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Epigenetic regulation of inflammatory pathways in cancer and aging

Edited by
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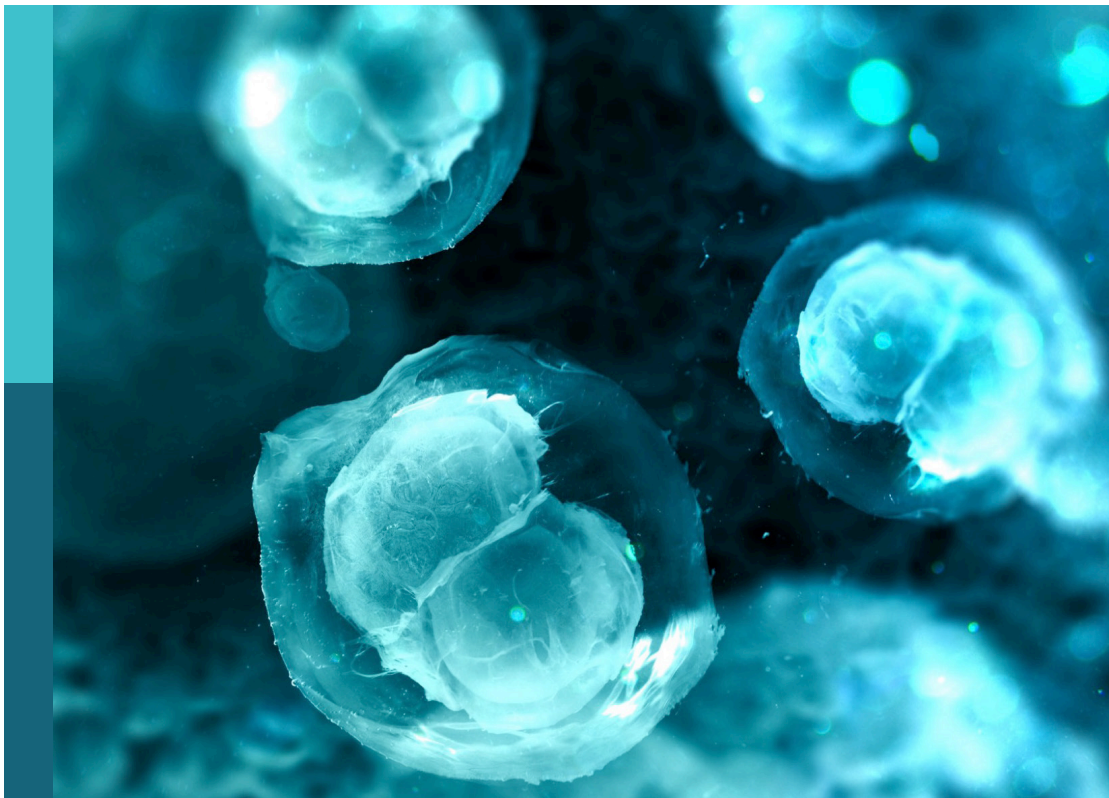

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Epigenetic regulation of inflammatory pathways in cancer and aging

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ERK1/2-EGR1-SRSF10 Axis Mediated Alternative Splicing Plays a Critical Role in Head and Neck Cancer

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Aberrant alternative splicing is recognized to promote cancer pathogenesis, but the underlying mechanism is yet to be clear. Here, in this study, we report the frequent upregulation of SRSF10 (serine and arginine-rich splicing factor 10), a member of an expanded family of SR splicing factors, in the head and neck cancer (HNC) patients sample in comparison to paired normal tissues. We observed that SRSF10 plays a crucial role in HNC tumorigenesis by affecting the pro-death, pro-survical splice variants of BCL2L1 (BCL2 Like 1: BCLx: Apoptosis Regulator) and the two splice variants of PKM (Pyruvate kinase M), PKM1 normal isoform to PKM2 cancer-specific isoform. SRSF10 is a unique splicing factor with a similar domain organization to that of SR proteins but functions differently as it acts as a sequence-specific splicing activator in its phosphorylated form. Although a body of research studied the role of SRSF10 in the splicing process, the regulatory mechanisms underlying SRSF10 upregulation in the tumor are not very clear. In this study, we aim to dissect the pathway that regulates the SRSF10 upregulation in HNC. Our results uncover the role of transcription factor EGR1 (Early Growth Response1) in elevating the SRSF10 expression; EGR1 binds to the promoter of SRSF10 and promotes TET1 binding leading to the CpG demethylation (hydroxymethylation) in the adjacent position of the EGR1 binding motif, which thereby instigate SRSF10 expression in HNC. Interestingly we also observed that the EGR1 level is in the sink with the ERK1/2 pathway, and therefore, inhibition of the ERK1/2 pathway leads to the decreased EGR1 and SRSF10 expression level. Together, this is the first report to the best of our knowledge where we characterize the ERK 1/2-EGR1-SRSF10 axis regulating the cancer-specific splicing, which plays a critical role in HNC and could be a therapeutic target for better management of HNC patients.

Keywords: alternative splicing, ERK/MAPK, HNC (Head and Neck Cancer), SRSF10 (Serine And Arginine Rich Splicing Factor 10), Egr1 (early growth response protein 1)

INTRODUCTION

Head and neck cancer (HNC) is a heterogeneous disease that includes a variety of tumors that originate from gingivobuccal complex (buccal mucosa, alveolus, retromolar trigone, and gingiva), tongue, lip, palate, and floor of the mouth (Nigro et al., 2017). According to the world health organization 2018 data, cancer is the second leading cause of death globally, accounting for one in six deaths. HNC is the sixth most common cancer worldwide (Kumar, 2017), with an incidence rate of 650,000 new cases every year (Kumar, 2017) and more than 350,000 deaths every year (Parkin et al., 2005). HNC arise from the mucosa of the oral cavity and are epithelial in origin, therefore, classified as squamous cell carcinomas. Mainly, HNC patients are detected at a late stage and thus are associated with poor survival rates (Garg and Karjodkar, 2012). An early clinical diagnosis of HNC may improve the survival rate with advancement in therapeutic options. Therefore, it is of great importance to identify new molecular targets for HNC treatment.

Almost all human genes undergo alternative splicing (AS), a highly regulated process, and studies evident that any deregulation in the AS process contributes to tumor progression (Srebrow and Kornblihtt, 2006), including HNC (Yadav et al., 2019). Cancer cells exhibit a remarkable alteration in the splicing process and generate specific splicing isoforms that not only act as drivers of cancer progression but contribute significantly to cancer hallmarks (Ladomery, 2013). The expression of cancer-specific isoform is very tightly regulated by RNA splicing regulators, which recently emerged as a new class of oncoproteins (Dvinge et al., 2016). The RNA splicing regulators involve the group of proteins called splicing factors that include mainly two groups of proteins SR (serine and arginine-rich proteins) family and HnRNP (heteronuclear ribonuclear proteins) family members. These splicing regulators bind to the specific sequence of the target genes and affect inclusion or exclusion of the exon, thereby regulates the expression of different transcripts from a single gene which further controls the key cellular process. A large number of splicing factors have been reported to be deregulated in multiple cancer types (Boukakis et al., 2010; Silipo et al., 2015) and thus found to be responsible for aberrant AS (Gupta et al., 2020). The deregulation of these splicing factors is governed by the triggering of several cellular signaling pathways, eventually leading to altered regulation of AS events (Blaustein et al., 2007). This highlights the importance of continued elucidation of the key signaling pathways, which contributes to the process of carcinogenesis.

Serine and Arginine Rich Splicing Factor 10 (SRSF10) is an atypical member of the SR protein family with a domain organization similar to SR proteins. SRSF10 is characterized recently (Cowper et al., 2001), and has been shown to act as a splicing repressor when activated by dephosphorylation (Shin et al., 2004). Later, the subsequent study reported that the phosphorylated form of SRSF10 could function as a splicing activator in a sequence-dependent manner. In continuation with these reports, SRSF10 was shown to play an important role in the AS process by regulating the exon inclusion both positively and negatively, which depends on its binding at pre mRNA relative to an alternative exon. The key role of SRSF10 is studied in the developmental process using several model systems (Li et al., 2014; Wei et al., 2015). It has been reported to affect colorectal cancer progression by modulating the AS of several genes (Zhou et al., 2014b). These studies strongly suggest that splicing factors play a peculiar role in specific tissue or cells. The function of SRSF10 overexpression in HNC has remained unexplored as well as the underlying molecular pathway that regulates the expression of SRSF10 has not yet been studied to the best of our knowledge.

Here in this study, we strongly highlight the tumorigenic role of SRSF10 in HNC. Importantly, we explored the key pathway for the over-expression of SRSF10 for the first time. Interestingly, we observed that inhibiting ERK signaling pathway results in the downregulation of SRSF10 by affecting the EGR1 expression level. Our data demonstrate that EGR1 regulates the expression level of SRSF10 by recruiting TET1 at the demethylated EGR1 binding site. Conclusively, we have dissected the ERK-EGR1-SRSF10 axis, which plays a critical role in HNC by directing the splicing of tumor-specific isoforms and could be a potential target for better management of HNC patients.

MATERIALS AND METHODS

Cell Culture

The two mammalian HNC cell lines were used in this study; BICR10 (Buccal mucosa squamous carcinoma) and H157 (human oral squamous cell carcinoma) were obtained from the European Collection of Authenticated Cell Culture (ECACC; Salisbury, United Kingdom) in May 2014. These two head and neck cell lines BICR10 (ECACC 04072103) and H157 (ECACC 07030901), were cultured in the ECACC recommended media, supplemented with the 10% Fetal Bovine Serum (Sigma, F7524), 2 mmol L-glutamine (Invitrogen, 25030081) and 100 units/ml of penicillin and streptomycin, and 0.5 μg/ml sodium hydrocortisone succinate at 37°C with 5% CO_2.

Head and Neck Cancer Sample Collection

Informed consent was obtained from head and neck patients undergoing surgery at Bansal Hospital, Bhopal, India. After surgery, tumor tissues with paired adjacent normal tissues were collected and snap-frozen immediately and stored at −80°C until use. One pair of tumor tissues and adjacent normal tissues were collected in RNAlater (Sigma, R0901) separately. The Institute Ethics Committee approved this study, and the clinical characteristics of patients used in this are presented in **Supplementary Table 2**.

Microarray Data Analysis

Gene expression profiles analyzed in this study were collected from Gene Expression Omnibus GEO (Barrett et al., 2013). Microarray platform with specific probes was mapped to the gene symbols with appropriate annotation files. The expression values of genes with more than one probe were averaged using DNA Chip Analyzer software and considered for the analysis. SRSF10 gene expression values were extracted from normalized and log2

transformed oral tumor profiles. The significant difference in the gene expression between normal and oral tumor was then calculated using the student's test (two tailed). *p*-value less than 0.05 was considered significant. GraphPad Prism was used to generate the box plots.

Oncomine Data Analysis

The expression of SRSF10 was examined in Oncomine (Rhodes et al., 2004), HNC profiles were selected for further investigation. The analyzed expression data and graphs were exported for representation.

Survival Data

Disease-free survival analysis was done using The Cancer Genome Atlas (TCGA) dataset on the GEPIA 2.0 online platform (Gene Expression Profiling Interactive Analysis). A survival curve was generated for patients across all HNSC subtypes using disease-free survival analysis. The patients were divided into high and low SRSF10 cohorts by keeping 30 and 70% as the cutoff value, respectively.

Overall survival analysis was performed by extracting clinical information of HNC patients from the GSE26549 dataset. The samples were divided into SRSF10_high and SRSF10_low group based on the mean of SRSF10 expression across all samples. Survival curve analysis was then performed using Log Rank test in GraphPad Prism (La Jolla, CA, United States).

RNA Interference

The BICR10 and H157 HNC cells were infected with the lentivirus containing small hairpin RNA (shRNA) purchased from Sigma (Saint Louis, United States) and specific to SRSF10 (sh SRSF10) and eGFP (sh control) using 8 μg/ml polybrene containing media. HNC cells were selected with 0.8 μg/ml puromycin for 2 days. Post selection with puromycin cells was used for further experiments.

Oligo Sequence of shRNAs

shRNA	Sequence
sheGFP	5′-CCGGTACAACAGCCACAACGTCTATCTCGAGATAGAC GTTGTGGCTGTTGTATTTTT-3′
shSRSF10_1	5′-CCGGGCCGAAGTTATGAAAGGAGGACTCGAGTCCTC CTTTCATAACTTCGGCTTTTTG-3′
shSRSF10_2	5′-CCGGCGGCGTGAATTTGGTCGTTATCTCGAGATAAC GACCAAATTCACGCCGTTTTTG-3′
shEGR1_1	5′-CCGGCATCTCTCTGAACAACGAGAACTCGAGTTCTC GTTGTTCAGAGAGATGTTTT-3′
shEGR1_2	5′-CCGGCTGTCTACTATTAAGGCCTTTCTCGAGAAAGG CCTTAATAGTAGACAGTTTTT-3′

Genomic DNA Isolation

Genomic DNA was isolated from BICR10 cells using mammalian genomic DNA isolation kit (Sigma, G1N70) and according to the manufacturer's instruction.

Cloning

The fragments of SRSF10 promoter region were generated by PCR using human genomic DNA as a template and subcloned into the *Nhe*I and *Hin*dIII sites of pGL3-Basic vector. The human genomic DNA was isolated from BICR10 cell lines. SRSF10 promoter fragments from −1,153 to +333, −922 to +333, −333 to +333, −200 to +333, −100 to +333, and +30 to +333 were amplified by using the primers as listed in **Supplementary Table 1**. PCR conditions is as follows: pre-degeneration for 3 min at 95°C, denaturation for 30 s at 95°C, annealing at 58°C for 30 s and extension at 72°C for 7 min. PCR reactions were carried out for 35 cycles, and PCR products were visualized in 1% agarose gels containing ethidium bromide under UV transillumination.

The PCR product and PGL3-Basic vehicle plasmid were digested with restriction enzyme *Hin*dIII (Takara bio science, 1615) and *Nhe*I (Takara bio science, 1622, 1241A) at 37°C for 2 h. The fragment of PCR product and PGL3-Basic vehicle plasmid was mixed with 1 μl T4 ligase buffer and 1 μl DNA ligase (New England Biolabs, M0202S) and added water to make up the volume up to 20 μl incubated at 22°C for 1 h and then transformed into *E. Coli*. The pGL3-SRSF10 promoter containing vector plasmid was extracted, and all constructs were verified by DNA sequencing.

The EGR1 overexpression plasmid was constructed by amplifying a 1.632 kb EGR1 fragment from BICR10 cDNA using Phusion high fidelity DNA polymerase (NEB, M053) and EGR1 primers (**Supplementary Table 1**). The product was cloned between the sites *Eco*RI (Takara bio science, 1611) and *Xho*I (Takara bio science, 1635) pFLAG-CMV 1a expression vector (Agilent Technologies, Santa Clara, CA, United States).

Luciferase Assay

BICR10 cells (5×10^4) were seeded in a 24-well plate and incubated in CO_2 incubator for 12 h. The cells were transfected with different SRSF10 promoter-luciferase construct as well as pRL-TK Renilla luciferase plasmids and again incubated in CO_2 incubator for 48 h. The cells were lysed with freshly prepared (150 μl/well) of passive lysis buffer. Transferred the 50 μl of each lysate into the wells of a white 96-well assay plate. Add 50 μl of luciferase lysis buffer per well and then incubated at room temperature (RT) for 2 min with shaking. The firefly luciferase activity was measured in a GloMax Multi Detection System (Promega). The relative luciferase activity can be determined by dividing the firefly luciferase activity with the Renilla luciferase activity. The relative values are represented as mean ± SD of triplicate values from a representative experiment.

Cell Viability/MTT Assay

BICR10 and H157 cells (2×10^6) were seeded in six-well cell culture plates. After 24 h, sh_SRSF10 and sh_control was transfected, and then selected the transfected cells with puromycin. After selecting these transfected cells with puromycin, the cells were seeded in 96-well culture plated (3×10^3/well) for 12, 24, 36, 48, and 72 h (in triplicate for each condition). The 20 μl of MTT (Sigma, M2128) stock solution (2 mg/ml) was added to each well in addition with 100 μl

media and incubated in a CO_2 incubator for at least 2 h. After incubation, the formazan crystals formed from MTT tetrazolium salt were solubilized using dimethyl sulfoxide. The numbers of viable cells were calculated by measuring the optical density using plate reader BioTek Eon (BioTek, Winooski, United States).

Wound Healing Assay

After selecting these transfected cells with puromycin, the cells were seeded in a 12-well plate, and upon reaching 100% confluence, the wound was created using a sterile 200 μl pipette tip and washed with 1XPBS two times to remove cell debris. The wounded area was marked in each well on the bottom of plates, and images were captured at 0, 12, 24 h with an inverted microscope. The wound width was measured using Image J software.

Invasion Assay

After the puromycin selection, 2×10^4 cells were then added to an upper chamber of a transwell (Corning, NY, United States) above a Matrigel layer (Corning, Bedford, MA, United States) and incubated for the next 24 h in a CO_2 incubator. The cells migrated to the lower chamber of transwell were then fixed in 4% paraformaldehyde solution and then stained with 0.05% crystal violet solution, and images were taken using an inverted microscope (Olympus, Tokyo, Japan).

Colony-Forming Assay

After puromycin selection, 1×10^3 cells were seeded in the fresh six-well cell culture plate and were maintained in 0.5 μg/ml puromycin-containing media for 12 days. Cells were then fixed using methanol and acetic acid (3:1) for 5 min and then washed with 1× PBS. Cells were then stained with 0.05% crystal violet for 30 min. Then, cells were washed with 1× PBS, and plates were dried for 30 min at room temperature and scanned. Colonies were counted using ImageJ software (La Jolla, CA, United States).

Immunoblotting

Cells were pelleted and lysed with urea lysis buffer (8M Urea: Sigma IU5378, 2M Thiourea: Sigma 11149, 10% CHAPS: Sigma C9426, 10% Dithiothreitol: Sigma D9779) and kept at 4°C for 30 min. After incubation, the lysate was centrifuged at 14,000 rpm for 2 h, and the supernatant was collected in a 1.5 ml microcentrifuge tube and stored at –80°C. The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The protein-containing PVDF membrane was blocked with 5% non-fat milk in tris buffered saline with 0.05% Tween 20 (TBST) for 30 min. The membrane were then probed with following primary antibodies: anti-SRSF10 (Sigma, HPA053831), anti-pERK (CST, 9101S), anti-ERK (CST, 9102S), anti-EGR1 (CST, 4154S), anti-flag (Novus Biologicals, NBP1-06712SS), anti-PKM2 (CST 4053S), anti-PKM1 (CST 7067S), and anti-GAPDH (CST 5174S). Anti-GAPDH was used as loading controls for protein assays. After 2 h of incubation with primary antibody at RT, membranes were then washed with 1× TBST then again incubated with secondary antibodies for 45 min at RT. The probed PVDF membranes were washed with TBST, and the bands were visualized using an odyssey membrane scanning system (Li-cor Biosciences, Bad Homburg, Germany).

Hydroxymethylation Dependent Immune Precipitation

Genomic DNA was isolated from BCR10 cells using genomic DNA isolation kit, and hydroxymethylation dependent immune precipitation (hMeDIP) assay were performed as per the protocol previously (Singh et al., 2017). Briefly, genomic DNA was first sonicated, and 3 μg of the sonicated DNA was incubated overnight at 4°C with 5-hydroxy-methyl cytosine antibody (Sigma, MABE251) and normal mouse IgG antibody (Calbiochem, NI03). 5% input and immunoprecipitated fractions were analyzed by qRT-PCR in duplicate using the SYBR Green master mix (Promega, A6002) and specific primers (**Supplementary Table 1**) across the promoter regions. Normalization was performed with input. Resultant values were then normalized relative to the mouse Ig control IP values for the primer set, and the student's *t*-test was used to identify the significance between two different groups. $p < 0.05$ was considered statistically significant.

Chromatin Immune Precipitation

Chromatin immune precipitation (ChIP) assay was performed as described previously (Singh et al., 2017). Briefly, the chromatin was sonicated, and about 25 μg of chromatin was immunoprecipitated using antibody of interest, followed by overnight incubation at 4°C. The following antibodies were used for ChIP: Anti-EGR1 (CST, 4154S), Anti TET1 (Novus, 1462), and Normal rabbit IgG (CST, 2729S). Immunoprecipitated fractions and 5% input were analyzed by quantitative real-time PCR in duplicate using the SYBR Green Master mix (Promega, A6002) and specific primers (**Supplementary Table 1**) across the promoter regions.

Lactate Assay

The BICR10 cells (3×10^5) were infected with lentivirus containing shRNA specific for SRSF10 gene in 6 well cell culture plates, and after 4 days, lactate assay was performed according to the manufacturers instruction. Briefly, an equal number of cells were homogenized in the presence of lactate assay buffer provided in lactate assay kit (Sigma, MAK064) and centrifuged at 13,000 *g* for 10 min. Lactate assay was then performed in 96-well plate, and lactate levels were measured with a plate reader at an optical density of 570 nm.

Glucose Uptake Assay

The BICR10 cells (3×10^5) were infected with lentivirus containing shRNA specific for SRSF10 gene in 6 well cell culture plates, and after 4 days glucose assay was performed according to the manufacturers instruction. Briefly, an equal number of cells were homogenized in the presence of glucose assay buffer provided in glucose uptake assay kit (Sigma, MAK083) and centrifuged at 13,000 *g* for 10 min. Glucose assay was then performed in a 96-well plate, and glucose levels was measured with a plate reader at an optical density of 570 nm.

Caspase 3/7 Assay

The BICR10 cells (3×10^5) were infected with lentivirus containing shRNA specific for SRSF10 gene in 6 well cell culture plates, and after 4 days, caspase activity was measured. In another set of experiment, post puromycin selection, 4 days later the cells were treated with 30 μM concentration of z-VAD-FMK pan-caspase inhibitor (Sigma, V116) after 24 h the caspase activity was measured. The caspase 3/7 activation was measured using the caspase 3/7 assay kit (Sigma, CASP3F) recommended by the manufacturer. Luminescence readings were taken using a Glomaz multi detection system (Promega).

