection consistent with the repression of early transcription (Balakrishnan and Milavetz, 2006), we first confirmed that this was the case. SV40 wild-type minichromosomes were isolated 2, 4, 6, and 8 h post-infection and analyzed by ChIP for the presence of RNAPII. As shown in **Figure 2A**, we observed a slow and continual decrease in the percentage of RNAPII bound to SV40 minichromosomes between 2 and 8 h post-infection. We next determined the percentage of minichromosomes isolated at 30 min, 2, 4, and 8 h which contained H3K9me1. We did not analyze for the presence of methylated H3K4 at these times because we have previously shown that minichromosomes contain very low levels of methylated H3K4 (Milavetz et al., 2012). As shown in **Figure 2B**, we did not observe an increase in the level of H3K9me1 as expected if it was associated with transcriptional repression. H3K9me1 remained present in approximately 1% or less of the minichromosomes at this time which was similar to the level that we previously reported present in the SV40 virus particles, 2.9 ± 1% (Milavetz et al., 2012), which was used for the infection.

Secondly, we determined whether infection by the mutant cs1085 which lacks Site I and fails to repress early transcription resulted in a changed pattern of histone methylation compared to wild-type virus during the same time. Again, we focused only on the methylated forms of H3K9 at this time because we have previously shown that there is very little if any methylated H3K4 at the very early times in question (Milavetz et al., 2012). SV40 minichromosomes were prepared at the indicated times, subjected to ChIP analyses and the percentage of minichromosomes containing each methylated form of H3K9 determined by real-time PCR. The data is represented as the percentage of minichromosomes

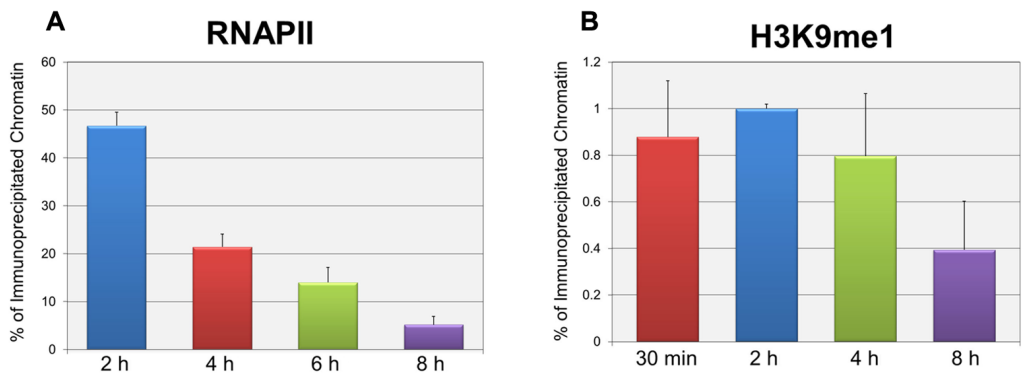

FIGURE 2 | Repression of active early transcription does not result in an increase in H3K9me1. SV40 wild-type minichromosomes were isolated between 30 min and 8 h post-infection, and subsequently subjected to ChIP analyses with antibodies to either RNA polymerase II (RNAPII) or H3K9me1. The percentage of the input minichromosomes containing either RNAPII **(A)** or H3K9me1 **(B)** was determined by real-time PCR for each time point analyzed. All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

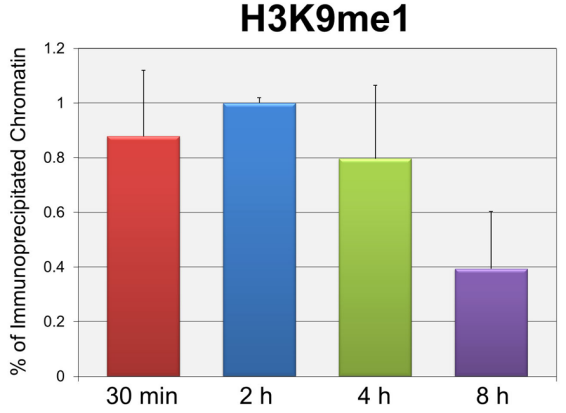

FIGURE 3 | H3K9me2 is significantly increased during active early transcription in the site I deletion mutant cs1085. Wild-type and cs1085 SV40 minichromosomes were isolated from appropriately infected cells at 30 min and 8 h post-infection. Isolated minichromosomes were subjected to ChIP analyses with antibodies against H3K9me1, H3K9me2, and H3K9me3, and the percentage of input minichromosomes containing each form of methylated H3 determined by real-time PCR. The results are displayed as the ratio of the percentage of minichromosomes isolated at 8 h which contain a particular modification divided by the percentage of minichromosomes isolated at 30 min which contain the same modification. Ratios greater than 1 indicate that a modification is increasing during the period from 30 min to 8 h, while a ratio less than 1 indicates that the modification is decreasing during this period of infection. All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

containing the modification present at 8 h of infection divided by the percentage present at 30 min of infection. A ratio less than 1 indicates that the percentage of minichromosomes carrying a particular methylated H3 is reduced over this period. As shown in **Figure 3**, we observed that for both the wild-type and cs1085 mutant we observed a reduction in the relative amount of H3K9me1 and H3K9 tri-methylation (H3K9me3) present in minichromosomes between 30 min and 8 h post-infection. However, while the amount of H3K9 di-methylation (H3K9me2) was reduced during this period in the wild-type virus, the amount was significantly increased in the cs1085 mutant. These results suggest that repression of early gene expression during active transcription occurs by a process in which the levels of H3K9me2 are kept low.

In order to independently confirm that Site I was responsible for the introduction of H3K9me1 at late times but not early times, we compared the level of H3K9me1 in an SV40 recombinant containing two copies of Site I (pBM131-1) to a parental recombinant containing only a single copy of Site I (pBM129-1). We hypothesized that if Site I was responsible for the introduction of H3K9me1 in a replication dependent manner, we would observe an increase in the percentage of H3K9me1 in the recombinant compared to the parental virus at late times but not at early times when replication was not occurring. For these studies we used recombinant viruses originally prepared to study the ability of SV40 regulatory sequences to phase nucleosomes and generate nucleosome free regions in SV40 chromatin. The parental recombinant and its construction as well as the recombinant containing two copies of Site I have been previously described (Hermansen et al., 1996). The structures of both of these constructs are shown in **Figure 3**. The parental construct pBM129-1 has a single copy of Site I in the regulatory region as in the wild-type virus (**Figure 4A**). pBM131-1 has two copies of Site I, one located as in pBM129-1 and a second copy present in the reporter region as shown in **Figure 4B**. The results of this analysis are graphically represented in **Figure 4C**. As shown at 8 h post-infection when Site I should be active down-regulating early transcription we observed a ratio of 0.50 ± 0.35 indicating that there was less methylation of H3K9me1 at this time in the recombinant carrying two copies of Site I than in the parental recombinant with only one copy. In contrast at 48 h post-infection when replication is occurring we observed a ratio of 1.66 ± 0.37 confirming that Site I is capable of directing the introduction of H3K9me1 when SV40 is replicated. Interestingly it is also apparent that the second copy of Site I can function during replication outside of its normal location within the virus genome.

Since the effect of repression on H3K9me1 was only seen at late times in infection, it seemed likely that it was either directly or indirectly related to the replication of SV40 DNA which was occurring at this time. In order to test his hypothesis we determined the effect of the inhibition of replication on the introduction of methylated H3K4 and H3K9. SV40 minichromosomes were prepared at 24 h post-infection when replication was beginning and at 48 h post-infection in the presence or absence of aphidicolin, a specific inhibitor of eukaryotic DNA replication (Ohashi et al., 1978). SV40 minichromosomes were then subjected to ChIP analysis with antibodies to methylated H3K4 and H3K9. We first investigated the introduction of methylated H3 during the increase in SV40 chromatin resulting from replication between 24 and 48 h post-infection. Since we generally observe a 50- to 200-fold increase in the pool size of SV40 minichromosomes between 24 and 48 h post-infection, we compared the increase in a particular form of modification to the increase in the amount of SV40 minichromosomes. We expected that this ratio would be 1 if both the SV40 minichromosomes and form of modification were increasing at the same rate, greater than 1 if the newly replicated minichromosomes were more likely to contain the form of modification, or less than 1 if the minichromosomes were increasing faster than the introduction of the modified histone H3. The results of this analysis are graphically represented in **Figure 5A**. Based upon the observed ratios, all methylated forms of H3K4 and H3K9 were being introduced into the newly replicated minichromosomes at a rate faster than the increase in SV40 chromatin. However, H3K4me2 and H3K9me3 appeared to be introduced at rates close to the rate of increase of chromatin (1.74 and 1.23, respectively), while the other methylated forms of H3 were introduced at rates much greater than 1.

Next, we determined whether the introduction of a particular form of methylated H3 was actually dependent upon ongoing DNA replication. If ongoing DNA replication was necessary for the introduction of a particular methylated form of H3, inhibition of replication with aphidicolin should also block the introduction of the methylated form of H3. In contrast if the introduction of a methylated form of H3 was due to some other biological process, one would expect little if any effect on the introduction of the methylated form of H3 following inhibition of replication. SV40 minichromosomes were isolated from cells treated

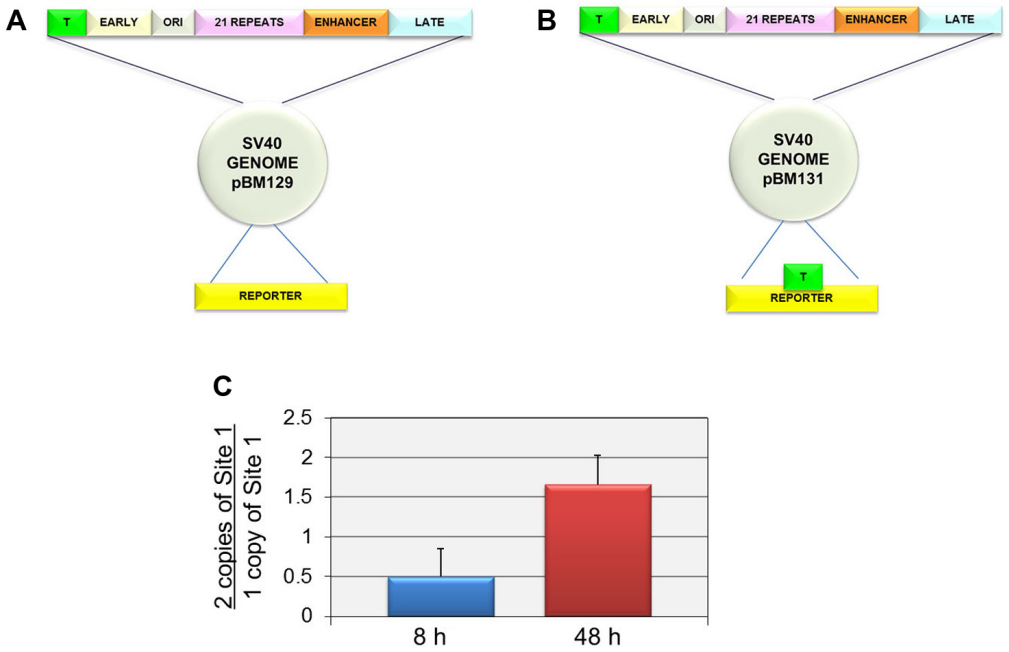

FIGURE 4 | Two copies of Site I directs the incorporation of more H3K9me1 compared to one copy of Site I in SV40 minichromosomes isolated at 48 h post-infection but not at 8 h post-infection. SV40 minichromosomes were prepared from cells infected with pBM129-1 (one copy of Site 1) or pBM131-1 (two copies of Site 1) at 8 and 48 h post-infection. The percentage of SV40 minichromosomes containing H3K9me1 was determined by ChIP analyses for each preparation of minichromosomes at each time point followed by real-time PCR. The results are displayed as the ratio of the percentage of minichromosomes containing two copies of Site I immunoprecipitated by antibody to H3K9me1 over the corresponding percentage for minichromosomes containing one copy of Site I. A schematic of the structure of the SV40 recombinants pBM129-1 is shown in **(A)** and pBM131-1 in **(B)**. pBM131-1 contains a second copy of Site I introduced into the reporter region of the basic recombinant, pBM 129-1. The results of this analysis are shown in **(C)**. All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

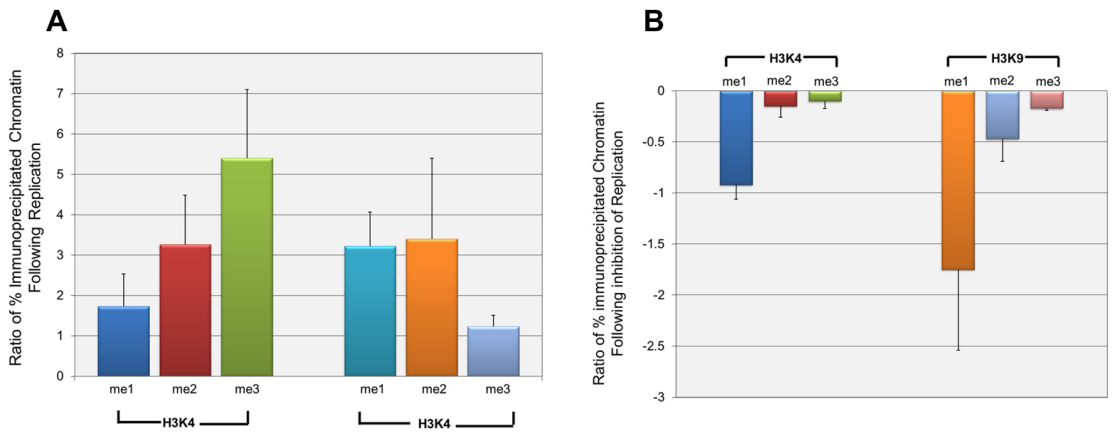

FIGURE 5 | H3K4me1 and H3K9me1 are introduced into wild-type SV40 minichromosomes primarily during active replication. Wild-type SV40 minichromosomes were isolated at 24, 48, and 48 h post-infection following treatment with the DNA replication inhibitor aphidicolin from 24 to 48 h post-infection. The percentages of SV40 minichromosomes containing methylated H3K4 and H3K9 were determined by ChIP analyses followed by real-time PCR. The relative increase of each methylated form of H3K4 and H3K9 following DNA replication from 24 h to 48 h post-infection is shown in **(A)**. The relative increase is shown as the ratio of the fold increase of a particular form of methylated H3 between 24 and 48 h post-infection divided by the corresponding fold increase in the amount of SV40 minichromosomes between these times. Ratios greater than 1 indicate that a particular methylated form of H3 is preferentially being introduced into newly replicated minichromosomes at a rate faster than the increase in the pool size of SV40 minichromosomes. The effects of the inhibition of DNA replication from 24 to 48 h post-infection on the introduction of methylated H3K4 and H3K9 are shown in **(B)**. The results are shown as the ratio of the fold decrease in the amount of a particular form of methylated H3 in minichromosomes following inhibition of DNA replication divided by the fold decrease in minichromosomes resulting from inhibition of replication. Ratios less than or equal to 1 indicate that a particular methylated form of H3 is inhibited to a greater or the same extent as the inhibition of replication of the total SV40 minichromosomes. All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

with aphidicolin from 24 to 48 h post-infection or from untreated cells at 48 h post-infection and subjected to ChIP analysis and real-time PCR. For each methylated form of H3, we then calculated the ratio of the decrease in methylated H3 to the decrease in the amount of SV40 minichromosomes following inhibition of replication. A ratio of 1 or greater would indicate that the introduction of methylated H3 was equal to or even greater than the reduction in the amount of SV40 chromatin, while a ratio near 0 would indicate that the introduction of methylated H3 was independent of DNA replication. The results of this analysis are graphically represented in **Figure 5B**. As shown in the figure the ratios for H3K9me1 (1.75) and H3K4me1 (0.92) were similar to or greater than 1 indicating that the introduction of these two methylated forms of H3 into SV40 chromatin were directly dependent upon DNA replication. The ratios for three of the methylated forms of H3 were very low including H3K4me2 (0.15), H3K4me3 (0.10), and H3K9me3 (0.17) indicating that these methylated forms of H3 were being introduced in the absence of direct DNA replication. The ratio for H3K9me2 (0.47) was intermediate between the other forms of methylated H3 suggesting that it was at least in part dependent upon replication. While we believe that the changes observed following aphidicolin treatment are primarily a result of the extensive inhibition of replication, we cannot exclude the possibility that indirect effects on transcription or induction of the DNA damage response following aphidicolin might also be contributing to changes in histone modifications.

DISCUSSION

In SV40 minichromosomes, repression of early gene expression by T-antigen binding to Site I in the viral regulatory region was shown to result in distinct epigenetic marks at early and late times post-infection. At early times when only early transcription was occurring T-antigen binding resulted in the inhibition of the introduction of H3K9me2, while at late times when replication was occurring T-antigen binding resulted in the introduction of H3K9me1. The latter was first shown in a previous publication (Milavetz et al., 2012).

These results raise interesting questions concerning the mechanisms responsible for the introduction of epigenetic marks at the two time points in infection. Clearly, T-antigen binding is required for the introduction of the majority of H3K9me1. However, T-antigen binding does not appear to be the only signal for the introduction of H3K9me1 since a low level of H3K9me1 is still present in SV40 minichromosomes in a mutant in which T-antigen binding cannot occur. While Site I is necessary for the late introduction of H3K9me1, the Site I does not have to be located in the regulatory region since a recombinant containing an extra copy of Site I near the terminus of transcription showed an increase in H3K9me1 at late times but not early times. The location independent increase in H3K9me1 in this recombinant suggests that Site I may be functioning like an enhancer to direct epigenetic changes (Calo and Wysocka, 2013).

It seems likely that the T-antigen directed introduction of H3K9me1 is mechanistically related to DNA replication. First, we have previously shown that at late times in infection H3K9me1 was specifically associated with SV40 minichromosomes actively undergoing replication using a two-step ChIP protocol (Balakrishnan and Milavetz, 2009) in which actively replicating minichromosomes were immunologically selected for subsequent analysis using an antibody to RPA70 a replication protein (Balakrishnan and Milavetz, 2009). Second, this association was confirmed by characterizing SV40 chromatin following inhibition of replication by aphidicolin. H3K9me1 appeared to be directly related to replication since it increased when replication occurred and was completely blocked when replication was blocked. Although H3K4me1 also appeared to be a direct result of replication the other methylated forms of H3K4 and H3K9 appeared to result from post-replication maturation. The introduction of H3K9me3 following replication has been shown to occur via a maturation process in which the H3K9me3 is introduced into previously replicated chromatin containing H3K9me1 (Loyola et al., 2009). It is not clear how the binding of T-antigen to Site I at early times results in the inhibition of the incorporation of H3K9me2. Potentially T-antigen might be disrupting the normal biological pathways linking H3K9me1 to H3K9me2 and H3K9me3.

These results are not consistent with a model of chromatin replication in which the pre-existing histone modifications present in the parental chromatin are duplicated in the daughter chromatin during replication (Corpet and Almouzni, 2009; Zhu and Reinberg, 2011). Instead these results suggest that in SV40 minichromosomes DNA replication can serve as an epigenetic switch in which newly replicated chromatin can be epigenetically modified in response to specific signals such as T-antigen binding to Site I. It seems unlikely that the H3K9me1 present during replication is simply a consequence of H3K9me1 being present in parental chromatin. If this were the case one would expect similar levels of H3K9me1 in both the cs1085 mutant and the wild-type virus since both contain H3K9me1 at early times. Secondly, a model in which pre-existing H3K9me1 drives the introduction of H3K9me1 following replication does not fit with the data obtained with the recombinant containing an extra copy of Site I. At early times the recombinant and its parental strain both contain similar levels of H3K9me1 yet at late times there is a significant increase in the amount of H3K9me1 present in replicated minichromosomes. This epigenetic switching hypothesis is consistent with a recent publication showing that replication of *Drosophila* chromatin occurs through a process in which pre-existing histone modifications are lost at the replication fork and histone modifications are re-introduced following replication by modifying complexes which remain closely associated with the replicating chromatin (Petruk et al., 2012). The results differ in that in the publication pre-existing modifying complexes are thought to drive the introduction of post-replicative histone modifications while in SV40 the post-replicative changes are driven by the binding of the repressive factor T-antigen.

The most likely reason for the epigenetic switch is to ensure that newly replicated minichromosomes are not capable of activation for early transcription at late times in infection. Allowing activation of early transcription as in the case of the mutant cs1085 has been shown to result in a significant reduction in the pool size of SV40 minichromosomes and yield of virus late in infection (Milavetz et al., 2012). This epigenetic switch may also play

a critical role in controlling the relative pool sizes of transcribing, replicating, and encapsidating SV40 minichromosomes.

While an epigenetic switch associated with replication appears to have a biological relevance for SV40 it is not yet clear whether a similar process functions in cellular chromatin. However, it is interesting to speculate that a similar process could act during cellular differentiation to prepare newly replicated chromatin for subsequent activation or repression of transcription in response to specific signals introduced during replication as part of the differentiation pathway.

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Role of extrachromosomal histone H2B on recognition of DNA viruses and cell damage

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Histones are essential components of chromatin structure, and histone modification plays an important role in various cellular functions including transcription, gene silencing, and immunity. Histones also play distinct roles in extrachromosomal settings. Extrachromosomal histone H2B acts as a cytosolic sensor to detect double-stranded DNA (dsDNA) fragments derived from infectious agents or damaged cells to activate innate and acquired immune responses in various cell types. It also physically interacts with interferon (IFN)-β promoter stimulator 1 (IPS-1), an essential adaptor molecule that activates innate immunity, through COOH-terminal importin 9-related adaptor organizing histone H2B and IPS-1 (CIAO), resulting in a distinct signaling complex that induces dsDNA-induced type I IFN production. Such a molecular platform acts as a cellular sensor to recognize aberrant dsDNA in cases of viral infection and cell damage. This mechanism may also play roles in autoimmunity, transplantation rejection, gene-mediated vaccines, and other therapeutic applications.

Keywords: DNA sensor, extrachromosomal histone, virus infection, DNA damage, epigenetic modifications

INTRODUCTION

Epigenetic modifications of histones, the primary protein component of chromatin, contribute to diverse homeostatic cellular activities such as transcriptional regulation, chromosome condensation (mitosis), apoptosis, and DNA repair (Bradbury, 1992; Koshland and Strunnikov, 1996; Rogakou et al., 2000; Fernandez-Capetillo et al., 2004). Histones are divided into two groups based on their principal functions. Histones H2A, H2B, H3, and H4 are known as the core histones. Two of each core histone form the histone octamer, which genomic DNA wraps around to form a nucleosome (Luger et al., 1997). Histone H1, the linker histone, binds and rearranges the DNA between nucleosome units (linker DNA) to assist chromatin compaction. Interestingly, histones are present in cytosol (Kobiyama et al., 2010) as well as in the nucleus, mitochondria (Konishi et al., 2003), and cell surface (Radic et al., 2004), particularly during viral infections, apoptosis, and cell damage. Histone H2B transits in and out of the nucleosome more rapidly than other core histones, such as H3 and H4. Thus, about 3% of total H2B is exchanged within 6 min ($t_{1/2}$), ∼40% within 130 min, and ∼50% by 8.5 h (Kimura, 2005). Histones have microbicidal activity in neutrophil extracellular traps (NETs), which are composed of DNA, elastase, and histones. Treatment of NETs with histone neutralizing antibodies resulted in reduced bactericidal activity against species such as *Shigella flexneri* and *Staphylococus aureus* (Brinkmann et al., 2004). Thus, these "extrachromosomal" histones play important roles in physiological conditions, including innate and adaptive immune responses. We recently reported that extrachromosomal histone H2B is involved in the recognition of cytosolic double-stranded DNA (dsDNA) generated by DNA viruses (non-self) and genomic DNA from damaged cells (self) (Kobiyama et al., 2010; Kawashima et al., 2011a).