Statical Analysis

Statistical analyses were performed using the GraphPad Prism5 (La Jolla, CA, United States). In the bar graph, Student's *t*-test was used to compare the differences between the two groups. The differences were considered statistically significant with the $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and non-significant difference ($p > 0.05$).

RESULTS

The Upregulation of the Splicing Factor SRSF10 in HNC Patient Samples Is Inversely Correlated With HNC Patient Survival

SRSF10 has been reported to function as a splicing activator in a sequence-specific manner (Zhou et al., 2014a). Here we selected various independent HNC cohorts from the GEO database (Barrett et al., 2013) and analyzed them for the altered expression of all the members of SR family splicing factor. We observed deregulation of a few of SR family members; among them, the SRSF10 was commonly up-regulated in all the HNC cohorts (**Figures 1A–C**). Further, to support our preliminary analysis, we analyzed the HNC profiles available in the Oncomine (Rhodes et al., 2004) and observed the upregulation of SRSF10 (**Supplementary Figures 1A–K**) in tumor tissues as compared to normal tissue obtained from the HNC patients. Next, we validated these *in silico* analysis in the HNC tissue samples obtained from the HNC patients receiving treatment at the Bansal Hospital, Bhopal, and observed the increased SRSF10 level by immunoblotting in HNC patient's tumor tissues as compared with the paired normal (**Figure 1D** and **Supplementary Figure 1L**). The immunoblotting included HNC tissue samples and corresponding normal tissues from 15 HNC patients. Statistical analysis showed that SRSF10 was significantly up-regulated in the HNC cancer patient samples compared with the paired normal tissues (**Figure 1E**). Moreover, the survival analysis using GEPIA 2.0 online platform (Gene Expression Profiling Interactive Analysis) with TCGA dataset (**Figure 1F**) showed that patients with low levels of SRSF10 expression had significantly longer survival than patients with higher SRSF10 expression (**Figure 1F**), this data includes patients with follow-up data till 200-months. We observed the similar results with Kaplan–Meier overall survival analysis using GEO dataset (GSE26549; **Supplementary Figure 1M**), where we used the patients with follow-up data till 10-months.

Together these observations strongly indicate that overexpression of SRSF10 was closely associated with the poor survival in HNC patients and also indicates that SRSF10 can act as an oncogenic driver in HNC. Thus, it highlights the irresistible need to identify the regulatory mechanism that underlies the increased SRSF10 level in HNC.

EGR1 Mediated Hydroxymethylation Leads to Increased SRSF10 Expression in HNC

The function of splicing factors is regulated either by affecting their post-translational modifications or affecting the expression level, thus modulating their target gene's splicing. Here in this study, we investigated the mechanism involved in the expression of SRSF10, the promoter region of the SRSF10 was dissected into a series of deletion fragments and constructed in a pGL3 basic vector, termed as pGL3-1153, pGL3-922, pGL3-333, pGL3-200, pGL3-100, and pGL3+30. The luciferase reporter assay was performed to detect the transcriptional activity of the fragments. In comparison to the pGL3-basic vector, the luciferase activity in all the constructs was increased, and the fragment –200 to +30 bp exhibited to decrease in the luciferase activity (**Figure 2A**), indicating the presence of several possible positive regulatory elements in this segment of the SRSF10 promoter, absence of which could diminish the expression of SRSF10. However, the screening of the fragment −200 to +30 bp highlights the presence of EGR1 (**Figure 2B**). Next, we analyzed the HNC profiles using Oncomine platform (**Supplementary Figures 2B,C**) as well as the HNC profiles available in the GEO database (**Figure 2C**) and observed the upregulation of (Early Growth Response1) EGR1 in HNC tumor tissues as compared to normal tissue. Interestingly, we also observed that the downregulation of EGR1 suppresses the expression of SRSF10 at the protein level (**Figure 2D**). To further understand the role of EGR1 in SRSF10 expression, we performed the ChIP using EGR1 antibody and primers specific to SRSF10 promoter fragment −200 to +30 (**Supplementary Figure 2A**). We observed the decrease in EGR1 enrichment at the SRSF10 promoter region in sh_EGR1 transfected cells in comparison to the sh_control HNC cell (**Figure 2E**). The earlier reports explain the regulatory mechanism with EGR1 mediated increase in the target genes expression as EGR1 mediated recruitment leads to the hypomethylation (hydroxymethylated) of the sites in neuronal cells (Sun et al., 2019). Therefore, we hypothesized that EGR1 leads to the increased SRSF10 expression via recruiting the TET1, which demethylate or hydroxymethylated the CpG moiety in the promoter region of SRSF10. To further understand, we performed the hMedIP experiment using the 5hmC antibody in sh_EGR1 transfected cells in comparison to sh_control cells and observed the significant downregulation of hydroxymethylation at the promoter region of SRSF10 (**Figure 2F**). Along the line to validate our hypothesis that EGR1 mediated recruitment of TET1 is responsible for the SRSF10 promoter demethylation (hydroxymethylation). We performed ChIP using TET1 antibody and observed the decrease in TET1 enrichment at the SRSF10

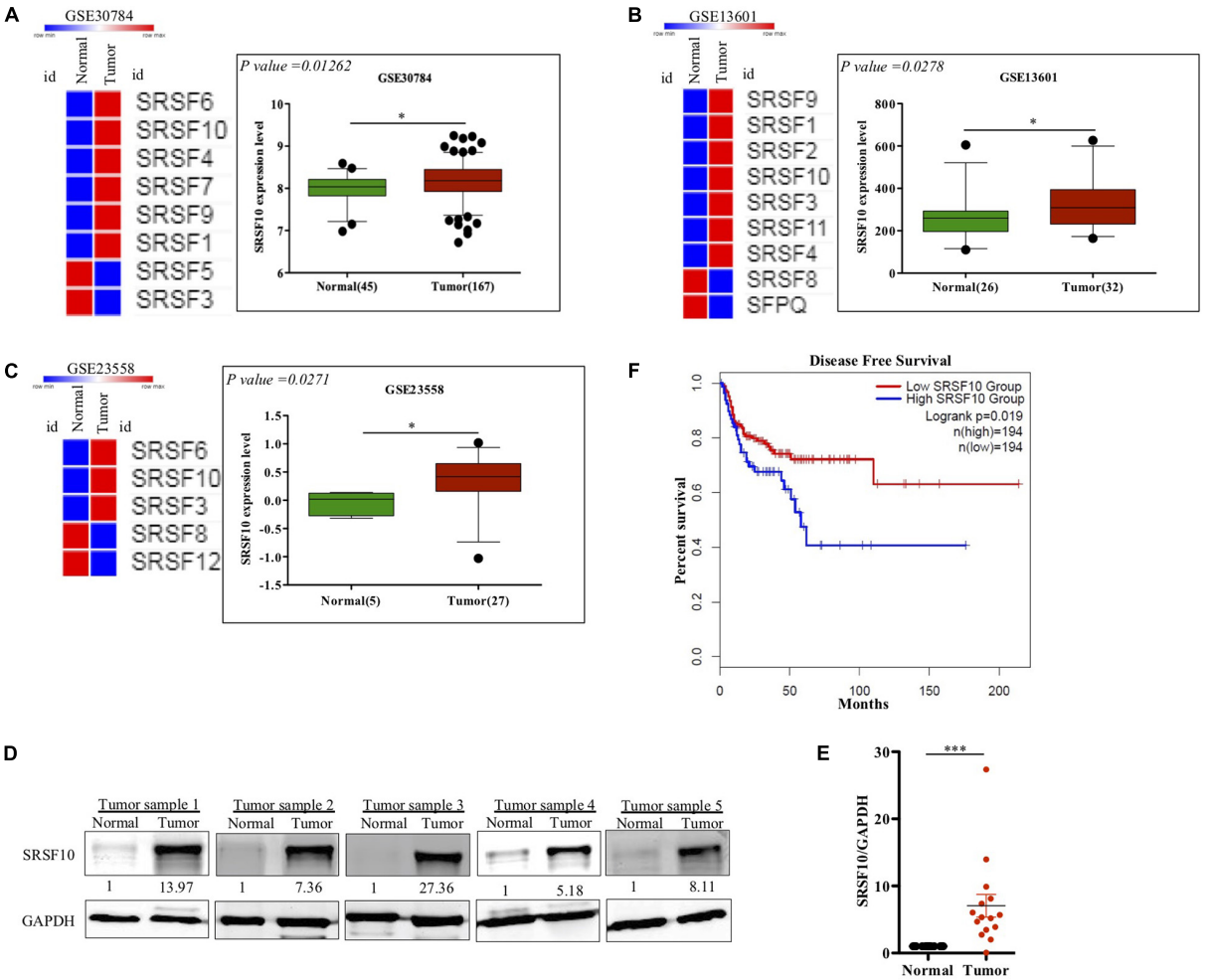

FIGURE 1 | Clinical relevance of SRSF10 expression in head and neck cancer: **(A–C)** Heat map of SR splicing factor proteins in head and neck cancer profiles (analyzed using GEO database) and the expression analysis of SRSF10 in the HNC profiles downloaded from GEO database, **(A)** GSE30784, **(B)** GSE13601, **(C)** GSE23558, and **(D)** Immunoblotting showing the SRSF10 expression at the protein level in 15 head and neck cancer patient samples and the paired normal (also see the **Supplementary Figure 1L**), **(E)** Quantification of SRSF10 immunoblots in 15 head and neck cancer patient samples and the paired normal which is normalized to GAPDH, **(F)** Kaplan–Meier curve showing significant association ($p = 0.019$) of disease-free patient survival with SRSF10 expression in TCGA dataset. GEO, Gene Expression Omnibus; GSE, Genomic Spatial Events. Error bars show the mean values ± SD and the differences were considered statistically significant with $^{*}P < 0.05$ and $^{***}P < 0.001$, ns non-significant ($P > 0.05$).

promoter region in sh_EGR1 transfected cells compared to sh_control HNC cell (**Figure 2G**).

Additionally, the upregulation of EGR1 is shown to be associated with the activation of the ERK/MAPK signaling pathway (Gregg and Fraizer, 2011). Next, to examine the role of the ERK/MAPK signaling pathway in the EGR1 to SRSF10 axis, we used a chemical compound purchased from the library of pharmacologically active compounds that target and inhibit the ERK signaling pathway. Interestingly, with the inhibition of the ERK/MAPK signaling pathway, we observed significant decrease in EGR1 expression as well as the decreased SRSF10 expression in an immunoblot analysis (**Figures 3A,B**), which was further confirmed by the luciferase reporter assay (**Figure 3C**). Further, to rule out whether the final effect of ERK/MAPK inhibition on SRSF10 expression is via EGR1 mediated demethylation at the SRSF10 promoter region. We performed the hMedIP experiment using 5hmC antibody and observed the significant decrease in hydroxymethylation in ERK inhibitor-treated sample in comparison to control (**Figure 3D**). Additionally, In order to validate the dependency of SRSF10 expression on EGR1, we overexpressed EGR1 in ERK inhibitor-treated BICR10 cells. The overexpression of EGR1 in ERK inhibitor treated cells rescued the expression of SRSF10 in ERK inhibitor_EGR1 cells as compared to ERK inhibitor_EV control cells (**Supplementary Figure 3A**). In continuation, we also examined the level of the hydroxymethylation level at SRSF10 promoter in ERK inhibitor_EGR1 samples and observed that hydroxymethylation was regained significantly by EGR1 overexpression in ERK inhibitor-treated cells in comparison to ERK inhibitor_EV cells (**Figure 3E**).

Furthermore, we also investigated the role of SRSF10 in tumorogenic properties in HNC cells using two independent

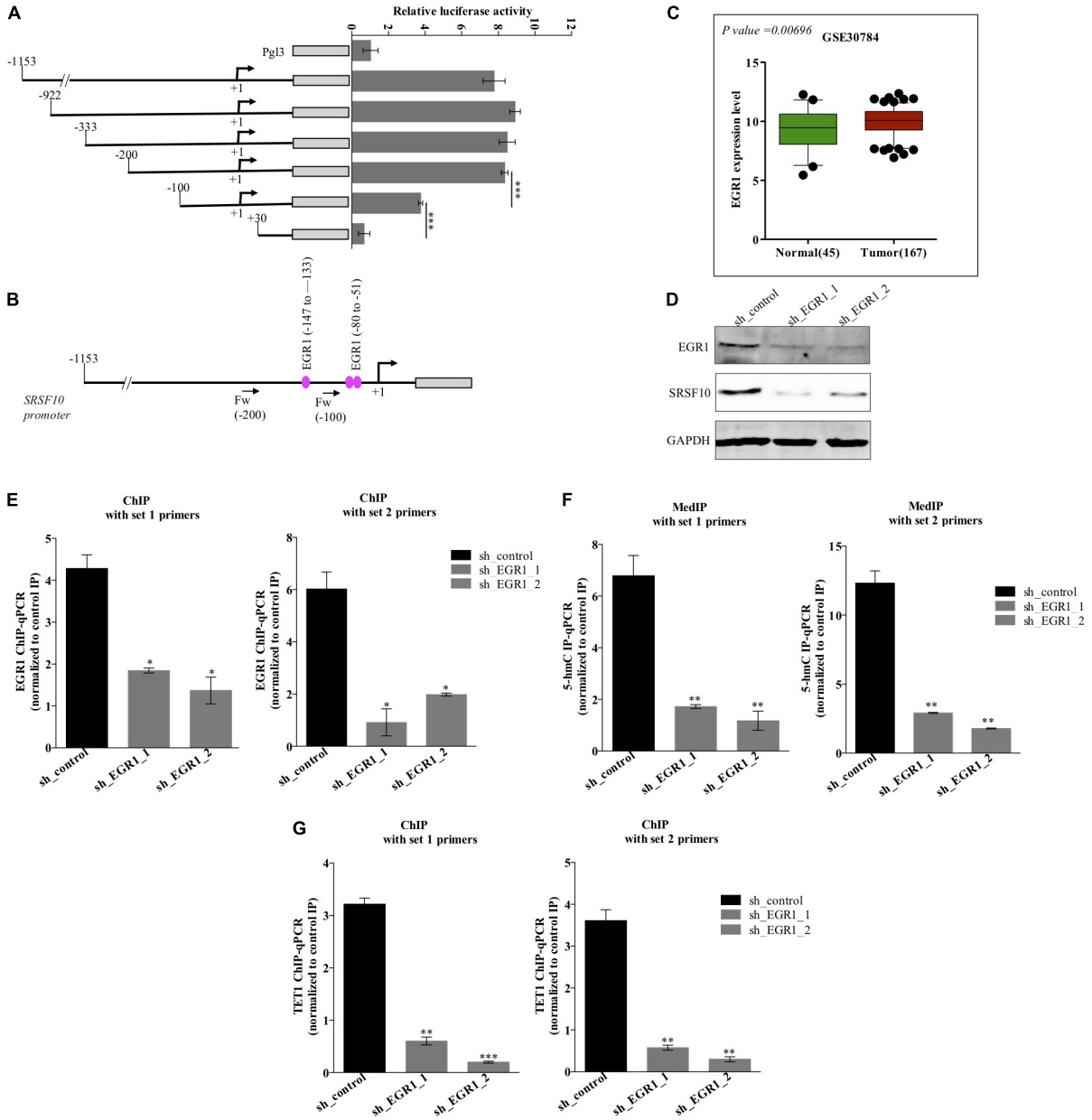

FIGURE 2 | Level of EGR1 regulates the expression of SRSF10 in HNC cells BICR10: **(A)** Luciferase assay with the deletion construct of SRSF10 promoter, **(B)** schematic representation showing the binding position for EGR1, **(C)** the expression analysis of EGR1 in the HNC profiles downloaded from GEO database (GEO30784), **(D)** Immunoblot showing the protein level of EGR1, SRSF10 in sh_EGR1 transfected cells versus sh_control in BICR10 cells, GAPDH act as a loading control. **(E)** EGR1-ChIP performed in sh_EGR1 transfected BICR10 cells in comparison to sh_control using indicated primers for SRSF10 promoter region, **(F)** hMedIP experiment performed in sh_EGR1 transfected BICR10 cells in comparison to sh_control using indicated primers for SRSF10 promoter region, **(G)** TET1-ChIP performed in sh_EGR1 transfected BICR10 cells in comparison to sh_control using indicated primers for SRSF10 promoter region. Error bar represents the mean values ± SD. Differences were considered statistically significant with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

shRNAs targeting SRSF10. Immunoblotting confirmed the sh_SRSF10 mediated SRSF10 depletion in comparison to sh_control in HNC cell lines (**Figure 4A** and **Supplementary Figure 4A**). Importantly, we observed significant growth inhibition in SRSF10 depleted cells as analyzed with the MTT assay (**Figure 4B** and **Supplementary Figure 4B**). SRSF10 depletion also remarkably reduce the wound healing capacity (**Figure 4C** and **Supplementary Figure 4C**), showing the effect of SRSF10 on cell migration and proliferation. The same is evident in the transwell cell migration assay, performed to analyze the effect of SRSF10 on single-cell motility. We analyzed that SRSF10 depletion reduced the number of invaded cells significantly in sh_SRSF10 transfected cells in comparison to sh_control cells (**Figure 4D**). Sequentially, to examine the role of SRSF10 in colony-forming capacity of HNC cells, we performed colony formation assay and observed decreased colony formation in

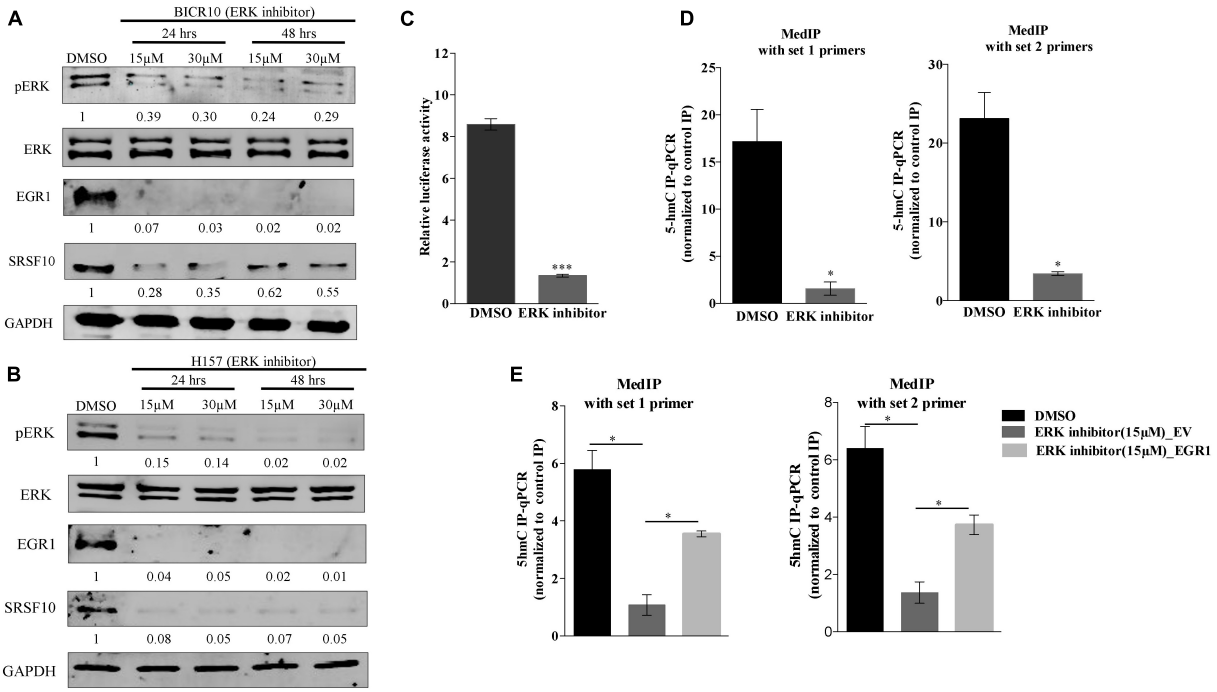

FIGURE 3 | ERK/MAPK signaling pathway regulates the EGR1 and SRSF10 expression: **(A)** Immunoblot showing the protein level of pERK, ERK, EGR1, and SRSF10 in ERK inhibitor-treated cells versus DMSO in BICR10 cells, GAPDH act as a loading control, **(B)** Immunoblot showing the protein level of pERK, ERK, EGR1, and SRSF10 in ERK inhibitor-treated cells versus DMSO in H157 cells, GAPDH act as a loading control, **(C)** Luciferase assay in ERK inhibited cells transfected with the SRSF10 promoter constructs, **(D)** hMedIP experiment performed in ERK inhibitor-treated cells versus DMSO in BICR10 cells, **(E)** hMedIP experiment performed in ERK inhibitor-treated cells versus ERK inhibitor-treated cells proceed by EGR1 overexpression in comparison to DMSO control in BICR10 cells using indicated primers for SRSF10 promoter region. Error bar represents the mean values ± SD. Differences were considered statistically significant with $^{*}P < 0.05$ and $^{***}P < 0.001$.

SRSF10 depleted BICR10 HNC cells in comparison to control cells (**Figure 4E**).

These results show that SRSF10 affects the proliferation, wound healing, invasion, and colony-forming capacity of HNC cells, and thus SRSF10 overexpression in HNC might play a critical role in HNC oncogenesis. More importantly, the overexpression of SRSF10 is under control of ERK/MAPK-EGR1 axis.

SRSF10 Upregulation Promotes HNC Progression by Favoring Cancer-Related Splicing Variants in HNC

SRSF10 was observed to play a critical role in myoblast differentiation (Wei et al., 2015) and adipocyte development (Li et al., 2014) via controlling the splicing of the critical genes, while the role of SRSF10 in HNC carcinogenesis is not yet clear. It has been shown earlier that the adipogenic defects caused by SRSF10 deficiency in mouse embryonic fibroblast, and the RNA seq data showed role of SRSF10 in mediating (pyruvate kinase M) PKM pre-mRNA splicing (Li et al., 2014). In continuation, another report explains SRSF10 mediated regulation of (BCLx apoptosis regulator) BCLx pre-mRNA splicing (Shkreta et al., 2016). These two studies caught our attention as we are aware that these two SRSF10 targets reported in the two different studies, are functionally associated with the cancer progression as the BCLx pre-mRNA splicing is related to the apoptosis of cells (Adams and Cory, 2007) and PKM pre-mRNA splicing is associated with the Warburg effect (Christofk et al., 2008).