DNA-MEDIATED IMMUNE RESPONSE

In 1963, Alick Isaacs found that nucleic acids, both DNA and RNA, strongly induce innate immune responses, such as type I interferon (IFN) production (Isaacs et al., 1963; Rotem et al., 1963). Although this finding generated a great deal of excitement in the field of immunology at that time, it was forgotten or largely ignored until it was shown that unmethylated CpG DNA stimulates immune cells to produce cytokines (Tokunaga et al., 1984; Krieg et al., 1995). As a result, most immunologists presumed that unmethylated CpG DNA was the essential element within self and non-self DNA that activated innate immunity. Toll-like receptor 9 (TLR9) was subsequently identified as a cellular receptor for unmethylated CpG DNA in the activation of innate immune responses in immune cells, such as dendritic cells (DCs), B cells, and macrophages (Hemmi et al., 2000, 2003). In the meantime, dsDNA independent of unmethylated CpG motifs or any other specific sequence was shown to up-regulate the expression of genes related to the immune response (Suzuki et al., 1999). In particular, genomic dsDNA released by injured cells induces maturation of antigen presenting cells and adaptive immune responses (Ishii et al., 2001).

Furthermore, TLR9-dependent and -independent IFN-α production is induced in response to herpes simplex virus-1 (HSV-1) infection (Hochrein et al., 2004). It was later confirmed that the right-handed helical structure (B-form) of DNA is the component responsible for induction of robust type I IFNs in both immune and non-immune cells through TLR9-independent recognition and signaling cascades (Ishii et al., 2006; Stetson and Medzhitov, 2006).

The harmful effects of aberrant DNA have been shown in relation to the function of enzymes that digest DNA (DNases). Thus, hepatic macrophages in DNase II-deficient mice failed to digest DNA from engulfed nuclei of erythroblasts and exhibited robust production of type I IFN, which resulted in severe anemia and development of rheumatoid arthritis (RA)-like symptoms in a TLR9-independent manner (Yoshida et al., 2005; Kawane et al., 2006). DNase I and DNase III knockout mice showed systemic lupus erythematosus (SLE)-like symptoms and inflammatory myocarditis, respectively (Napirei et al., 2000; Yasutomo et al., 2001; Morita et al., 2004). The functional mutations of DNase I and DNase III in humans have also been associated with several autoimmune disorders, such as SLE (Yasutomo et al., 2001; Lee-Kirsch et al., 2007b), Aicardi–Goutieres syndrome (Crow et al., 2006), familial chilblain lupus (Lee-Kirsch et al., 2007a), and retinal vasculopathy with cerebral leukodystrophy (Richards et al., 2007). Thus, DNA-induced immune responses are involved in the prevention of both microbial infection and autoimmune responses. These findings also suggest that normal cells are equipped with innate machinery that senses and removes aberrant genomic DNA fragments before they produce pathological effects.

CYTOSOLIC SENSORS FOR DNA FRAGMENTS AND THEIR METABOLITES

Several proteins have been identified as DNA sensors that recognize aberrant cytosolic DNA fragments and their metabolites. These sensors are involved in the elimination of invasive pathogens and the induction of inflammation. In most cases, recognition of cytosolic DNA by these sensors results in induction of innate immune responses through several key proteins such as stimulator of interferon genes (STING) and TANK-binding kinase 1 (TBK1) (Ishii et al., 2006; Ishikawa and Barber, 2008). STING and TBK1 are also essential factors in the immunogenicity of plasmid DNA vaccines (Ishii et al., 2008; Ishikawa et al., 2009). The underlying mechanisms for the immunological advantages of DNA vaccines have not been fully elucidated. However, it has been suggested that the detection of the double-stranded structure of plasmid DNA by cytosolic DNA sensors contributes to an enhanced adaptive immune response to the vaccine antigen.

Z-DNA binding protein 1/DNA-dependent activator of IFN-regulatory factors (ZBP-1/DAI) was the first reported cytosolic DNA sensor (Takaoka et al., 2007). ZBP-1/DAI contains two Z-DNA binding domains and a D3 domain, all of which are essential for its activation. Overexpression of ZBP-1/DAI enhanced dsDNA-mediated gene expression and knockdown of ZBP-1/DAI impaired IFN-β production by HSV-1 infection, but not Newcastle disease virus (NDV) infection, in a mouse fibroblast cell line (Takaoka et al., 2007). However, fibroblasts from ZBP-1/DAI deficient mice normally responded to dsDNA, and the mice also showed normal immunogenicity to plasmid DNA vaccinations (Ishii et al., 2008).

In 1993, it was reported that electroporated DNA induces cell death in murine macrophages (Stacey et al., 1993). Recently, absence in melanoma 2 (AIM2) was identified as a cytosolic DNA sensor for activation of the inflammasome, a large multimolecular complex that regulates activation of the enzyme caspase-1, to induce IL-1β production and DNA-induced cell death. AIM2 is a member of the hematopoietic IFN-inducible nuclear protein with a 200-amino-acid repeat (HIN-200) family, which contains a pyrin domain and a DNA-binding HIN-200 domain. AIM2 recognizes cytosolic DNA and interacts with inflammasome-related molecules to induce pyroptosis, a type of programed cell death characterized by activation of caspase-1 and IL-1β production upon inflammatory antimicrobial responses. Deficiency of AIM2 results in an enhancement of susceptibility to bacteria and DNA viruses (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

Interferon gamma inducible protein 16 (IFI16) is a member of the pyrin and HIN domain-containing (PYHIN) protein family that contains a pyrin domain and two DNA-binding HIN domains. IFI16 directly binds viral DNA in the cytosol and induces IFN-β production through STING (Unterholzner et al., 2010). Small interfering RNA (siRNA) for IFI16 inhibited DNA-induced but not RNA-induced IFN-β production. Knockdown of p204, a mouse ortholog of IFI16, impaired activation of transcription factors and gene inductions upon DNA virus infection.

Although retinoic acid-inducible gene I (RIG-I) was initially identified as a cytosolic RNA receptor, it is also involved in the recognition of cytosolic dsDNA. Thus, knockdown of RIG-I in human hepatocellular carcinoma cell line, HuH-7, attenuated dsDNA-induced type I IFN production. Subsequently, it was shown that poly(dA·dT)·poly(dT·dA) and DNA virus-derived DNAs were converted into 5′-triphosphate RNA by RNA polymerase III to induce RIG-I-mediated type I IFN production. This IFN production induced by intracellular bacteria was abolished by a specific inhibitor of RNA polymerase III, which in turn resulted in a promotion of bacterial growth (Chiu et al., 2009).

High mobility group box protein 1 (HMGB1), initially identified as a non-histone DNA-binding and chromatin-associated protein, is involved in DNA organization and transcriptional regulation (Goodwin et al., 1973; Bustin, 1999). Although most of HMGB1 is localized to the nucleus, HMGB1 acts as an "alarmin" to promote inflammation upon its release from the nucleus during necrosis (Scaffidi et al., 2002). In addition, extracellular HMGB1 is involved in the pathogenesis of autoimmune diseases, as evidenced by the presence of anti-HMGB1 autoantibodies in sera from RA and drug-induced SLE patients (Wittemann et al., 1990; Ayer et al., 1994). The HMGBs (HMGB1, HMGB2, and HMGB3) also bind immunogenic nucleic acids, e.g., virus-derived RNAs and genomic DNAs, and activate innate immune signaling through receptor for advanced glycation and end products (RAGE). In fact, knockdown of HMGBs resulted in a reduction of innate immune responses against immunogenic nucleic acids (Yanai et al., 2009).

In human cells, various types of DNA reportedly induce type III IFNs, especially IFN-λ1 (or interleukin29; IL29). Ku70,

whose original functions were reported as DNA repair, V(D)J recombination and telomerase maintenance, was identified as a cytosolic DNA sensor that is responsible for the induction of IFN-λ1 (Zhang et al., 2011a). Knockdown of Ku70 suppressed IFN-λ1 activation in human cells. Whereas other known DNA sensors are involved in type I IFN production, Ku70 is unique in the production of type III IFN upon dsDNA stimulation.

Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) was initially identified as an RNA-binding protein, but it was eventually recognized as a receptor for both exogenous DNA and RNA (Yang et al., 2010). LRRFIP contains a DNA-binding domain, and is responsible for the production of IFN-β through interaction with β-catenin and recruitment of acetyltransferase p300 in cases of vesicular stomatitis virus (VSV) and *Listeria monocytogenes* infection.

RNA and DNA helicases are members of the DEADbox family, the name of which was derived from one of the conserved amino-acid sequences in the proteins. Members of the DExD/H-box (where x can be any amino acid) helicase superfamily, such as DHX9 and DHX36, were identified as cytosolic CpG DNA sensors for the induction of type I IFN production in plasmacytoid DCs (Kim et al., 2010). Another helicase, DDX41, a member of the DEXDc family, was identified as an intracellular dsDNA sensor that is responsible for type I IFN production in myeloid DCs (Zhang et al., 2011b). After stimulation with dsDNA, DDX41 interacts with STING in the microsome, mitochondria, and mitochondria-associated endoplasmic reticulum membrane fractions. DDX41 also recognizes bacterial second messenger cyclic di-GMP and cyclic di-AMP, and activates type I IFN production by interacting with STING, leading to TBK1-IRF3 activation (Parvatiyar et al., 2012).

DNA transfection or DNA virus infection leads to a production of cyclic GMP-AMP (cGAMP) via the function of cGAMP synthase, cGAS, which belongs to the nucleotidyltransferase family, and an endogenous second messenger to induce innate immune responses. cGAS binds to DNA in the cytoplasm and catalyzes cGAMP synthesis to function as a cytosolic dsDNA sensor that induces type I IFNs (Sun et al., 2013). It was also shown that cGAMP directly interacts with STING to activate IRF3, and knockdown of cGAS results in the suppression of IFN-β production induced by dsDNA transfection or DNA virus infection (Sun et al., 2013).

These studies were performed using different types of cells, synthetic DNAs, bacteria, and viruses as shown in **Table 1**. Therefore, it should be noted that multiple recognition machineries for sensing cytosolic DNA and DNA metabolites might differ among species and/or cell types.

EXTRACHROMOSOMAL HISTONE H2B IS INVOLVED IN DNA SENSING

To identify molecules responsible for cytosolic dsDNA-mediated type I IFN production, we screened a cDNA expression library using HEK293T cells stably transfected with a luciferase gene cassette under an IFN-β promoter. Among >960,000 independent clones examined, a single clone encompassing the histone H2B ORF exhibited a striking enhancement of dsDNA-induced IFN-β promoter activation (Kobiyama et al., 2010). In a separate set of experiments, cellular proteins that bind dsDNA were purified from rat thyroid cell line FRTL-5, cells previously proven to respond well to dsDNA (Suzuki et al., 1999). Protein extracts were passed through ssDNA sepharose and absorbed onto dsDNA sepharose columns before electrospray ionization (ESI)-MS/MS mass spectrometry analysis. Among the molecules identified, histone H2B showed a significantly high MASCOT (probability) score (Kawashima et al., 2011a). Thus, two independent approaches implied that extrachromosomal H2B functionally mediates IFN-β promoter activation in human kidney cells following dsDNA stimulation and physically associates with dsDNA in rat thyroid cells.

Type I IFN production induced by dsDNA was significantly suppressed in HEK293 cells treated with H2B siRNA, but not by those treated with siRNAs for other core histones. Although most histone H2B localizes in the nucleus, it appears to sense DNA in the cytoplasm by interacting with IFN-β promoter stimulator 1 (IPS-1) (Kobiyama et al., 2010), an essential adaptor molecule for signal activation triggered by cytoplasmic dsRNA and single stranded 5′-triphosphate RNA (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Human, but not mouse, IPS-1 was involved in the dsDNA-mediated signal transduction (Kumar et al., 2006; Ishii et al., 2008). Therefore, histone H2B interacts with IPS-1 in the cytoplasm following dsDNA stimulation only in human cells.

Yeast two-hybrid screening identified KIAA1192 as a molecule that interacts directly with histone H2B; therefore, it was renamed CIAO (C-terminal importin 9-related adaptor organizing histone H2B and IPS-1) based on its novel role. While high similarities of amino acid sequences were detected between human and mouse H2B (>70.1%) and between human and mouse CIAO (99.2%), the amino acid sequence of IPS-1 was largely different between human and mouse (30.3%). The observed interaction of CIAO and IPS-1 only in human molecules is a possible reflection of this difference in IPS-1 sequence (Kobiyama et al., 2010). These results strongly suggest that there is species-specific involvement of IPS-1 in dsDNA-mediated signaling.

We further examined the role of histone H2B on cell-autonomous antiviral responses. Knockdown of histone H2B suppressed IFN-β production and STAT1 phosphorylation when DNA viruses, in this case modified vaccinia virus Ankara (MVA), were infected (Kobiyama et al., 2010). Multiplication of adenovirus type 5 was significantly enhanced in the H2B knockdown cells, while multiplication of RNA viruses, such as encephalomyocarditis virus (EMCV), was not affected by the presence or absence of histone H2B (**Figure 1A**). Multiplication of other DNA viruses, such as human papilloma viruses (HPV11 and HPV16) and adenovirus serotype 5, was significantly enhanced in cells to which histone H2B siRNA was transfected. These results suggested that extrachromosomal histone H2B is involved in the sensing of DNA viruses and mediates cell-autonomous antiviral immune responses in human cells. The human immunodeficiency virus (HIV) is a lentivirus, a class of retrovirus, which has two copies of positive single stranded RNA that codes viral genes. Upon infection in target cells, the viral RNA genome is reverse transcribed into dsDNA in the peri-integration complex (PIC). When

Table 1 | Cytosolic DNA sensors.

DNA sensor	Localization	Pathogens	Nucleic acid ligand	Reference
ZBP-1/DAI	Cytoplasm	HSV	Poly(dA:dT), ISD	Takaoka et al. (2007)
AIM2	Cytoplasm	VV, MCMV, *L. monocytogenes*, *F. tularensis*	Calf thymus DNA, poly(dA:dT)	Burckstummer et al. (2009), Fernandes-Alnemri et al. (2009), Hornung et al. (2009), Roberts et al. (2009)
IFI16	Cytoplasm	VV, HSV-1	Poly(dA:dT)	Unterholzner et al. (2010)
RNA pol III/RIG-I	Cytoplasm	*L. pneumophila*, AdV, HSV-1, EBV	Poly(dA:dT)	Chiu et al. (2009)
HMGB1	Nucleus, extracellular	VSV, HSV-1	dsDNA, dsRNA, ssDNA, ssRNA	Yanai et al. (2009)
Ku70	Cytoplasm	HIV?	Plasmid DNA	Zhang et al. (2011a)
LRRFIP1	Cytoplasm	*L. monocytogenes*, VSV	Poly(dA:dT)	Yang et al. (2010)
DDX41	Cytoplasm	*L. monocytogenes*, AdV, HSV-1, VV	Poly(dA:dT), c-d-GMP, c-d-AMP	Zhang et al. (2011b), Parvatiyar et al. (2012)
cGAS	Cytoplasm	HSV-1	cGAMP	Sun et al. (2013)
Histone H2B	Nucleus, cytoplasm	HPV, AdV, HIV	Poly(dA:dT), genomic DNA	Kobiyama et al. (2010), Kawashima et al. (2011a)

HSV, herpes simplex virus; VV, vaccinia virus; MCMV, mouse cytomegalovirus; AdV, adenovirus; EBV, Epstein–Barr virus; VSV, vesicular stomatitis virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; dA:dT, poly(dA-dT)·poly(dT-dA); ISD, immunostimulatory DNA.

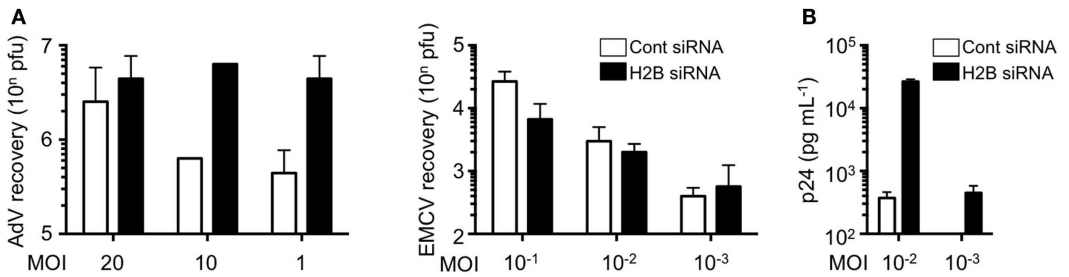

FIGURE 1 | Histone H2B is a key factor for the suppression of viral replication. **(A)** HEK293 and HeLa cells were pretreated with control siRNA (Cont siRNA) or histone H2B siRNA (H2B siRNA). The cells were infected with AdV type 5 or EMCV. Twenty-four hours after infection, viral multiplication was determined by a plaque assay. **(B)** Magic 5 cells were pretreated with Cont siRNA or H2B siRNA. The cells were infected with HIV-1 IIIB for 3 h. Seventy-four hours after infection, viral multiplication was determined by HIV-1 p24 ELISA using culture supernatant.

histone H2B was knocked-down in CCR5-expressing HeLa/CD4$^+$ cell clone 1–10 (Magic 5) cells, HIV-1 replication was significantly enhanced (**Figure 1B**). These data clearly indicate that histone H2B discriminates between foreign DNA and RNA upon viral infection to evoke IPS-1-mediated signaling through association with a novel adaptor protein, CIAO. It has also been suggested that human IPS-1 has evolutionarily gained the potential to transmit dsDNA-initiated, histone H2B-mediated signaling to combat human viruses that produce DNA intermediates within the cell. Whether histone H2B has a role in infection in mice, probably by interacting with molecules other than IPS-1, is currently unknown.

We next examined the involvement of genomic DNA-mediated immune responses in light of a possible role in the triggering of autoimmune disorders. When FRTL-5 thyroid cells were exposed to progressively higher levels of electric pulsing, in the absence of pathogens or immune cells, genomic DNA was released to the cytoplasm, which was associated with activation of the expression of certain genes, such as those encoding type I IFN and chemokines. More importantly, the expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules was also induced in thyroid cells (Suzuki et al., 1999; Kawashima et al., 2011a), suggesting that the autoimmune target cell itself might present autoantigens upon cell damage (Kawashima et al., 2011b). It has been assumed that autoimmune thyroid diseases, such as Graves's disease and Hashimoto's thyroiditis, develop by a combination of genetic susceptibility and environmental factors. The data suggested that thyroid cell injury results in the release of genomic DNA fragments into the cytosol, which are recognized by extrachromosomal histone H2B to activate genes involved in both innate and acquired immune responses. Such responses may relate to the development of thyroiditis that in turn may increase the chance to present self-antigens to immune cells and initiate autoimmune reactions. Thus, our findings suggest that extrachromosomal histone H2B acts as a cytosolic DNA sensor for both self and non-self DNA, and that this recognition mechanism may be involved in preventing microbial infections and triggering of autoimmune disorders.

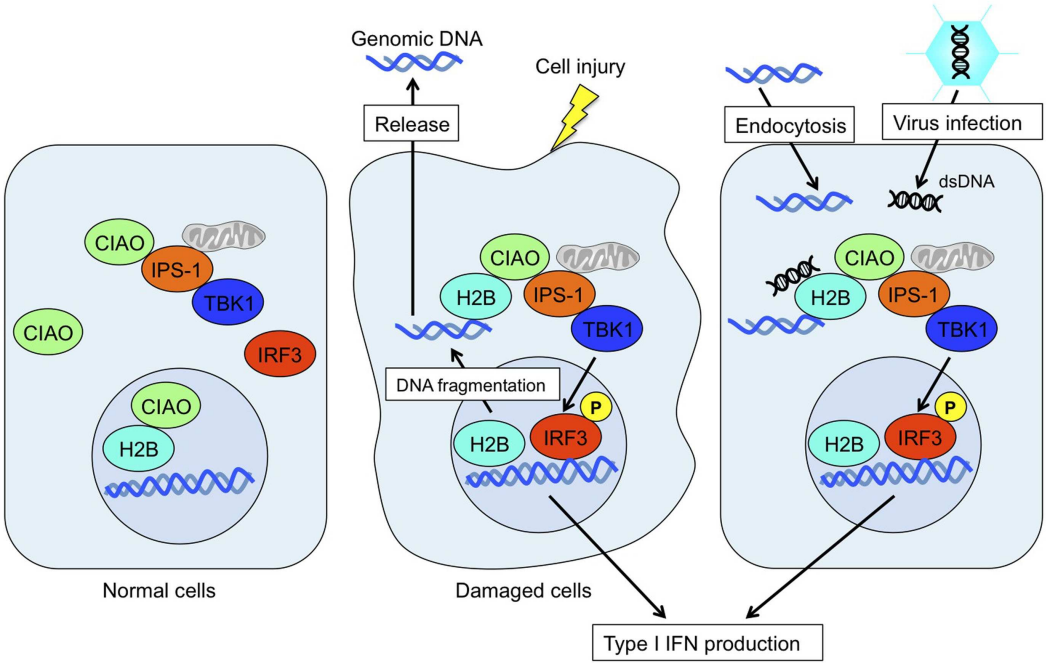

FIGURE 2 | Mode of extrachromosomal histone H2B-mediated innate immune responses. Under normal conditions, histone H2B primarily localizes to the nucleus. In cases of cell damage or viral infection, histone H2B recognizes aberrant self- or non-self-derived dsDNA and forms an H2B-CIAO-IPS-1 interaction complex in the cytoplasm, which in turn activates TBK1 and induces IRF3 phosphorylation to produce type I IFN.

EPIGENETIC MODIFICATION AND VIRUS INFECTION

Epigenetic modifications, including histone modifications and chromatin remodeling, regulate cellular processes that require access to genomic DNA. DNA viruses utilize the chromatin-mediated regulation of gene transcription and DNA replication of the host cell (Liang et al., 2009). In the case of herpes viruses, chromatin modulation is a regulatory factor of viral latency and reactivation cycles. Infection of cells with herpes virus results in the deposition of nucleosomes bearing repressive K9 methylation of histone H3 (H3-K9) on the viral genome. Inhibition of lysine-specific demethylase (LSD1) results in an accumulation of repressive chromatin and blockage of viral gene expression (Liang et al., 2009). In the case of HIV-1, histone H3-K9 methyltransferase G9a is responsible for chromatin-mediated HIV-1 transcriptional latency through methylation of H3 (Imai et al., 2010). In addition, K9 methylation of histone H3 is involved in repression of the human cytomegalovirus gene (Ioudinkova et al., 2006). Thus, since viruses utilize the host gene regulation system for their replication, its modification blocks initial gene expression of a DNA virus, including adenovirus (Liang et al., 2013).

Histone H2B can also be modified by acetylation (Schiltz et al., 1999), GlcNAcylation (Fujiki et al., 2011), phosphorylation (Fernandez-Capetillo et al., 2004), sumoylation (Nathan et al., 2006), and ubiquitination (Zhu et al., 2005), but not by citrullination and methylation. Thus, histone H2B acetylation (K12 and K15) is involved in transcriptional activation (Schiltz et al., 1999; Kawasaki et al., 2000), and phosphorylation of histone H2B (S14) is an epigenetic marker of apoptotic cells (Cheung et al., 2003). Deacetylation of K15 is essential for H2B S14 phosphorylation, and inhibition of deacetylation suppresses internucleosomal DNA degradation (Ajiro et al., 2010). Histone H2B is phosphorylated by irradiation, which accumulates in irradiation-induced foci (Fernandez-Capetillo et al., 2004). Ubiquitination of histone H2B is involved in DNA breaks (Wu et al., 2009). Since our findings suggest that histone H2B was involved in the recognition of both virus- and host-derived DNA, modification of histone H2B may also affect immune responses.

CONCLUDING REMARKS

It was long believed that the sole function of histones is to wrap genomic DNA for nucleosome assemblage. However, recent studies suggest a potential role for histones in other physiological functions in extrachromosomal settings. Histone H2A.X is phosphorylated in response to dsDNA breaks and recruited to the site of the break (Redon et al., 2002). Histone H3.3 accumulates in condensed chromatin where gene transcription is activated (Janicki et al., 2004). Also, histone H1.2 is a cytochrome *c*-releasing factor that appears in the cytoplasm after exposure to X-ray-irradiation (Konishi et al., 2003). More striking evidence is that extracellular histones have a cytotoxic ability and act as major mediators of death in cases of sepsis (Xu et al., 2009). In addition, human histone H2A and H2B have microbicidal activity, and are involved in killing promastigotes of *Leishmania amazonensis (L. amazonensis)*, *L. major*, *L. braziliensis*, and *L. mexicana*. Exposure to histones markedly decreased the infectivity of promastigotes in murine macrophages *in vitro* (Wang et al., 2011). These data strongly suggest that extrachromosomal and extracellular histones

work as an alarmin to maintain cellular homeostasis by changing their modifications and subcellular localizations. Thus, extrachromosomal histone H2B acts as a sensor for dsDNA aberrantly present within the cell, alerting cells to dangerous situations, such as infection, apoptosis, DNA breaks, and cell injury (**Figure 2**). This mechanism may also play an important role in autoimmunity, transplantation rejection, gene-mediated vaccines, and other therapeutic applications.

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Epigenetic modification of the Epstein–Barr virus BZLF1 promoter regulates viral reactivation from latency

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The Epstein–Barr virus (EBV) is an oncogenic human gamma-herpesvirus that predominantly establishes latent infection in B lymphocytes. Viral genomes exist as extrachromosomal episomes with a nucleosomal structure. Maintenance of virus latency or execution of reactivation is controlled by the expression of BZLF1, a viral immediate-early gene product, tightly controlled at the transcriptional level. In this article, we review how BZLF1 transcription is controlled, in other words how virus reactivation is regulated, especially in terms of epigenetics. We recently found that histone H3 lysine 27 trimethylation (H3K27me3) and H4K20me3 markers are crucial for suppression of BZLF1 in latent Raji cells. In addition, H3K9me2/3, heterochromatin protein 1, and H2A ubiquitination are associated with latency, whereas positive markers, such as higher histone acetylation and H3K4me3, are concomitant with reactivation. Since lytic replication eventually causes cell cycle arrest and cell death, development of oncolytic therapy for EBV-positive cancers is conceivable using epigenetic disruptors. In addition, we note the difficulties in analyzing roles of epigenetics in EBV, including issues like cell type dependence and virus copy numbers.

Keywords: epigenetics, Epstein–Barr virus, reactivation, latency, BZLF1 gene

INTRODUCTION

The Epstein–Barr virus (EBV), a human gamma-herpesvirus that predominantly establishes latent infection in B lymphocytes, is associated with various disease entities, including Burkitt's lymphoma, post-transplant lymphoproliferative disorder (PTLD), Hodgkin's disease, gastric cancer, and nasopharyngeal carcinoma (NPC). Only a small percentage of infected cells switch from the latent stage into the lytic cycle and produce progeny viruses. Transitions and differences in EBV infection cycling between lytic and latent states are closely tied, not only with the virus production and spread, but also with disease progression and malignancy of EBV-positive cancers, and thus detailed analysis of molecular mechanisms that govern the EBV latent-to-lytic switch is of fundamental importance.

LATENCY AND REACTIVATION OF EBV IS REGULATED BY AN ABUNDANCE OF BZLF1

Although the mechanism of EBV reactivation *in vivo* is not fully understood, it is known to be elicited *in vitro* by treatment of latently infected B cells with some chemical or biological reagents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophores, sodium butyrate, and anti-immunoglobulin (Ig). Stimulation of the EBV lytic cascade by these reagents leads to expression of two viral immediate-early genes, BZLF1 (also known as Zta, EB1, ZEBRA, or Z) and BRLF1 (Rta or R). The BZLF1 protein is a transcriptional activator that shares structural similarities to basic leucine zipper (b-Zip) family transcriptional factors and BZLF1 expression alone can trigger the entire reactivation cascade (Speck et al., 1997; Amon and Farrell, 2005; Tsurumi et al., 2005). BZLF1 has a very interesting and unique characteristic trial. In cells latently infected with EBV, the viral lytic promoters are strongly repressed by repressive epigenetic marks, including heavy 5′-CG-3′ dinucleotide (CpG) DNA methylation (Fernandez et al., 2009), but BZLF1 can preferentially bind to and activate the methylated promoters (Bhende et al., 2004; Dickerson et al., 2009; Flower et al., 2011). Therefore, BZLF1 serves as the molecular switch for EBV reactivation from latency. Actually, induction of BZLF1 (20- to 50-folds) by anti-IgG or other chemical inducers (see **Figure 1**) can cause efficient viral gene expression, viral DNA replication and progeny production, at least in Akata or B95-8.

POSITIVE/NEGATIVE CONTROL OF BZLF1 EXPRESSION BY TRANSCRIPTION FACTORS

Expression of the BZLF1 gene is tightly controlled at the transcriptional level. The BZLF1 promoter (Zp) normally exhibits low basal activity and is activated in response to TPA or the other reagents listed above. The promoter is activated by transcriptional factors including myocyte enhancer factor 2 (MEF2; Liu et al., 1997b) and Sp1/3 (Liu et al., 1997a). Cellular b-Zip type transcription factors, such as the cyclic AMP-response element-binding protein (CREB), activating transcription factor (ATF), activator protein-1 (AP-1; Ruf and Rawlins, 1995; Liu et al., 1998; Murata et al., 2009, 2011) or a spliced form of the X-box binding protein 1 [XBP-1(s); Bhende et al., 2007], also play crucial roles in the promoter activation. We previously showed the importance of CREB and its calcineurin-dependent activation by transducer of regulated CREB 2 (TORC2; Murata et al., 2009). Once produced, BZLF1 itself can bind to and activate its own promoter (Flemington and Speck, 1990; Murata et al., 2010). Most of the positive

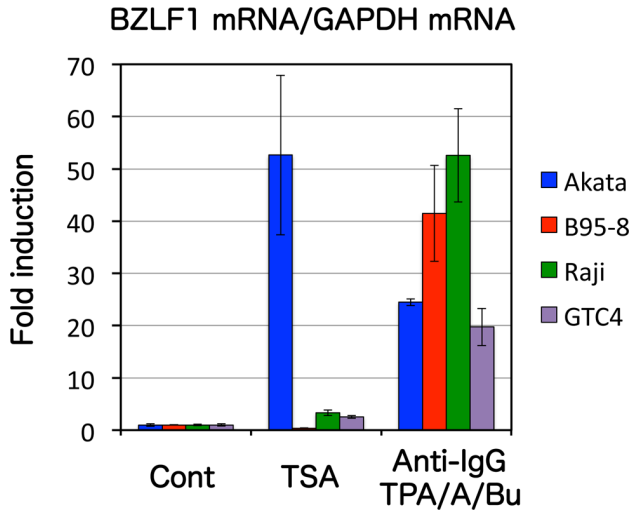

FIGURE 1 | Effects of an HDAC inhibitor TSA on BZLF1 expression differ with the type of EBV-positive cell. Akata, Raji, B95-8, and GTC-4 cells were treated with either vehicle (Cont) or 300 nM TSA. Anti-IgG (for Akata) or TPA/A23187/butyrate (for B95-8, Raji, and GTC-4) served as positive controls, as these substances induce BZLF1. After 24 h, RNAs were collected and real-time RT-PCR was carried out to measure the levels of BZLF1 mRNA, the results being shown as bars after normalization to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. TSA alone treatment induced BZLF1 expression in Akata, but did not appreciably enhance in other cells.

factors have been demonstrated or are presumed to up-regulate the BZLF1 promoter by recruiting transcriptional coactivators, such as histone acetylases. On the other hand, the activity of Zp is restricted by repressive factors including Jun dimerization protein 2 (JDP2; Murata et al., 2011), zinc finger E-box binding factor (ZEB; Yu et al., 2007), Yin Yang 1 (YY1; Montalvo et al., 1995), and an unidentified repressor that binds to the ZIIR motif (Liu et al., 1998).

EPIGENETICS OF THE BZLF1 PROMOTER ASSOCIATED WITH LATENCY AND REACTIVATION

In the previous section, we noted that BZLF1 promoter activity is regulated positively or negatively by transcription factors and cofactors. The question then arises of how those host transcription factors regulate BZLF1 transcription, which eventually leads to EBV reactivation? The answer is through epigenetic changes that mediate transcription factors and BZLF1 expression.

With regard to epigenetics, CpG DNA methylation could be one possible cause of BZLF1 promoter repression, as this is frequently associated with constitutive heterochromatin, where transcription is tightly suppressed, irreversibly. However, Fernandez et al. (2009) showed that CpG methylation levels at BZLF1 promoters in various EBV-positive cell lines are exceptionally low, although most of the viral genome in the latent phase is highly methylated. Likewise, very little CpG DNA methylation was found in the promoter region of the lytic switch gene, ORF50/K-Rta, for Kaposi sarcoma-associated herpesvirus (KSHV), another oncogenic gamma-herpesvirus (Gunther and Grundhoff, 2010).

However, treatment of EBV-positive cells with 5-aza-2′-deoxycytidine (5-Aza), a potent inhibitor of DNA methyltransferase, induces BZLF1 transcription (Murata et al., 2012; see **Figure 3**). It is speculated that 5-Aza activates EBV lytic gene expression by an unknown mechanism that does not involve decreased CpG DNA methylation levels (Countryman et al., 2008). This is because 5-Aza induces BZLF1 expression within a very short period of time (15 min or less), although it must take days to bring about hypo-methylation of the CpG DNA, since 5-Aza is a DNA methyltransferase inhibitor and it does not actively trim off nor abolish methylation without *de novo* DNA amplification. In fact, accumulating data indicate that the mechanism of gene inductions by 5-Aza or its analogs is very complicated, and does not necessarily depend on DNA demethylation. The inhibitors can activate gene expressions through DNA damage (Link et al., 2008; Wang et al., 2008), degradation of a certain proteins (Zheng et al., 2012), or histone reorganization (Wozniak et al., 2007; Komashko and Farnham, 2010). Therefore, it is quite likely that the consequence of 5-Aza is a side effect, although the possibility cannot be denied that DNA methylation is present at Zp at least to some extent, and plays a role in BZLF1 gene suppression (Li et al., 2012).

Possible epigenetic modifications which might silence the promoter include histone changes. From a historical perspective, the best-characterized epigenetic histone marker of BZLF1 promoter is acetylation. Histone acetylation causes destabilization of chromatin, leading to a loose, open structure of the promoter, so that it becomes easily accessible to basic transcription factors. Histone acetylation of EBV Zp first came to light because histone deacetylase (HDAC) inhibitors were found to cause reactivation of EBV (Luka et al., 1979; Jenkins et al., 2000). Histone acetylation levels are low in latency, and are induced upon reactivation (Murata et al., 2012). In fact, silencing of the BZLF1 promoter in latently infected cells is mediated by and solely dependent on low levels of histone acetylation, at least in some cell lines such as Akata, since inhibitors of HDAC, like sodium butyrate or trichostatin A (TSA), can reverse the silencing (Miller et al., 2007; Murata et al., 2012; **Figure 1**). However, treatment with butyrate or TSA alone does not efficiently induce BZLF1 transcription in cell lines like B95-8 or Raji, suggesting that the molecular mechanisms that govern the suppression of BZLF1 transcription in these cells must be more complex than simply reduction in the acetylation level of the promoter (Countryman et al., 2008; Murata et al., 2012; **Figure 1**).

In order to analyze mechanisms that govern BZLF1 transcription other than histone acetylation in such a cell line, we first examined various epigenetic histone modifications in the Zp of EBV DNA. Chromatin immunoprecipitation (ChIP) assays revealed that suppressive histone markers including histone H3 lysine 27 trimethylation (H3K27me3), H3K9me2/3 and H4K20me3 are present in the Zp of latent Raji cells, while high levels of histone acetylation and H3K4me3 markers correlate with reactivation of the virus (**Figure 2**; Murata et al., 2012).

H3K27me3 is a suppressive histone modification, characteristic of facultative heterochromatin, a form of heterochromatin where expression of a wide variety of genes is considerably silenced by specific histone modifications (Kondo, 2009). With specific signaling, histone modifications of this type of heterochromatin can be

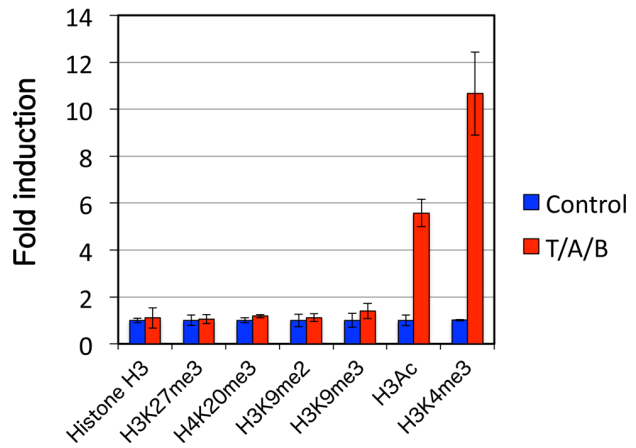

FIGURE 2 | Histone modification pattern of EBV BZLF1 promoter upon lytic reactivation. Raji cells were treated with control vehicle (blue) or TPA/A23187/butyrate (T/A/B, red) at 20 ng/ml, 1 μM and 5 mM, respectively. ChIP experiments were performed using antibodies indicated below, followed by real-time PCR. Active marks (H3Ac, H3K4me3) were elevated while levels of suppressive marks (H3K27me3, H4K20me3, and H3K9me2/3) remained unchanged.

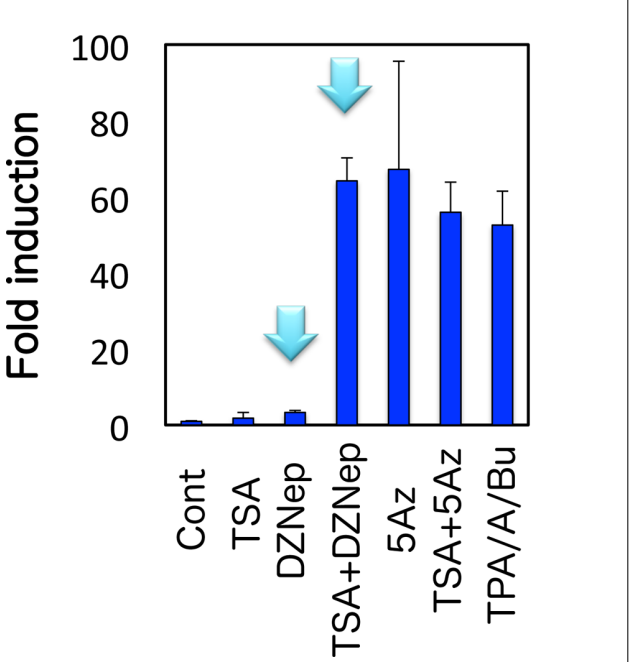

FIGURE 3 | Effects of pharmacological inhibitors on BZLF1 expression in Raji cells. Raji cells were treated with vehicle (Cont), 10 μM DZNep, 300 nM TSA, 1 μM 5-Aza alone or in combinations as indicated. As a positive control (TPA/A/Bu), Raji cells were treated with TPA/A23187/butyrate at 20 ng/ml, 1 μM and 5 mM, respectively. For DZNep or 5-aza-2′-deoxycytidine (5-Aza) treatment, cells were exposed to the reagent daily for 3 days. Treatment with other chemicals was for 24 h. Real-time RT-PCR was carried out to measure the levels of BZLF1 mRNA, which were then normalized to GAPDH mRNA levels. The light-blue arrows indicate that DZNep alone did not efficiently induce BZLF1, but could markedly enhance the expression if treated in combination with TSA.

reversed so that it becomes transcriptionally active, unlike constitutive heterochromatin. The presence of H3K27me3 methylation was recently reported by other groups in EBV Zp (Ramasubramanyan et al., 2012) and KSHV ORF50/K-Rta (Gunther and Grundhoff, 2010; Toth et al., 2010). To test if H3K27me3 modification is involved in the BZLF1 suppression during latency, we here used an inhibitor of the modification, 3-deazaneplanocin A (DZNep; Tan et al., 2007; Miranda et al., 2009). While treatment of Raji cells with either DZNep or TSA alone had only minor effects on BZLF1 levels (1.8- and 3.3-fold increase, respectively), use of the two inhibitors in combination (TSA + DZNep) stimulated the expression 64.2-fold (Murata et al., 2012; **Figure 3**). This result suggests that not only histone deacetylation but also histone H3K27me3 serve to inhibit BZLF1 transcription, at least in Raji cells. H3K27me3 methylation is mediated by enhancer of zeste 2 (Ezh2), a member of polycomb repressor complex 2 (PRC2; Cao et al., 2002). To further verify the involvement of H3K27me3 in BZLF1 gene repression, we then knocked down Ezh2. Silencing increased BZLF1 levels by 2.5-fold even without TSA, and addition of TSA elevated this to 10.9-fold (Murata et al., 2012). Furthermore, we confirmed these inhibitors and small interfering RNA (siRNA) treatment actually caused expected changes in epigenetic marks (see Figures 7 and 9 in Murata et al., 2012). An importance of histone H3K27me3 in the maintenance of latency was also recently demonstrated for KSHV ORF50/K-Rta (Toth et al., 2010). These results point to involvement of Ezh2 methyltransferase and the histone H3K27me3 marker in silencing of BZLF1 gene expression during EBV latency. In addition, we would like to note that histone acetylation is also needed for efficient expression of BZLF1.

It has been reported that, in addition to histone H3K27me3, H4K20me3 histone modification is also inhibited by DZNep (Tan et al., 2007; Miranda et al., 2009), and we found H4K20me3 is present in the Zp of latent Raji cells. In order to specifically examine the effect of the H4K20me3 methylation on silencing of the BZLF1 gene, Suv420h1, the methyltransferase responsible for the modification, was knocked down by siRNA technology. Remarkable induction of the BZLF1 gene by Suv420h1 knockdown and TSA corresponded with reduction of H4K20me3 levels and elevation of active H3K9Ac and H3K4me3 markers (see Figures 7 and 10 in Murata et al., 2012). Therefore, we conclude that silencing of the BZLF1 promoter in Raji cells is similarly brought about by histone H4K20me3 methylation.

Because DZNep exhibited potent inducing effects on BZLF1 gene transcription, we also tested BIX01294, a specific inhibitor of G9a, the methyltransferase responsible for histone H3K9me2 methylation, which is another typical marker of facultative heterochromatin. Paradoxically, treatment of Raji cells or other EBV-positive cells with BIX01294 alone or in combination with TSA, DZNep, or 5-Aza, did not increase the BZLF1 expression at all, or caused very modest increase at most, even though H3K9me2 is present at the Zp at significantly high level (Murata et al., 2012). The data imply that K3K9me2 may not play an important role in the suppression of BZLF1, at least in Raji cells.

A representative constitutive heterochromatin marker histone H3K9me3, too, has been reported to be definitely present during latency in EBV Zp (Murata et al., 2012) and KSHV ORF50/K-Rta promoters (Gunther and Grundhoff, 2010; Toth et al., 2010). Although Toth and others observed that H3K9me3 in the KSHV ORF50/K-Rta promoter decreased upon induction, suggesting that the modification is involved in silencing of the immediate-early gene, we failed to see an equivalent decline in EBV Zp (Murata et al., 2012; **Figure 2**). We speculate that this inconsistency was related to the use of Raji cells in our experiments, since the Raji genome has a deletion of the BALF2 gene, essential for lytic viral DNA synthesis. Furthermore, treatment of latent Raji cells with chaetocin, an Suv39H1 histone H3K9me3 methyltransferase inhibitor, did not induce BZLF1 expression, even in combination with other epigenetic inhibitors, such as TSA, DZNep, or 5-Aza. Therefore, histone H3K9me3 modification is a feature of EBV Zp in latency, but we still do not have conclusive evidence that it plays a role in the maintenance of latency, at least in Raji cells. Since other methyltransferases, such as SETDB1/ESET, can also catalyze histone H3K9me3 modification, they may be acting to suppress BZLF1 gene in the presence of chaetocin.

Histone H3K4me3 is enriched in the promoter regions of transcriptionally active genes in euchromatin, and thus serves as an active chromatin marker. It elicits transcription by recruiting factors like chromodomain-containing and plant homeodomain (PHD) finger proteins, as well as chromatin remodeling factors. Lytic induction of Raji cells markedly elevated the active histone marker, H3K4me3, in the Zp, while the level was low in latency (Murata et al., 2012; **Figure 2**). Enhancement of H3K4me3 upon induction has been reported for KSHV (Gunther and Grundhoff, 2010; Toth et al., 2010) in addition to other herpesviruses, indicating that histone H3K4me3 methylation, like histone acetylation, plays an important and universal role in lytic gene expression of herpesviruses.

Further to the epigenetic modifications described above, already published by us or other groups, we have confirmed in our preliminary experiments that other epigenetic alterations are associated with EBV latency and reactivation. In the mammalian genome, approximately 10% of histone H2A is mono-ubiquitinated at Lys 119, in association with transcriptional suppression, and then de-ubiquitinated upon transcriptional activation. We have found EBV Zp of latent Raji cells to be labeled with high levels of mono-ubiquitinated H2A, although massive reduction did not occur on induction. A similar ChIP result was obtained when heterochromatin protein 1 (HP1) was monitored. Because HP1 binds to methylated histone H3K9, the presence of HP1 serves to strengthen significance of H3K9 methylation at the promoter. Thus, BZLF1 promoter has various repressive epigenetic modifications, and also acquires cofactors associated for the gene suppression. Physiological relevance of those factors is being analyzed.