Next, we validated the expression of BCLx isoforms (**Supplementary Figure 3B**) and PKM isoforms (**Supplementary Figure 3C**) in the HNC tissue samples obtained from patients under treatment at the Bansal Hospital, Bhopal, and we observed the higher PKM2 and low PKM1 expression in HNC tumor tissue samples in comparison to paired normal tissue samples at the RNA level (**Figure 5A**). Similarly we observed higher BCLxL (cancer-specific isoform) and low BCLxs (normal isoform) expression (**Figure 5B**) in HNC tumor tissue samples in comparison to paired normal tissues at the RNA level. Further, as we know, the PKM2 isoform of the PKM gene is associated with the Warburg effect (Rajala et al., 2016), and an increase in the Warburg effect is indicated with the increase in lactate production and glucose uptake (Heiden et al., 2009). Thus, we hypothesized that SRSF10 regulates the PKM splicing favoring the PKM2 isoform, leading to the cancer progression via the Warburg effect. Next, we examined the effect of SRSF10 downregulation on lactate production and glucose uptake and observed the lower lactate production (**Figure 5C**) and decreased glucose uptake (**Figure 5D**) with the SRSF10 depletion as we expected. Similarly, the BCLx gene is associated

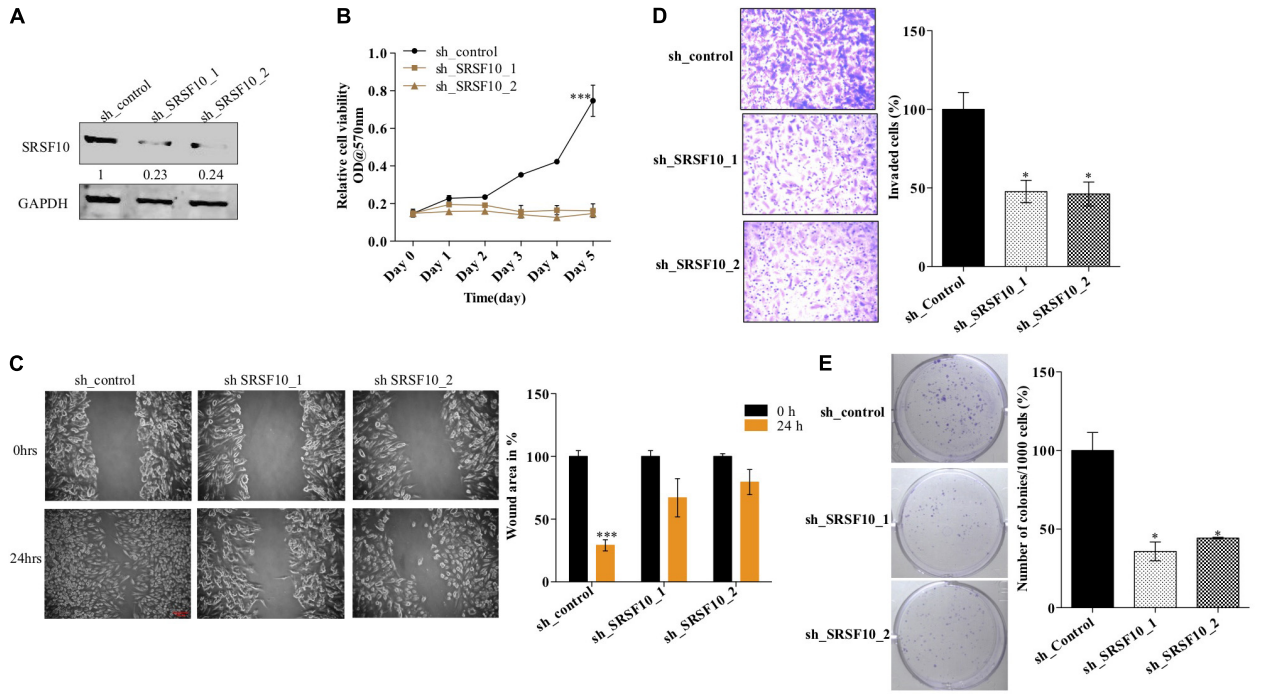

FIGURE 4 | SRSF10 affects the proliferation in head and neck cancer cell BICR10: SRSF10 expression was depleted in HNC cell lines, and differential proliferation status of these cells was analyzed, **(A)** Immunoblot showing the protein level of SRSF10 in sh_SRSF10 transfected cell in comparison to sh_control cells, GAPDH acts as a loading control, **(B)** relative cell proliferation was analyzed through MTT assay, **(C)** cell migration was analyzed through wound healing assay, **(left)** wound was observed under the microscope and the **(right)** quantification of wound width, **(D)** cell invasion assay in BICR10 cells, **(left)** representative image of invasion assay **(right)** quantification of cell count in different groups, **(E)** clonogenic assay in BICR10 cells, **(left)** representative image of clone forming cells, **(right)** quantification of cloning efficiency of BICR10 cells. Error bar represents the mean values ± SD. Differences were considered statistically significant with $^{*}P < 0.05$ and $^{***}P < 0.001$.

with the apoptotic pathway, and here we examined the effect of SRSF10 downregulation on apoptosis via measuring the caspase activity using caspase assay and observed the increase in caspase activity with the SRSF10 depletion (**Figure 5E**). To rule out the promotive effect of SRSF10 downregulation in caspase assay is via BCLx pre-mRNA splicing switch from anti-apoptotic BCLxL to pro-apoptotic isoform BCLxs mediated caspase activation. We used a well-known zVAD-FMK pan-caspase inhibitor, and treated the sh_SRSF10 transfected cells, then measured the caspase activity using caspase assay (**Figure 5F**). We observed significant decrease in the caspase activity in pan caspase inhibitor treated sh_SRSF10 transfected cells in comparison to the sh_SRSF10_DMSO treated cells.

These observations suggest the oncogenic role of SRSF10 may partially be explained by its effect on BCLx and PKM splicing switch, which affects the Warburg effect and apoptosis and thus the growth of HNC cells.

Next, to examine the SRSF10 occupancy on BCLx and PKM gene, we performed RNA immune precipitation using the SRSF10 antibody in HNC cells, and interestingly we observed the SRSF10 enrichment at PKM RNA (**Figure 6A** and **Supplementary Figure 5A**) and BCLx RNA (**Figure 6B**). Further, we explored the role of SRSF10 in PKM (**Figures 6C,D**) as well as BCLx (**Figures 6E,F**) pre-mRNA splicing. We depleted the SRSF10 in HNC cells, where we observed the switch in splicing from cancer-specific isoform to normal isoform in SRSF10 down-regulated cells in comparison to the control cells at the mRNA and protein level (**Figures 6C–F** and **Supplementary Figures 5B–E**). We also observed the splicing switch from cancer-specific isoform to normal isoform in EGR1 depleted cells (**Supplementary Figures 5F,G**). These results collectively support the hypothesis that EGR1 plays an important role in the oncogenic effect of SRSF10 in HNC by regulating the splicing of its target genes which are known to be associated with cancer progression.

Together, these results suggest the ERK1/2-EGR1-SRSF10 axis in the generation of PKM2 and BCL-xL, cancer-specific splice isoforms as shown in the schematic diagram (**Figure 6G**).

DISCUSSION

Alternative splicing is a highly regulated process that contributes to the proteome diversity in eukaryotic organisms. The process of AS is found to be deregulated in cancer which, in turn, favors the tumor progression (Ladomery, 2013). Expression of cancer-specific isoforms of various genes in cancer cells is majorly due to epigenetic modifications at the gene locus (Singh et al., 2017) as well as due to the deregulation of splicing factors which include the SR protein family and HnRNP family members

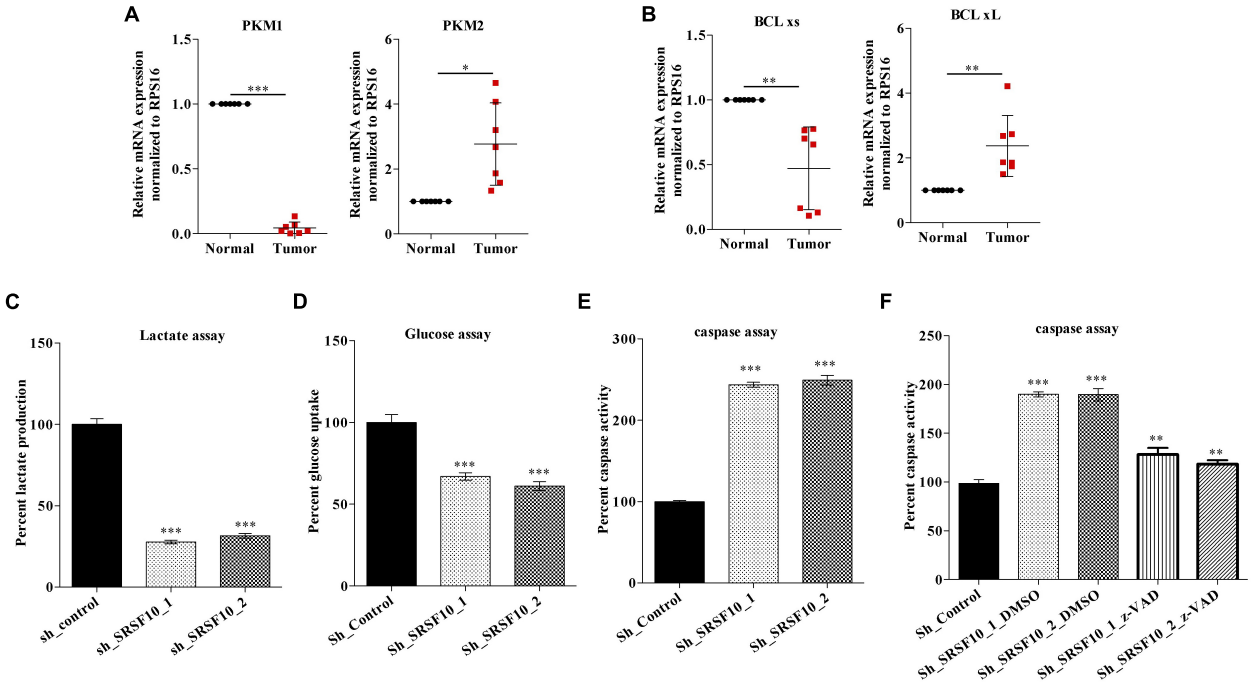

FIGURE 5 | Clinical relevance of PKM and BCLx gene and effect of SRSF10 depletion on the tumorogenic potential of HNC: **(A,B)** RPS16 normalized qRT-PCR in paired normal and tumor HNC patient's samples using the splicing primers for **(A)** PKM genes and **(B)** BCLx gene. **(C,D)** Percentage of decreased **(C)** lactate production and **(D)** glucose uptake in sh_SRSF10 transfected cells in comparison to sh_control cells, **(E)** Percentage of increase in caspase activity in sh_SRSF10 transfected cells in comparison to sh_control cells, **(F)** Percentage of caspase activity in sh_SRSF10 transfected cells in comparison to sh_control and effect of pan caspase inhibitor (z-VAD-FMK) in BICR10 cells. Three independent experiments were conducted, and the representative data are shown here with the mean values ± SD. *P* value using two-tailed student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and ns = non-significant.

(Gupta et al., 2020). In fact, a large number of splicing factors have been reported to be deregulated in multiple cancer types, and have been found to be responsible for aberrant AS. Our study is focused on SRSF10, which is a new member of an expanded family of SR splicing factors and acts as a sequence-dependent splicing regulator (Zhou et al., 2014a). Since the characterization of the SRSF10 splicing factor in 2001 (Cowper et al., 2001), many researchers have explained the role of SRSF10 in several model systems, where they showed its importance in the developmental processes of different model systems like adipocyte development (Li et al., 2014) and myoblast development (Wei et al., 2015). While SRSF10 has been reported in different model systems including colon cancer (Zhou et al., 2014b), cervical cancer (Liu et al., 2018) for its role in the regulation of AS, its deregulation in HNC has remained to be elucidated, which is the sixth most common cancer worldwide (Parkin et al., 2005). Here in this study, we identified SRSF10 overexpression in HNC for the first time, and SRSF10 expression level is inversely related to the patient's survival as the percent survival of the SRSF10-high group was significantly lower than the SRSF10-low group.

Our study also demonstrated that the downregulation of the SRF10 reduces the cells proliferation, migration, invasive property as well as the colony-forming ability of HNC cells, these *in vitro* analysis further support that SRSF10 plays an essential role in HNC cell growth. Our study provide a strong evidence that SRSF10 directly regulates the AS of BCLx and PKM pre-mRNA as shown by RNA immune-precipitation. Further, to analyze the role of SRSF10 in PKM and BCLx pre-mRNA splicing, we examined the PKM and BCLx pre-mRNA-splicing pattern in SRSF10 depleted HNC cells and observed the splicing switch of PKM and BCLx gene from cancer-specific (PKM2 and BCLxL) isoform to normal isoform (PKM1 and BCLxs).

We then concluded that the tumorigenic effect of SRSF10 is mediated via modulating the splicing of the genes like PKM and BCLx. Interestingly, the PKM2 isoform of the PKM gene is associated with the Warburg effect or aerobic glycolysis (Christofk et al., 2008). Warburg effect is a hallmark of cancer which is characterized by increased glucose uptake and lactate production (DeBerardinis et al., 2008) where the PKM gene regulates the key step of glycolysis, and thus the splicing switch from PKM1 to cancer-specific PKM2 isoforms plays a key role in the Warburg effect (Christofk et al., 2008; Dayton et al., 2016). Similarly, the BCLx gene is associated with apoptosis where the small isoform of BCLx that is BCLxs isoform is reported to promote the apoptosis and known as pro-apoptotic, and BCLxL isoform is the long isoform of the BCLx gene and is involved in the anti-apoptotic pathway (Boise et al., 1993; Adams and Cory, 2007). Together, these results suggest that SRSF10 promotes the cancer-specific isoforms of genes like PKM and BCLx thus play a crucial role in HNC.

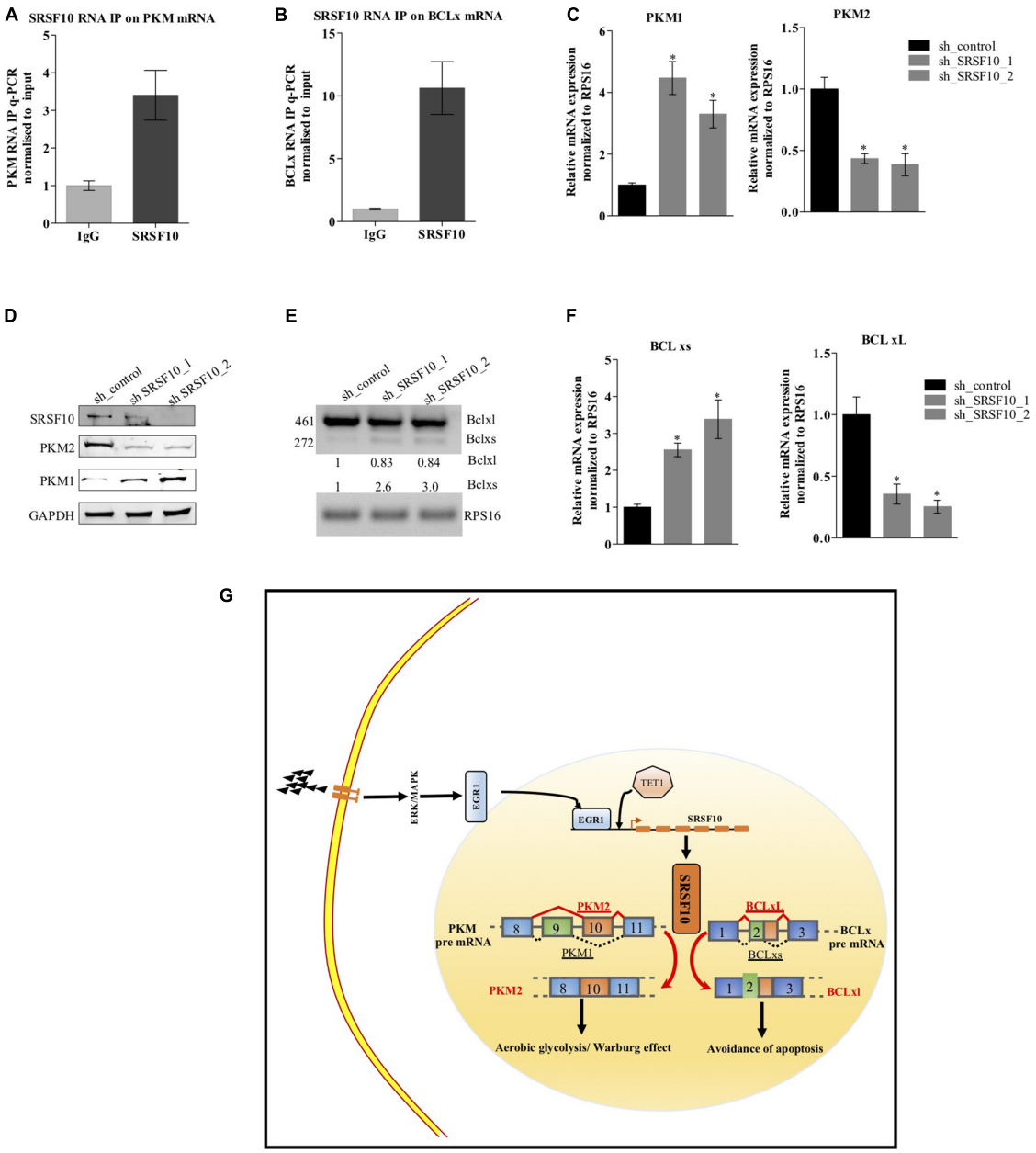

FIGURE 6 | Effect of SRSF10 downregulation on splicing of PKM and BCLx gene in BICR10 cells: **(A)** qRT-PCR performed after RIP using SRSF10 antibody with constitutive primers for PKM gene, **(B)** qRT-PCR performed after RIP using SRSF10 antibody with constitutive primers for BCLx gene, **(C)** RPS16 normalized qRT-PCR in sh_SRSF10 transfected cells in comparison to sh_control using splicing primers for PKM gene, **(D)** Immunoblot showing the protein level of SRSF10, PKM1, PKM2 in sh_SRSF10 transfected cells versus sh_control in BICR10 cells, GAPDH act as a loading control, **(E)** semi-q PCR showing the two isoforms of BCLx in sh_SRSF10 transfected cells in comparison to sh_control, **(F)** RPS16 normalized qRT-PCR in sh_SRSF10 transfected cells in comparison to sh_control using splicing primers for BCLx gene, **(G)** Schematic model. Three independent experiments were conducted, and the representative data are shown here with the mean values ± SD. *P* value using two-tailed student's *t*-test, $^*P < 0.05$ and ns = non-significant.

To date, the role of SRSF10 in different model systems has been shown but what leads to an increase in the expression of SRSF10 in tumor samples in comparison to normal is not yet clear.

In this study, we dissected the mechanism responsible for the increased expression level of SRSF10. To study the cause of increased SRSF10 expression, we performed luciferase assay with deletion constructs of the SRSF10 promoter region, which lead to the EGR1 gene, a transcription factor. Interestingly, EGR1 is shown to function as an oncogene in prostate cancer (Virolle et al., 2003), and EGR1 mediated expression of its target genes involves EGR1mediated recruitment of TET1 at the

EGR1 binding site which further promote the demethylation (hydroxymethylation), elevating the expression of the EGR1 target gene in neuronal cells (Sun et al., 2019). Interestingly, in HNC cells we observed similar regulatory mechanism of EGR1 binding at the SRSF10 promoter region and EGR1 mediated recruitment of the TET1 at the EGR1 binding site leading to demethylation or hydroxymethylation of the EGR1 site, thus increasing the expression of SRSF10. Though, our results explain the role of EGR1 as a regulator of SRSF10 expression via TET1 recruitment, but further studies will be needed to understand if the TET1 acts as a docking site for other co-factors to co-operate with EGR1.

Next, with the literature support we analyzed the role of ERK/MAPK pathway in the regulation of EGR1 expression level. We observed the decrease in EGR1 and SRSF10 expression at the protein level with the inhibition of ERK1/2 phosphorylation. The effect of ERK/MAPK inhibition on SRSF10 expression was also confirmed with luciferase activity, and the expression level of SRSF10 was rescued with the overexpression of EGR1 in ERK inhibitor-treated cells. Together, these results support the ERK/MAPK-EGR1-SRSF10 axis is crucial for HNC progression and provide an alternative strategy for drug targets.

Collectively, these results suggest the ERK 1/2-EGR1-SRSF10 axis, which could explain the SRSF10 overexpression and its regulation in line with the ERK1/2 pathway via the EGR1 transcription factor. To confirm the axis of the ERK pathway to SRSF10 via EGR1, we performed the rescue experiment with the EGR1 overexpression construct where we overexpressed the EGR1 in ERK1/2 inhibitor-treated cells and observed the increase in SRSF10 protein expression level, which indicated that the ERK1/2-EGR1-SRSF10 axis plays a role in modulating the SRSF10 targeted splicing.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/, GSE26549, GSE30784, GSE13601, and GSE23558.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute Ethics Committee, Indian Institute of Science Education and Research Bhopal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SS and SY designed the experiments, wrote the manuscript, and analyzed the data. SY performed the western blotting, luciferase assay, ChIP, MedIP, RIP, qPCR, MTT assay, wound healing, Lactate assay, Glucose assay, Caspase assay, invasion assay, colony formation assay, bioinformatics analysis, and wrote the manuscript. DP performed the Kaplan–Meier survival curve analysis. AS provided the clinical samples and histopathological information. SS, NK, and SG contributed to conceptualization, formal analysis, visualization and wrote, review, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.713661/full#supplementary-material

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REVIEW
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Crosstalk Between Inflammatory Signaling and Methylation in Cancer

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Inflammation is an intricate immune response against infection and tissue damage. While the initial immune response is important for preventing tumorigenesis, chronic inflammation is implicated in cancer pathogenesis. It has been linked to various stages of tumor development including transformation, proliferation, angiogenesis, and metastasis. Immune cells, through the production of inflammatory mediators such as cytokines, chemokines, transforming growth factors, and adhesion molecules contribute to the survival, growth, and progression of the tumor in its microenvironment. The aberrant expression and secretion of pro-inflammatory and growth factors by the tumor cells result in the recruitment of immune cells, thus creating a mutual crosstalk. The reciprocal signaling between the tumor cells and the immune cells creates and maintains a successful tumor niche. Many inflammatory factors are regulated by epigenetic mechanisms including DNA methylation and histone modifications. In particular, DNA and histone methylation are crucial forms of transcriptional regulation and aberrant methylation has been associated with deregulated gene expression in oncogenesis. Such deregulations have been reported in both solid tumors and hematological malignancies. With technological advancements to study genome-wide epigenetic landscapes, it is now possible to identify molecular mechanisms underlying altered inflammatory profiles in cancer. In this review, we discuss the role of DNA and histone methylation in regulation of inflammatory pathways in human cancers and review the merits and challenges of targeting inflammatory mediators as well as epigenetic regulators in cancer.