Interestingly, we found there is a prominent binding site of CCCTC-binding factor (CTCF), a transcriptional regulator and insulator, in the Zp of EBV. Binding of CTCF correlates with binding of Rad21, a subunit of cohesion. Other groups also recently confirmed such binding to EBV Zp (Holdorf et al., 2011; Arvey et al., 2012). Since it is known that CTCF/cohesin regulate transcription by creating long range chromatin loops and/or by acting as insulators, roles of such factor binding in latency and reactivation of EBV is of great interest. Binding of CTCF/cohesin to the KSHV ORF50/K-Rta promoter and a contribution to the suppression of reactivation have already been established (Chen et al., 2012), but their role in EBV reactivation may be different, as the binding sites of CTCF/cohesin in the ORF50/K-Rta promoter appear redundant while there is only one major peak of CTCF/cohesin in the EBV BZLF1 promoter (Holdorf et al., 2011; Arvey et al., 2012). We recently made recombinant EBV with point mutation at the CTCF binding site of the BZLF1 promoter, but our preliminary data showed that disruption of CTCF binding to the peak did not notably influence on BZLF1 levels, if any, at least in HEK293 cells.

EPIGENETIC AGENTS AS MOLECULAR TARGETS FOR ANTI-VIRAL/CANCER DRUGS

Because there are very limited numbers of anti-EBV drugs developed or being developed to date, including acyclic nucleoside analogs, such as acyclovir or ganciclovir, and kinase inhibitors, such as maribavir (Wang et al., 2009), the search and development of effective anti-viral drugs for patients with infectious mononucleosis, caused by primary and acute EBV infection in adolescence, are important tasks. Because histone acetylation plays a crucial role in EBV reactivation, inhibitors of histone acetyl transferase (HAT) have potential in this regard. Inhibition of histone demethylase LSD1 by monoamine oxidase inhibitors is reported to block alpha herpesvirus lytic replication and reactivation from latency (Liang et al., 2009).

Interestingly, as execution of the viral lytic program arrests cell cycle progression in infected cells (Kudoh et al., 2003), induction of EBV lytic replication in EBV-positive cancers by epigenetic inhibitors, such as HDAC inhibitors, 5-Aza, and/or DZNep, may offer clinical application as a type of oncolytic therapy in the future (Feng et al., 2004; Jung et al., 2007). Because treatment like this must induce efficient production of progeny viruses, anti-viral drugs, such as ganciclovir, should obviously be used in combination to both induce apoptosis and prevent viral spreading.

PARTICULARITY AND DIFFICULTIES OF ANALYZING EBV EPIGENETICS

It must be emphasized that responses of BZLF1 promoter activity to certain epigenetic inhibitors depend largely on the cell type. To take one example, levels of BZLF1 mRNA expression in Akata cells are markedly induced by TSA treatment alone, whereas the virus in other cells, including B95-8 or Raji, does not appear to respond (Murata et al., 2012; **Figure 1**). We have demonstrated, in Raji cells, that BZLF1 expression is suppressed by histone H3K27me3 and H4K20me3, in addition to low level histone acetylation, whilst in Akata cells, only low level histone acetylation accounts for repression of the gene induction (Murata et al., 2012). Curiously however, the Zp of the Akata cell line, is modified with histone H3K27me3 and H4K20me3, almost as efficiently as Raji (Murata et al., 2012). Then, why do the suppressive H3K27me3 and H4K20me3 markers not actually prevent BZLF1 expression in Akata cells?

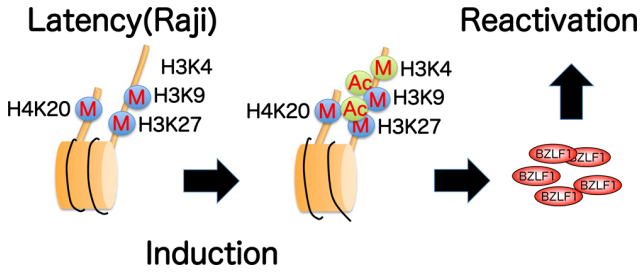

FIGURE 4 | Summary of epigenetic histone modifications in the BZLF1 promoter of Raji cells. Repressive histone H3K9, H3K27, H4K20 methylations (marked with blue circles) are present in latency, and are not appreciably decreased even after induction. High levels of active markers, such as H3K4 methylation and histone acetylation (green circles), are notably associated with lytic induction.

Another question is why treatment with TPA, A23187, and sodium butyrate did not affect repression markers, such as H3K9me2/3, H3K27me3, or H4K20me3, at all in Raji, whereas they significantly elicited expression of BZLF1 (Murata et al., 2012)? It is considered in general that such suppressive markers must be diminished for transcriptional activation.

We believe these inconsistencies can be explained in terms of latent EBV genome copy numbers. To take an example, it is known that about 5–100 copies of the episomal EBV genome are present per latent cell. For the first question, let us suppose there are 10 copies of the latent EBV genome in one Akata cell, and nine copies are modified and repressed by suppressive H3K27me3 and H4K20me3 markers, the remaining copy being unmodified. This means the virus in Akata cells retains high sensitivity to TSA alone. For the second question, if TPA/A23187/butyrate treatment of Raji cells induces reduction of such repressive histone methylation in only a few copies but still allows efficient expression of BZLF1, reduction of the repressive modification must be difficult to detect, because the histone methylations in the majority of the genome copies are intact. In contrast, induction of active histone markers, like histone acetylation or H3K4me3 methylation, can clearly be observed.

In addition, the presence of epigenetic markers, like H3K27me3 or H3K4me3, may not in itself be sufficient for suppression or activation. Adaptor or mediator complexes, such as polycomb-group proteins or PHD finger proteins must be recruited to the promoter regions and appropriately act to compact or open the chromatin structure. Therefore, we suggest that only presence or absence of a certain epigenetic alteration in any regulatory region of EBV does not necessarily mean that it is critical. For determination of actual significance, functional assays, such as use of specific inhibitors and knockdown of epigenetic enzymes, are essential.

SUMMARY

We recently found (Murata et al., 2012) that histone H3K27me3 and H4K20me3 markers are crucial for maintenance of EBV latency, while histone acetylation and H3K4me3 are associated with reactivation from latency, at least in Raji cells (**Figure 4**). Although there may be differences in response between cell types, these data provide primary evidence for potential in anti-viral/cancer drug development.

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Gene expression analysis of Toll-like receptor pathways in heterophils from genetic chicken lines that differ in their susceptibility to *Salmonella enteritidis*

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Previously conducted studies using two chicken lines (A and B) show that line A birds have increased resistance to a number of bacterial and protozoan challenges and that heterophils isolated from line A birds are functionally more responsive. Furthermore, when stimulated with Toll-like receptor (TLR) agonists, heterophils from line A expressed a totally different cytokine and chemokine mRNA expression pattern than heterophils from line B. A large-scale gene expression profile using an Agilent 44K microarray on heterophils isolated from line A and line B also revealed significantly differential expression in many immune-related genes following *Salmonella enteritidis* (SE) stimulation, which included genes involved in the TLR pathway. Therefore, we hypothesize the differences between the lines result from distinctive TLR pathway signaling cascades that mediate heterophil function and, thus, innate immune responsiveness to SE. Using quantitative RT-PCR on mRNA from heterophils isolated from control and SE-stimulated heterophils of each line, we profiled the expression of all chicken homologous genes identified in a reference TLR pathway. Several differentially expressed genes found were involved in the TLR-induced My88-dependent pathway, showing higher gene expression in line A than line B heterophils following SE stimulation. These genes included the TLR genes *TLR4*, *TLR15*, *TLR21*, *MD-2*, the adaptor proteins *Toll-interleukin 1 receptor domain-containing adaptor protein* (*TIRAP*), *Tumor necrosis factor-receptor associated factor 3* (*TRAF3*), the IκB kinases transforming growth factor-β-*activating kinase 1* (*TAK1*), *IKKε and IKKα*, the transcription factors *NFkB2 and interferon regulatory factor 7*, *phosphatidylinositol-3 kinase* (*PI-3K*), and the mitogen-activated protein kinase *p38*. These results indicate that higher expression of TLR signaling activation of both MyD88-dependent and TRIF-dependent pathways are more beneficial to avian heterophil-mediated innate immunity and a complicated regulation of downstream adaptors is involved in stronger induction of a TLR-mediated innate response in the resistant line A. These findings identify new targets for genetic selection of chickens to increase resistance to bacterial infections.

Keywords: Toll-like receptors, heterophils, microarray, chickens, genetic selection

INTRODUCTION

Host genetics plays an indispensable role in response to *Salmonella* colonization of chickens. For the past several years, we have been profiling the phenotype of two parental broiler lines (A and B) with regard to their resistance or susceptibility against bacterial (*Salmonella enteritidis*, Ferro et al., 2004; Swaggerty et al., 2005a; *Enterococcus gallinarum*, Swaggerty et al., 2005b; *Campylobacter jejuni*, Li et al., 2008; and protozoan *Eimeria*, Swaggerty et al., 2011) challenges. In all cases, line A chickens are more resistant to the pathogen challenges than line B chickens. Mechanistically, this resistance was mediated by the predominant avian granulocyte, the heterophil, with heterophils from Line A functionally more responsive and capable of producing a differential cytokine/chemokine profile compared with line B (Ferro et al., 2004; Swaggerty et al., 2004, 2005a). However, we focused all of these studies on downstream events and/or end products (cell effector functions and cytokine/chemokine gene expression), which led us to ask whether the differences were initiated at either the level of receptor recognition or downstream signaling events induced by ligation of the receptors.

Recognition of potential pathogenic microbes by the innate immune system is the function of a class of cellular receptors known as pattern-recognition receptors (PRRs), which include Toll-like receptors (TLRs). The TLR superfamily represents an evolutionarily conserved signaling system that is a decisive determinant of the innate immune and inflammatory responses. The innate system uses these germ-line encoded receptors to detect evolutionarily conserved microbial proteins, lipids, and nucleic

acids (microbial-associated molecular patterns, MAMPs; Fearon and Locksley, 1996). Microbial product-induced activation leads to the activation of intracellular signaling pathways that initiate microbial killing mechanisms, the production of pro- and/or anti-inflammatory cytokines, and up-regulation of co-stimulatory molecules required for antigen presentation to the acquired immune system (Medzhitov and Janeway, 1997). A broad TLR expression profile has been reported in heterophils which suggest that heterophils may play a major role as first-line effector cells through the TLR-induced signaling pathway (Kogut et al., 2005, 2006).

Toll-like receptors are evolutionarily conserved microbial sensing receptors that are able to detect microbial lipids, proteins, and nucleic acids (Takeuchi and Akira, 2010). The avian genome encodes 10 functional TLRs that are located either on the cell surface or within endosomes (Brownlie and Allan, 2011). TLRs located on the cell surface induce the transcriptional activation of pro-inflammatory cytokines, chemokines, and antimicrobial proteins and are mediated by nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways through initial activation of the adaptor protein Myeloid differentiation factor 88 (MyD88). Receptor engagement induces MyD88 to activate IL-1R associated kinase-4 (IRAK4), which in turn activates other IRAK family members. These IRAK members then activate the E3 ubiquitin protein ligase TNFR-associated factor 6 (TRAF6) that links with members of an E2 ubiquitin-conjugating enzyme complex. Following a series of ubiquitination steps, transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) complexes with TAK1-binding proteins that then activates the IκB kinase (IKK) complex and MAPK pathway. The IKK complex phosphorylates and ubiquitinates the NF-κB inhibitor IκBα, marking it for degradation. NF-κB is released from IκBα and translocates to the nucleus to initiate the expression of pro-inflammatory cytokines (O'Neill, 2008; Takeuchi and Akira, 2010). The MAPK pathway activates the transcription factor activator protein-1 (AP-1), which is also responsible for the expression of the pro-inflammatory cytokines.

With all of this information at hand, we hypothesized that the differences between the two genetic lines of chickens result from distinctive TLR recognition and/or signaling pathway cascades that mediate heterophil function and; thus, innate responsiveness to various bacterial and protozoan infections in chickens. Therefore, using a chicken genome Agilent microarray and quantitative real-time PCR (qRT-PCR) analysis, we evaluated TLR pathway gene expression differences between heterophils from the two lines of chickens with and without infection with *S. enterica* serotype Enteritidis (SE).

MATERIALS AND METHODS

EXPERIMENTAL CHICKENS

The two distinct parental broiler lines used in this study were obtained from a commercial company. To maintain confidentiality, the lines were designated as A and B. At the day of hatch, chickens were placed in floor pens (8 feet × 8 feet) containing wood shavings, provided supplemental heat, water, and a balanced, unmedicated corn and soybean meal based chick starter diet *ad libitum*. The feed was calculated to contain 23% protein and 3200 kcal metabolized energy/kg of diet, and all other nutrient rations met or exceeded the standards established by the National Research Council (1994).

BACTERIA

A poultry isolate of SE (#97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA, USA). SE was cultured in tryptic soy broth (Difco Laboratories, Becton Dickinson, Co., Sparks, MD, USA) overnight at 41°C. Stock SE (1×10^9 cfu/ml) was prepared as previously described (Kogut et al., 2010).

HETEROPHIL ISOLATION

Heterophils were isolated from the peripheral blood of 100 chickens per line 6 days post-hatch. Following blood collection, heterophils were isolated as previously described (Kogut et al., 2012). Briefly, blood from chickens was collected in vacutainer tubes containing disodium ethylenediaminetetraacetic acid (EDTA; BD vacutainer, Franklin Lakes, NJ, USA) and mixed thoroughly. The blood and EDTA for each line was pooled and diluted 1:1 with RPMI 1640 media containing 1% methylcellulose and centrifuged at $40 \times g$ for 15 min at 4°C. The supernatant was transferred to a new conical tube and diluted with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (1:1), layered onto discontinuous Histopaque® gradients (specific gravity 1.077 over 1.119) and centrifuged at $190 \times g$ for 1 h at 4°C. The Histopaque® layers were collected, washed with RPMI 1640 (1:1) and pelleted at 485 *g* for 15 min at 4°C. The cells were then re-suspended in fresh RPMI 1640, counted on a hemacytometer, and diluted to 1×10^7/ml in RPMI. All tissue culture reagents and chemicals obtained from Sigma Chemical Company, St. Louis, MO, USA, unless noted otherwise.

TOTAL RNA ISOLATION

Heterophils (1×10^7) were treated with 300 μl RPMI or SE (1×10^9 cfu/ml), for 1 h at 39°C on a rotary shaker. Treated heterophils were pelleted, washed with RPMI ($485 \times g$ for 15 min at 4°C), the supernatant discarded, the cells re-suspended in lysis buffer (Qiagen RNeasy mini RNA extraction kit, Qiagen, Inc., Valencia, CA, USA), and frozen. The lysed cells were transferred to QIAshredder homogenizer columns and centrifuged for 2 min at $\geq 8000 \times g$. Total RNA was extracted from the homogenized lysate according to the manufacturer's instructions, eluted with 50 μl RNase-free water and stored at −80°C.

MICROARRAY EXPERIMENT DESIGN

A dual color, balanced design was used to provide four different comparisons: line A infected (AI)/AC, line B infected (BI)/BC, AC/BC, and AI/BI (C, non-infected controls; I, SE-infected). Only the between line comparisons are reported here; i.e. AC/BC and AI/BI. Within line comparisons have been describe previously (Chiang et al., 2008). Four biological replicates were conducted in each comparison and the dye balance was used throughout in order to prevent the dye bias during the sample labeling.

LABELING AND HYBRIDIZATION

The integrity of total RNA samples was confirmed using Agilent Bioanalyzer 2100 Lab-on-chip system (Agilent Technologies, Palo

Alto, CA, USA). Five hundred nanograms (ng) of total RNA were reverse-transcribed to cDNA during which a T7 sequence was introduced into cDNA. T7 RNA polymerase-driven RNA synthesis was used for the preparation and labeling of RNA with Cy3 (or Cy5) dye. The fluorescent cRNA probes were purified using Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA), and an equal amount (825 ng) of Cy3 and Cy5 labeled cRNA probes were hybridized on a 44 K chicken Agilent array (GEO accession: GSE9416). The hybridized slides were washed using a commercial kit package (Agilent Technologies, Palo Alto, CA, USA) and then scanned using Genepix 4100A scanner (Molecular Devices Corporation, Sunnyvale, CA, USA) with the tolerance of saturation setting of 0.005%.

MICROARRAY DATA COLLECTION AND ANALYSIS

For each channel, the median of the signal intensity and local background values were used. A Locally Weighted Linear Regression (LOWESS) normalization was applied to remove signal intensity-dependent dye bias for each array using R program. The normalized data was analyzed using SAS 9.1.3 (SAS Institute, Inc., Cary, NC, USA) with mixed model analysis. The mixed model used to identify significantly differentially expressed genes was:

$$Y_{ijklm} = \mu + T_i + L_j + D_k + S_l + T^*L_{ij} + e_{ijklm}$$

Where Y_{ijklm} represents each normalized signal intensity; μ is an overall mean value; T_i is the main effect of treatment (SE infection) i; L_j is the main effect of chicken line j; D_k is the main effect of dye k; S_l is the random effect of slide l; T^*_{Lij} is the interaction between treatment and line; and e_{ijklm} is a stochastic error (assumed to be normally distributed with mean 0 and variance σ^2). An approximate F test on least-square means was used to estimate the significance of difference for each gene in each comparison where $P < 0.001$ was considered to be statistically different. The false discovery rate (Q value) was calculated for each P-value using R program according to the method described by Storey and Tibshirani (2003).

QUANTITATIVE REAL-TIME PCR

Genes having more than one probe with inconsistent gene regulation expression were further confirmed by qRT-PCR. The anti-coagulated blood from 100 chickens/line was pooled, and the heterophils were isolated from each line as described above. A total of three separate heterophil isolations were made for separate pools of replicated qRT-PCR. The qRT-PCR assay was conducted three times with pooled heterophils (heterophils pooled from 100 chickens from each line with or without SE). At least three replicates were conducted for each gene with the heterophils from each pool of chickens. The data from these three repeated experiments were pooled for presentation and statistical analysis. Total RNA (300 ng) from each sample, AI, line A non-infected (AN), BI, and line B non-infected (BN), were used for cDNA synthesis with random hexamer primer of a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. The cDNAs were quantified by qRT-PCR using ABI prism 7900HT system (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). The specific oligonucleotide primers (**Table 1**) were designed by the PRIMER3 program (Rozen and Skaletsky, 2000). The conditions of qRT-PCR amplification were: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 59°C for 1 min. The chicken β-actin gene was

Table 1 | Primers used for qRT-PCR.

Gene name	Accession no.	Primer sequence (5′–3′)
β-Actin	NM_205518	F[a]: ACGTCTCACTGGATTTCGAGCAGG
		R[b]: TGCATCCTGTCAGCAATGCCAG
TLR1-1	AY633574	F: CTGTCTTGCCAATCTGTC
		R: GTGAAGGCTCCGTGTATT
TLR1-2	NM_001098854	F: AGCTGCAGGACTTCCTGCGC
		R: TTGTCTGCGTCCACTGCCAC
TLR2-1	AB050005	F: TTAAAAGGGTGTCCAGGAG
		R: GTCCAAACCCATGAAAGAGC
TLR2-2	AB046533	F: AGGCACTTGAGATGGAGCAC
		R: CCTGTTATGGGCCAGGTTTA
TLR3	CR407213	F: CTGCTGCTTCCTTCGTAAGT
		R: GCCAAACAGATTTCCAATCG
TLR4	NM_001030693	F: TGCACAGGACAGAACATCTCTGGA
		R: AGCTCCTGCAGGGTATTCAAGTGT
TLR5	CR353090	F: CTCACCTCTCTCTCAGGGTTTT
		R: TGGGTACACACAGTACCTGTCA
TLR7	AJ720504	F: CCTCGATCTCAACCCTACTTCT
		R: CAGTATCTTTTCCTCACCACACA
TLR15	NM_001037835	F:GTTCTCTCTCCCAGTTTTGTAAATAGC
		R: GTGGTTCATTGGTTGTTTTTAGGAC
TLR21	NM_001030558	F: AGAAGGTGTCGGAGGATGGTG
		R:GGGCTCCAAATGCTGACTGC
MD2	BX932484	F: TCCATCTGGCACGCTGCTGT
		R: GTCGTCGGTCCCGCTGCAAA
MyD88	NM_001030962	F: AAGTTGGGCCACGACTACCT
		R: CAGAAAGGGTTGTTAAGCACTG
TRIF	EF025853	F: TCAGCCATTCTCCGTCCTCTTC
		R: GGTCAGCAGAAGGATAAGGAAAGC
TIRAP	DQ019929	F: CTCATAGCACCACCAGCCACTC
		R: GGGTAATCCTTCCTGTCAATGTCC
IRAK4	AJ720408	F: AATTGCTTGGTTTCTCAAGTG
		R: GCAATTTCACACCTTGTGTTC
TRAF6	CK607050	F: AGTAAATACGAGTGCCCGATCT
		R: TTAGCGAAGTTGTCTGGAAAAA
TRAF3	BX935958	F: CCAGCTCTCAGCAGCAGGAGACA
		R: TCAGCACGAGGACACGGAAGC
IKKα	AJ720520	F: CTTTCATCTATGGCAACTCCTG
		R: ATGTCCAAACCAAGACGTGAT
IKKε	BU133261	F: GTGGACGTGGTGGCCGACTG
		R: GGCGGTTGTGTCCCCTCTGC
TAK1	CR524033	F: GGGCAAAGCAACTCGCCACT
		R: TGATGTGCCTGGCCGTATTTTTCA
NF-κB2	D16367	F: GGTCGACGATGGCTGTGCGG
		R: GAGGGTCGGTGTGCGTCACC
IRF7	U20338	F: AGACCAACTTCCGCTGCGCC
		R: GGCATCCCCTGTGTGTGCCC
p38	CR339030	F: TTGGTTCCACAACTCCAGCACAG
		R: CCGCATCCAGCACCAGCATGT

[a] *Forward primer.*

[b] *Reverse primer.*

used as the internal control. Dissociation curves were performed at the end of amplification for validating data quality. Each individual sample was run in triplicate and the average critical threshold cycle (Ct) was used for calculating relative quantification by fold change and statistical significance. Data analysis was conducted by two tailed, paired Student's *t*-test using Microsoft® Excel 2003 version (Microsoft Corporation, 2003). The $P < 0.05$ was considered significant.

RESULTS

GLOBAL TRANSCRIPTION OF TLR GENE EXPRESSION BETWEEN LINES A AND B

All TLR were expressed in non-infected heterophils from both lines of birds, but only TLR4 (Line A, 1.54-fold change) and TLR7 (line B, −0.64-fold change) were differentially expressed ($p < 0.05$; **Table 2**). Upon infection with SE, there were no significant differences in TLR expression in heterophils between lines A and B with one exception, TLR15 (**Table 2**) where *TLR* 15 was differentially expressed in the heterophils from line A following infection with SE when compared to heterophils from line B chickens.