Keywords: cancer, inflammation, epigenetics, DNA methylation, histone methylation 2

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INTRODUCTION

Inflammation

The immune system protects the human body from different infections and can respond to cellular damage. Chiefly, the immune system plays a central role in clearing infection, healing an injury, and restoring tissue homeostasis. Inflammation is a complex immune defense response triggered to neutralize an invading infection and is characterized by redness, swelling, and pain (Coussens and Werb, 2002). Inflammation is mediated and regulated by different cytokines. Pro-inflammatory and anti-inflammatory cytokines function in an opposing manner, the former triggering the inflammatory reaction whereas the latter reduces the response. The fate of the cell depends on the balance between the pro- and anti-inflammatory immune signals. Acute inflammatory response

is beneficial to the host and a well-balanced immune response can be largely anti-tumorigenic (Yasmin et al., 2015). Chronic activation of inflammatory response is, however, linked to pro-tumorigenic conditions and cancer. Inflammation and cancer are closely linked. Individuals with chronic inflammatory diseases have a higher risk of developing cancer (Nelson et al., 2004; Garcea et al., 2005; Vagefi and Longo, 2005; Peek and Crabtree, 2006). Studies suggest that around 20% of cancers are associated with chronic inflammation that is linked to different stages of oncogenesis: cellular transformation, tumor progression, invasion, angiogenesis, and metastasis (Coussens and Werb, 2002; Mantovani, 2005; Vendramini-Costa and Carvalho, 2012).

Abbreviations: 5mC-5, methylcytosine; ADP, adenosine diphosphate; AIM2, absent in melanoma 2; ALK, anaplastic lymphoma kinase; ALK, anaplastic lymphoma kinase; AML, acute myeloid leukemia; ANXA2, annexin A2; AP1, activator protein 1; B-ALL, B-cell acute lymphoblastic leukemia; BMDC, bone marrow-derived dendritic cells; CADM1, cell adhesion molecule 1; CAF, cancer-associated fibroblast; CagA, cytotoxin-associated gene A; CBP, CREB-binding protein; CCL2, C-C motif chemokine ligand 2; CDH1, cadherin-1; CDH13, cadherin 13; CHFR, checkpoint with forkhead and ring finger domains; CoREST1, corepressor for RE1 silencing transcription factor/neural-restrictive silencing factor; COX, cyclooxygenase; CRC, colorectal cancer; CRP, C-reactive protein; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; DC, dendritic cell; DNMT, DNA methyltransferase; DTNB, dystrobrevin beta; DZNep, 3-deazaneplanocin A; EBV, Epstein–Barr virus; EEF2, eukaryotic elongation factor 2; EEF2, eukaryotic translation elongation factor 2; EGFR, epidermal growth factor receptor; EGFR, epidermal growth factor receptor; ep-CAM, epithelial cell adhesion molecule; Erk, extracellular signal regulated kinase; EWAS, epigenome wide association studies; Ezh2, enhancer of zeste 2; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; FOXM1, forkhead box protein M1; HBx, hepatitis B X protein; HCC, hepatocellular carcinoma; HPV, human papilloma virus; IFN, interferon; IL, interleukin; JAK, Janus kinase; JARID2, Jumonji, AT rich interactive domain 2; JMJD3, Jumonji domain-containing protein D3; KMT, lysine methyltransferase; KMT1B, lysine N-methyltransferase 1B; LAG3, lymphocyte-activation gene 3; LOX, lipoxygenase; LSD1, lysine-specific histone demethylase 1A; LTF, lactotransferrin; LY86, lymphocyte antigen 86; MAL, myelin and lymphocyte protein; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MCP-I, monocyte chemoattractant protein-1; MDSC, monocyte-derived suppressor cell; MEN1, multiple endocrine neoplasia type1; MGMT, O^6-methylguanine DNA methyltransferase; MHC, major histocompatibility complex; MICA, MHC class I polypeptide–related sequence A; MMP-9, matrix metallopeptidase 9; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; NK, natural killer; *NLRC5*, NOD-like receptor family CARD domain containing 5; NO, nitric oxide; NOS2, nitric oxide synthase; NSAID, nonsteroidal anti-inflammatory drug; NSCLC, non-small cell lung cancer; NTRK, neurotrophic tropomyosin receptor kinase; NTRK, neurotrophin receptor kinase; OSCC, oral squamous cell carcinoma; PAI-1, plasminogen activator inhibitor 1; PAX6, paired box 6; PcG, polycomb group; PD-L1, programmed death-ligand 1; PGE2, prostaglandin E2; PI3K, phosphatidylinositol 3-kinase; PPAR-γ, peroxisome proliferator-activated receptor gamma; PRC2, polycomb repressive complex 2; PTM, post translational modification; PTPRC, Protein Tyrosine Phosphatase Receptor Type C; PTPRR, Protein Tyrosine Phosphatase Receptor Type R; RARβ, retinoic acid receptor beta; RASSF1, Ras association domain-containing protein 1; RbBP5, RB binding protein 5, histone lysine methyltransferase complex subunit; RIPK3, receptor interacting serine/threonine kinase 3; S100A9, S100 calcium-binding protein A9; SAM, S-adenyl methionine; SETDB1, SET domain bifurcated histone lysine methyltransferase 1; Smad3, mothers against decapentaplegic homolog 3; SOCS1, suppressor of cytokine signaling 1; SRC, tyrosine-protein kinase; SSI, systematic inflammation; STAT3, signal transducer and activator of transcription 3; T reg, regulatory T cell; TAM, tumor associated macrophage; TET1, ten-eleven translocation methylcytosine dioxygenase 1; TGF, tumor growth factor; Th, T helper; TME, tumor microenvironment; TNFR2, tumor necrosis factor receptor 2; TNFs, tumor necrosis factors; TOLLIP, toll interacting protein; ULBP, UL16 binding protein 1; USP2, ubiquitin specific peptidase 2; VEGF, vascular endothelial growth factor; YAP1, yes-associated protein 1.

The process by which a normal cell is transformed into a pre-malignant cell is known as tumor initiation. The proliferation of genetically altered cells and chronic inflammation promotes tumor growth by inhibiting apoptosis and accelerating angiogenesis. Tumor progression and metastasis, which involves additional genetic changes, increased tumor size, and spreading of the tumor from the local site to different secondary sites, is influenced by inflammation. Thus, there is a close link and a continuous crosstalk between inflammation and cancer at all stages of tumorigenesis (Grivennikov et al., 2010).

Genetic and epigenetic alterations trigger transformation of normal cells to cancer cells (Baylin and Jones, 2016). Inflammatory signaling pathways that get activated in different cancers is an important connecting link between chronic inflammation and oncogenesis. The molecular circuits that lead to sustained activation of inflammatory factors are still being explored. Several epidemiological and molecular studies link cancer and inflammation. Proinflammatory cytokines including chemokines and adhesion molecules cause chronic inflammation. Proinflammatory genes like Tumor necrosis factors (TNFs) and members of its superfamily, members of interleukin family, vascular endothelial growth factor (VEGF), matrix metalloprotease 9 (MMP-9), 5-lysyl oxidase (5-LOX), and cyclooxygenase-2 (COX-2) are important players of apoptosis, angiogenesis, proliferation, invasion, and metastasis (Yasmin et al., 2015). Many signaling pathways, including IL-6/STAT3 (interleukin-6/signal transducer and activator of transcription 3), play crucial roles in cancer initiation and progression. Inflammatory cytokines like IL-6 and interferons (IFNs) activate STAT3 and induce its translocation to the nucleus, where it binds to specific regulatory sites to activate gene expression. In oncogenic conditions, STAT3 is constitutively activated leading to sustained expression of its downstream targets, which are involved in cell proliferation, invasion, and differentiation (Yu et al., 2009, p. 3). Thus, persistent activation of inflammatory mediators can cause tumor progression and may be triggered by events of aberrant epigenetic changes.

Epigenetic alterations are essential hallmarks to cancer initiation and progression. However, what triggers epigenetic changes in cancer is still being investigated. Mechanistic insights of regulation of inflammatory signaling by epigenetic alteration need an in-depth exploration to design effective therapeutic targets for different cancers. These targets include various mediators of inflammatory networks.

Markers and Mediators of Inflammation

The inflammatory response to infection or injury comprises a whole host of immune cells, secreted factors, signaling pathways, and markers. It is important to identify the major markers, mediators, and orchestrators of this intricate network, and how they relate to each other in order to appreciate the nature and complexity of the epigenetic control of the network (**Figure 1**).

Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha (TNFα) is a member of the tumor necrosis factor (TNF) family and a major signaling molecule involved in the inflammatory network. It is a potent cytokine

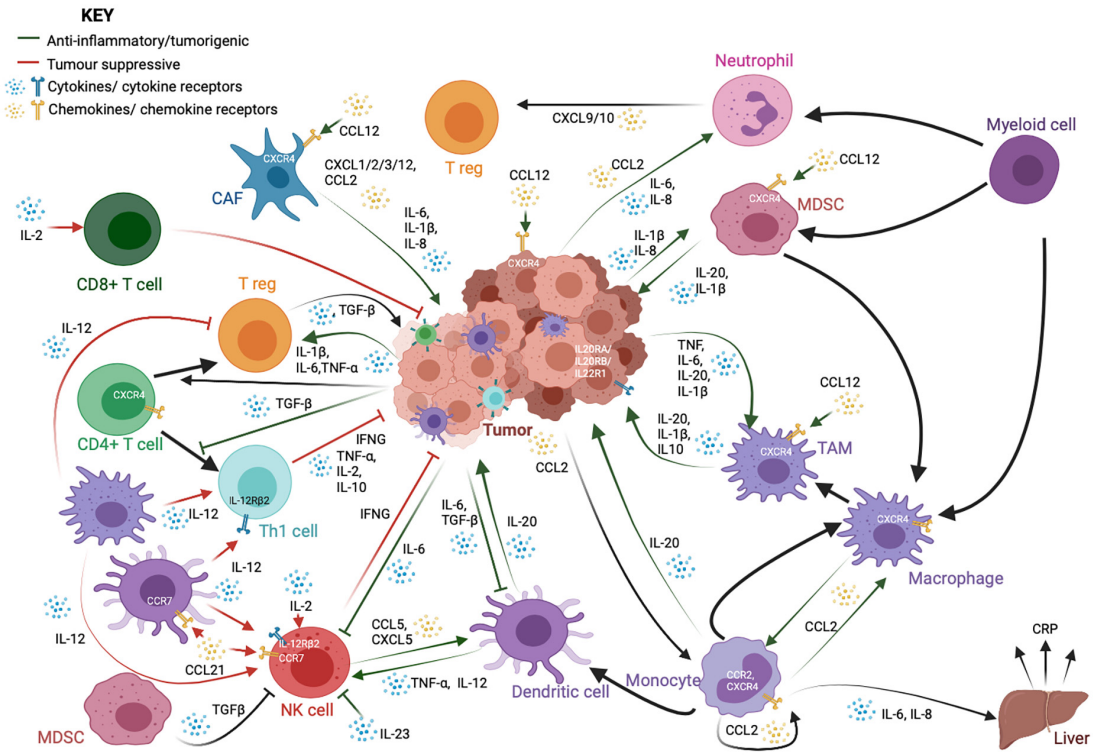

FIGURE 1 | A schematic showing the complex interactions between cancer cells and immune cells, particularly those in the tumor microenvironment. While immune cells, such as cytotoxic $CD8^+$ T-cells, T helper (Th1) and natural killer (NK) cells, are important for anti-tumor responses and cancer cell clearance, tumors and cancer-associated fibroblasts (CAFs) often employ a variety of signaling molecules that result in the dysregulation of a variety of both lymphoid and myeloid-derived immune cells. The crosstalk between these cells further dampens the anti-tumor response and exacerbates oncogenic phenotypes such as cancer cell proliferation and metastasis. This signaling network is reliant upon a whole host of cytokine and chemokine ligands and their receptors. These cytokines and chemokines could also be present in the tumor microenvironment as a result of chronic inflammation, and release from the tumor and infiltrating tumor cells could lead to persistent inflammation in turn. The factors shown here are only a part of the many factors involved in cell-to-cell crosstalk. They have been highlighted here because their expression is regulated by methylation.

involved in the acute phase of inflammation that can trigger a cascade of signaling, resulting in the production of adhesion molecules that cause migration of neutrophils to the site of infection. TNFα is produced primarily by macrophages and has chemotactic roles. It signals through two transmembrane receptors, TNFR1 and TNFR2, and plays a key role in cell survival, proliferation, and apoptosis. As a master regulator in the cytokine cascade, TNFα levels are under tight control. This regulation is context- and tissue-specific, but several epigenetic mechanisms have been identified to be critical. TNFα is found to be aberrantly expressed in many diseases, including autoimmune diseases and cancer (Parameswaran and Patial, 2010; Chu, 2013). It has been shown to have both tumor-suppressive and tumor-promoting roles (Waters et al., 2013; Montfort et al., 2019).

Interleukins

Interleukins (ILs) (from IL-1 to IL-38) are cytokines produced during inflammatory processes, primarily by macrophages and monocytes at the site of inflammation. They drive the production of acute phase proteins linked to inflammation. ILs are also well-known to be deregulated in a whole range of inflammation-linked pathologies, including cancer (Gabay, 2006; Akdis et al., 2016). In cancer, IL-20, which is typically secreted by monocytes, macrophages, and dendritic cells, promotes pro-inflammatory signaling, metastasis, and proliferation. The IL20 family receptors are found to be expressed on a variety of cancer cell lines (Rutz et al., 2014; Niess et al., 2018; **Figure 1**). IL-1β is likewise produced by both tumor cells as well as infiltrating monocyte-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), thereby recruiting and activating other myeloid cells and regulatory T cells (T regs), as well as promoting tumor cell proliferation and angiogenesis (Bent et al., 2018). IL1β and other pro-inflammatory cytokines, such as IL-6 and IL-8, which are known to promote inflammatory signaling, tumor growth, and metastasis, are also produced by cancer-associated fibroblasts (CAFs) (Tanaka et al., 2014; David et al., 2016; Bent et al., 2018). IL-6 also modulates the activity of macrophages, T regs, natural killer (NK) cells, and antigen-presenting dendritic cells (DCs) (Jones and Jenkins, 2018). IL-12, produced mainly by macrophages and DCs, is thought to be tumor suppressive through its ability to stimulate Interferon-γ (IFNγ) production by Th1 cells and NK cells. IL-2 is likewise primarily tumor suppressive in nature, through its activation of T cells and NK cells (Choudhry et al., 2018). IL-23 is overexpressed in

several cancer models and promotes tumorigenesis through suppression of NK cell activity, activation of IL-17 signaling, and the upregulation of MMP9 and VEGF (Ngiow et al., 2013; Yan et al., 2018; **Figure 1**).

Chemokines

Chemokines constitute a family of secreted chemotactic proteins, including 50 known endogenous ligands and 20 known receptors that signal through cell surface G protein-coupled chemokine receptors and can stimulate the migration of cells, especially leukocytes. Chemokines can be secreted by a wide variety of cells and play a pivotal role in the development of the immune system as well as in inflammatory responses (Griffith et al., 2014; Hughes and Nibbs, 2018). Pro-inflammatory cytokines, such as CXCL1/2/3/12 and CCL2 are produced by CAFs in the tumor microenvironment (Bent et al., 2018). Neutrophils can produce CXCL9 and CXCL10 to recruit T-cells to the microenvironment (David et al., 2016). The CXCL12/CXCR4 axis is important for tumor cell survival and metastasis, CAF activation, and recruitment of monocyte-derived cells (Chatterjee et al., 2014) CCL21 signaling; on the other hand, it can recruit lymphocytes, NK cells, and antigen presenting cells with anti-tumor activity (Lin et al., 2014; **Figure 1**).

Interferon-γ

Inflammation is a critical part of the immune response to harmful pathogens. Therefore, paradoxically, although chronic inflammation may play a role in tumorigenesis and tumor progression, impaired acute inflammation in response to viruses may in fact be instrumental in tumor development. One example of an inflammatory molecule critical for prevention of oncogenic signaling is IFNγ, which is the principal macrophage-activating cytokine produced by a whole host of immune cells in response to foreign antigens, particularly viruses. IFNγ is required for the expression of human major histocompatibility complex (MHC) class I and class II proteins, and therefore, plays a critical role in tumor immunogenicity. Unsurprisingly therefore, its expression is reduced in various forms of cancer (Schroder et al., 2004; Castro et al., 2018).

Transforming Growth Factor Beta

Transforming growth factor beta (TGF-β) is a key pleiotropic cytokine involved in many pathways, including inflammation and immune response. It regulates lymphocyte proliferation, differentiation, and survival and also controls inflammatory responses through the regulation of chemotaxis, activation, and survival of a variety of immune cells, including lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes (Li et al., 2006). In cancer, TGF-β signaling typically provides a favorable microenvironment for tumor growth through regulation of infiltration of inflammatory cells and cancer associated fibroblasts (Yang et al., 2010). It promotes expansion of T regs, inhibits expansion and/or activity of effector T cells, DCs, and NK cells and regulates macrophages and neutrophils (Batlle and Massagué, 2019; **Figure 1**). It can induce the expression of DNMTs and, therefore, can significantly affect the global methylome of cancer cells.

Signal Transducers and Activators of Transcription

Signal transducers and activators of transcription (STATs) are transcription factors involved in many signaling networks that often involve ILs and Janus kinases (JAKs). They are critical components of the response to infection. Activated by interferon signaling, STATs promote inflammation in a myriad of ways, including through the induction of chemokine expression, reactive oxygen species and NO, and the regulation of the development and death of hematopoietic cells (Pfitzner et al., 2004; Kaplan, 2013).

Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a major transcription factor at the nexus of inflammatory signaling and cancer. NF-κB is a critical regulator of both adaptive and innate immunity through its ability to induce the secretion of pro-inflammatory genes and regulation of the inflammasome. Its downstream targets include TNFα and IL6 (Liu et al., 2017; Xia et al., 2018). NF-κB is often deregulated in cancers.

Cyclooxygenase-2

Cyclooxygenase-2 is one of the two closely related enzymes responsible for converting arachidonic acid to prostaglandins, including prostaglandin E2 (PGE2). COX-2 is induced in response to inflammatory stimuli and is involved in the production of those PGEs that mediate pain and support inflammation (Simon, 1999). COX-2 derived PGE2 has been linked to various stages of the process of tumorigenesis and progression. COX-2 can be induced by pro-inflammatory TNF-α, IL-1, and IFN-γ, and suppresses anti-inflammatory IL-4, IL-13, and IL-10 (Harizi, 2015).

C-Reactive Protein

C-reactive protein (CRP) is a protein produced in acute response to inflammation. It is a sensitive marker of chronic low-grade inflammation that can be detected in the blood. CRP also plays key roles in apoptosis, phagocytosis, the complement pathway, production of nitric (NO), and the production of interleukin-6 and tumor necrosis factor-α (Sproston and Ashworth, 2018).

Suppressor of Cytokine Signaling 1

Suppressor of cytokine signaling (SOCS) proteins are suppressors of cytokine signaling, particularly through the JAK/STAT pathway, and negative regulators of inflammatory responses (Duncan et al., 2017; Liau et al., 2018).

Epigenetics

Activating inflammatory signaling cascades in response to cues need to be tightly regulated. Epigenetic mechanisms play a critical role in regulating inflammatory signaling pathways. They provide a means by which the expression of genes in specific pathways can be turned on or off reversibly and in a controlled manner.

Epigenetic mechanisms can take multiple forms. Prominent among these are DNA and histone modifications. Both these forms of regulation affect the interactions between DNA and the

nucleosomes. Tight DNA-histone interactions prevent binding of transcriptional machinery, whereas more relaxed interactions lead to increased accessibility and facilitate active transcription. Through DNA and histone modifications, these interactions, and consequently, gene transcription, can be regulated at specific loci.

DNA Methylation

CpG islands are dinucleotide repeats that are abundantly present in mammalian genomes and are associated, in the unmethylated form, with gene promoters. DNA methylation involves the conversion of cytosine residues in DNA to 5-methylcytosine (5mC) by the transfer of a methyl group from the cofactor S-adenyl methionine (SAM). This process is carried out by a family of enzymes known as DNA methyltransferases (DNMTs) comprising DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. 5mC is a major repressive mark, because it prevents DNA transcription either through the recruitment of repressive complexes or through prevention of transcription factor binding. Therefore, DNA methylation is a critical form of transcriptional silencing in many physiological and developmental processes. The opposite function, that is, DNA demethylation, is carried out by a group of enzymes known as tet-eleven translocation (TET) proteins. These proteins catalyze the conversion of 5mC to 5mC into 5-hydroxymethylcytosine (5hmC). This is further oxidized to form 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can then be converted back to unmodified cytosine (Tahiliani et al., 2009; He et al., 2011; Ito et al., 2011). The opposing functions of DNMTs and demethylases are necessary for many processes including heterochromatin maintenance, tissue-specific gene expression, genomic imprinting, X-chromosome inactivation and transcriptional silencing of retroviral elements. Deregulation of the expression or function of either of these groups of enzymes can have widespread consequences on cells (Jin et al., 2011; Jones, 2012; Greenberg and Bourc'his, 2019). Global and gene-specific hypomethylation, as well as regional hypermethylation, have been implicated in cancer (Ehrlich, 2002; Szyf, 2003).