REAL-TIME PCR ANALYSIS OF TLR GENE EXPRESSION BETWEEN LINES A AND B

The expressions of TLR on heterophils from each line were verified by qRT-PCR analysis (**Table 3**). When comparing the non-infected control heterophils between lines, only Line A heterophils showed differentially expressed TLRs, specifically TLR4 and, the chicken specific, TLR21. Upon infection with SE, both TLR4 and TLR21, as well as TLR15 were significantly up-regulated when compared to heterophils from line B chickens.

GLOBAL TRANSCRIPTION OF TLR SIGNALING PATHWAY GENES BETWEEN LINES A AND B

Using Agilent 44K microarray analysis, we determined the global transcriptome of the TLR signaling pathways between lines A and B before and after infection with SE (**Table 4**). After analyzing 16 conserved TLR pathway genes identified from the chicken genome, we found only one gene in each line that was differentially expressed in the non-infected heterophils. Specifically, we found that *RIP1* was constitutively up-regulated in non-infected line B heterophils; whereas interferon regulatory factor 7 (IRF7) was the only gene up-regulated in non-infected heterophils from line A chickens. However, upon infection with SE, we found a significant up-regulation in six other TLR signaling pathway genes in the heterophils from line A chickens: *MD-2*, *TIRAP*, *IKK*ε, *NF-κB2*, *p38 MAPK 11*, and *p38 MAPK 12* in addition to *IRF7* when compared to line B heterophils ($p < 0.01$). Only the *RIP1* was found to be significantly up-regulated in the line B heterophils when compared to line A heterophils ($p < 0.05$).

REAL-TIME PCR ANALYSIS OF TLR SIGNALING PATHWAY GENES BETWEEN LINES A AND B

Regulation of the TLR signaling pathway genes were verified by qRT-PCR analysis (**Table 5**). As observed in the microarray analysis, only the *RIP1* gene was constitutively up-regulated in line B heterophils. Similarly, as seen in the microarray analysis, *IRF7* was constitutively up-regulated in heterophils from line A. However, the qRT-PCR data showed that NF-κB2 was constitutively up-regulated in line A heterophils. As observed in the microarray, upon infection with SE, only the *RIP1* was found to be significantly up-regulated in the line B heterophils when compared to line A heterophils. Likewise, *MD-2*, *TIRAP*, *IKK*ε, *NF-κB2*, *p38 MAPK 11*, *p38 MAPK 12*, *IRF7* were up-regulated in heterophils from line A when compared to line B heterophils. More interestingly, using qRT-PCR analysis, we found five more TLR pathway genes that were up-regulated in line A heterophils (*IRAK4*, *TRAF3*, *TAK1*, *IKK*α, and *PI-3K*) when compared to line B heterophils.

DISCUSSION

There are 10 known avian TLRs (TLR1b, 1b, 2a, 2b, 3, 4, 5, 7, 15, and 21; reviewed in (Brownlie and Allan, 2011). We have shown previously that all 10 TLRs are found on chicken heterophils and can be functionally activated *in vitro* with either TLR agonists or intact bacterial cells (Farnell et al., 2003a,b; He and Kogut, 2003; He et al., 2003; Kogut et al., 2005, 2006, 2008; Nerren et al., 2009, 2010). The results from the present microarray and

Table 2 | Fold change of Toll-like receptor genes between heterophils isolated from line A and B chickens using microarray analysis.

Gene name	Accession no.	AC/BC Fold change	AI/BI Fold change	Description
TLR1 type 1	AJ20806	NS	NS	Toll-like receptor 1 type 1
TLR1 type 2	BU405042	NS	NS	Toll-like receptor 1 type 2
TLR2 type 1	AB050005	NS	NS	Toll-like receptor 2 type 1
TLR2 type 2	AB046533	NS	NS	Toll-like receptor 2 type 2
TLR3	CR407213	NS	NS	Toll-like receptor 3
TLR4	NM_001030693	1.54 ± 0.04	NS	Toll-like receptor 4
TLR5	CR353090	NS	NS	Toll-like receptor 5
TLR7	AJ720504	-0.64 ± 0.01	NS	Toll-like receptor 7
TLR15	BU265392	NS	1.62 ± 0.06	Toll-like receptor 15
TLR21	DQ198090	NS	NS	Toll-like receptor 21

Positive values mean genes have a higher expression in line A.

Negative values mean genes have a higher expression in line B.

NS, no significance in gene expression between line A and line B ($p > 0.05$).

qRT-PCR analysis confirm that the mRNA for all of the known avian TLRs were constitutively expressed in both genetic lines of chickens with only TLR4 (line A) and TLR7 (line B) differentially up-regulated in the non-infected heterophils between the two lines. However, upon infection with SE, three TLR mRNA were significantly up-regulated in heterophils from line A: TLR4, TLR15, and TLR21. These results are noteworthy for two reasons: (a) this is the first report of an up-regulation of TLR21 in chicken heterophils exposed to *Salmonella* and (b) these results are the first to illustrate a striking difference in *Salmonella* recognition by TLRs in heterophils between these genetically distinct parental broiler lines of chickens. These results establish a genetic paradigm for a coordinated TLR response mechanism of the avian heterophil against *Salmonella*. *Salmonella* has at least four TLR ligands: lipopolysaccharide (LPS), lipoproteins, flagellin, and CpG DNA, which activate TLR4, TLR2, TLR5, and TLR21, respectively. Recognition of *Salmonella* lipoproteins and flagellin is apparently similar between the two lines of chickens since we found no significant differences in mRNA expression for TLR2 and TLR5 between line A and B. The agonist(s) for TLR15 are still unknown at this time, but in an earlier series of experiments, we have shown that intact heat-killed Gram-negative or Gram-positive bacteria, but not known TLR agonists induced a significant increase in TLR15 mRNA expression in heterophils (Nerren et al., 2010). Clearly, recognition of the ligand(s) by TLR15 is critical for inducing downstream signaling against *Salmonella* infection in line A chickens. Shaughnessy et al. (2009) found a transient increase in TLR21 mRNA expression in the cecum of *Salmonella*-infected chickens 6 h, but not 20–48 h after infection. Interestingly, just the opposite was found in TLR15 mRNA expression where a transient decrease was found early (6 h) but a significant increase was measured by 48 h post-infection with *Salmonella*.

We also found an up-regulation in expression of the myeloid differentiation (MD)-2 gene that codes for a protein essential for regulating LPS signaling through TLR4 (Dobrovolskaia and Vogel, 2002). MD-2 binds on TLR4 and then the TLR4-MD-2 complex moves to the cell surface. LPS binds MD-2 triggering changes in MD-2 conformation that are detected by TLR4. Engagement of TLR4 activates intracellular signaling *via* the adapter MyD88 (O'Neill, 2006). These results provide further proof of the role of TLR4 in avian heterophil recognition of SE.

Intact bacteria are capable of activating multiple TLR since they typically express a variety of MAMPs on a given cell. Thus, as observed from the present results, multiple TLRs are engaged by the heterophils from both lines of chickens. However, the integrated balance of TLR4, TLR15, and TLR21 to recognize and activate intracellular signaling events in heterophils from line A presumably dictate the resistant phenotype that we have previously

Table 3 | Fold change of Toll-like receptor genes between heterophils isolated from line A and B chickens using qRT-PCR analysis.

Gene name	Accession no.	AC/BC Fold change	AI/BI Fold change
TLR1 type 1	AJ20806	NS	NS
TLR1 type 2	BU405042	NS	NS
TLR2 type 1	AB050005	NS	NS
TLR2 type 2	AB046533	NS	NS
TLR3	CR407213	NS	NS
TLR4	NM_001030693	2.09 ± 0.08	1.28 ± 0.03
TLR5	CR353090	NS	NS
TLR7	AJ720504	NS	NS
TLR15	BU265392	NS	1.98
TLR21	DQ198090	1.72 ± 0.11	1.54 ± 0.07

Positive values mean genes have a higher expression in line A.
Negative values mean genes have a higher expression in line B.
NS, no significance in gene expression between line A and line B ($p > 0.05$).

Table 4 | Fold change of Toll-like receptor pathway genes between heterophils isolated from line A and B chickens using microarray analysis.

Gene name *gene description*	Accession no.	AC/BC Fold change	AI/BI Fold change
MD-2	BX932484	NS	1.54 ± 0.04
MyD88	AJ851640	NS	NS
TIRAP *Toll/interleukin 1 receptor (TIR) domain-containing adaptor protein*	BX933959	NS	1.31 ± 0.07
IRAK4	AJ720408	NS	NS
TRAF3 T*umor necrosis factor-receptor associated factor 3*	BX935958	NS	NS
TAK1 *TGF-β-activating kinase 1*	CR524033	NS	NS
RIP1 *Receptor-interacting protein-1 serine/threonine kinase*	AB108485	-0.68 ± 0.03	-0.72 ± 0.04
IKKε	BU133261	NS	2.52 ± 0.12
IKKα	M74544	NS	NS
NF-κB1	BU479586	NS	NS
NF-κB2	D16367	NS	1.30 ± 0.10
PI-3K	AJ720776	NS	NS
p38 (MAPK 12)	CR339030	NS	1.71 ± 0.12
IRF7 *interferon regulatory factor 7*	U20338	2.40 ± 0.11	2.03 ± 0.08

Positive values mean genes have a higher expression in line A.
Negative values mean genes have a higher expression in line B.
NS, no significance in gene expression between line A and line B ($p > 0.05$).

Table 5 | Fold change of Toll-like receptor pathway genes between heterophils isolated from line A and B chickens using qRT-PCR analysis.

Gene name	Accession no.	AC/BC Fold change	AI/BI Fold change
MD-2	BX932484	NS	4.41 ± 0.15
MyD88	AJ851640	NS	NS
TIRAP	BX933959	NS	5.37 ± 0.24
IRAK4	AJ720408	NS	4.88 ± 0.21
TRAF3	BX935958	NS	3.68 ± 0.30
TAK1	CR524033	NS	2.65 ± 0.22
RIP1	AB108485	-1.94 ± 0.44	-2.77 ± 0.27
IKKε	BU133261	NS	3.47 ± 0.19
IKKα	M74544	NS	7.26 ± 0.31
NF-κB1	BU479586	NS	NS
NF-κB2	D16367	1.26 ± 0.08	5.91 ± 0.41
PI-3K	AJ720776	NS	4.43 ± 0.28
p38 (MAPK 12)	CR339030	NS	7.62 ± 0.48
IRF7	U20338	3.79 ± 0.22	7.51 ± 0.35

Positive values mean genes have a higher expression in line A.
Negative values mean genes have a higher expression in line B.
NS, no significance in gene expression between line A and line B ($p > 0.05$).

observed (Ferro et al., 2004; Swaggerty et al., 2005a). The results from these experiments support complementary roles by recognizing different MAMPs on SE that induce redundant, but synergistic effector mechanisms previously noted for heterophils from line A chickens (Swaggerty et al., 2004, 2005b; Kogut et al., 2006). These redundant mechanisms for microbial recognition and activation of these heterophil-mediated responses serve to provide the resistant phenotype of line A against diverse pathogen challenges (Ferro et al., 2004; Swaggerty et al., 2005a,b, 2011; Li et al., 2008).

Activated TLRs signaling initiates with the recruitment of TIR-domain-containing adaptor molecules (MyD88, TRIF, TIRAP, IRAK) which act as important messengers to activate downstream kinases (IKK complex, MAPKs, TBK1) and transcription factors (NF-κB, AP-1, IRF3, IFR7), which produce effecter molecules including cytokines, chemokines, inflammatory enzymes such as iNOS and oxidase, and type I interferons (Kawai and Akira, 2010). Overall, there are two types of TLR signaling pathways: MyD88-dependent and TRIF-dependent (Beutler, 2009; Kawai and Akira, 2010). MyD88 signaling has generally been linked to NF-κB and MAPK signaling, whereas TRIF-dependent pathway (MyD88-independent pathway) not only mediates pro-inflammatory cytokine production, but also mediates type I interferon production. Studies have also shown crosstalk and overlap between these two pathways depending on the cell type involved (Beutler, 2009). In mammals, all TLRs except TLR3 utilize MyD88-dependent signaling, whereas TLR4 and TLR3 utilize TRIF-dependent signaling. TLR4 is unique in that it utilizes both MyD88- and TRIF-dependent pathways. The MyD88-dependent pathway requires both MyD88 and TIRAP to activate NF-κB, whereas, TRIF-dependent signaling are controlled by TRIF and TRAM. Whether the absence of TRAM on the chicken genome (Brownlie and Allan, 2011) has an effect on the control of the TRIF-dependent pathway is unknown, but results from the present experiments suggest that both the MyD88-dependent and TRIF-dependent pathways are activated in the heterophils from line A during the interaction with SE. Consequently, the absence of TRAM from the chicken genome does not appear to be detrimental.

Previously, we demonstrated a large-scale gene expression profiling on heterophils isolated from broilers with different genetic backgrounds (*Salmonella*-resistant line A and -susceptible line B). Many immune-related genes showed significantly differential expression following SE stimulation, which includes genes involved in the TLR pathway (Chiang et al., 2008). In addition, global analysis data suggested a similar TLR regulatory network might exist in both lines where a possible MyD88-independent pathway may participate in the regulation of host innate immunity (Chiang et al., 2008). Therefore, for the present studies, using the mammalian TLR pathway as a reference, an inferred chicken TLR pathway consisting of 72 chicken genes was constructed to compare gene expression between SE-infected to non-infected heterophils from each line. Of these 72 TLR reference genes, we found virtually no significantly differentially expressed genes between lines in the non-infected heterophils. However, upon infection with SE, we found 11 of the TLR reference genes that were significantly up-regulated in heterophils from line A when compared to line B (*MD-2*, *TIRAP*, *IRAK4*, *TRAF3*, *TAK1*, *IKKε*, *IKKα*, *NF-κB2*, *PI-3K*, *p38*, and *IRF7*). It is evident from the data in these studies that heterophil response from line A birds to SE involves the coordination of genes from *all of the components* of the intracellular TLR signaling pathway: receptors (*TLR4*, *TLR15*, *TLR21*), adaptors (*TRAF3*, *TIRAP*, *IRAK4*), kinases (*Iκκα*, *Iκκε*, *TAK1*, *p38*), transcription factors (*NF-κB2*, *IRF7*), and effector molecules (*IL-6*, *IL-12A*, *CCL4*, *CCL5*, *IFN-α*). Furthermore, it is evident that SE infection of the line A heterophils induce the activation of both a MyD88-dependent and a TRIF-dependent TLR signaling pathways (**Figure 1**).

Tumor necrosis factor-receptor associated factor 3 is an important adaptor that transmits upstream activation signals to protein kinases that phosphorylate transcription factors to induce the production of type I IFNs. TRAF3 plays roles in both TLR-dependent and TLR-independent signaling pathways involved in type I IFN production. TIRAP plays a crucial role in MyD88-dependent signal transduction by TLR2 and TLR4, acting as a bridging adaptor to recruit MyD88 (Jenkins and Mansell, 2010). TRAF3 is recruited to TLR adapters, MyD88 and TRIF, and associates with IRF3/7 kinases, TBK1 and IKK-ε, and IRAK1 when these proteins are over expressed in HEK-293T transformed epithelial cells (Oganesyan et al., 2006). Thus, during TLR signaling, TRAF3 serves as a cytoplasmic adapter and transmit upstream signals to downstream kinases involved in type I IFN production. IRAK4 has an essential role in TLR-mediated signaling by associating with MyD88 and induces IRAK1 phosphorylation, recruitment of TRAF6, and engagement of TAK1 (Li, 2008; O'Neill, 2008), which activates MAPKs and transcription factors NF-κB and AP-1, resulting in transcription of genes encoding inflammatory mediators. The kinase activity of IRAK4 plays a critical role in TLR-mediated immune responses. Inactivation of IRAK4 kinase activity leads to (a) reduced mRNA stability and diminished production of cytokines and chemokines in response to LPS stimulation and

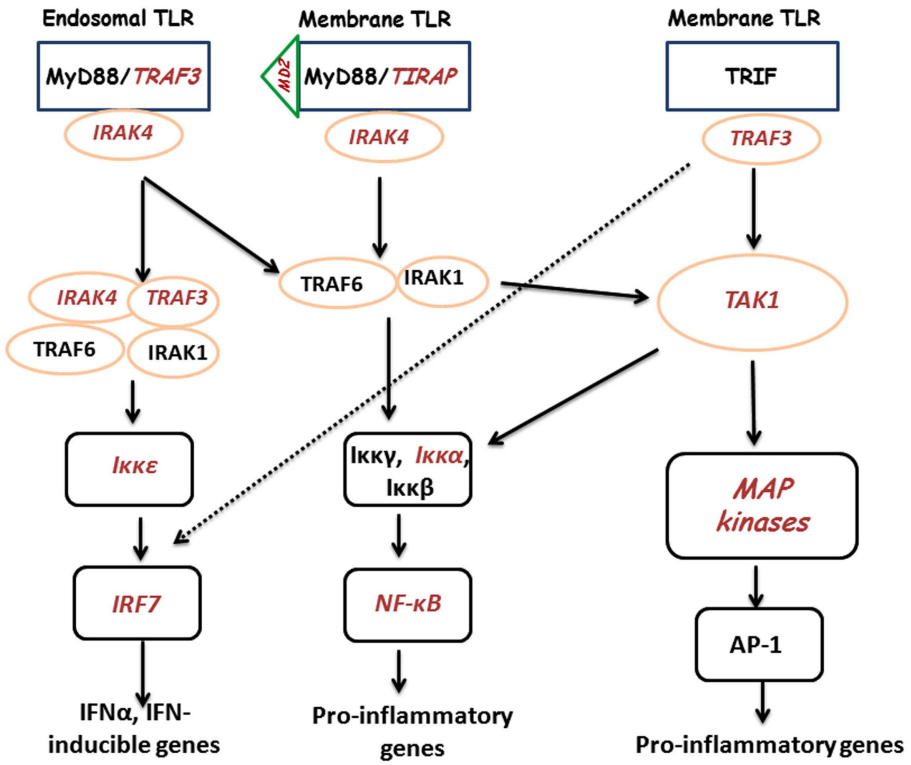

FIGURE 1 | Cartoon of TLR signaling pathways in heterophils from line A chickens following interaction with SE. TLRs send signal through TIRAP and MyD88 or TRIF to IRAK/TRAF6 to activate downstream kinases. The signals are delivered to kinases including MAPKs, IKKs, and TBK1, to activate transcription factors, NF-κB, AP-1, and IRF. Transcription factors bind to specific DNA sequences and produce effecter molecules such as cytokines, inflammatory enzymes, chemokines, and type I interferons (IFNs). Components in red italics were significantly up-regulated.

(b) both TLR7- and TLR9-mediated cytokine and type I IFN production was abolished IRAK4 kinase-inactive knock-in mice (Kim et al., 2007). TAK1 is activated by phosphorylation via TLR2 and/or four-mediated pathway, whose kinase activity is required for NF-κB activation. TAK1 regulates NF-κB-inducing kinase activity that activates IKKα/β downstream of MyD88 and TRAF6. TAK1 is also a MAP kinase kinase kinase for p38 that is critical for the production of pro-inflammatory cytokines (Irie et al., 2000).

Stimulation of TLRs results in the downstream activation of the cytoplasmic Toll/IL-1 receptor (TIR) domain portion of the TLR, which then recruits MyD88/IRAK/TRAF6 and activates the MAPK superfamily cascade (Dalpke and Heeg, 2002; O'Neill, 2002; Akira, 2003) and the transcription factors, NF-κB and AP-1 that leads to the expression of genes that participate in the innate immune response including pro-inflammatory cytokines. The MAPK superfamily of serine/threonine kinases consists of at least three distinct families: p38, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) that play a major role in cellular activation of a variety of cell types. In mammalian cells, the phosphorylation of the MAPK superfamily has been established as the hallmark of cellular activation following TLR engagement (O'Neill, 2002). We have found that heterophils, when stimulated with specific TLR agonists, activate the p38 and ERK1/2 MAPK signaling cascades leading to the up-regulation of pro-inflammatory cytokine gene expression (Kogut et al., 2005, 2008) and increased responsiveness of line A heterophils were mediated, by an increased ability to the p38 MAPK pathway and specific transcription factors, all of which directly affect the innate immune response (Swaggerty et al., 2011). These results were all confirmed by the present experiments where the gene for *p38* was differentially up-regulated in heterophils from line A heterophils infected with SE when compared to line B heterophils.

RIP1 is an adaptor serine/threonine kinase associated with the signaling complex of death receptors (DRs) including Fas, TNFR1, and TRAIL-Rs which can initiate apoptosis. In addition, RIP1 can bind to TRAF 2 (Hsu et al., 1996) and help recruit IKK (Li et al., 1999; Zhang et al., 2000). RIP1 was found to be significantly down-regulated in line A heterophils when compared to line B heterophils. These results suggest that line A heterophils do not use apoptosis as a immune mechanism to remove *Salmonella* nor do they require RIP1 for NF-κB activation.

IκB kinase and IKK-related kinases play critical roles in regulating the immune response through NF-κB and IFN regulatory factor-dependent signaling transduction cascades. In response to pro-inflammatory stimuli, such as TNFα, IL-1, and TLR agonists (such as LPS), two kinases, TAK1 and mitogen-activated protein/ERK kinase kinase 3, are recruited into the proximity

of the IKK complex by interacting with several receptor associated proteins, thereby phosphorylating and activating both IKKα and IKKβ in the cytoplasm. A major consequence of IKKα/IKKβ activation is the initiation of NF-κB-mediated transcriptional activation (Fitzgerald et al., 2003; Hacker and Karin, 2006). Unlike IKKα and IKKβ, which are major catalysts of the NF-κB pathway, IKKε, and TBK1 have restricted functions in the NF-κB activation pathway. They are activated by TLR agonists and viral ssRNA in the cytoplasm and mainly function as mediators of type I IFN gene expression, which contributes to the antiviral response by their activation of the IRF3 and IRF7, which are transcriptional factors with diverse roles in immunity and cellular response to viral infections. Thus, IKKε and TBK1 are important mediators of antiviral response and, together with IKKα and IKKβ, coordinate and organize the host immune defense (Fitzgerald et al., 2003; Hacker and Karin, 2006). IKKα is the other catalytic kinase of the classic IKK complex (along with IKKβ). In contrast to IKKβ's effect on IκB phosphorylation in the canonical pathway, IKKα might have a crucial function to facilitate NF-κB-dependent gene transcription instead of IκB phosphorylation due to its lower ability to induce IκB phosphorylation. IKKε is the other non-canonical IKK involved in regulating the activation of the IRF3 and NF-κB signaling pathways (Fitzgerald et al., 2003). Upon activation in response to TLR agonists and viral infection, IKKε phosphorylates IRF3 and IRF7 and triggers IRF3/IRF7 nuclear translocation, which results in the up-regulation of type I IFN expressions.