Histone Methylation

Like DNA methylation, histone methylation is also a crucial regulatory process. Histone residues, particularly lysine (K) and arginine (R) on histone subunit 3 (H3), can acquire a large variety of post-translational modifications (PTMs) that can affect the structure of the subunit and the function of the nucleosome as a whole. Among these PTMs, which include methylation, acetylation, formylation, propionylation, butyrylation, crotonylation, malonylation, succinylation, hydroxylation, ubiquitination, sumoylation, adenosine diphosphate (ADP)-ribosylation, citrullination, and glycosylation, histone methylation/demethylation is perhaps the most well studied.

Lysine and arginine residues can accept between 1 and 3 methyl (me) groups, added sequentially, from SAM. The effect of histone methylation depends on the number of groups added and the residue modified. Some marks, such as H3K9me1/2 and H3K27me3, are repressive in nature, whereas others, such as H3K4me2, facilitate transcriptional activation. Enzymes that catalyze the addition of methyl groups are known as lysine methyltransferases (KMTs), whereas those that have the opposite function are known as demethylases. Specific enzymes catalyze the formation or removal of only specific marks. Likewise, epigenetic "readers" that recognize these marks and relay the effects to transcriptional complexes are also specific in their recognition of marks. Therefore, the entire process is a tightly regulated and involves many different enzymes (Bannister and Kouzarides, 2011; Greer and Shi, 2012; Zhao and Shilatifard, 2019).

Due to the importance of DNA and methylation as forms of regulation of gene expression, the deregulation of the enzymes involved in these processes has profound effects on the physiology of a cell, including in the context of cancer. Deregulated expression of many epigenetic modifiers, including methyltransferases and demethylases, have been linked to perturbed gene expression profiles, oncogenic phenotypes, and poor survival outcomes in cancer patients. Histone demethylases are capable of removing methyl groups from both histones and proteins. KDM1 family of demethylases is composed of KDM1A and KDM1B. KDM1A also known as LSD1 (Lysine-specific demethylase 1) (Shi et al., 2004) removes methyl groups via amine oxidase domain activity using FAD cofactor. The second group of histone demethylases is the Jumonji C (JmJC) domain containing demethylases that remove trimethylation mark (D'Oto et al., 2016). Cancer cells can also hijack the activity of these enzymes to alter the expression of genes involved in inflammatory signaling cascades.

In this article, we review the regulation of inflammatory signaling through both histone and DNA methylation in cancer. We discuss the various signaling cascades that cancer cells employ, through the use of altered histone and DNA methylation, to adopt an inflammatory phenotype that allows survival, colonization, and metastasis. It is important to note that the reverse also occurs; epigenetic profiles can change in response to inflammation. We also discuss targeting inflammation using small molecule inhibitors of various key players as an alternative to direct targeting of components of signaling pathways. Finally, we highlight recent progress, future challenges, and what we can learn from other diseases that can help with development of therapeutics in cancer.

DNA AND HISTONE METHYLATION IN REGULATION OF INFLAMMATORY SIGNALING PATHWAYS

Global reorganization of epigenetic modifications is an important part of cancer initiation and progression, including in the switching on of pro-inflammatory signaling programs in cancers cells and infiltrating tumor cells.

As a major repressive mark and regulator of gene expression, DNA methylation is of critical importance in switching on and off inflammatory signaling pathways in response to cues. Several studies have indicated interactions between DNA methylation and circulating inflammatory proteins (Ahsan et al., 2017; Myte et al., 2019). Epigenome wide association studies (EWAS),

global DNA methylation patterns, and candidate gene analysis suggested that global genome hypomethylation is linked to inflammation (Gonzalez-Jaramillo et al., 2019). Several groups have studied the association between global DNA methylation (LINE-1 methylation) patterns and CRP levels. Some have reported no association between global DNA methylation and CRP levels (Baccarelli et al., 2010; Zhang et al., 2012), while others have indicated a link between lower methylation and higher CRP levels (Perng et al., 2012). In addition, meta-analysis of several EWAS has shown that many specific differentially methylated CpG islands are significantly linked to CRP expression and chronic low-grade inflammation. Serum levels of CRP were linked either positively or negatively to various CpG sites, notably with one site near the transcription start site of the Absent in melanoma 2 (AIM2 gene), a protein induced by interferon-gamma and involved in the innate immune response (Ligthart et al., 2016). There appears to be a link between higher CRP levels and lower levels of AIM2 and IL6 methylation, as well as between higher CRP levels and higher levels of suppressor of cytokine signaling 1 (SOCS1), LY86 and EEF2 methylation [reviewed by Gonzalez-Jaramillo et al. (2019)].

Histone methylation is also important in propagating inflammatory cues. H3K9 methyl transferases and demethylases balance the methylation status of H3K9. Jmjd3, a Jumonji family member, is responsible for the deletion of histone marks and control of differentiation and cell identity in macrophages. Thus, Jmjd3 protein functions as a link between inflammation and reprogramming of the epigenome (Ishii et al., 2009). Macrophages exposed to bacterial products and inflammatory cytokines induce Jmdj3, which in turn binds to polycomb group (PcG) target genes and regulates their H3K27me3 levels and the transcriptional activity (De Santa et al., 2007). Activation of Jmjd3 on continuous IL-4 treatment triggers release of H3K27me3 repressive marks from STAT6 promoter. Jmjd3 is positively regulated by activated STAT6 through promoter binding. Jmjd3 also triggers expression of specific inflammatory genes by removal of H3K27me mark (Bayarsaihan, 2011).

DNA and histone methylation defects affect inflammatory signaling in a myriad of ways in various forms of cancer. Cooperative interactions between DNA methylation and histone methylation during severe systematic inflammation (SSI) was shown in TNFα promoter in blood leucocytes (Gazzar et al., 2008). Here, we summarize the known changes to these pathways in individual forms of cancer, to show the many ways in which they are deregulated and underline the need to take into account the various factors involved in inflammation and tumor progression when trying to treat certain forms of cancer (**Table 1** and **Figure 2**).

TABLE 1 | DNA and histone methylation in regulation of inflammatory signaling pathways.

Target	Cancer/Cells	Effect of methylation	References
Lung cancer			
IL-20RA	NSCLC	↓(DNA hypermethylation)	Tessema et al., 2008; Baird et al., 2011
IL-1β	NSCLC	↓(DNA hypermethylation)	Tekpli et al., 2013
IL-6	NSCLC	↓(DNA hypermethylation)	Tekpli et al., 2013
IL-8	NSCLC	↓(DNA hypermethylation)	Tekpli et al., 2013
IL23A, IL23R	NSCLC	↓(DNA hypermethylation)	Baird et al., 2013
IL-12Rβ2	NSCLC	↓(DNA hypermethylation)	Suzuki et al., 2007
HIC1	NSCLC	↓(DNA hypermethylation)	Wang X. et al., 2016
IFNγ	CD4+ T cells	↓(DNA hypermethylation)	Wang F. et al., 2013
TGFβRII	NSCLC	↓(DNA hypermethylation)	Zhang et al., 2004
NF-κB	NSCLC	↑(Histone hypomethylation)	Chen et al., 2018
TGFβ	Non-invasive LC	↓(Histone hypermethylation)	Du et al., 2018
IL-2	NSCLC	↓(Histone hypermethylation)	Wakabayashi et al., 2011; Wu et al., 2014
JARID2	NSCLC	↓(Histone hypermethylation)	Chen et al., 2010
Digestive cancers			
CXCL14	Gastric cancer, CRC	↓(DNA hypermethylation)	Cao et al., 2013; Hu et al., 2013
CXCR4	Pancreatic cancer	↓(DNA hypermethylation)	Sato et al., 2005
FOXM1	Pancreatic cancer	↑(Histone hypomethylation)	Zhou Z. et al., 2019
PD-L1	Pancreatic cancer	↓(Histone hypermethylation)	Winograd et al., 2015; Lu et al., 2017
PTPRC	CRC	↑(DNA hypomethylation)	Magzoub et al., 2019
S100A9	CRC	↑(DNA hypomethylation)	Magzoub et al., 2019
LTF	CRC	↓(DNA hypermethylation)	Magzoub et al., 2019
CXCL9/10	CRC	↓(Histone hypermethylation)	Nagarsheth et al., 2016
CXCR4	CRC	↑(Histone hypomethylation)	Ghanem et al., 2014; Liu et al., 2015
COX-2	Gastric cancer	↓(DNA hypermethylation)	Toyota et al., 2000; Song et al., 2001; Kikuchi et al., 2002; Hur et al., 2003
SOCS1	HCC	↓(DNA hypermethylation)	Herath et al., 2006
YAP1	HCC	↓(Histone hypermethylation)	Xu et al., 2013
Ash2	HCC	↑(Histone hypomethylation)	Barcena-Varela et al., 2019
ULBP1	HCC	↓(Histone hypermethylation)	Bugide et al., 2018
MICA	HCC	↓(Histone hypermethylation)	Bugide et al., 2018
Cancers of the blood			
NF-κB	CLL	↑(DNA hypermethylation of miR-9-3 and miR-708)	Wang L. Q. et al., 2013; Baer et al., 2015

(Continued)

Lung Cancer

Lung cancer is one of the most common and lethal cancers. Small-cell lung cancer accounts for about 10–15% of global incidence, while non-small-cell lung cancers (NSCLCs) account for the majority, with subsets of patients showing mutations in various genes, including Epidermal growth factor receptor (EGFR), Anaplastic lymphoma kinase (ALK), ROS1, and

TABLE 1 | (Continued)

Target	Cancer/Cells	Effect of methylation	References
TNFβ	Leukemia, Lymphoma	↑(DNA hypomethylation)	Kochanek et al., 1991
IL-12Rβ2	B-ALL	↓(DNA hypermethylation)	Airoldi et al., 2006
SOCS1	Multiple Myeloma	↓(DNA hypermethylation)	Amodio et al., 2013
IL-3R α	AML	↑(Histone hypomethylation)	Agger et al., 2016
Breast cancer			
CXCR4	Breast cancer	↑(DNA hypomethylation)	Ramos et al., 2011
CXCL12	Breast cancer	↓(DNA hypermethylation)	Ramos et al., 2011
TNFR2	Breast cancer	↑(DNA hypomethylation)	Smith et al., 2014
IL-6	Breast cancer	↑(DNA hypomethylation)	Smith et al., 2014
LSD1	Breast cancer	↑(Histone demethylation)	Shi et al., 2004, p. 1
TNF	Breast cancer	↓(Histone hypermethylation)	Mabe et al., 2020
Cancers of the reproductive systems			
IFN-γ	Cervical cancer	↓(DNA hypermethylation)	Ma et al., 2020
IFN-κ	Cervical cancer	↓(DNA hypermethylation)	Rincon-Orozco et al., 2009; Alfaro-Mora et al., 2019
TNF	Ovarian Cancer	↑(DNA hypomethylation)	Gong et al., 2020
CXCR4	Ovarian Cancer	↑(DNA hypomethylation)	Gong et al., 2020
TGF-β	Prostate Cancer	↓(Histone hypermethylation)	Li et al., 2016
Other cancers			
IL-6	ESCC	↑(DNA hypomethylation)	Lima et al., 2011
IL-1α	ESCC	↓(DNA hypermethylation)	Lima et al., 2011
TGFβRII	ESCC	↓(DNA hypermethylation)	Ma et al., 2020
CXCR4	Melanoma	↓(DNA hypermethylation)	Mori et al., 2005
CCR7	Melanoma	↓(DNA hypermethylation)	Mori et al., 2005

Summarized here are the inflammatory genes regulated by DNA and/or histone methylation in various forms of cancer. In column 3, ↓ indicates downregulated expression, while ↑ indicates upregulated expression. Means through which upregulation or downregulation are achieved are indicated in brackets in Column 3.

Neurotrophin receptor kinase (NTRK). Despite considerable progress in developing biomarkers and stratifications for treatment strategies, NSCLC continues to be a leading cause of death worldwide (Cersosimo, 2002; Duma et al., 2019).

DNA Methylation

The expression of IL-20 and its receptors are often found to be dysregulated in NSCLC. IL20 can signal through IL-20RA/RB or IL20-RB/IL22-R1 receptor complexes. These genes are epigenetically regulated through several mechanisms, including DNA methylation. IL-20RB and IL-22R1 were found to be overexpressed at both the mRNA and protein levels when compared to healthy counterparts. Conversely, loss of IL-20RA expression, linked to promoter hypermethylation, was found when compared to normal bronchial epithelial cells. This may suggest that the IL20-RB/IL22-R1 complex is the main complex through which NSCLC cells signal. IL-20RA has been previously linked to anti-angiogenic effects in NSCLC, and restoration of IL20 signaling through IL20RA was shown to downregulate VEGF expression (Tessema et al., 2008; Baird et al., 2011). A study by Tekpli et al., has shown that pro-inflammatory IL-1β, IL-6, and IL-8 genes all show differential DNA methylation patterns in NSCLC when compared to adjacent non-cancerous tissue or bronchial epithelial cells, and there is an inverse relationship between DNA methylation and gene transcription for IL6 and IL1β. Interestingly, all these cytokines were found to be hypermethylated and downregulated in tumor tissues when compared to non-tumor tissues (Tekpli et al., 2013). IL23A, a member of the IL6 family of cytokines, is pro-proliferative in NSCLC, and treatment with 5-aza-2'deoxycitidine (5-Aza) showed an increase in the expression of IL23A, indicating that it is transcriptionally silenced through DNA methylation. Similarly, the expression of the IL23 receptor (IL23R) was also increased upon 5-Aza treatment in the A549 lung adenocarcinoma cell line (Baird et al., 2013). In lung adenocarcinomas, aberrant methylation of the IL-12Rβ2 gene was linked to loss of expression in cell lines and primary tumors and poor prognosis among adenocarcinoma patients. Treatment with a demethylating agent was able to restore expression in these cell lines (Suzuki et al., 2007). In addition, the hypermethylation and loss of Hypermethylated in cancer 1 (HIC1) was shown to lie upstream of upregulation of IL6 and activation of the IL6/STAT3 axis (Wang X. et al., 2016).

Co-culturing SPC-A1 lung cancer cells and healthy CD4+ T cells induced DNMT expression and IFNγ promoter hypermethylation in CD4+ T cells, indicating a tumor-induced, DNA methylation-dependent suppression of IFNγ in lung cancer and highlighting the crosstalk between these processes (Wang F. et al., 2013).

TGFβRII, which is thought to function as a tumor suppressor in many solid tumors, where TGF-β signaling is important for inhibition of epithelial cell growth, is downregulated in NSCLC through methylation of its promoter (Zhang et al., 2004).

Histone Methylation

A number of NSCLCs show high levels of Enhancer of Zeste 2 (EZH2), the enzymatic subunit of polycomb repressive complex 2 (PRC2). Tumors from mice administered with EZH2 inhibitors singly or in conjunction with chemotherapy were sensitive to EZH2 inhibition. Along with it, there was an amplification of an inflammatory program involving NF-KB signaling. Combinatorial therapy of EZH2 inhibitors with anti-inflammatory agents can provide a promising therapy for a subset of Kras-driven NSCLC (Chen et al., 2018).

In non-invasive lung tumor cells, TGF-β facilitates the association of methyltransferase SET Domain Bifurcated Histone Lysine Methyltransferase 1 (SETDB1) with Smad3 which in turn mediates H3K9me in Snail promoter, downregulating its expression. In invasive lung tumor cells undergoing TGF-β-induced EMT, Snail promoter is de-repressed due to the repression of SETDB1 (Du et al., 2018). The interaction of SETDB1 with Smad3 reduces metastasis in lung cancer by downregulating IL-2 and the calcium-dependent RNA-binding protein annexin A2 (ANXA2), which interacts with c-myc mRNA (Wakabayashi et al., 2011; Wu et al., 2014).

Jumonji and At-rich interaction domain containing 2 (JARID2), a component of the PRC2 complex is activated by TGF-β and downregulates expression of E-cadherin in lung

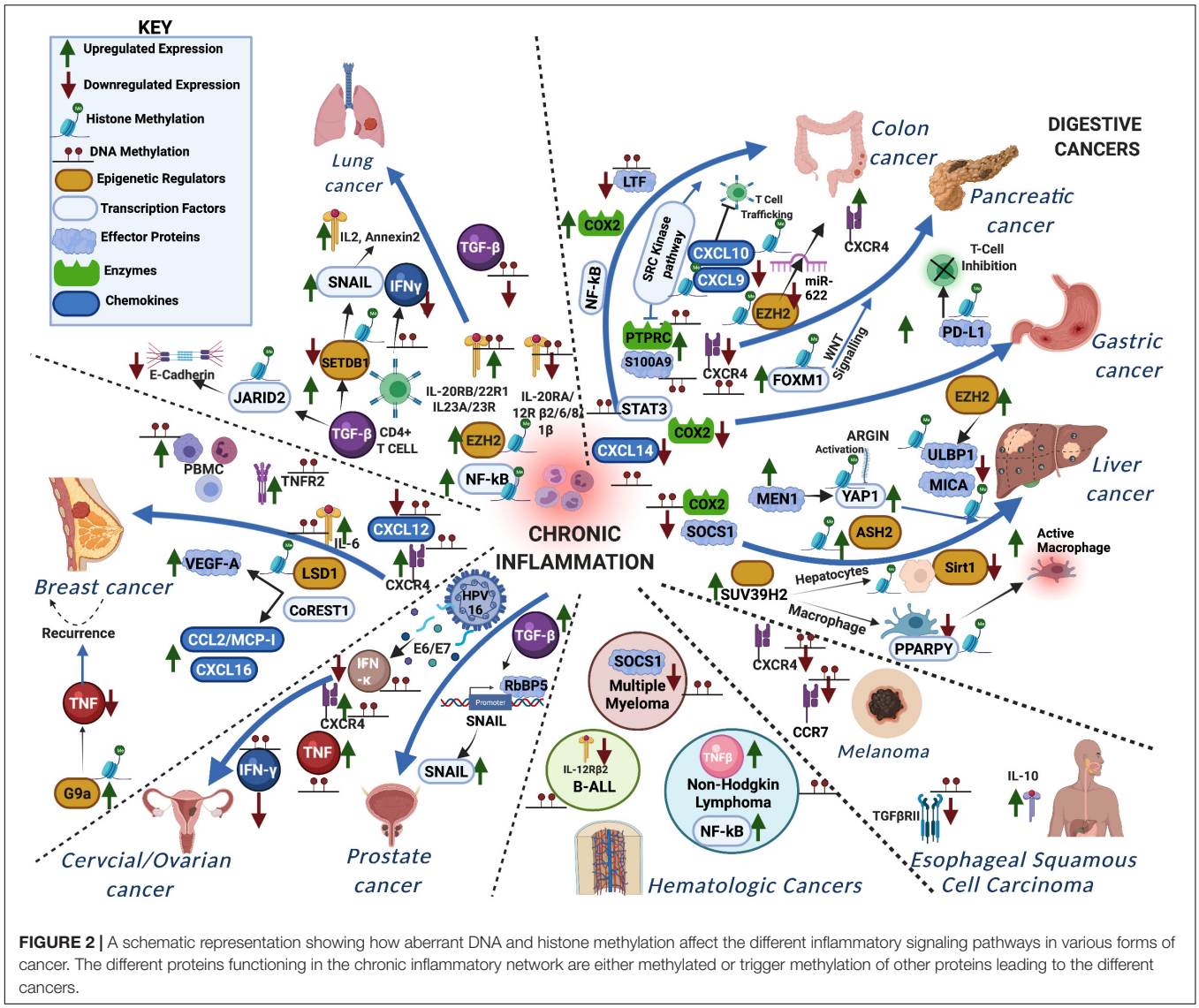

FIGURE 2 | A schematic representation showing how aberrant DNA and histone methylation affect the different inflammatory signaling pathways in various forms of cancer. The different proteins functioning in the chronic inflammatory network are either methylated or trigger methylation of other proteins leading to the different cancers.

cancer cells. JARID2 occupies promoters of CDH1 and miR-200 family members which in turn controls the recruitment of PRC and G9a methyltransferase, promoting methylation of H3K27 and H3K9 (Li et al., 2016). The pro-metastatic effect of G9a is contributed by gene silencing of epithelial cell adhesion molecules (ep-CAM), increasing the invasive capacity of lung tumor cells (Chen et al., 2010).

Digestive Cancers

Digestive cancers, including liver cancer, gastric cancer, pancreatic cancer, and colon/colorectal cancers, are a leading cause of cancer-related deaths worldwide. Hepatocellular carcinoma (HCC) is one of the most common cancers and is chiefly caused by infectious diseases like viral hepatitis B or C or through food toxins. In the early stages, inflammatory responses like cytokine secretion or proliferation play important roles in subsequent development of HCC. Chronic inflammatory responses like liver cirrhosis and necrosis are important in the later stages and advancement of HCC. Epigenetic regulation is important in both the early and late stages of HCC.

DNA Methylation

The chemokine CXCL14 is downregulated in gastric cancer cells when compared to healthy tissue, due at least in part to aberrant hypermethylation in exon 1 of the gene (Hu et al., 2013), and in colorectal cancer, where hypermethylation and loss of CXCL14 expression are linked to proliferation, migration, invasion, and EMT through NF-κB (Cao et al., 2013). The chemokine receptor CXCR4 is hypermethylated in pancreatic cancer cell lines and primary pancreatic adenocarcinomas, while it is unmethylated in healthy pancreas tissue. The reason for CXCR4 downregulation in pancreatic cancer is unclear. It has been suggested that the *de novo* methylation of the CXCR4 locus may simply be a part of genome-wide process in a distinct subgroup of pancreatic cancers characterized by a profound methylator phenotype and that alternative pathways

to CXCR4/CXCL12 may be utilized for tumor progression (Sato et al., 2005).

Nuclear factor kappa-light-chain-enhancer of activated B cells and STAT3 are both critical in the progression of chronic inflammation to malignancy in CRC, primarily through the maintenance of a favorable microenvironment for tumorigenesis through secretion of a myriad of pro-inflammatory cytokines (Yang et al., 2019).

In addition, the Protein Tyrosine Phosphatase Receptor Type C (PTPRC) gene, which disrupts normal T- and B-cell signaling through SRC kinase pathways, and the S100 Calcium Binding Protein A9 (S100A9) gene, which is implicated in many conditions associated with inflammation, are both hypomethylated. At the same time, the promoter of the LTF (Lactotransferrin) gene, which restricts the inflammatory reaction in CRC, is hypermethylated (Magzoub et al., 2019).

While some studies have linked high COX-2 expression to worse outcomes in CRC (Soumaoro et al., 2004; Ogino et al., 2008; Wang and Dubois, 2010), there is a subset of gastric cancers and CRCs where the expression of COX-2 is lost through hypermethylation of the promoter (Toyota et al., 2000; Song et al., 2001; Kikuchi et al., 2002; Hur et al., 2003). Consequently, treatment with 5-Aza restores COX-2 expression and sensitivity to IL1β signaling (Song et al., 2001). Interestingly, in HCC, downregulation of COX-2 was linked to reduced survival of patients (Fernández-Alvarez et al., 2012).

Furthermore, hypermethylation and loss of SOCS1 expression are a common occurrence in HCC (Herath et al., 2006).

Histone Methylation

Higher levels of H3K4 trimethylation are associated with a poor prognosis of HCC (Chen et al., 2020). Menin is a scaffold protein encoded by multiple endocrine neoplasia type1 (MEN1) gene. MEN1 undergoes heterozygous ablation in female mice and causes a reduction in chemical carcinogen-induced liver carcinogenesis and suppresses the activation of inflammatory pathways. Chromatin immunoprecipitation assays revealed that menin and H3K4me3 occupancy at the YAP1 promoter was markedly increased in HCC tissues (Xu et al., 2013). Abundantly expressed proteoglycan, Argin in the HCC tissue can activate the YAP gene and cause metastasis and invasion. On the other hand, low levels of H3K4 dimethylation in HCC are associated with Ash2, an H3K4 methylating enzyme (Barcena-Varela et al., 2019). A study showed that EZH2 overexpression is associated directly with promoters of natural killer (NK) cells ligand like ULBP1 and MICA in HCC cells and promotes the occupancy of H3K27me3 repressive marks in these promoters (Bugide et al., 2018).

Suppressor of variegation 3–9 homolog 2 (SUV39H2) or KMT1B-mediated H3K9me3 accelerates hepatocarcinogenesis by contributing to non-alcoholic steatohepatitis in mice. KMT1B represses Sirt1 transcription in hepatocytes, whereas KMT1B suppresses PPARϒ in macrophages which favors proinflammatory active macrophage (M1) phenotype over anti-inflammatory alternatively active macrophage (M2) phenotype, thus elevating hepatic inflammation (Fan et al., 2017). HBV X protein (HBx) decreases levels of H3K27me3 silence modification while increasing levels of activating histone modification H3K27me1 in the host EpCAM promoter, which is involved in HBV-mediated hepatocarcinogenesis (Zhang et al., 2015, 2016).

In colon cancer, production of the Th1-type chemokines, CXC chemokine ligand 9 (CXCL9), and CXCL10, which mediates T cell trafficking, is inhibited by H3K27me3 in their gene promoters (Nagarsheth et al., 2016). However, CXC chemokine receptor 4 (CXCR4) is upregulated by EZH2-mediated loss of miR-622, thus favoring evasion of immune surveillance by interaction with CXCL12 (Ghanem et al., 2014; Liu et al., 2015).

Upregulation of FOXM1 by KMT4-induced H3K79me2 significantly reduces antitumor responses like bone marrow-derived dendritic cell (BMDC) maturation, T cell activation, and cytokine secretion via the Wnt5a signaling pathway in pancreatic cancer (Zhou Z. et al., 2019). Further expression of PD-L1, a T-cell inhibitory receptor ligand causing immunosuppression, was upregulated in pancreatic cancer due to upregulation of H3K27me3 levels in CD274 promoter triggered by KMT2A overexpression (Winograd et al., 2015; Lu et al., 2017).

Cancers of the Blood

Cancers of the blood, the most common of which include leukemia, lymphoma, and myeloma, have seen a gradual increase in survival rates over time. Nevertheless, these cancers can still present various difficulties in treatment, and chemotherapy can lead to devastating long-term side effects, as well as relapse, in patients. Pro-inflammatory signaling molecules in the blood can affect a wide range of cells and trigger an intricate network of signaling, especially as hematopoiesis and leukocyte functions are dependent on cytokines and chemokines (Rosu-Myles et al., 2000; Allart-Vorelli et al., 2015).

DNA Methylation

In contrast to normal human granulocytes, monocytes, or HeLa cells, the TNFβ gene showed hypomethylation chronic myeloid leukemia (Kochanek et al., 1991).

In pediatric B-cell acute lymphoblastic leukemia (B-ALL) cells, methylation of a CpG island in exon 1 in the IL-12Rβ2 gene was found to be responsible for silencing of this gene. IL12 is an especially important anti-inflammatory, anti-tumorigenic signaling molecule that has been linked to restricting tumor growth through its anti-proliferative, anti-metastatic, and anti-angiogenic functions in various types of cancers (Dias et al., 1998; Duda et al., 2000; Pistoia et al., 2009). Therefore, inhibition of IL12 signaling through the ablation of one of its receptor subunits gives B-ALL cells a survival advantage (Airoldi et al., 2006).

In multiple myeloma, SOCS1 expression is suppressed through promoter hypermethylation, which can be reversed through the activity of miR29b (Amodio et al., 2013).

In chronic lymphocytic leukemia (CLL), NF-κB signaling is regulated by multiple miRNAs, including miR-9-3a and miR708, both of which are downregulated through DNA hypermethylation (Wang L. Q. et al., 2013; Baer et al., 2015). This leads to aberrant NF-κB signaling.

Histone Methylation

Rearrangement of mixed-lineage leukemia genes in acute myeloid leukemia (AML) leads to aggressive hematopoietic

malignancies. The IL-3Rα expression is dependent on Jmjd2/Kdm4 through a mechanism involving H3K9me3 removal from the gene promoter (Agger et al., 2016). It has also been shown that methyltransferase SUV39H1 is directly involved in resistance to TGF-β signaling in AML. Deregulation of TGF-β direct targets p21 and p15 through SUV39H1-mediated H3K9me3 marks leads to inhibition of cell cycle arrest and gives leukemic cells a proliferative advantage (Ruscetti et al., 2005). Furthermore, SETDB1 promotes heterochromatin formation and immune evasion in AML. Knockdown of SETDB1 leads to induction of the interferon immune response, thus qualifying SETDB1 as a fundamental leukemic survival protein (Schultz et al., 2002; Monaghan et al., 2019).

Breast Cancer

Breast cancer is one of the common cancers reported worldwide. Clinically, breast cancers are categorized into three basic therapeutic groups, which include: estrogen receptor (ER) positive group, which is the most diverse and numerous, the HER2 amplified group with effective therapeutic targeting of Her2, and the triple-negative breast cancer with only chemotherapy options and have a high incidence of BRAC1 mutation in the patients.

DNA Methylation

The interaction between the chemokine CXCR4 and its ligand, CXCL12, is linked to cell proliferation, survival, invasion, and metastasis in various forms of cancer, such as breast cancer, where the upregulation of CXCR4 in cancer tissue is also accompanied by peak expression of its ligand CXCX12, at sites of common metastasis (Müller et al., 2001). CXCR4 is known to be hypomethylated and overexpressed, while CXCL12 is hypermethylated and absent in breast cancer cell lines and primary tumors themselves. Patients with both CXCL12 hypermethylation and CXCR4 hypomethylation showed shorter overall survival and disease-free survival (Ramos et al., 2011).

Interestingly, chemotherapy in breast cancer patients was shown to be associated with significantly decreased methylation at eight CpG sites in peripheral blood mononuclear cells and increased levels of TNFR2 and IL-6 (Smith et al., 2014). Inflammation and fatigue are known to be common effects of chemotherapy.

Histone Methylation

One of the first identified demethylases, LSD1, facilitates demethylation of H3K4/K9 and is associated with nuclear receptors. H3K4 demethylation by LSD1 was previously shown to inhibit inflammation (Shi et al., 2004, p. 1). CoREST1 on coordination with LSD1 promotes expression of VEGF-A and proinflammatory factors CCL2/MCP-I and CXCL16 and contributes to angiogenesis and tumor inflammatory responses in breast cancer (Zhao and Shilatifard, 2019). A recent study showed that G9a promotes breast cancer recurrence by inhibiting pro-inflammatory signaling pathway. G9a activity is essential to downplay the expression of pro-inflammatory cytokines, including TNF, by H3K9 methylation in the gene promoters. G9a repression triggers re-expression of the pro-inflammatory cytokines leading to activation of p53 and necroptosis. The study showed that receptor interacting protein kinase-3 (RIPK3) expression is upregulated in recurrent tumors, which makes it sensitive to necroptosis following G9a suppression in breast cancer. Histone methyl-modifying enzymes are now being considered as potential therapeutic targets against cancer, by inhibiting the inflammatory response (Mabe et al., 2020).

Cancers of the Reproductive Systems

Of the cancers of the reproductive systems, the most common are endometrial, ovarian, and cervical cancers in females and prostate and testicular cancers in males. While less is known about the link between inflammation and epigenetics in these cancers, there is growing evidence to show the presence of this link and the similarities with other cancers. In particular, cervical cancer in females, which is often linked to infection by the human papillomavirus (HPV), which indicates the intricate relationship between infection, inflammation, and cancer; in cervical cancer, anti-viral inflammation is required to prevent cancer development, but it is also necessary to limit the effects of inflammation on cervical tissue (Cohen et al., 2019).

DNA Methylation

In cervical cancer tissues, methylation of the IFN-γ gene is significantly higher than in healthy cervical tissue, resulting in reduced expression (Ma et al., 2020). The viral oncoprotein E6/E7, produced by human papillomavirus 16 (HPV16), can also induce DNMT activation, DNA methylation, and downregulation of IFN-κ, which is also downregulated in cervical cancer biopsies compared to healthy tissue (Rincon-Orozco et al., 2009; Alfaro-Mora et al., 2019). In ovarian cancer, many genes, including the pro-inflammatory TNF and CXCR4, are hypomethylated and overexpressed (Gong et al., 2020).

Histone Methylation

Previous studies show that in prostate cancers, TGF-β promotes H3K4me3 and Retinoblastoma binding protein 5 (RbBP5) recruitment to the Snail promoter by association with Smad2/3 and CBP, leading to enhanced Snail expression in the cancer cells (Li et al., 2016).

Other Cancers

In esophageal squamous cell carcinoma (ESCC), differentially methylated CpG sites between cancer tissue and healthy tissue were found in genes in the IL10 signaling pathway; IL-6 was found to be hypomethylated, while IL-1α was found to be hypermethylated (Lima et al., 2011). TGFβRII is likewise downregulated through promoter hypermethylation in ESCC, where it can regulate proliferation of ESCC cell line by G2/M arrest; treatment with 5-aza was able to restore its expression (Ma et al., 2020).

CXC chemokine receptor 4 is also hypermethylated and transcriptionally repressed in melanoma, as is CCR7; treatment with 5-Aza increases the expression of both these chemokines. In line with this, 5-Aza treated cells also showed increased migration in response to treatment with CCL21 and CXCL12, the ligands

for CCR7 and CXCR4, respectively, indicating a functional rescue upon demethylation (Mori et al., 2005).

TARGETING INFLAMMATION IN CANCER

DNA Methylation: Targeting Options and Therapeutics

The vast majority of genes that are deregulated through aberrant DNA methylation, including those that are involved in inflammatory signaling, tend to be hypermethylated. Consequently, inhibitors of DNA methylation are well developed and the development of DNMT inhibitors for the treatment of cancer has also made some progress. There are multiple generations and iterations of DNMT inhibitors that are now being developed or are in use. This includes Azacitidine, Decitabine, Zebularine, (-)-epigallocatechin-3-gallate, MG98, RG108, and Procainamide (Brueckner et al., 2007; Gravina et al., 2010; Gnyszka et al., 2013). However, the full extent of the effects of many of these drugs is not known, and problems include serious side effects, the development of resistance, and partial or no response in a group of patients. There also tends to be a change in global CpG methylation patterns as the result of using DNA inhibitors, which may pose a problem if new sets of genes are deregulated (Giri and Aittokallio, 2019). Another point to consider is that, although DNA hypermethylation underlies the deregulation of most inflammatory signaling genes, there are some that are hypomethylated rather than hypermethylated. The use of DNA inhibitors is, therefore, limited.

Nevertheless, better understanding of the mechanism of action and global effects of these inhibitors will allow us to implement more suitable dosing regimens in combination with other drugs.

Histone Methylation: Targeting Options and Therapeutics

Histone methylation can contribute as potential therapeutic targets in digestive cancers. Inhibition of EZH2-mediated H3K27me and G9a-mediated H3K9me/H3K are being considered as important strategic targets. GSK343, the drug that suppresses EZH2 activity and reduces H3K27me3 expression was shown to potentially recover intestinal inflammation and also delayed onset of colitis-associated cancer (Zhou J. et al., 2019). DZNep (3-deazenplanocin A) which is a chemical inhibitor of S-adenosylhomocysteine hydrolase, suppresses histone methyltransferases including EZH2, and leads to marked reduction in cell proliferation and migration in colorectal cancer (Cheng et al., 2012). DZNep can also alter miR-663a and miR-4787-5p expression in turn suppressing TGFb1-induced EMT signaling in pancreatic cancers (Mody et al., 2016). EZH2 inhibitor, GSK126 can promote infiltration of functional CD8 T-cells by epigenetic reprogramming and significantly decrease HCC growth (Wei et al., 2019). GSK126 increases the number of myeloid-derived suppressor cells (MDSCs) and decreases CD4+ and IFNϒ CD8+ T-cells, which is associated with antitumor immunity in colorectal cancer, via EZH2-mediated H3k27me3 levels (Huang et al., 2019).

In the context of EMT, TGF-b1 treatment in gastric cancer cells promotes the expression of JARID1A demethylase, which is recruited by p-SMAD3 to CDH1 promoter, leading to gene silencing and promoting malignancy (Liang et al., 2015). EZH2 methyltransferase inhibitors that are now in clinical trials can be used with extracellular signal-regulated kinase (Erk) inhibitors to suppress TGF-β induced EMT (Olea-Flores et al., 2019).

THE CROSSTALK: INFLAMMATION-INDUCED CHANGES IN METHYLATION

It is important to recognize that there also exists a crosstalk between inflammation and altered epigenetics. While altered epigenetic function may be the cause of altered expression and function of many inflammatory signaling networks, there is equal evidence, if not more, to show that the opposite is also true: inflammation and inflammatory signaling can cause aberrant methylation. It is crucial to recognize that targeting epigenetic regulators using small molecule modulators may not always be useful in targeting inflammatory networks; sometimes these lie upstream of the methylation aberrations and may have other underlying causes that may need to be identified for treatment.

Perhaps the best-known type of cancer linked to chronic inflammation is *Helicobacter pylori*-linked gastric cancer. *H. pylori* inject cytotoxin-associated antigen A (CagA) into host cells and cause inflammatory stress within gastric mucosa through activation of pathways including NK-κB, activator protein-1 (AP1), phosphoinositide 3-kinase (PI3K), STAT3, Wnt/beta-catenin, and COX-2. Inflammation-induced DMNT upregulation is thought to lead to the deregulation of many downstream targets. Studies have demonstrated the presence of aberrant methylation at the promoter of multiple genes in the gastric mucosa cells during *H. pylori* infection, including methylation-induced production of pro-inflammatory genes such as NOS2, IL1B, and TNF (Maeda et al., 2017). Compared to gastric mucosa upon *H. pylori* eradication, as well as compared to non-cancerous gastric tissue, there is hypermethylation at many of these loci. In a gerbil model, these changes are thought to be linked, not to the infection itself, but to the infection-induced inflammatory response, as methylation changes temporally reflected the expression levels of inflammation-related genes such as CXCL2, IL-1β, NOS2, and TNFα, and treatment with an anti-inflammatory drug (cyclosporin A) led to a blockade in the DNA methylation pattern changes previously seen without affecting colonization by *H. pylori* (Kurkjian et al., 2008; Niwa et al., 2010). The changes in DNA methylation seen in gastric epithelial cells were induced by bacteria-induced macrophage-produced NO, once again showing the link between inflammatory signaling and the induction of aberrant DNA methylation (Katayama et al., 2009, p. 3). In gastric cancer, *H. pylori* and Epstein-Barr virus (EBV)-induced chronic inflammation is known to induce aberrant methylation in the gastric mucosa, resulting in gene profile changes that promote

tumorigenesis, including changes in the expression of target genes such as *p16INK4A, LOX, CDH1, IL-1β, IL-8, NOS2,* and *TNF* (Matsusaka et al., 2014).

In inflammatory bowel disease and colitis-associated CRC, which is characterized by chronic inflammation, there is an altered methylation profile when compared to sporadic CRC. In colitis-associated cancer, NF-κB-induced release of TNF and IL6 from immune cells is thought to induce NF-κB and STAT3-dependent altered signaling in epithelial cells of the gastric mucosa, including increased DNMT expression and changes to the methylome of the epithelial cells (Hartnett and Egan, 2012). DNMT expression was found to be higher than in sporadic CRC, and this increase was found to be linked to IL6 signaling. In addition, IL6-induced upregulation of DMNT1 has been linked to the hypermethylation and downregulation of SOCS3 (Li et al., 2012). IL6-induced upregulation of DNMT1 was also found to result in hypermethylation and silencing of genes linked to tumor suppression, adhesion, and apoptosis, including PAI-1, IL-4, Maspin, and IRF-7 (Foran et al., 2010). IL6 has also been shown to induce the cytochrome P450 gene CYP1B1 through DNA methylation-dependent suppression of microRNA miR27b (Patel et al., 2014). Hence, it is thought that inflammation induces a novel epigenetic profile, which thereby affects progression of CRC (Foran et al., 2010; Abu-Remaileh et al., 2015; Pekow et al., 2019). Altered methylation profile as the result of chronic inflammation is likewise the case in HCC (Stoyanov et al., 2015), where crosstalk between inflammation and epigenetic mechanisms can result in a positive feedback loop that advances inflammation and tumor progression (Sceusi et al., 2011; Martin and Herceg, 2012).

Interestingly, while some studies have reported the downregulation of COX-2 by promoter methylation in HCC, others have shown, using a transgenic COX-2 mouse model, that COX-2 is sufficient to induce HCC in a number of ways, including through the induction of hypermethylation and downregulation of TET1. TET1 converts 5m to 5-hydroxymethylcytosine (5hmC), so downregulation of TET1 results in hypermethylation and repression of several tumor suppressor genes (Chen et al., 2017).

In the IL-6-responsive human multiple myeloma cell line KAS 6/1, IL6 maintains promoter methylation of the tumor suppressor gene p53 through an induction of DMNT1 (Hodge et al., 2005). In addition, the circulating levels of IL6 appear to be associated with the methylation of several candidate genes, including MGMT, RARβ, RASSF1A, CDH13, SOCS1, USP2, TMEM49, SMAD3, DTNB, and IL-6 itself, across tumor specimens and peripheral blood samples. Furthermore, histone methyltransferase EZH2 is induced by IL-6 in IL-6 dependent MM cell lines (Croonquist and Van Ness, 2005).

Higher levels of methylation of EEF2 and SOCS1 were associated with higher levels of TNFα [reviewed by Gonzalez-Jaramillo et al. (2019)]. In AML, it has been suggested that stem-cell-like epitypes that lack a dominant driver mutation may be making use of pro-inflammatory signaling for AML cell survival and proliferation and that DNA methylation clustering can be used to identify a subset that makes use of the STAT inflammatory pathway (Giacopelli et al., 2021).

In triple-negative breast cancer, acetylated STAT3 associates with DMNT1 and promotes aberrant promoter hypermethylation (Lee et al., 2012). In basal-like breast cancer, NF-κB drives the repression of the Ten-eleven translocation enzymes TET1, which induces DNA demethylation by converting 5mC to 5hmC. This is also associated with activation of immune pathways and with tumor infiltration by immune cells. This link between NF-κB, immune cell infiltration, and TET1 suppression, leading to global changes in the methylome, is also found in melanoma, lung cancer, and thyroid cancer (Collignon et al., 2018).

In ovarian cancer cells, TGF-β-induced methylation of CpG islands located in or near promoters of genes involved in EMT and cancer progression was achieved through TGF-β-dependent upregulation of DNMTs (Cardenas et al., 2014). Interestingly, TGF-β can also inhibit DNMT1 and DNMT3a expression and global DNMT activity. In a rat model, it was shown to upregulate Collagen type I alpha I (COL1A1) by this mechanism (Pan et al., 2013). In cervical cancer, NO-dependent inflammation is known to deregulate DNA methylation of many target genes, including PTPRR (Su et al., 2017). Other hypermethylated targets include cell adhesion molecule 1 (CADM1) gene and T lymphocyte maturation associated protein (MAL) (Holubekova et al., 2020).

Changes in methylation patterns can be brought about by sex steroid hormones, treatment with which was shown to alter methylation patterns and expression of a variety of inflammatory signaling molecules in prostate cancer. Hypomethylated genes included CXCL12, CXCL5, CCL25, IL1F8, IL13RAI, STAT5A, CXCR4, and TLR5; while hypermethylated genes included ELA2, TOLLIP, LAG3, CD276, and MALT1 (Wang S. et al., 2016).

In oral squamous cell carcinoma (OSCC), IL6-induced inflammation alters global LINE1 hypomethylation, while also resulting in the hypermethylation of tumor suppressor genes such as CHFR, GATA5, and PAX6. Thus, inflammation-induced alterations to DNA methylation is thought to have important implications in tumor progression (Gasche et al., 2011).

IL1β is known to be highly expressed in the tumor microenvironment of various cancer types, and drives many malignant processes such as initiation, proliferation, and metastasis. IL1 is also key for angiogenesis and tumor growth and has been linked to metastases in various forms of cancer (Elaraj et al., 2006; Voronov et al., 2007). Similarly, IL8 is also known to be constitutively produced by cancer cells and cell lines and plays a role in tumor growth and metastasis (Xie, 2001). IL6 is a key pro-inflammatory cytokine that drives chronic inflammation in a number of ways and has been linked to poor survival outcomes in various forms of cancer (De Vita et al., 1998). Similarly, in a mouse model of gastric cancer, IL1β signaling was linked to promoter methylation and transcriptional repression of E-cadherin, a gene that is critical in preventing cell migration and metastasis (Huang et al., 2016). NF-κB promotes the expression of programmed death ligand 1 (PD-L1) through demethylation of its promoter. PD-L1 mediates a negative feedback of lymphocyte activation, a loop that is exploited by tumors for immune evasion and survival. The upregulation of PD-L1 is also linked to EMT (Antonangeli et al., 2020, p. 1). An EWAS-identified locus in the NLR Family CARD Domain Containing 5 (*NLRC5)* gene was

associated with CXCL11, CXCL9, IL-12, and IL-18 levels (Ahsan et al., 2017). NLRC5 is involved in interferon-linked innate immunity through its ability to regulate MHC Class I receptors.