Increasing evidence supports the involvement of the phosphatidylinositol-3 kinase (PI-3K) pathway in the regulation of activation of IRFs by TLRs. The PI-3K pathway can be activated by various TLR ligands and can negatively or positively regulate TLR responses, depending on cell types and the ligands. Both TLR9 and -3 activate the PI-3K/Akt/mTOR pathway leading to the activation of IRF7, -3, and -5 and mTOR kinase activity is required for the interaction between MyD88 and IRF7 (Fukeo and Kayasu, 2003; Cao et al., 2008; Schmitz et al., 2008; Ning et al., 2011). PI-3K is critical for the nuclear translocation of IRF7 and type I IFN production in response to TLR7/9 activation, and mTOR kinase activity is required for the interaction between MyD88 and IRF7 (Schmitz et al., 2008; Ning et al., 2011).

Nuclear factor-κB transcriptional factors are central regulators and transcriptional factors in response to pathogens and viruses. NF-κB transcription factors are important in the regulation of immune and inflammatory responses (Karin and Ben-Neriah, 2000). NF-κB is composed of dimeric complexes of members of the Rel/NF-κB family of polypeptides. This family comprises Rel-A, c-Rel, Rel-B, NF-B1/p50, and NF-B2/p52. NF-κB dimers in non-stimulated cells interact with one of a family of cytoplasmic inhibitory proteins (IκBs) which prevent nuclear entry. This family includes $I\kappa B_{\alpha}$, IBβ and IBε together with the precursor forms of NF-κB1 (p105) and NF-κB2 (p100). NF-κB1 p105 and NF-κB2 p100 are proteolytically processed by the proteasome to produce p50 and p52, respectively. Following agonist stimulation, IκBα, IκBβ, and IκBε and p105 are phosphorylated by the IKK complex, triggering their ubiquitylation and degradation by the proteasome. Associated NF-κB dimers are then released to translocate into the nucleus and modulate gene expression. Proteolysis of NF-κB2 p100 is regulated by the IKKα subunit of the IKK complex, which triggers processing to generate p52 which can then undergo nuclear translocation (Karin and Ben-Neriah, 2000). IRF7 is the master regulator of type I IFNs against pathogenic infections, which activate IRF7 by triggering signaling cascades from PRRs that recognize pathogenic nucleic acids. Activation of IRF7 is a prerequisite for its functions as a transcription factor. Inactive IRF7 resides in the cytoplasm as a latent form when pathogenic infection triggers IRF7 phosphorylation and translocation into the nucleus, where with other co-activators it forms a transcriptional complex that binds to the promoter regions of target genes to activate transcription. Core signaling components involved in signaling cascades leading to activation of IRFs include the adaptors TRIF and MyD88, IRAK1/4, TRAF6, and TRAF3, and the IKKs, IKKε, and TBK1, depending on the activating TLR. IRF7 is activated by pathogenic nuclei acids through pathways mediated by TLR3, -7, and -9 (Ning et al., 2011).

Our results indicate that higher expression of a combination of TLRs and a complicated regulation of downstream adaptors, kinases, and transcription factors are involved in a stronger induction of heterophil-mediated innate immune response; thus, is more beneficial to the resistant line. These findings lay the foundation for future studies on the genetic selection for the regulatory gene network in chicken TLR pathways and immune modulation of SE infection in chickens. Furthermore, the basic TLR signaling pathways regulating innate immunity are central to many infections in poultry. Based on the present and past studies we have conducted to profile the immune gene expression of lines A and B to *Campylobacter* (Li et al., 2010, 2011, 2012), perhaps future studies can be directed toward genotype-specific strategies to control such infections affecting the poultry industry.

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DNA methylation fluctuation induced by virus infection differs between MD-resistant and -susceptible chickens

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Marek's disease (MD) is a lymphoproliferative disease induced by Marek's disease virus (MDV) infection. To augment vaccination measures in MD control, host genetic resistant to MD becomes obviously more and more important. To elucidate the mechanism of MD-resistance, most of researches were focused on the genetic differences between resistant and susceptible chickens. However, epigenetic features between MD resistant and susceptible chickens are poorly characterized. Using bisulfite pyrosequencing method, we found some candidate genes have higher promoter methylation in the MD-susceptible ($L7_2$) chickens than in the MD-resistant ($L6_3$) chickens. The hypermethylated genes, involved in cellular component organization, responding to stimulus, cell adhesion, and immune system process, may play important role in susceptibility to disease by deregulation of these genes. MDV infection induced the expression changes of all three methyltransferases genes (*DNMT1*, *DNMT3a*, and *DNMT3b*) in both lines of chickens. The *DNMT1* was up-regulated in $L7_2$, whereas the *DNMT3b* was down-regulated in $L6_3$ at 21 dpi. Interestingly, a dynamic change of promoter methylation was observed during MDV life cycle. Some genes, including *HDAC9*, *GH*, *STAT1*, *CIITA*, *FABP3*, *LATS2*, and *H2Ac*, showed differential methylation behaviors between the two lines of chickens. In summary, the findings from this study suggested that DNA methylation heterogeneity and MDV infection induced methylation alterations differences existed between the two lines of chickens. Therefore, it is suggested that epigenetic mechanisms may be involved in modulating the resistance and/or susceptibility to MD in chickens.

Keywords: chicken, Marek's disease, MD-resistance, MD-susceptibility, DNA methylation

INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by Marek's disease virus (MDV) with pathological features including mononuclear cell-infiltration in the peripheral nerves, skin, and muscle (Davison and Nair, 2004). MDV is classified into the *Mardivirus* genus due to its genome content (Davison, 2002) and biological effect on lymphocytes like EBV (Epstein, 2001). MDV life cycle in its host can be divided into four phases, including an early cytolytic phase from 2 to 7 days post infection (dpi), a latency phase around 7–10 dpi, a late cytolytic phase starting from 18 dpi and a proliferation phase after 28 dpi (Calnek, 1986, 2001). Although MD is controlled by vaccination, the virulence of MDV has being evolved over time and resulted in more severe brain edema and acute deaths even after vaccination (Witter, 1997; Osterrieder et al., 2006). MD remains a problem in the poultry industry worldwide (Churchill et al., 1969). Since the inheritance and resistance to MD was first observed (Asmundson and Biely, 1932), MD-resistant and -susceptible chickens have been bred by those including Stone (lines 6 and 7; Bacon et al., 2000), Hutt, and Cole (lines N and P; Davison and Nair, 2004). Nowadays, the selection of genetically disease resistant chickens is especially important in MD control. A better understanding in the mechanisms of MD-resistance and -susceptibility should be of great value in developing better strategies to further prevent and control MD.

In recent years, most of the studies are focused on genetic variations between MD-resistant and susceptible chickens (Gilmour et al., 1976; Fredericksen et al., 1977; Kaiser et al., 2003; Sarson et al., 2008a). However, little is done on epigenetic differences between the two kinds of chickens. Epigenetics is the study of alterations in phenotypes that are not brought about by changes in DNA sequences, but by factors including DNA methylation, histone modifications, and so on (Allis et al., 2006). DNA methylation is known as a post-replication modification found on the 5-C position of cytosine mainly in CpG dinucleotides, generated and maintained by three methyltransferases – DNMT1, DNMT3a, and DNMT3b (Allis et al., 2006). In mammals, DNA methylation was found playing important role in development, imprinting, carcinogenesis, and other diseases (Feinberg and Tycko, 2004; Feng et al., 2010). Notably, we found two DNA mutations in *DNMT3b* (Yu et al., 2008a) and a higher promoter methylation level of *ALVE* and *TVB* in the spleen of MD-susceptible chickens ($L7_2$) compared to that of MD-resistant chickens ($L6_3$; Yu et al., 2008b), and the methylation level in *CD4* promoter region was

down-regulated in the former but not in the later at 21 dpi (Luo et al., 2011).

To advance the understanding of functional patterns of DNA methylation in disease resistance or susceptibility, we extended the scope of examination to 18 interested genes, which include *STAT1*, *CIITA*, *NK-lysin*, *CD44*, *IL12*, and *GH1* that the expression levels of these gene are alterable upon MDV challenge (Liu et al., 2001; Abdul-Careem et al., 2006; Parcells and Burgess, 2008; Sarson et al., 2008a,b; Heidari et al., 2010; Thanthrige-Don et al., 2010). Some of the 18 genes were also chosen based on our previous temporal microarray data, which include *FABP3*, *HDAC9*, *IL28RA*, *MON2*, and *THBS2* (Luo et al., 2011; Yu et al., 2011).

MATERIALS AND METHODS

ANIMALS, CHALLENGE TRIAL, AND SAMPLE COLLECTION

Specific pathogen free chickens from two highly inbred White Leghorn lines, the $L6_3$ and $L7_2$, were used. Chickens from each of the lines were divided into two groups. One group was challenged with a very virulent plus MDV (vv + MDV), 648A passage 40, intra-abdominally at day 5 post hatch at a 500 plaque-forming unit (PFU) dosage, the other was not challenged and was assigned as the control group. Fresh spleen samples were respectively collected at 5, 10, and 21 dpi from both groups, and placed in RNAlater (Qiagen, Valencia, CA, USA) immediately, and then stored at −80°C.

All of the experimental chickens were challenged and maintained in a BSL-2 facility at the Avian Disease and Oncology Laboratory (ADOL), East Lansing, Michigan. The chickens were handled closely following animal usage procedures established by the ADOL ACUC committee.

DNA EXTRACTION, BISULFITE TREATMENT, AND PYROSEQUENCING

DNA was extracted from 20 ∼ 30 mg spleen by NucleoSpin® Tissue Kits (Macherey-Nagel, Bethlehem, PA, USA). Bisulfite treatment of 1 μg DNA per chicken was performed using EZ DNA Methylation-Gold Kit™(ZYMO Research, Irvine, CA, USA). Primers for PCR and pyrosequencing were designed with PSQ Assay Design software (Biotage, Charlotte, NC, USA; **Table A1** in Appendix). For cost saving purposes, a universal primer (5′-GGGACACCGCTGATCGTTTA-3′) was used in the PCR assays (Yu et al., 2008a). PCR was carried out using Hotstar Taq DNA polymerase (Qiagen, Valencia, CA, USA) in 20 μl reactions in iCycler (Bio-Rad, Hercules, CA, USA) Detection System as follows: samples were denatured at 95°C for 15 min, followed by 50 cycles at 95°C for 30 s, 55–60°C for 30 s, 72°C for 30 s, and then extended at 72°C for 10 min. DNA methylation level analysis was performed on the Pyro Q-CpG system (PyroMark ID, Biotage, Charlotte, NC, USA) as previously described (Colella et al., 2003; Yu et al., 2008a).

RNA EXTRACTION AND QUANTITATIVE REAL-TIME RT-PCR

RNA from 30 ∼ 50 mg spleen was extracted using the RNeasy Mini Kit (Qiagen, USA). Reverse transcription was carried out in 20 μl with 1 μg of total RNA by using SuperScriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT)12–18 primers (Invitrogen, Carlsbad, CA, USA). Primers (**Table A2** in Appendix) for quantitative real-time RT-PCR were designed using Primer3 online primer designer system[1]. Quantitative real-time RT-PCR was performed on the iCycler iQ PCR system (Bio-Rad, USA) in a final volume of 20 μl using QuantiTect SYBR Green PCR Kit (Qiagen) with following procedures: denatured at 95°C for 15 min, followed by 40 cycles at 95°C for 30 s, 55–60°C for 30 s, 72°C for 30 s, then extended at 72°C for 10 min. Each reaction was replicated. The housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used to normalize the loading amount of cDNA.

FUNCTIONAL ANALYSIS AND STATISTICS

The GO Biological Process analysis of the genes was analyzed by PANTHER[2]. Student's *t*-test was used to analyze the differences of the promoter methylation level and the gene expression before and after MDV infection.

RESULTS

DIFFERENTIAL METHYLATION PATTERNS BETWEEN THE $L6_3$ AND $L7_2$

To determine the different methylation levels of genes between the MD-resistant $L6_3$ and the MD-susceptible $L7_2$ chickens, we analyzed the DNA methylation status of promoters for 18 genes by bisulfite pyrosequencing method. The results showed that most of the 18 genes, including *LATS2*, *MON2*, *IL28RA*, *STAT1*, *CD44*, *H2Ac*, *TNFSF10*, *IL12*, *FABP3*, and *CIITA*, were hypomethylated (methylation level <40%); few of them, *ITGB5*, *THBS2*, and *HDAC9*, had intermediate methylation level (between 40% and 60%), and the rest (*IGF2*, *GH1*, *NK-lysin*, and *TGFβ3*) had hypermethylation methylation level (>60%) in the control groups of both lines (**Table A3** in Appendix). However, some of the CpGs of *CD82* had a very low methylation level (<10%) and others had an intermediate methylation level (**Table A3** in Appendix).

Differential promoter methylation levels were observed for *ITGB5*, *THBS2*, *HDAC9*, *IL12*, *CD44*, *H2AC*, and *TNFSF10* between the $L6_3$ and $L7_2$, As showed in **Figure 1**, the methylation levels in all the tested CpG sites of the *ITGB5*, *THBS2*, *HDAC9*, *IL12*, *H2AC* were significantly higher in $L7_2$ than in $L6_3$ ($P < 0.05$; **Figures 1A–E**). However, some of the CpG sites in *CD44* (CpG 2 and 4) and *TNFSF10* (CpG 5) had higher level of methylation ($P < 0.05$), while some others (*CD44* CpG 3; *TNFSF10* CpG 1 and 3) had lower methylation levels in $L6_3$ than $L7_2$ ($P < 0.05$; **Figures 1F,G**).

To test if the differential promoter methylation levels of these genes are related with gene expression, we randomly chose two genes, *ITGB5* and *H2Ac*, and did quantitative RT-PCR. We found that the expression levels of the two genes, whose promoter methylation is higher in $L7_2$ chicken, is lower in these chickens (**Figure 2**).

Functional analysis of the genes (**Figure 3**) showed that, in comparison to the whole gene set we examined in this experiment, genes with lower methylation levels in $L6_3$ are mainly enriched in cellular component organization, response to stimulus, cell adhesion, and immune system process. In contrast, an under-enrichment of these genes was shown in cell communication, transport, system process, reproduction, and

[1]http://frodo.wi.mit.edu/
[2]http://www.pantherdb.org/

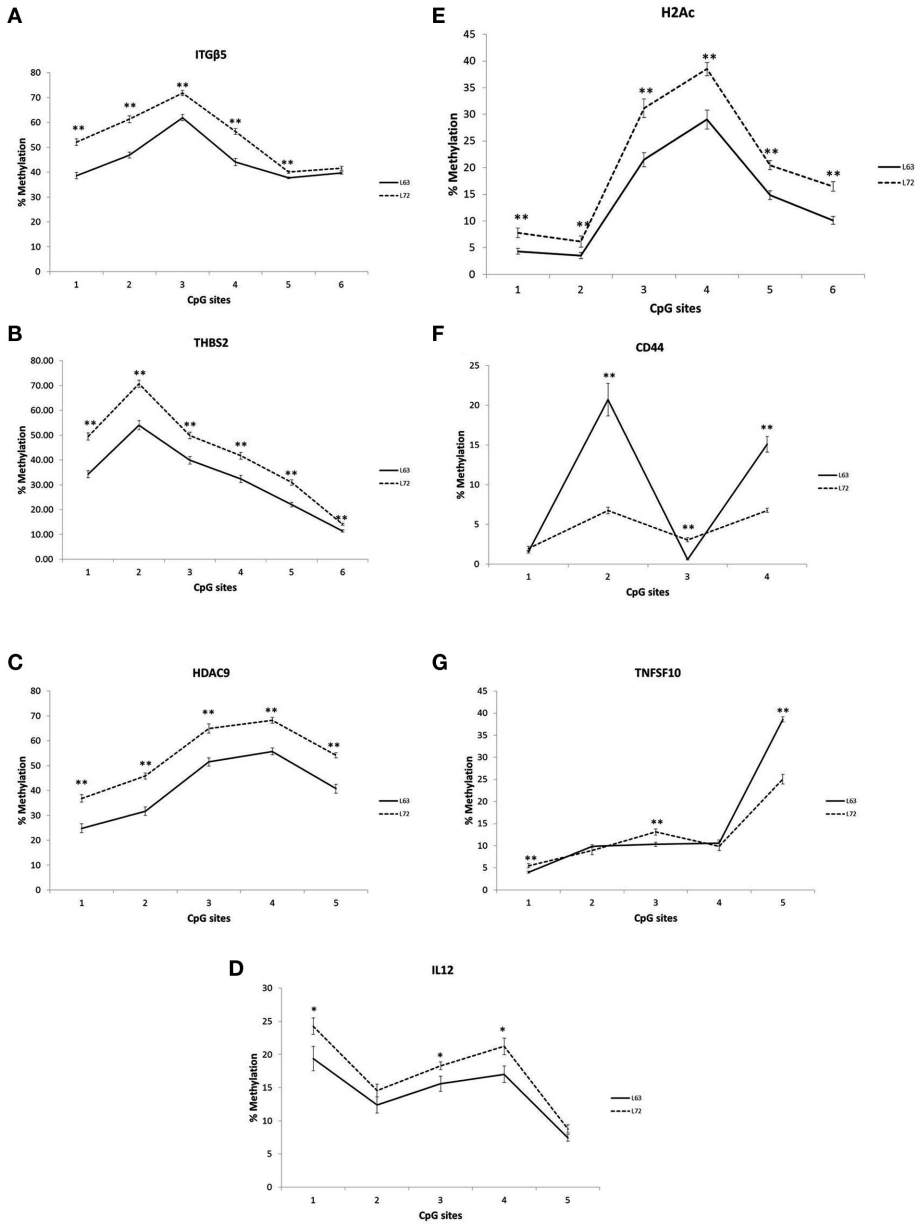

FIGURE 1 | Differentiated promoter methylation levels between the $L6_3$ and $L7_2$ chickens observed in *ITG*β5 (A), *THBS2* (B), *HDAC9* (C), *IL12* (D), *H2Ac* (E), *CD44* (F), and *TNFSF10* (G). The promoter methylation levels of the genes in spleen were examined by pyrosequencing. The absolute methylation level for each CpG site from pyrosequencing result was plotted. Solid line: $L6_3$; dashed line: $L7_2$. $^*P < 0.05$, $^{**}P < 0.01$. $N = 12$ for each group. Error bar = SEM.

developmental process. For genes with a varied methylation levels between $L6_3$ and $L7_2$, they are over-represented in functions of cell adhesion and immune system process. However, for the genes with similar methylation between the $L6_3$ and $L7_2$, no under or over-represented biological functions was identified.

DIFFERENTIAL DNMT1, DNMT3a, AND DNMT3b EXPRESSION INDUCED BY MDV CHALLENGE

To explore how MDV challenge induces DNA methylation alteration, we first checked if the expressions of the methylation agents, three methyltransferases (*DNMT1*, *DNMT3a*, and *DNMT3b*), were influenced over three time points (5, 10, and 21 dpi), which represent the early cytolytic, latent, and later cytolytic phase of the virus life cycle in the host cells, respectively. Interestingly, similar trends of expression changes were observed at 5 and 10 dpi for all three DNMTs in the MDV challenged chickens of both lines (**Figure 4**), while at 21 dpi, the changes were much more complicated. At 21 dpi, the *DNMT1* was significantly up-regulated in the infected $L7_2$ chickens compared to the $L7_2$ control group ($P < 0.05$). The *DNMT1* was remained

unchanged, however, between the infected and uninfected $L6_3$ groups ($P > 0.05$; **Figure 4A**). For *DNMT3a*, no expression difference was observed at 21 dpi between the infected and non-infected groups of both lines ($P > 0.05$; **Figure 4B**). However, the *DNMT3b* was significantly down-regulated in the infected group of $L6_3$ at 21 dpi ($P < 0.05$), but no differential expression was observed in $L7_2$ (**Figure 4C**). Overall, the expression levels of all the three DNMTs were significantly inducible by MDV infection, but with varied alteration trends and extents were found over different time points and between the infected and non-infected groups as well as between the chicken lines.

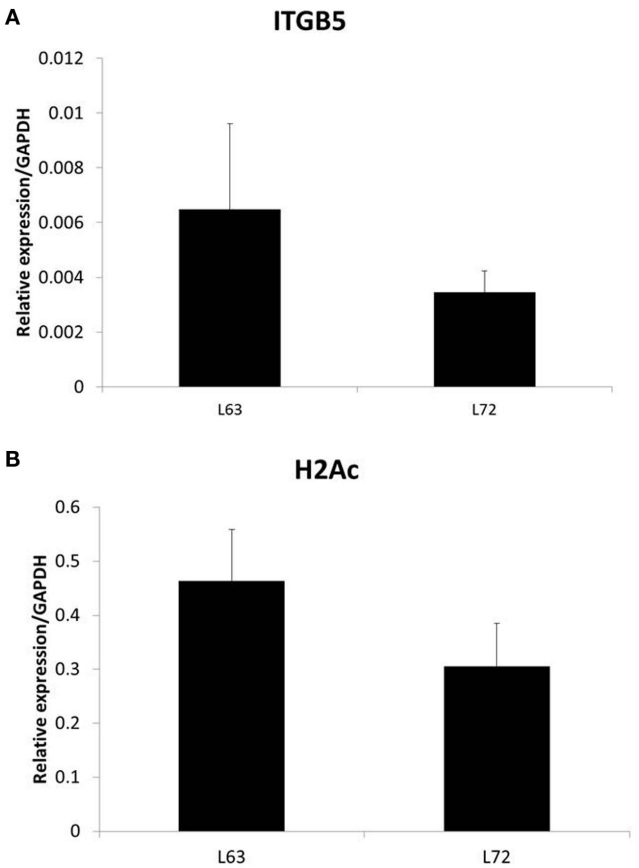

FIGURE 2 | Expression of *ITGB5* (A) and *H2Ac* (B) in non-infected chicken samples. $N = 4$ for each group. Error bar = STD.

ABERRANT METHYLATION LEVEL INDUCED BY MDV INFECTION

To further study DNA methylation dynamic response to MDV infection, we tested the promoter methylation of the 18 genes on 5, 10, and 21 dpi. Pairwise comparison was performed between the infected and non-infected age-matched sample groups of each chicken line for each of the CpG sites. Significant methylation level changes ($P < 0.05$) were detected at one or more CpG sites in all of the genes except *THBS2* gene after MDV challenge. The methylation level changes of the examined genes were under 30%. The MDV-induced DNA methylation changes for *CIITA*, *NK-lysin*, *FABP3*, and *ITGB5* were 10% above their unchallenged counterpart for each of the CpG sites. More than 10% methylation change was found in *HDAC9* at 5 dpi and 7–10% changes at 21 dpi in $L7_2$. Most of the genes (12/17) had significant methylation change ($P < 0.05$) at more than one time point (**Table A4** in Appendix; **Figure 5**), except for *IL12*, *TNFSF10*, and *ITGB5*, which were only changed at 5 dpi, and *CD44*, *LATS2*, *CIITA*, which were only changed at 21 dpi. In contrast between the two lines of chickens, more genes in $L6_3$ had significant methylation changes at 5 dpi, while more genes were observed with significant methylation changes in $L7_2$ at 10 and 21 dpi (**Figure 6**).

DIFFERENTIALLY METHYLATION CHANGES DUE TO MDV CHALLENGE

To compare the contents of the methylation change between $L6_3$ and $L7_2$, the mean methylation change of all the CpG sites was

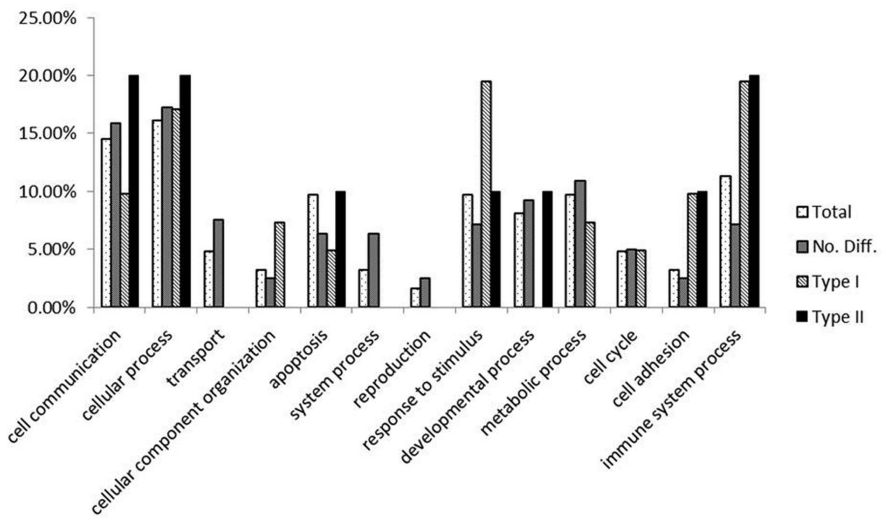

FIGURE 3 | Functional analysis of the genes by PANTHER.

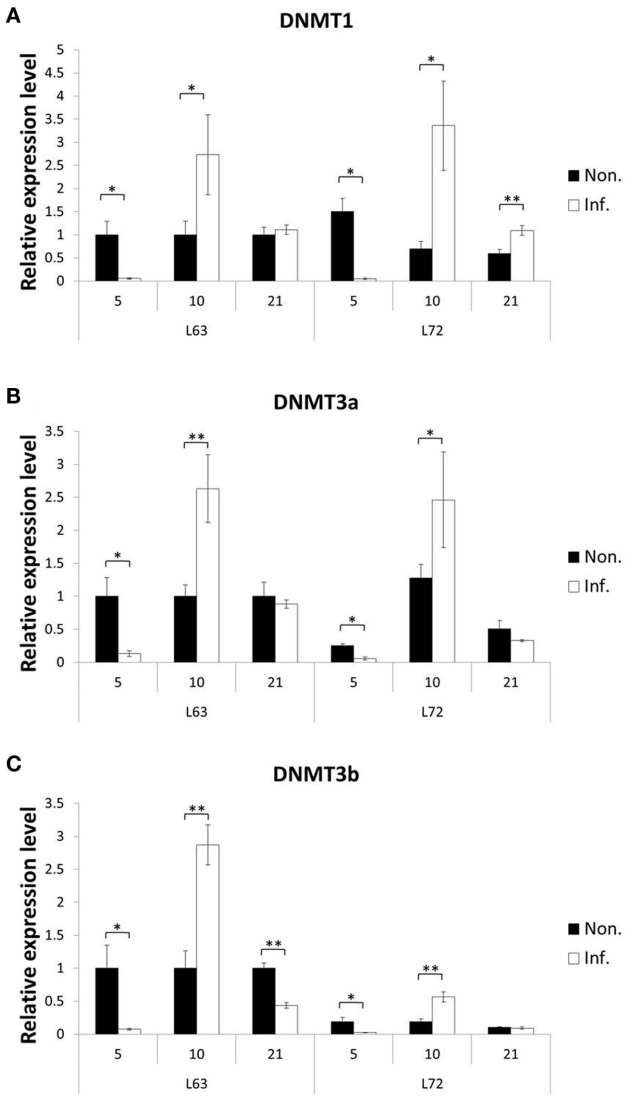

FIGURE 4 | Expression of DNA methyltrasferase genes after MDV infection. The expression of three DNA methyltrasferase genes, *DNMT1* **(A)**, *DNMT3a* **(B)**, and *DNMT3b* **(C)**, were tested by quantitative real-time RT-PCR at 5, 10, and 21 dpi in chicken spleen both resistant and susceptible to MD. The relative expression level was normalized to GAPDH gene. Black: non-infected; white: infected. $*P < 0.05$, $**P < 0.01$. $N = 4$ for each group. Error bar = SEM.

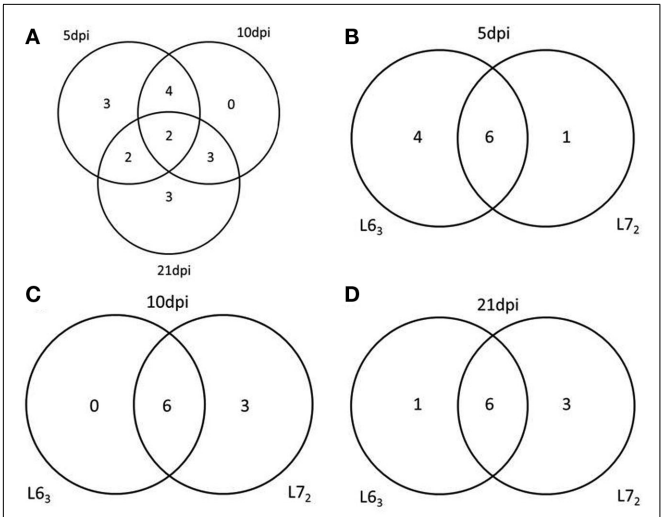

FIGURE 5 | Venn Diagrams of the number of genes have the methylation change at different time points and in different chicken lines. (A) The number of genes has the methylation change at 5, 10, and 21 dpi. (B–D) The number of genes has the methylation change between $L6_3$ and $L7_2$ at 5, 10, and 21 dpi respectively.

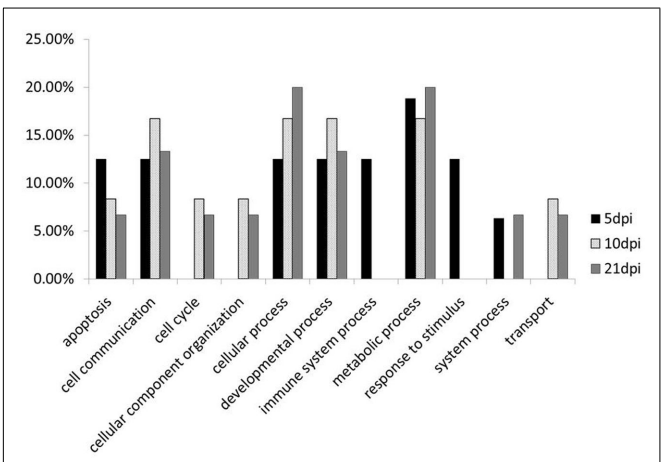

FIGURE 6 | Functional analysis of the genes have different methylation change between $L6_3$ and $L7_2$ at 5, 10, and 21 dpi by PANTHER.

calculated for each gene. Seven out of the 18 genes (*HDAC9*, *GH*, *STAT1*, *CIITA*, *FABP3*, *LATS2*, and *H2Ac*) showed significant differentially averaged methylation changes ($P < 0.05$) between the two lines of chickens (**Table 1**; **Figure A1** in Appendix). Functional analysis of the genes with temporal methylation changes revealed that the genes, related to apoptosis, immune system process, and response to stimulus, were over-represented at 5 dpi (**Figure 6**). However, genes, involved in enrichment of cell communication, were shown at 10 dpi; Genes, involved in functionality of cell cycle, cellular component organization, and transport, were over-represented at 10 and 21 dpi.

DISCUSSION

The development of disease resistance has long been a very important strategy for control of diseases in farm animals (Bishop et al., 2010; Luo et al., 2012). A better understanding on the mechanisms of disease resistance will facilitate breeding of more disease resistant animals, help to better control diseases in farm animal and also provide better models to learn disease control strategies for humans. Since the establishment of the non-MHC associated MD-resistant and -susceptible chicken lines (Line 6 and Line 7), lots of experiments have been done to elucidate the genetic mechanism of MD-resistance between the two lines of chickens (Gilmour et al., 1976; Fredericksen et al., 1977; Kaiser et al., 2003; Sarson et al., 2008a). However, not until recently, our lab started

Table 1 | Differential DNA methylation change between $L6_3$ and $L7_2$ after MDV challenge.

Time points (dpi)	Gene name	DNA methylation level change		*P* value
		$L6_3$	$L7_2$	
5	*GH*	−4.71	2.95*	0.0146
	CIITA	1.95	−7.51*	0.0298
	STAT1	−0.95**	0.42	0.0244
	H2Ac	1.11**	2.62**	0.0306
10	*FABP3*	4.37**	−9.21**	0.0002
	LATS2	−0.01	0.48**	0.0409
	H2Ac	2.53**	−0.56	0.0044
21	*HDAC9*	3.16**	7.92**	0.0273
	GH	0.83	6.07**	0.0096
	FABP3	−1.76	4.70**	0.0117
	H2Ac	0.78**	−1.36	0.0211

*$P < 0.05$, **$P < 0.01$.

to explore their epigenetic differences between the chicken lines, which provides evidence that DNA methylation may be involved in MD-resistance or -susceptibility (Yu et al., 2008a,b; Luo et al., 2011). As we know, although the functions of DNA methylation in development, imprinting *etc.* were reported in mammals, it's still unclear about its function in disease resistance. Previous study in human (Jelinek et al., 2011) and plant (Akimoto et al., 2007) showed that individuals with a higher DNA methylation level in some particular genes are susceptible to diseases or bacterial infection, which is consistent with our finding that a higher methylation level of several genes (*ITGB5*, *THBS2*, *HDAC9*, *IL12*, and *H2Ac*) were shown in MD-susceptible ($L7_2$) chickens. However, variable methylation level of *CD44* and *TNFSF10* between $L6_3$ and $L7_2$ indicated that the hypermethylation in susceptible chickens is not genome-widely. Functioning classification showed that the hypermethylated genes in susceptible chicken are showing functions of cellular component organization, response to stimulus, cell adhesion, and immune system process. Interestingly, hypermethylation of genes functioning in regulating cell adhesion was very important for the development of various cancers in human (Katto and Mahlknecht, 2011). Furthermore, expression analysis of the hypermethylated genes in the susceptible chickens showed a lower expression of these genes. The results indicated that there are specific pathways that may involve in MD-susceptibility or -resistance through hyper- or hypo-methylation of the genes included. In the future, a genome-wide DNA methylation research will be designed, which will help us explore the mechanisms further.

In previous study, the DNA methyltransferase (DNMTs) were usually found up-regulated by virus infection in human cells, like SV40 (Chuang et al., 1997) and EBV (Tsai et al., 2002). However, dynamic change of *DNMTs* expression was observed *in vivo* during MD life cycle in chicken. The *DNMTs* were first down-regulated at 5 dpi and then up-regulated at 10 dpi in both $L6_3$ and $L7_2$ chickens. Furthermore, different regulations of *DNMTs* were observed between the MD-resistant and -susceptible chickens at 21 dpi, indicating that late cytolytic phase is a critical time for *DNMTs* function in DNA methylation process or tumorigenesis. However, the *DNMTs* expression change was not necessary for the change of the methylation level change in the genes we studied. The correlation between *DNMTs* expression and methylation is upon chickens and time point. There are several reasons for that: First, other epigenetic mechanisms involve in the methylation change during MDV infection; second, the changed dosages of *DNMTs* are not efficient for the change of methylation on these genes; third, other functions of DNMTs involve. Except for establishing and maintaining the DNA methylation in cells, DNMTs also have other functions. The finding that *DNMT1* was only up-regulated in MD-susceptible chicken is consistent with the observation that DNMT1 is necessary for establishing and maintaining the transformation state of cells (Bakin and Curran, 1999; Robert et al., 2003). Similarly, *DNMT3B* deficient mouse embryo fibroblasts were found resistant to virus induced transformation (Soejima et al., 2003), which is consistent with our finding that the down-regulation of *DNMT3b* was only shown in MD-resistant chicken.

Abnormal DNA methylation is a common feature of human cancer. The fact is that DNA methylation started to be changed from very early stage of transformation process and a stepwise or dynamic change was happened during carcinogenesis (Ehrlich, 2009; Novak et al., 2009). Furthermore, DNA methylation modifications at the promoter regions of genes play a critical role in the intricate host–virus interaction network (Young et al., 2000; Zheng et al., 2008). From our results, the dynamic DNA methylation change during MD progression not only indicated an interaction between MDV and host gene, but also revealed the genes with aberrant methylation level may also involve in virus induced transformation process. During MDV life cycle in chicken spleen, 5 dpi is the early cytolytic phase when B cells and some T cells were targeted by MDV (Osterrieder et al., 2006). Virus infection in this stage provokes some apoptosis, lymphoid lesion, and inflammation responses in the immune organ (Morimura et al., 1996; Baigent and Davison, 1999). Different methylation change in genes enriched in apoptosis, immune system process, and response to stimulus suggested that the expression of these genes maybe differentially regulated between the MD-resistant and -susceptible chickens, which show different response to MDV infection. Although 10 and 21 dpi represent the latency and later cytolytic or transformation stage of MDV infection, it's very difficult to differentiate them very clearly *in vivo* since the latently infected cells can be mixed with the transformed cells (Davison and Nair, 2004). So we found some function enrichments like cell communication and transport are shared at 10 and 21 dpi. Genes over-represented in cell cycle and cell communications have different DNA methylation changes in $L6_3$ and $L7_2$ chicken. Since genes involve in cell cycle and cell communication play important role in carcinogenesis (Yamasaki et al., 1995; Hanahan and Weinberg, 2000, 2011), these results suggested that DNA methylation may participate in MD-resistance by disrupting pathways intriguing tumor formation.

In conclusion, we found DNA methylation heterogeneity between the MD-resistant $L6_3$ and -susceptible $L7_2$ chickens. The hypermethylation of genes involved in cellular component organization, response to stimulus, cell adhesion, and immune system process may play important role in MD-susceptibility. Different from other viruses, MDV induces a dynamic expression change in *DNMTs*. Differential methylation changes are observed between resistant and susceptible chickens after MDV infection. All in all, the differential DNA methylation levels and DNA methylation level change induced by MDV challenge between the lines of chickens suggested that DNA methylation may play a role in host resistance and/or susceptibility to MD.

AUTHORS' CONTRIBUTIONS

Juan Luo extracted DNA and RNA, performed the DNA methylation and mRNA expression experiments, analyzed the data and wrote the paper. Ying Yu extracted some of the DNA and performed some of the DNA methylation analysis. Fei Tian extracted DNA and RNA. Shuang Chang conducted the challenge trials and collected samples. Huanmin Zhang revised the paper. Jiuzhou Song designed the experiments and revised the paper.

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APPENDIX

Table A1 | Primers for pyrosequencing analysis of promoter methylation.

Genes	Accession No.	Primers	Sequence
ITGB5	NM_204483	F	5′-GGGACACCGCTGATCGTTTA YGTGYGGAGTTYGTAGAGAT-3′
		R	5′-CCCTTAAAAACTATCTCRTTCCA-3′
		Sequencing	5′-TCTCRTTCCAATTATACAC-3′
		Assay	5′-RACRCTACCACCCRCTACRT-3′
CD82	NM_001008470	F	5′-AGCGTTGYGAGTTTTATAGAAGTG-3′
		R	5′-GGGACACCGCTGATCGTTTA AACCCTCRCTCRACTACTTTACC-3′
		Sequencing	5′-AAGTGAGAATAATGTAATGG-3′
		Assay	5′-TAGYGGTTAGTAGTTYGGTATTTYGTTGTTATYGTAGYGTTGTAATYGTT-3′
HDAC9	NM_001030981	F	5′-TTGGGATATGGGTTGTCGAAAT-3′
		R	5′-GGGACACCGCTGATCGTTTA GCTAATACTCTCGTTCGCAACATS′
		Sequencing	5′-TGGGTTGTCGAAATAGTT-3′
		Assay	5′-TYGYGGGATTGTTGTGYGTGGGYGYGGTAGAAATTATGTTGCGAACGAGA-3′
STAT1	NM001012914	F	5′-TGTAAYGAAGTAAAATAGGYGAGA-3′
		R	5′-GGGACACCGCTGATCGTTTA TCAACCTACACTACRCAACCTAA-3′
		Sequencing	5′-TAAAATAGGCGAGATATAAG-3′
		Assay	5′-TAYGYGAGTYGTTYGYGAGGTAGGGTCGTT-3′
TGFB5	NM_205454	F	5′-GYGAGGATATTTATTTGGAAGAG-3′
		R	5′-GGGACACCGCTGATCGTTTA CCCAAAAAATATCACCTCCAAT-3′
		Sequencing	5′-GAGTTTGGGTTGGGTA-3′
		Assay	5′-TAYGTAGTATTYGGAATTTTGTTYGAAATAGGTTGGTGTTGTTTTTTTTTG YGGAGGATA YGTTAAAGYG-3′
Nk-lysin	NM001044680	F	5′-GYGTTAGTTGAATTTTAGAGTTTAAAG-3′
		R	5′-GGGACACCGCTGATCGTTTA TTTATAAATTTTTCTCCACTACTACTAAT-3′
		Sequencing	5′-AATTTTAGAGTTTAAAGGGA-3′
		Assay	5′-GYGGAGAYGGAGTATAATATTATAYGTATTATTAAYGTTAYGTAGTTTTT-3′
IL28RA	XM 417841	F	5′-GGGACACCGCTGATCGTTTAAGGATGTGCGAGGTAGAATATTG-3′
		R	5′-CAAACCCTACAACAACCACATAAT-3′
		Sequencing	5′-CCCTACAACAACCACATAA-3′
		Assay	5′-TCRCTATATACTAACCRCCACRTTCCCAACAACRCACTAACRACTACAAC0A ATATTCTACCTCGCAC-3′
M0N2	NM 001199605	F	5′-TTATTGCGGTAGGGGTTAATATT-3′
		R	5′-GGGACACCGCTGATCGTTTA CAAACTAAACGCTATCCTAAACT-3′
		Sequencing	5′-CGGTAGGGGTTAATATTTT-3′
		Assay	5′-YGGGAGAYGTTAGYGGYGGGGATGGYGTTTTGTAGAGAGTAGTTTAGGATA-3′
THBS2	NM 001001755	F	5′-GGGACACCGCTGATCGTTTA GGGTGTATGTAGAAAAGGGAATGT-3′
		R	5′-TTCAACACGATACTATTCCTACCC-3′
		Sequencing	5′-ACATAACTACATCTCCATAT-3′
		Assay	5′-ACRTACRCTCCCACAATAAATAAAACAAACRACRACCRCTTAAACRTACAA ACATTCCCTTTTCTACATA C-3′
CD44	NM_204860	F	5′-GTTTTTTTAAAATTTGTGTGGTTGT-3′
		R	5′-GGGACACCGCTGATCGTTTA AAACTCCATCAAAAATCACACC-3′
		Sequencing	5′-GGTTGTTTAGTTAGAATTTA-3′
		Assay	5′-YGGTTTTTYGYGGTTTTTTTTGTTTTGTTTCGTAAT-3′
IL12	NM_213588	F	5′-GTCGATGTCGTGTTTTGTTATGT-3′
		R	5′-GGGACACCGCTGATCGTTTA CCACGAAATTCCCAACTCTCA-3′
		Sequencing	5′-TTTTGTTCGATGAAATTG-3′
		Assay	5′-ATGYGGGATYGGTGGTTGTYGTAGGAGTTGYGTTGTTTTTATGTYGGTGG AGGAGTAGG AGTTTTTTTTT-3′
LATS2	XM417143	F	5′-GGGACACCGCTGATCGTTTATTTTGGTAGAAAGTTGGTGTGAAT-3′
		R	5′-CACCATATAACACTTCCCTACCTC-3′
		Sequencing	5′-CCTACCTCACAAAAACC-3′
		Assay	5′-TCRCCCRTCTTACAAACRATTCACCRTCTCRCCATCTTCTCCCCCRCTCCT TCAACTCRACRAATTCACA CCAACTTTCTACCAAAA-3′

(Continued)

Table A1 | Continued

Genes	Accession No.	Primers	Sequence
GH	NM_204359	F	5′-GGGACACCGCTGATCGTTTA GATTGGTGTGGAAAGGAGGAAGA-3′
		R	5′-CAAAAACAAATCGAACCCACAAC-3′
		Sequencing	5′-CTCCTACAATTATCCATCC-3′
		Assay	5′-CACRTTCTACCTCRTACRACTCAAAAATAAATATACTAAAACT-3′
IGF2	NM_001030342	F	5′-AAGTATAACGTGTGGTAGAAGAAGAGTT-3′
		R	5′-GGGACACCGCTGATCGTTTA TCGCCCTAACTTCCTCAACTACT-3′
		Sequencing	5′-CGTGTGGTAGAAGAAGAGT-3′
		Assay	5′-TYGTAGYGGTTGTAGYGGGAGGTGTTAGGTATTTTGYGTGTTYGTYGGTAT YGGTGGTAGGCGGAGGGG TTGTAAGT-3′
TNFSF10	NM 204379	F	5′-GAGGGGAGGTTTAGGTTGGATATT-3′
		R	5′-GGGACACCGCTGATCGTTTA ACCGCCCACATCCCTCAATA-3′
		Sequencing	5′-GGGGTGGAGTAGTGGTATA-3′
		Assay	5′-GTYGTTYGGGGAGYGGTGGAGTTATYGTTTTTGGAAGTGTTTAGAGTYGTGGGGATGTGGTATTGAGGG ATGT-3′
H2Ac	NM_001079475	F	5′-AGTGGGGGACGTGCGAAATA-3′
		R	5′-GGGACACCGCTGATCGTTTA CCCCGCCCTTCCTCTTTTATAAC-3′
		Sequencing	5′-TTATTGGGTAGATTTGGAT-3′
		Assay	5′-TYGYGGYGTTATTGGTYGGAGYGAGTGAGAGAGTATATYGGTTAATYGGAAAGYGAGTYG GGTYGTTGYGGGAGGTTATAAAAGAGGAAG GGCG-3′
CIITA	NC 006101.2	F	5′-CGGGAATTTTTACGTTAGGTTTATAGTG-3′
		R	5′-GGGACACCGCTGATCGTTTAAACGCGAAACGAAAAAACTCCT-3′
		Sequencing	5′-TTTTTACGTTAGGTTTATAG-3′
		Assay	5′-TGTYGTYGYGGTATTTTAGTYGTTYGGTYGGGTTGYGGGGYGGTTTYGTT TTTTTTGGGGGYGGTTGTGGG AGCGGAGGAGTTTTTT-3′
FABP3	NM 001030889	F	5′-AGAGGGGGAAATTGAGGTA-3′
		R	5′-GGGACACCGCTGATCGTTTA AACACACACACACGATCC-3′
		Sequencing	5′-GGGGGAAATTGAGGTA-3′
		Assay	5′-YGGGAGYGTTYGTGGGGATAYGYGGGATCGTGTGTGTGTGTTGGGGGT-3′

Y stands for C/T, and R stands for G/A. Bold Y or R in the assay sequence is the CpG sites analyzed in each region.