CONCLUSION

The crosstalk between inflammation and epigenetic rewiring is one that is coming to be increasingly understood as posing unique challenges and opportunities for therapeutic development in cancer. Epigenetic rewiring by cancer cells allows them to manipulate and exploit several processes and phenomena that give them a survival advantage. Epigenetic modulators constitute a singularly useful class of enzymes for cancer cells, given their potentially wide range of targets and their reversible effects. A single modulator can have a host of target genes involved in a whole host of processes, so that alteration of that modulator alone can be used to achieve a wide range of downstream effects. This property of epigenetic modulators, however, also makes them useful targets for development of therapeutics. Inflammatory signaling networks are often deregulated in cancer through epigenetic means, meaning that the modulators can be targeted to treat inflammation, along with other cancer phenotypes regulated by that modulator. However, as discussed above, the opposite also happens; epigenetic rewiring may be a result, rather than the cause, of inflammatory dysregulation, so that it may be necessary to develop other means to target this oncogenic phenotype. It is also necessary to consider the global effects of epigenetic drugs. It has been shown that the use of DNMT inhibitors, for example, causes altered global gene profiles, which may result in the undesired activation or suppression of an entirely new set of genes (Giri and Aittokallio, 2019). Understanding the overall effects of these drugs requires further investigations.

Many cancers make use of similar alterations to give themselves a survival advantage, and it is therefore unsurprising that certain factors are overexpressed or underexpressed across an array of different cancers through similar mechanisms. For example, BRAK/CXCL14 is a chemokine that is constitutively produced by most tissue types but has been found to be depleted in a variety of human cancers and tumor cell lines. As a potent inhibitor of angiogenesis as well as a powerful chemoattractant for monocytes and dendritic cells, it is downregulated by cancers to allow the critical processes of angiogenesis and tumor infiltration by immune cells (Shellenberger et al., 2004; Shurin et al., 2005). The downregulation of CXCL14 is achieved, at least in part, through the hypermethylation of CpG island sequences in the CXCL14 gene promoter. Consequently, treatment using 5-Aza results in increased CXCL14 production and chemoattractant activity of conditioned medium (Song et al., 2010, p. 14). Similarities between various forms of cancer allow us to take the lessons and therapeutics developed from one form and attempt to apply it to others.

However, cytokines and chemokines have pleiotropic roles and may be exploited in different ways by different cancers to achieve survival, growth, and metastasis. For example, the promoter of IL10 has been shown to be either hypermethylated or hypomethylated depending on the type of cancer. A study that examined the methylation status of the IL10 family of genes across colon, kidney, lung, stomach, and breast cancer datasets found that these genes are typically hypomethylated (Shen et al., 2012), but this is not always the case. This underlines to need to exercise caution when trying to apply the principles and strategies used for one form of cancer to deal with another.

Even within a single type of cancer, there may be subsets of populations that show differential expression of certain genes. For example, while COX-2 is generally upregulated in CRCs, there is a small population in which it is downregulated through promoter hypermethylation, and this downregulation, in fact, is linked to poorer survival outcomes (Toyota et al., 2000; Song et al., 2001; Kikuchi et al., 2002; Hur et al., 2003; Soumaoro et al., 2004; Ogino et al., 2008; Wang and Dubois, 2010; Fernández-Alvarez et al., 2012). Using DNMT inhibitors would, therefore, exacerbate this effect in this group of patients.

Another thing to consider is the fact that the mechanisms by which histone methylation regulates gene expression are still debated. Detailed mechanisms linking H3K4me3 to upregulation in gene expression are still to be explored. Crosstalk between histone arginine methylation and lysine methylation are important subjects to be explored in the contexts of cancer and inflammation. The in-depth mechanisms of these interactions and upstream events along with the recruitment of the histone modifiers should be looked in greater detail for development of potent epi-drugs. Furthermore, with global changes in epigenetic marks, it can be difficult to disentangle cause from effect. H3K4me3 may be the "cause" rather than "effect" of upregulated gene expression. Likewise, as previously mentioned, it has been suggested that the *de novo* methylation may simply be a part of genome-wide process in a distinct subgroup of cancers characterized by a profound methylator phenotype (Sato et al., 2005). In other words, it may not always be possible to recognize the functional effect of certain methylation signatures through genome-wide screens, and changes in methylation may not necessarily lead to biologically significant changes in gene expression or activity, but may simply be a result of other changes.

Finally, it is important to recognize the interplay between groups of enzymes that perform opposing functions. For example, both DNMTs and TETs have been implicated in cancer, as both global hypomethylation and regional hypermethylation have been identified (Ehrlich, 2002; Szyf, 2003). It is important to understand both the global as well as the gene-specific roles of these two antagonistic groups of enzymes before attempting to target one or the other for therapy. After all, hypomethylation can in theory be attributed to either reduced DNMT activity or increased TET activity, and the opposite holds true for hypermethylation.

FUTURE PERSPECTIVES

The latest technological advancements in whole-genome transcriptomics analysis and epigenomic profiling will be crucial in the development of targeted therapeutic strategies. Despite their shortcomings, methylation signatures may still

have some prognostic value. DNA methylation is critical for lineage specificity and cell differentiation, particularly for hematopoiesis and for the development of the myeloid-derived suppressor cells that are generally produced in response to tumor secreted factors, and which are linked to cancer-associated inflammation. Immunomethylomics, or methylation profiling of the immune cells using DNA from archival peripheral blood may be developed as a potential prognostic tool for solid tumors as well as in lymphatic/hematopoietic cancers, in which differential methylation and differential variability in methylation are associated with tumor progression and outcomes (Kelsey and Wiencke, 2018; Domingo-Relloso et al., 2021). In addition, integrating expanded DNA methylation data with somatic mutation data and gene expression has allowed the identification of "triple-evidenced" genes, which are differentially expressed, differentially methylated, and associated with somatic mutation in different forms of cancer, which could be further investigated as prognostic markers or therapeutic targets (Fan et al., 2019). Large, representative datasets make the identification of targets much more robust and reliable, indicating the need for collaboration, sharing, and meta-analysis of datasets across populations that may differ in terms of age, race, sex, and indeed, various other factors. This will be needed in order to extract meaningful, reproducible data that are both widely applicable and specific to certain populations. For instance, it is known that some populations are more susceptible to chronic inflammation and certain types of cancers, and these factors must be taken into consideration (Ranjit et al., 2007; Stepanikova et al., 2017; Özdemir and Dotto, 2017; Schmeer and Tarrence, 2018; Meaney et al., 2019; Yeyeodu et al., 2019).

Much of what we understand about inflammation and inflammatory pathways in cancer comes from research on other diseases in which inflammation is the most defining characteristic. Many allergic, autoimmune, and age-related conditions are characterized by major aberrations in inflammatory networks that can inform research on inflammation in cancer as well. The use of drugs that are used to treat inflammation in other pathologies may well be used along with existing cancer therapeutics in order to take a more holistic treatment approach. For example, it has been shown in lung cancer that the combined treatment of aspirin, a commonly used anti-inflammatory drug, and radiotherapy, resulted in a synergistic reduction of cell viability, partly through downregulation of COX-2 (Sun et al., 2018). Drugs that target inflammatory enzymes, such as non-steroidal anti-inflammatory drugs (NSAIDs), which target COX2, could also have a more complex role to play if inflammation lies upstream of epigenetic changes and altered genome profiles. In addition to drugs, dietary anti-inflammatory natural compounds, such as Vitamin C, D, and E all have effects on both inflammation and epigenetics, particularly DNA methylation (Saccone et al., 2015; Castellano-Castillo et al., 2018; Gerecke et al., 2018; Zappe et al., 2018; Yang et al., 2019). However, further work will be required to understand which combinations of drugs work together.

AUTHOR CONTRIBUTIONS

DD and NK contributed equally to the writing of this article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Diverse Roles of F-BoxProtein3 in Regulation of Various Cellular Functions

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Accumulated evidence shows that the F-box protein 3 (FBXO3) has multiple biological functions, including regulation of immune pathologies, neuropathic diseases and antiviral response. In this review article, we focus on the role of FBXO3 in inflammatory disorders and human malignancies. We also describe the substrates of FBXO3, which contribute to inflammatory disorders and cancers. We highlight that the high expression of FBXO3 is frequently observed in rheumatoid arthritis, leukemia, pituitary adenoma, and oral squamous cell carcinoma. Moreover, we discuss the regulation of FBXO3 by both carcinogens and cancer preventive agents. Our review provides a comprehensive understanding of the role of FBXO3 in various biological systems and elucidates how FBXO3 regulates substrate ubiquitination and degradation during various physiological and pathological processes. Therefore, FBXO3 can be a novel target in the treatment of human diseases including carcinomas.

Keywords: Fbxo3, carcinomas, inflammation, rheumatoid arthritis, ubiquitination

INTRODUCTION

Ubiquitination is one type of post-translational modifications (PTMs), which regulates cellular protein concentrations in eukaryotic organisms (Senft et al., 2018). The ubiquitin protease system selectively targets multiple proteins for degradation through the use of activating (E1), conjugating (E2) and ligating (E3) enzymes (Popovic et al., 2014). In particular, E3 ubiquitin ligases determine substrate specificity for ubiquitination and then transfer ubiquitin chains to the substrate, which leads to substrate degradation in the 26S proteasome (Grabbe et al., 2011). Ubiquitination is involved in the regulation of almost all cellular activities, including embryonic development, cell proliferation, apoptosis, autophagy, signal transduction and DNA repair (Grabbe et al., 2011; Popovic et al., 2014). In recent years, Bortezomib as a proteasome inhibitor has been approved for the treatment of multiple myeloma and mantle cell lymphoma (Palumbo et al., 2016). Targeting specific substrates for ubiquitination has become a new clinical therapeutic strategy.

The ubiquitin protein ligase complex Skp1-Cullin1-F-Box (SCF) is composed of four subunits: F-box protein, SKP1, CULLIN1, and RBX1 (Skaar et al., 2014). Twenty years after the discovery of the F-box protein family, around 70 different F-box proteins have now been identified in mammals (Skaar et al., 2013). F-box proteins are classified into three categories based on the type of C-terminal interaction: FBXW (containing WD40 repeats), FBXL (containing leucine-rich repeats), and FBXO (containing neither, but with other domains) (Wang et al., 2014). The F-box protein directs the SCF complex to specific substrates for ubiquitination. Emerging evidence has demonstrated that F-box

proteins are associated with the aggressiveness of human tumors, cell cycle regulation, and regulation of the epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs) as well as drug resistance (Song et al., 2019; Yan et al., 2020). The F-box protein 3 (FBXO3), also known as FBX3, F-box only protein 3, F-box protein FBX3, and FBX, is encoded by a gene located on chromosome 11p13. Human FBXO3 gene has 13 splicing variants and belongs to the F-box protein family. Recently, studies have shown that FBXO3 participates in immune pathologies, neuropathic diseases, antiviral response, inflammatory disorders and human malignancies (Mallampalli et al., 2013; Lin et al., 2015; Shao et al., 2016; Hung et al., 2019). In the following paragraphs, we describe how FBXO3 contributes to inflammatory disorders and cancers, including leukemia, pituitary adenoma, oral squamous cell carcinoma and breast cancer. Moreover, we describe the regulatory mechanism of FBXO3 by carcinogens and cancer preventive agents.

Structure of FBXO3

The human FBXO3 protein has 471 amino acids, with a molecular mass of 54,561 Da. Alternative splicing of FBXO3 gene generates two transcript variants diverging at the 3′ end. FBXO3 has two domains at its C-terminal: F-box domain (positions 10–56) and ApaG (Adenine tetraphosphate adenine G) domain (positions 278–408). ApaG domain is involved in mediating the ubiquitination and degradation of F-box and leucine-rich repeat protein 2 (FBXL2), resulting in cytokine gene transcription and promoting the progression of inflammation (Mallampalli et al., 2013). The relevant ApaG domain consists of an immunoglobin/fibronectin III-type fold and a classical β-sheet core. The central β-sheet core is a potential target in drug discovery, which aims at regulating inflammation and malignancies (Krzysiak et al., 2016). The FBXO3 has three described isoforms: isoform 1 (Q9UK99-1), isoform 2 (Q9UK99-2, 414–415: EM →VS, 416–471: Missing), and isoform 3 (Q9UK99-3, 36–40: Missing, 414–415: EM →VS, 416–471: Missing). The FBXO3 protein recognizes specific substrates for ubiquitination and degradation. Additional structural studies are needed to elucidate the functions of each domain of the FBXO3 protein in its various physiological and pathological processes.

FBXO3 Regulates Inflammation

Following infection with a virulent pathogen, there is an excessive release of cytokines from proinflammatory cells, including macrophages, lymphocytes and polymorpho nuclear leukocytes (Dinarello, 2007; Sheu et al., 2010). This process, known as a "cytokine storm", leads to hypercytokinemia where in hypercytokines it increases capillary permeability and tissue edema causing fever, pain, multiple organ failure, and even death (Nathan, 2002; Aird, 2003). TRAF proteins are cytokine signaling adapter proteins that are critically involved in inflammation and programmed cell death (Inoue et al., 2000; Mallampalli et al., 2013). FBXO3 proteins are critically involved in inflammation and target FBXL2 for degradation, partly promoting TNF receptor-associated factor (TRAF) signal transduction and cytokine gene transcription (Inoue et al., 2000; Mallampalli et al., 2013). Chen et al. reported that mice with FBXO3 knockout that are infected with *Pseudomonas aeruginosa* showed reduced lavage cytokine levels, protein concentrations, and proinflammatory cell counts in the lung tissue. This suggests that FBXO3 knockdown attenuates lung injury induced by *Pseudomonas* and reduces mortality (Chen et al., 2013). Typically, patients with sepsis die of organ dysfunction because of an unusually strong reaction response to an infection (Pantzaris et al., 2021). The researchers also found that subjects with sepsis had more TRAF and FBXO3 proteins and less FBXL2 protein in circulating white blood cells compared with control subjects. Moreover, the circulating FBXO3 and TRAF proteins in sepsis patients had positive correlations with cytokine responses (Chen et al., 2013).

Studies of the FBXO3 C-terminal structure demonstrate that the classical ApaG molecular features are indispensable for mediating FBXL2 ubiquitination and for promoting the release of cytokines (Krzysiak et al., 2016). This leads to the development of BC-1215 compound, which is a highly selective small molecule as a FBXO3 antagonist targeting the ApaG domain (Chen et al., 2013). BC-1215 decreases FBXO3-FBXL2 interaction in a dose-dependent manner and protects FBXL2 from FBXO3-induced degradation, which effectively lowers the expression of TRAF1-TRAF6 proteins. The addition of BC-1215 reduces proinflammatory cytokines and modestly inhibits bacterial growth in a mouse model of cecal ligation and perforation to induce sepsis (Chen et al., 2013). Treatment with BC-1215 or knockdown of FBXO3 were found to attenuate the inflammation of lung tissue induced by *Pseudomonas* and the H1N1 influenza virus, ear injury induced by tetradecanoylphorbol, and active colitis induced by acetate dextran sulfate sodium (Chen et al., 2013; Mallampalli et al., 2013). Furthermore, the downregulation of FBXO3 levels attenuates lung edema induced by ischemia-reperfusion (I/R) (Hung et al., 2019).

Oxygen glucose deprivation/re-oxygenation (OGD/R) model is often used for ischemia studies via oxygen and glucose deprivation and then reoxygenation to mimic ischemia/reperfusion injury condition. In the OGD/R model, miR-142-3p directly targets FBXO3 to ameliorate inflammation and apoptosis in SH-SY5Y cells (Li and Ma, 2020). The inflammasome is a complex composed of multiple proteins that regulate the maturation and release of pro-inflammatory cytokines, such as IL-1β and IL-18. Lipopolysaccharide (LPS) exposure attenuates FBXL2-induced NALP3 inflammasome ubiquitination by activating FBXO3, thereby increasing the secretion of IL-1β and IL-18 in inflammatory cells (Han et al., 2015). FBXO3 also potentiates vascular inflammation and increases atherosclerosis. Depletion of FBXO3 protein in macrophages eliminates oxidatively modified low-density lipoprotein-induced inflammation without affecting oxidized low-density lipoprotein uptake by macrophages (Chandra et al., 2019). Treatment with BC-1215 reduces the release of IL-1β and TNF-α (Chandra et al., 2019), which alleviates FBXO3-induced vascular inflammation and atherosclerosis. These data suggest that FBXO3 is a novel target of drug design that aims to alleviate atherosclerosis driven by pro-inflammatory cytokines. This benefit might extend beyond low-density lipoprotein reduction.

FBXO3 Regulates Neuropathic Pain

Rab3-interactive molecule-1α (RIM1α) is essential for C-terminal regions-associated vesicle exocytosis (Schoch et al., 2006) and spinal plasticity in the presynaptic site of the dorsal horn, which contributes to the development of neuropathic pain. The voltage-gated N-type Ca^{2+} channel (Cav2.2) has been demonstrated that promoted neuropathic pain in mice model (Saegusa and Tanabe, 2014). Besides, it has been reported that RIM is related to Cav2.2 in neuropathic pain via promotion of vesicle exocytosis (Coppola et al., 2001; Hibino et al., 2002). One study demonstrated that FBXO3-dependent FBXL2 ubiquitination promotes RIM1α/CaV2.2 cascade in neuropathic pain based on spinal plasticity (Lin et al., 2015). FBXO3 degrades FBXL2 and leads to deubiquitination of RIM1, resulting in enhanced RIM1 interaction with the CaV2.2, which contributes to chronic pain due to upregulating CaV2.2 (Lai et al., 2016).

The roles of NcK-interacting kinase (TNIK) in neuropathic pain development is coupling TNIK–GluR1 and leads to subcellular redistribution of GluR1-AMPA receptors (AMPARs) (Hussain et al., 2010). TRAF2 enhances TNIK/GluR1 phosphorylation-dependent subcellular GluR1-AMPARs redistribution, leading to spinal nerve ligation-induced allodynia (Lin et al., 2015). Allodynia is a kind of pain due to a hypersensitive reaction to a normal stimulus. Similar to RIM1α, TRAF2 is also regulated by FBXO3-dependent FBXL2 (Chen et al., 2013; Mallampalli et al., 2013). FBXO3 is involved in neuropathic allodynia via its effects on degradation of FBXL2 and upregulation of TRAF2, and administration of BC-1215 ameliorates this allodynia (Lin et al., 2015). While the research in this field is still infancy, FBXO3 might provide a potential drug target for neuropathic pain relief.

FBXO3 Regulates Autoimmunity Functions

Autoimmune regulator (AIRE) as a transcription factor is crucial for the maintenance of self-tolerance (Finnish-German, 1997; Nagamine et al., 1997). Impairment of AIRE activity is implicated in disturbed negative selection of T cells that are specific for self-antigens, which leads to lymphocytic infiltration of affected organs and causes disorders in immunological homeostasis. These ultimately result in autoimmune diseases, including type 1 diabetes mellitus, thymomas, and autoimmune thyroid diseases (Peterson and Peltonen, 2005; Sabater et al., 2005; Perheentupa, 2006; Gavanescu et al., 2007). FBXO3 has been reported to regulate autoimmunity by promoting the ubiquitination and transcriptional activity of the AIRE (Shao et al., 2016). AIRE is phosphorylated on the Thr-68 and Ser-156 residues near its N-terminus allowing it to bind to FBXO3 and becomes ubiquitinated. The ubiquitination of AIRE increases the activity of tissue-specific antigens (TSA) genes and enhances the recruitment of positive transcription elongation factor b (P-TEFb) to target genes (Shao et al., 2016), which influences the maturation of thymocytes (Peterson et al., 2008).

FBXO3 Negatively Regulates Antiviral Response

Rift Valley fever virus (RVFV) infection can cause animal-derived diseases, which are transmitted to people mainly through mosquito bites or contact with infected livestock (Bird et al., 2009; Boshra et al., 2011). RVFV is prevalent in Africa and has caused frequent outbreaks that result in devastating loss of lives and properties. It is known that interferon activation is essential for antiviral response. One group showed that FBXO3 inhibits the antiviral response in host cells. As the main virulence factor of RVFV, the nonstructural protein NSs recruits FBXO3 to degrade the transcription factor TFIIH-p62 of host cells (Kainulainen et al., 2014). This facilitates the pathogenesis of RVFV by inhibiting transcriptional upregulation of the innate immunity and hindering the antiviral type I interferon (IFN-I) system to allow uncontrolled viral replication (Bouloy et al., 2001; Billecocq et al., 2004). NSs interacts with the full-length FBXO3 as well as with a truncated isoform that lacks the C-terminal acidic and poly(R)-rich domains (Kainulainen et al., 2014).

The genome structures of type 1 IFN in fish are similar with mammals (Kirsten et al., 2018). IRF3 and IRF7 are transcription factors of the IFN regulatory factor (IRF) family that induce IFN expression (Lu et al., 2019). IFN induces transcription of downstream antiviral genes through activation of JAK-STAT signaling pathway (Cheng et al., 2019). IRF3 and IRF7 degradation and IFN signaling activation are critical for FBXO3-mediated antiviral response in zebrafish. FBXO3 negatively regulates antiviral response by promoting K27-linked ubiquitination and proteasomal degradation of IRF3 and IRF7 in an F-box domain-independent manner (Li et al., 2020). In zebrafish model, FBXO3 deletion induces the expression of key antiviral genes and shows higher resistance to virus infection in liver and spleen (Li et al., 2020).

Role of FBXO3 in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease because immune system hurts healthy cells, leading to erosion of bone and cartilage in joints, especially in the hands, knees and wrists, which causes pain, stiffness, swelling and dysfunctions in the joints (Wang et al., 2019). To investigate the role of FBXO3 in RA, Masuda et al. examined the expression of a diverse array of genes through *in situ* hybridization (Masuda et al., 2002). They concluded that some proliferation-related molecules, including FBXO3, displayed higher expression levels in RA synovial tissues compared to normal synovial tissues (Masuda et al., 2002). The involvement of FBXO3 in RA has not been clearly demonstrated. It is known that F-box proteins regulate the cell cycle (Winston et al., 1999), FBXO3 might be potentially involved in survival and proliferation of RA synovial fibroblast (RA-SF).