Table A2 | Primers for quantitative real-time RT-PCR.

Genes	Primers	Sequence
ITGβ5	F	5′-GTTTGGGGAGACCTGTGAGA-3′
	R	5′-TCATCCTTGCAGTGCTTTTG-3′
H2Ac	F	5′-CGGAAAGCAGGGCGGGAAG-3′
	R	5′-GTCAGGTACTCCAGCACGG-3′
DNMT1	F	5′-CCACCAAAAGGAAATCAGAG-3′
	R	5′-TAATCCTCTTCTCATCTTGCT-3′
DNMT3a	F	5′-ATGAACGAGAAGGAAGACATC-3′
	R	5′-GCAAAGAGGTGGCGGATCAC-3′
DNMT3b	F	5′-CGTTACTTCTGGGGCAACCTC-3′
	R	5′-ATGACAGGGATGCTCCAGGAC-3′
GAPDH	F	5′-GAGGGTAGTGAAGGCTGCTG-3′
	R	5′-ACCAGGAAACAAGCTTGACG-3′

Table A3 | Promoter Methylation levels of $L6_3$ and $L7_2$ not challenged with MDV.

Genes	Lines	CpG sites								Note
		1	2	3	4	5	6	7	8	Hypo.
LATS2	$L6_3$	0.73 ± 0.91	0.12 ± 0.41	3.60 ± 2.33	0.56 ± 0.84	1.19 ± 1.31	0.71 ± 1.30	1.13 ± 2.49	0.32 ± 0.64	
	$L7_2$	0.78 ± 0.97	0.67 ± 0.88	3.20 ± 1.32	0.78 ± 0.89	1.30 ± 1.22	0.81 ± 1.27	2.09 ± 2.80	0.56 ± 0.70	
MON2	$L6_3$	1.04 ± 1.37	1.50 ± 1.48	1.59 ± 1.80	0.99 ± 1.80	0.57 ± 1010	N/A/A	N/A/A	N/A/A	
	$L7_2$	1.94 ± 1.11	1.34 ± 1.50	2.47 ± 3.25	1.53 ± 2.09	1.01 ± 1.30	N/A/A	N/A/A	N/A/A	
IL28RA	$L6_3$	4.77 ± 1.14	7.91 ± 1.85	2.09 ± 1.47	5.00 ± 1.25	3.50 ± 0.81	N/A/A	N/A/A	N/A/A	
	$L7_2$	3.90 ± 1.08	7.95 ± 1.34	2.49 ± 0.56	5.10 ± 1.75	3.90 ± 1.38	N/A/A	N/A/A	N/A/A	
STAT1	$L6_3$	1.41 ± 1.31	2.22 ± 1.22	2.97 ± 1.48	2.59 ± 1.76	1.26 ± 1.17	1.98 ± 1.27	N/A/A	N/A/A	
	$L7_2$	1.06 ± 1.28	3.26 ± 1.93	2.91 ± 1.65	3.10 ± 0.70	0.71 ± 1.07	1.50 ± 1.47	N/A/A	N/A/A	
CD44	$L6_3$	1.53 ± 0.60	20.72 ± 7.08	0.58 ± 0.16	15.10 ± 3.39	N/A/A	N/A/A	N/A/A	N/A/A	
	$L7_2$	1.94 ± 1.02	6.73 ± 1.43	3.04 ± 0.91	6.76 ± 0.93	N/A/A	N/A/A	N/A/A	N/A/A	
H2Ac	$L6_3$	4.33 ± 0.57	3.55 ± 0.59	21.51 ± 1.32	29.03 ± 1.76	14.83 ± 0.81	10.09 ± 0.74	N/A/A	N/A/A	
	$L7_2$	7.81 ± 0.91	6.16 ± 1.02	31.18 ± 1.75	38.50 ± 1.25	20.48 ± 0.87	16.50 ± 0.87	N/A/A	N/A/A	
TNFSF10	$L6_3$	4.02 ± 0.90	9.92 ± 1.09	10.33 ± 1.67	10.63 ± 2.35	38.60 ± 2.04	N/A/A	N/A/A	N/A/A	
	$L7_2$	5.48 ± 1.58	8.93 ± 3.23	13.15 ± 2.42	9.91 ± 3.54	25.07 ± 3.75	N/A/A	N/A/A	N/A/A	
IL12	$L6_3$	19.38 ± 6.42	12.38 ± 4.22	15.60 ± 3.94	17.02 ± 4.29	7.43 ± 1.76	N/A/A	N/A/A	N/A/A	
	$L7_2$	24.25 ± 4.29	14.57 ± 3.30	18.30 ± 2.01	21.22 ± 4.25	8.78 ± 2.16	N/A/A	N/A/A	N/A/A	
FABP3	$L6_3$	35.99 ± 7.36	35.24 ± 4.20	48.05 ± 7.12	32.69 ± 4.39	4.16 ± 1.81	20.43 ± 8.12	N/A/A	N/A/A	
	$L7_2$	28.22 ± 6.60	30.27 ± 8.05	39.98 ± 12.15	28.38 ± 7.52	4.85 ± 1.46	17.03 ± 5.14	N/A/A	N/A/A	
CIITA	$L6_3$	25.57 ± 4.55	29.20 ± 6.72	4.26 ± 3.64	3.22 ± 2.96	N/A/A	N/A/A	N/A/A	N/A/A	
	$L7_2$	19.76 ± 7.59	24.70 ± 8.94	4.56 ± 4.72	4.30 ± 5.96	N/A/A	N/A/A	N/A/A	N/A/A	
ITGB5	$L6_3$	38.62 ± 4.45	46.81 ± 4.08	61.94 ± 4.47	44.06 ± 4.88	37.74 ± 1.32	39.63 ± 1.57	N/A/A	N/A/A	Inter.
	$L7_2$	52.04 ± 4.62	61.32 ± 4.76	71.83 ± 3.54	56.37 ± 4.09	40.05 ± 1.86	41.53 ± 5.97	N/A/A	N/A/A	
THBS2	$L6_3$	34.31 ± 5.01	54.07 ± 6.46	32.32 ± 4.90	22.03 ± 3.13	11.37 ± 1.85	N/A/A	N/A/A	N/A/A	
	$L7_2$	49.45 ± 5.16	70.68 ± 5.04	41.68 ± 4.63	30.91 ± 3.82	14.08 ± 1.45	N/A/A	N/A/A	N/A/A	
HDAC9	$L6_3$	24.83 ± 6.00	31.65 ± 6.03	51.50 ± 5.82	55.74 ± 4.78	40.77 ± 6.22	N/A/A	N/A/A	N/A/A	
	$L7_2$	36.80 ± 5.36	45.83 ± 4.20	64.86 ± 6.47	68.25 ± 3.94	54.15 ± 3.48	N/A/A	N/A/A	N/A/A	
IGF2	$L6_3$	89.69 ± 4.08	89.25 ± 2.35	88.05 ± 3.85	77.81 ± 3.72	79.39 ± 3.46	49.77 ± 3.85	68.19 ± 7.40	88.95 ± 1.92	Hyper.
	$L7_2$	91.55 ± 3.95	92.06 ± 2.54	89.78 ± 2.74	81.15 ± 4.68	82.37 ± 10.41	52.64 ± 5.77	72.41 ± 6.71	89.97 ± 2.02	
GH1	$L6_3$	63.12 ± 2.75	48.64 ± 2.51	80.64 ± 2.54	N/A/A	N/A/A	N/A/A	N/A/A	N/A/A	
	$L7_2$	61.26 ± 2.19	45.13 ± 1.90	79.23 ± 1.91	N/A/A	N/A/A	N/A/A	N/A/A	N/A/A	
NK-lysin	$L6_3$	89.47 ± 2.33	42.98 ± 5.29	82.46 ± 2.20	78.32 ± 1.72	62.63 ± 2.16	N/A/A	N/A/A	N/A/A	
	$L7_2$	91.79 ± 2.63	40.57 ± 5.47	80.53 ± 2.45	78.14 ± 1.88	66.56 ± 1.52	N/A/A	N/A/A	N/A/A	
TGFB3	$L6_3$	88.04 ± 1.60	90.98 ± 2.19	90.39 ± 2.28	80.86 ± 1.60	68.43 ± 2.13	74.39 ± 3.46	N/A/A	N/A/A	
	$L7_2$	89.88 ± 2.59	92.91 ± 2.58	93.51 ± 2.66	82.90 ± 2.09	72.94 ± 3.09	77.00 ± 2.68	N/A/A	N/A/A	
CD82	$L6_3$	3.86 ± 1.56	5.24 ± 1.86	3.97 ± 1.61	3.98 ± 0.98	3.65 ± 2.02	1.88 ± 1.36	40.48 ± 5.54	52.78 ± 3.63	Hypo +
	$L7_2$	3.16 ± 0.99	4.41 ± 1.31	3.59 ± 1.12	4.41 ± 1.40	2.69 ± 1.23	1.85 ± 1.27	41.22 ± 5.29	51.61 ± 2.67	Inter.

Methylation level shown in each cell = mean ± STD.

Hypo., hypomethylation; Hyper., hypermethylation; Inter., intermediate methylation.

N/A, data not available.

N = 12 for each group.

Table A4 | Promoter methylation level change at different CpG sites of genes after MDV challenge.

Gene name	Time points (dpi)	Lines	% Methylation level change after MDV infection of different CpG sites							
			1	2	3	4	5	6	7	8
GH	5	$L6_3$	−5.09**	−7.44**	−1.60					
		$L7_2$	3.29	1.50	4.07					
	10	$L6_3$	2.73*	−1.00	0.95					
		$L7_2$	3.75*	1.04	−0.33					
	21	$L6_3$	0.50	2.08*	−0.09					
		$L7_2$	6.87*	7.10**	4.23**					
CD44	5	$L6_3$	−0.23	0.71	−0.02	0.40				
		$L7_2$	0.40	−4.25	−1.26	0.36				
	10	$L6_3$	−0.56	2.39	−0.13	−1.63				
		$L7_2$	0.32	0.29	1.34	0.59				
	21	$L6_3$	0.03	−3.30	0.21	0.16				
		$L7_2$	−0.77	−3.17**	−1.57	1.39				
CIITA	5	$L6_3$	4.41	−3.23	1.50	5.11				
		$L7_2$	−11.86*	−12.67	−2.78	−2.74				
	10	$L6_3$	−2.21	0.29	0.59	−1.60				
		$L7_2$	1.04	0.28	1.19	0.24				
	21	$L6_3$	−10.17**	−6.12*	0.34	1.27				
		$L7_2$	−6.95**	−5.83*	0.36	−1.18				
NK-lysin	5	$L6_3$	4.24*	18.21**	6.25**	6.72**	1.20			
		$L7_2$	2.69	29.14**	4.23	4.82*	−0.40			
	10	$L6_3$	1.61	8.62	1.63	3.46	1.28			
		$L7_2$	−1.03	7.60	1.63	1.33	2.90			
	21	$L6_3$	−0.92*	−3.59	−1.22	−1.55	−1.69**			
		$L7_2$	−1.69**	−2.27	−2.06	−2.00*	−1.99			
THBS2	5	$L6_3$	8.06	7.03	5.51	3.73	4.83			
		$L7_2$	−1.75	5.07	1.58	8.38	2.97			
	10	$L6_3$	5.74	6.32	0.61	2.03	0.05			
		$L7_2$	−1.25	1.46	0.33	−0.43	1.35			
	21	$L6_3$	−1.69	−1.38	2.49	1.50	2.07			
		$L7_2$	1.31	1.06	−0.20	−6.24	−0.06			
M0N2	5	$L6_3$	0.63	−1.33	−2.87*	−1.69	−0.10			
		$L7_2$	−1.43	2.22	0.35	−3.06	−0.09			
	10	$L6_3$	1.86	−0.62	0.59	0.75	0.41			
		$L7_2$	−1.17	−0.76	−5.96*	0.42	−0.52			
	21	$L6_3$	−0.33	0.43	0.56	0.29	−0.33			
		$L7_2$	0.94	−1.18	−0.63	0.64	−0.06			
HDAC9	5	$L6_3$	5.28	8.65	6.56	6.04	7.54			
		$L7_2$	3.37	11.67**	−4.57	0.48	−3.20			
	10	$L6_3$	−4.07	1.41	3.86	−2.11	3.13			
		$L7_2$	−1.01	0.17	3.47	0.07	−1.60			
	21	$L6_3$	3.18	1.08	3.80	2.90	4.86			
		$L7_2$	12.56**	7.00**	4.48	4.59	10.99**			
FABP3	5	$L6_3$	7.07	9.34*	15.81*	8.58*	3.22*			
		$L7_2$	7.75	7.22	10.96	8.09	0.13			
	10	$L6_3$	3.43	4.52	8.48	4.50	0.92			
		$L7_2$	−9.44*	−11.67**	−11.80**	−10.82**	−2.31**			
	21	$L6_3$	−8.35	−1.06	−2.23	0.03	2.81			
		$L7_2$	5.40	4.11	7.09	4.15	2.74			

(Continued)

Table A4 | Continued

Gene name	Time points (dpi)	Lines	% Methylation level change after MDV infection of different CpG sites							
			1	2	3	4	5	6	7	8
IL28RA	5	$L6_3$	−1.87	−3.97*	−0.54	−0.92	−0.46			
		$L7_2$	−2.75*	−0.64	−1.12**	−1.80	1.50			
	10	$L6_3$	−2.05**	−0.60	−0.77	−0.65	0.02			
		$L7_2$	−1.67*	−1.01	0.07	−1.15	0.70			
	21	$L6_3$	0.81	0.03	1.20	1.41	0.04			
		$L7_2$	0.11	0.65	−0.58	−0.12	0.21			
IL12	5	$L6_3$	3.19*	4.27	−0.42	0.66	−1.46*			
		$L7_2$	−0.68	−0.68	0.13	−4.91	−1.43			
	10	$L6_3$	0.39	−0.88	0.60	−3.44	−0.28			
		$L7_2$	−1.07	−4.03	−1.05	−5.39	−1.00			
	21	$L6_3$	−2.02	−3.49	−3.58	0.12	−0.32			
		$L7_2$	3.06	1.85	−0.17	−3.05	1.08			
TNFSF10	5	$L6_3$	0.23	−1.73*	−1.70	−1.24	−1.11			
		$L7_2$	−0.25	−2.79*	−3.72*	−1.43	−2.24			
	10	$L6_3$	1.23	0.48	−0.19	0.81	−2.94			
		$L7_2$	−1.01	0.68	−1.19	0.06	1.80			
	21	$L6_3$	0.38	0.12	0.19	−1.29	−1.54			
		$L7_2$	−1.37	2.79	−2.24	2.07	3.67			
ITGB5	5	$L6_3$	21.99**	24.91**	25.41**	21.58**	1.54*	0.38		
		$L7_2$	16.68**	20.25**	17.04**	15.07**	1.73	−0.77		
	10	$L6_3$	3.73	−0.14	−3.03	−1.15	−0.24	−2.14		
		$L7_2$	−0.81	−2.01	−0.54	−1.81	−1.25	−2.76		
	21	$L6_3$	6.85	7.81	5.15	6.66	2.51	1.05		
		$L7_2$	8.92	5.84	5.91	7.19	2.30	1.75		
STAT1	5	$L6_3$	−1.18	−1.20	−1.13	−1.69	−0.30	−0.19		
		$L7_2$	0.77	−1.10	0.23	−0.48	2.05*	1.07		
	10	$L6_3$	1.51*	−0.35	−0.55	2.24**	−0.62	0.64		
		$L7_2$	0.12	−0.86	0.01	1.39**	−0.36	0.53		
	21	$L6_3$	0.95	−1.35	−1.98	−0.10	3.69*	1.46		
		$L7_2$	0.58	−3.86*	−1.37	−1.86*	1.42	2.08*		
TGFB3	5	$L6_3$	−0.44	4.22**	5.32	0.25**	6.90	5.63		
		$L7_2$	3.62	2.66	2.97*	2.38	2.15	−1.95		
	10	$L6_3$	1.34	0.84	−0.85**	3.36**	5.54	3.20		
		$L7_2$	−0.57	−2.24	−0.10	1.58	4.74	1.38		
	21	$L6_3$	2.38	3.58	3.92*	−0.33	−1.25	−4.07		
		$L7_2$	−0.19	0.69	−0.34	−1.03	0.80	−0.13		
H2AC	5	$L6_3$	0.63	0.65*	2.26	1.26	1.26	0.60		
		$L7_2$	2.79	1.25	4.58	3.69*	1.83	1.55		
	10	$L6_3$	1.14	1.13	4.78	4.98	2.27*	0.85**		
		$L7_2$	−0.15	0.14	−1.76**	−1.40	0.12	−0.28		
	21	$L6_3$	0.60	0.25	0.95	1.61	0.22	1.04		
		$L7_2$	−1.47*	0.87	−3.09**	−3.88**	−0.72	0.13		
CD82	5	$L6_3$	1.85	2.32	0.33	0.50	0.18	−0.47	−4.79	−2.36
		$L7_2$	2.06	0.04	2.27	0.39	−0.57	−0.15	−5.52	−2.09
	10	$L6_3$	−1.87*	−2.07**	−1.42	0.92	−2.17	3.88*	1.60	−0.24
		$L7_2$	0.55	−0.80	−1.08	0.00	0.86	2.65	0.09	1.27
	21	$L6_3$	0.21	0.88*	−0.18	0.47	−0.50	0.44	−0.85	−1.30*
		$L7_2$	2.01**	0.94*	1.19	3.10**	0.06	1.48	−1.77	−0.47

(Continued)

Table A4 | Continued

Gene name	Time points (dpi)	Lines	% Methylation level change after MDV infection of different CpG sites							
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
IGF2	5	$L6_3$	0.02*	2.83	0.16	3.50	−0.32	3.14	6.32*	2.45*
		$L7_2$	2.19	1.47	0.82	3.70	−1.45	3.28	5.75	0.53
	10	$L6_3$	−1.36	1.05	1.93	2.18	2.69	3.44	0.49	0.30
		$L7_2$	−4.09	−1.95	−1.67	3.24	4.55*	3.60	−0.21	0.02
	21	$L6_3$	0.34	0.64	−0.12	0.79	1.62	2.33	−3.51	0.22
		$L7_2$	−1.23	0.10	−1.18	−1.32	4.91	5.46	2.08	−0.60
LATS2	5	$L6_3$	−0.21	1.04	−0.87	1.08	0.09	1.14	1.57	0.52
		$L7_2$	0.32	0.25	−0.33	0.81	0.94	1.42	−1.65	0.48
	10	$L6_3$	−0.02	0.27	−0.26	0.24	−0.87	−0.23	0.85	−0.03
		$L7_2$	0.77	0.74	0.82	0.42	−0.27	0.55	0.41	0.38
	21	$L6_3$	1.64*	0.67	0.80	0.62	0.69	0.48	1.31	0.60
		$L7_2$	1.90*	0.74	−0.22	0.70	0.35	0.02	1.15	0.84

$*P < 0.05$, $**P < 0.01$.

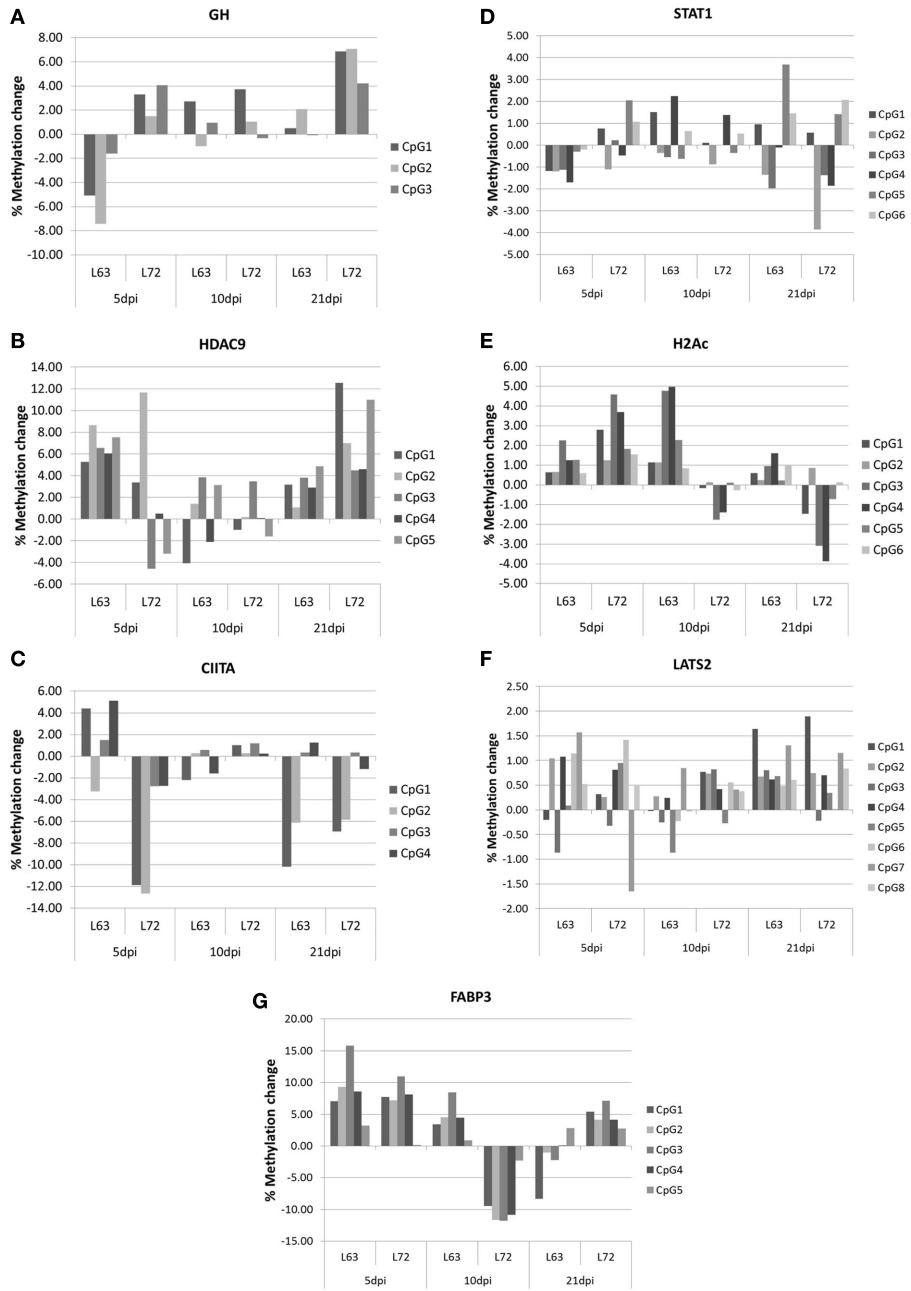

FIGURE A1 | Promoter methylation change during MDV life cycle in *GH* (A), *HDAC9* (B), *CIITA* (C), *STAT1* (D), *H2Ac* (E), *LATS2* (F), and *FABP3* (G). The methylation change within lines was the methylation changed after MDV infection compared with the promoter methylation level before MDV infection. $N = 4$ for each group.