Role of FBXO3 in Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) has a poor prognosis, and most of patients with ALS die within 3–7 years after involving the respiratory muscles (Talbott et al., 2016). The pathogenic mechanism is still unclear, and no effective treatment is available. Chromosome 21 open reading frame 2 (C21ORF2) interacts with never in mitosis gene A related kinase 1 (NEK1) that involve in DNA damage repair and regulate cell cycle, which is highly related to ALS development (Fang et al., 2015; Chia et al.,

2018). Watanabe et al. showed that Fbxo3 targeted C21ORF2 for ubiquitination and degradation (Watanabe et al., 2020). Because NEK1 was stabilized by C21ORF2, depletion of FBXO3 stabilized both C21ORF2 and NEK1. NEK1-mediated phosphorylation of C21ORF2 can protect it from proteasome-dependent degradation due to attenuating the interaction between FBXO3 and C21ORF2 (Watanabe et al., 2020). Inhibition of NEK1 activity and increased degradation of C21ORF2 by FBXO3 may be potential approaches for treatment of patients with ALS.

Role of FBXO3 in Human Cancers

FBXO3 Regulates Pathogenesis in Several Types of Cancers

FBXO3 has been demonstrated to participate in the occurrence and progression of a variety of human cancers. It is noteworthy that in acute promyelocytic leukemia, the PML gene is the site of the t (15, 17) chromosomal translocation wherein it is fused to the retinoic acid receptor (RAR) gene, leading to the generation of PML-RAR fusion protein (de Thé et al., 1990; de Thé et al., 1991; Goddard et al., 1991; Kakizuka et al., 1991). It has been shown that PML activates transcription by preventing FBXO3-catalyzed ubiquitination of HIPK and p300 (Shima et al., 2008). Conversely, PML-RAR enhances FBXO3-induced degradation of HIPK2 and p300 in a dose-dependent manner to inhibit transcription, which might contribute to the pathogenesis of leukemia (Shima et al., 2008). Another study by Laura et al. analyzed the relationship between FBXO3 and pituitary adenoma and showed that FBXO3 enhances the degradation of the aryl hydrocarbon receptor-interacting protein (AIP) through the rapid ubiquitin proteasome pathway, which has direct implications for the phenotype. Through this, a novel pathogenic mechanism of pituitary adenoma was generated (Hernández-Ramírez et al., 2016). In a work done by Cha et al., which employed a combination of array-based comparative genomic hybridization and multiplex ligation-dependent probe amplification, it was found that the highest deletion frequencies in FBXO3 might be related to the occurrence and progression of oral squamous cell carcinoma (Cha et al., 2011).

The FBXO3-ΔNp63α is critical for TGF-β-induced tumor metastasis (Hao et al., 2019). ΔNp63 has been identified as a subtype of p63 that leads to regulation of cell proliferation, cell adhesion, EMT, and inhibition of tumor metastasis (Bergholz and Xiao, 2012). FBXO3 promotes breast cancer metastasis through K48-linked polyubiquitination of the ΔNp63α. This process is independent of Smad but dependent of Erk (Niu et al., 2021). Upregulation of FBXO3 by activation of TGF-β results in the degradation of ΔNp63α with concomitant decreased expression of E-cadherin and desmoplakin (DPL) (Niu et al., 2021). In addition, the high expression of FBXO3 indicates poor prognosis in patients with breast cancer (Niu et al., 2021). Taken together, FBXO3 targets different substrates to participate in carcinogenesis.

FBXO3 Regulates Smurf1 and BMP Pathway

The Smad ubiquitination regulatory factor 1 (Smurf1) is a member of the HECT-type E3 ubiquitin ligases and based on its C2-WW-HECT architecture, which belongs to the neural precursor cell-expressed and developmentally downregulated gene 4 (Nedd4) family of lipases (Fu et al., 2020; Wang et al., 2020). Smurf1 regulates several biological pathways, including the transforming growth factor beta (TGF-β), the bone morphogenetic protein (BMP), the non-canonical Wnt pathway, and the mitogen-activated protein kinase (MAPK) pathway (Fu et al., 2020). Smurf1 is also related to cell growth and migration, embryonic development, immune responses, and tumorigenesis. FBXO3 targets all the Nedd4 family members including Smurf1 for degradation (Li et al., 2015). FBXO3 upregulates BMP pathway by mediating Smurf1 ubiquitination *in vivo* as well as *in vitro* and FBXO3 significantly promotes the poly-ubiquitination of Smurf1 (Li et al., 2015).

FBXO3 Regulates DNA Damage

The mouse homolog of diaphanous 2 (mDia2) belongs to the diaphanous-related formin 1 (mDia1) family. mDia2 influences the remodeling of actin and microtubule cytoskeletons after transformation to its active conformation (Chesarone et al., 2010), and plays a crucial role in cell invasion and cytokinesis (Lizárraga et al., 2009; Daou et al., 2014). FBXO3 forms a complex with mDia2 and p53, and co-expression of mDia2 and FBXO3 promotes p53-dependent apoptosis in an actin-nucleation-independent manner (Isogai et al., 2015). As a tumor suppressor, p53 regulates cell growth through cellular apoptotic programs and DNA repair. FBXO3 knockdown attenuates p53-mediated apoptosis upon DNA damage (Bieging and Attardi, 2012).

FBXO3 Regulates Cell Apoptosis

Studies have shown that FBXO3 contributes to tumor progression but also increases tumor cell apoptosis. Recruitment of histone deacetylases by oncoproteins is a key inciting event for cancer progression (Hess-Stumpp, 2005; Minucci and Pelicci, 2006). In lung squamous cell carcinoma, Kong et al. demonstrated that treatment with the histone deacetylase inhibitor belinostat (PXD101) transcriptionally upregulates FBXO3 and FBXW10, which directly target son of sevenless (SOS), an upstream regulator of the MAPK pathway, to inhibit growth of tumor cells (Kong et al., 2017). Thus, the induction of tumor cell apoptosis is increased, and drug resistance to cisplatin is reduced. This suggests that targeting FBXO3 might be a novel strategy for cancer treatment.

FBXO3 Is Regulates by miRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that regulate target genes at the post-transcriptional level (Winkle et al., 2021). Notably, non-coding RNAs are essential for maintaining cellular homeostasis and perform their functions by regulating cell proliferation, migration, invasion, metastasis, and apoptosis (Goodall and Wickramasinghe, 2021; Jiang et al., 2020). The levels of some miRNAs have been shown to correlate with various cancers through negatively regulating genes including F-box proteins (Lin et al., 2019). Analyses using the TargetScan online computational algorithm (www.targetscan.org) and luciferase reporter genes showed that FBXO3 is identified as a

TABLE 1 | The roles of FBXO3 in various biological functions.

Functions	Targets	References
Regulates inflammation	FBXL2	Mallampalli et al. (2013)
Neuropathic pain	FBXL2	Lin et al. (2015)
Immunoregulation	AIRE	Shao et al. (2016)
Negatively regulates antiviral response	TFIIH-p62	Kainulainen et al. (2014)
Rheumatoid arthritis	N/A	Masuda et al. (2002)
Leukemia	HIPK, p300	Shima et al. (2008)
Pituitary adenoma	AIP	Hernández-Ramírez et al. (2016)
Oral squamous cell carcinoma	N/A	Cha et al. (2011)
Breast cancer	ΔNp63	Niu et al. (2021)
Tumorigenesis	Smurf1	Li et al. (2015)

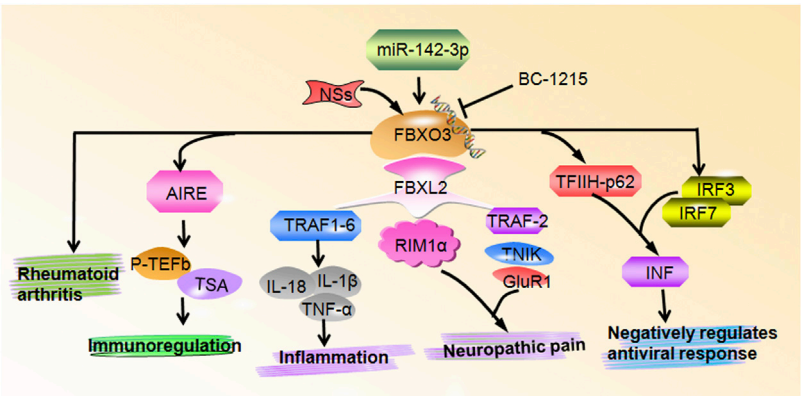

FIGURE 1 | FBXO3 regulates multiple biological functions. FBXO3 controls numerous cellular signaling pathways and genes to participate in inflammation, neuropathic pain, immunoregulation, negatively regulates antiviral response and rheumatoid arthritis.

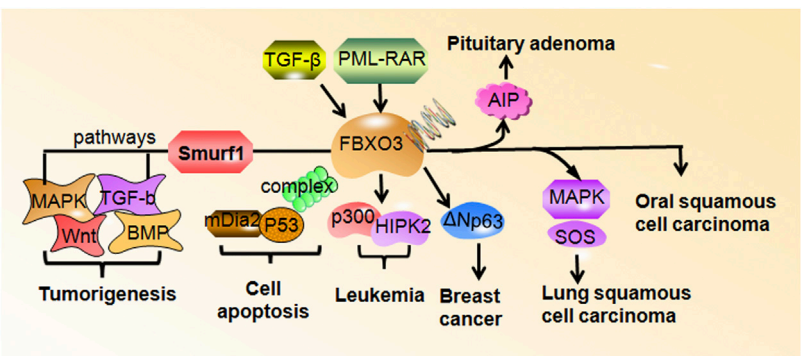

FIGURE 2 | FBXO3 regulates downstream targets in human cancers. FBXO3 targets numerous genes to participate in tumorigenesis and cell apoptosis. FBXO3 is regulated by TGF-βand PML-RAR. The high expression of FBXO3 is frequent in rheumatoid arthritis, leukemia, pituitary adenoma, oral squamous cell carcinoma.

target of miR-142-3p (Li and Ma, 2020). It has been shown that miR-142-3p plays key roles in tumorigenesis and cancer progression and is expressed at lower levels in breast tumor tissues than in those from normal individuals (Xu et al., 2020). miR-142-3p negatively regulates the canonical Wnt signaling pathway to regulate human breast cancer stem cells (Isobe et al., 2014). Several studies have also found that miR-142-3p is an important regulatory element in hepatocellular carcinoma, cervical cancer, non-small cell lung carcinoma, and glioblastoma (Chai et al., 2014; Xiao and Liu, 2015; Shrestha et al., 2017; Dong and Song, 2021). Furthermore, miR-142-3p has also been linked to inhibition of tumor progression and invasion. Thus, miR-142-3p might be useful in targeting cancer stem cells (Ghafouri-Fard et al., 2021). However, the relation between miR-142-3p to

FBXO3 in human cancer cells is not yet clear and needs further research.

CONCLUSION AND PERSPECTIVE

FBXO3 is involved in numerous biological functions and has an important impact on promoting inflammation, immune regulation, the inhibition of IFN-I that triggers virus replication, and the processes of neuropathic pain and rheumatoid arthritis (**Table 1** and **Figure 1**). Among these, the most studies determine the role of FBXO3 in the pathophysiological mechanism of inflammation. According to previous researches, FBXO3 is a critical modulator of inflammation which can inhibit LPS stimulation of inflammatory responses by promoting and inhibiting the degradation of FBXL2 and TRAFs, respectively. A new area being explored in cancer research is the role of ubiquitination in inflammasome biology. Leucine-rich repeat receptors (NLRs) and melanoma 2 (AIM2)-like receptors (ALRs) families are important in the assembly of the inflammasome complex (Schroder and Tschopp, 2010). Among these, NLRP1, NLRP3, and NLRC4 are linked to inflammatory diseases and colorectal cancer (Jin et al., 2007; Man and Kanneganti, 2015). The formulation of new immunotherapy to regulate inflammasomes governed by ubiquitination can provide a novel strategy for the treatment of diseases.

A review of literature shows contradicting reports on the effect of FBXO3 on tumor development (**Figure 2**). The expression of FBXO3 is increased in oral squamous cell carcinoma, acute promyelocytic leukemia, pituitary adenoma, and breast cancer. However, co-expression of FBXO3 and p53 promotes apoptosis of tumor cells (Bieging and Attardi, 2012). In addition, FBXO3 in combination with chemotherapeutic drugs can reduce drug resistance and increase chemical sensitization. These independent findings corroborate the potential roles of FBXO3 in the processes of anti-tumor activity and progression of tumors (Kong et al., 2017). While this may complicate the treatment, the specificity of FBXO3 makes it an attractive therapeutic target. However, the association of the expression of FBXO3 with tumor size, tumor stage, deep of infiltration, and prognosis in cancer patients has not yet been well established. The specific mechanism is also not clear, and there is still a lack of clinical data on FBXO3-related tumors.

In order to fully understand the role of FBXO3 in tumorigenesis, the following questions need to be addressed. What are the expression levels of FBXO3 in many human cancers other than oral squamous cell carcinoma, acute promyelocytic leukemia, and pituitary adenoma? What are the carcinogenic or anticancer signaling pathways that trigger FBXO3-induced oncogenesis? What are the targeted proteins of FBXO3 that are critically involved in tumor progression? What are other factors regulating the expression of FBXO3 in tumor cells? Is the high expression of FBXO3 associated with poor prognosis in various types of cancers? To find answers to these questions, FBXO3 knockout or knock-in transgenic mouse models could be used to validate the mechanism of FBXO3 in regulating the progression of human cancer. It is also important to look into various databases. Currently, the research on FBXO3 is still in its infancy, and further investigation is needed to develop better treatments using FBXO3 as a molecular target.

AUTHOR CONTRIBUTIONS

ZZ, ZB, JY, and PG searched the literature and made the figures and tables. ZZ drafted the manuscript. PW and DC edited and revised the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Epigenetic Regulation of Inflammatory Signaling and Inflammation-Induced Cancer

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Epigenetics comprise a diverse array of reversible and dynamic modifications to the cell's genome without implicating any DNA sequence alterations. Both the external environment surrounding the organism, as well as the internal microenvironment of cells and tissues, contribute to these epigenetic processes that play critical roles in cell fate specification and organismal development. On the other hand, dysregulation of epigenetic activities can initiate and sustain carcinogenesis, which is often augmented by inflammation. Chronic inflammation, one of the major hallmarks of cancer, stems from proinflammatory cytokines that are secreted by tumor and tumor-associated cells in the tumor microenvironment. At the same time, inflammatory signaling can establish positive and negative feedback circuits with chromatin to modulate changes in the global epigenetic landscape. In this review, we provide an in-depth discussion of the interconnected crosstalk between epigenetics and inflammation, specifically how epigenetic mechanisms at different hierarchical levels of the genome control inflammatory gene transcription, which in turn enact changes within the cell's epigenomic profile, especially in the context of inflammation-induced cancer.

Keywords: cancer, inflammation, epigenetics, histone modifications, high-order genome organization, super-enhancer, senescence

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INTRODUCTION

Chromatin structure serves as the foundation for regulating transcriptional processes, and chromatin-based alterations constitute one of the fundamental molecular mechanisms that govern cellular physiology, ranging from growth and differentiation to DNA damage repair and apoptosis. The regulation of chromatin structure via epigenetic changes, including histone modifications, chromatin remodeling and higher-order chromosomal interactions, controls the accessibility of chromatin for binding by transcription factors (TFs) and other transcriptional machinery in response to internal and external stimuli. Additionally, chromatin regulating factors interact dynamically with the epigenome to coordinate precise spatiotemporal gene expression programs that undergird cell identity and function. Misregulation of chromatin homeostasis can activate inflammatory signaling pathways that lead to the onset and development of cancer (Marazzi et al., 2018).

Inflammation is a beneficial immune defense response to curtail pathogenic infection and tissue damage. However, prolonged activation of inflammatory signaling results in chronic inflammation that can induce malignant cellular transformation. Indeed, inflammation and carcinogenesis are

closely interconnected, and patients debilitated with chronic inflammatory diseases bear an increased risk of developing cancer (Garcea et al., 2005; Vagefi and Longo, 2005; Peek and Crabtree, 2006). Significant progress has emerged in recent years investigating the complex crosstalk between inflammation and tumorigenesis, switching from a cancer-centric concept to a more comprehensive view of tumor ecology that consists of epigenetically plastic cancer cells and stromal cells, which include diverse immune cells, fibroblasts and vascular cells (Greten and Grivennikov, 2019). Moreover, chronic inflammation favors a tumor-permissive microenvironment that blocks anti-tumorigenic immunity and promotes tumor development. Tumor-educated immune cells and stromal cells enable tumor immune escape and cancer progression by upregulating immune checkpoint genes and producing pathogenic immunoglobulins and cytokines (Ren et al., 2012; Simon and Labarriere, 2017; Gu et al., 2019). Therefore, immune checkpoint blockade has recently become a popular and effective form of cancer therapy.

Besides immune cells, host microbiota can contribute to a chronic inflammatory environment, which supports tumor incidence, growth and metastasis, as previously documented in gastric and colorectal cancers (Xavier et al., 2020). Interestingly, microbial organisms also act as integral components of tumor tissues in various other cancer types, such as melanoma and glioma, as well as pancreatic, breast, lung and ovarian tumors (Nejman et al., 2020). Accordingly, perturbation of tumor-resident microbiota by antibiotics elicits a predominantly inhibitory effect on breast cancer distal metastases (Fu et al., 2022). Collectively, inflammation is integral in sculpting the gene expression trajectories of stromal and cancer cells within the tumor microenvironment to favor oncogenesis, which in turn re-shapes the epigenetic landscape of immune cells and induces tumor-promoting inflammatory states to establish a positive feedback cycle for further perpetuating cancer progression.

Oncogenic and inflammatory responses are regulated by common factors and signaling pathways. A classic example is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), a central transcription factor that is commonly activated in both tumor and immune cells to produce inflammatory cytokines, chemokines and growth factors, such as IL-1β, IL-6 and CCL2. Upon stimulation with the proinflammatory cues tumor necrosis factor alpha (TNFα) and lipopolysaccharide (LPS), p65, the core component of NF-kB, translocates into the nucleus, binds directly onto chromatin and induces its structural remodeling to orchestrate downstream transcriptional outputs (Brown et al., 2014). During this transactivation process, p65 also recruits and interacts with several chromatin regulators, such as epigenetic reader proteins (e.g., BRD4) and histone modifying enzymes (e.g., acetyltransferases CBP/p300) (Mukherjee et al., 2013; Hajmirza et al., 2018). Furthermore, NF-kB, in cooperation with BRD4, facilitates super-enhancer formation to trigger the production of proinflammatory transcripts (Brown et al., 2014). These observations illustrate the importance of transcription factors in directing inflammatory activation via epigenetic alterations.

In this review, we focus on the epigenetic regulation of inflammatory signaling in the context of cancer. We first describe how various chromatin modifications and histone variants function in mediating inflammatory responses. Next, we delineate the roles of chromatin structure modulation, super-enhancers and higher-order genome organization in contributing to key inflammatory transcription programs and inflammation-related oncogenic processes such as epithelial-mesenchymal transition (EMT) and senescence. Finally, we illustrate the bidirectional effects between epigenetic alterations and inflammation, as well as highlight the therapeutic application of anti-inflammatory and epigenetic drugs to combat cancer.

CHROMATIN MODIFICATIONS

Chromatin, a principal component of the nucleus, is organized around a fundamental repeating structure known as the nucleosome, each comprising eight core histone proteins (two each of histone H2A, H2B, H3 and H4) that scaffold the tight packaging of DNA. Protruding out of the nucleosomal structure includes the N-terminal tail of every histone and the C-terminal tail of histone H2A that permit post-translational modifications. These epigenetic changes affect chromatin structure and accessibility, thereby playing instrumental roles in regulating gene transcription in disease onset and progression, including inflammation in cancer (Bannister and Kouzarides, 2011).

Histone acetylation/deacetylation and methylation/demethylation are among the most predominant histone modifications that occur on all core histones, and they modulate inflammatory responses in both cancer and immune cells. Aside from these two histone modifications, histone phosphorylation and ubiquitination have also gradually gained attention for their crucial roles in regulating transcription and chromatin structure. As the roles of histone and DNA methylation/demethylation in cancer and inflammation have been recently and extensively reviewed (Das et al., 2021), here we focus on the mechanistic basis of histone acetylation/deacetylation, phosphorylation and ubiquitination, and how they mediate inflammatory signaling in cancer.

Histone Acetylation and Deacetylation

Histone acetylation, one of the most prevalent histone post-translational modifications, is dynamically regulated by two protein families of opposing functions: histone acetyltransferases (HATs) and deacetylases (HDACs). HATs acetylate lysine residues of histones by transferring acetyl groups from acetyl-coenzyme A, thereby reducing the positive charge of lysine and weakening the interplay between DNA and histones (Racey and Byvoet, 1971; Bannister and Kouzarides, 2011). In contrast, HDACs remove acetyl groups from ε-N-acetyl lysine on histones (Li G. et al., 2020). The enzymatic activities of HATs and HDACs alter chromatin configuration and contribute primarily to gene activation and repression, respectively (Peserico and Simone, 2011).

HATs have been traditionally classified into two classes, type A and type B, based on their cellular localization. HAT1 (also

known as KAT1), HAT2 and HAT4 constitute the solely B-type HATs, which are originally isolated from cytoplasmic extracts as they are enzymes found in the cytoplasm (Kleff et al., 1995; Parthun et al., 1996; Yang et al., 2011). They acetylate newly synthesized and free histones, particularly free histone H4, which contributes to chromatin assembly (Parthun et al., 1996; Yang et al., 2011). However, some reports have demonstrated that B-type HATs can localize to the nuclear compartment, albeit with poorly understood functions (Ruiz-Garcia et al., 1998; Ai and Parthun, 2004; Parthun, 2012). The role of B-type HATs in cancer and inflammation is also not well investigated. On the other hand, A-type HATs are a more diverse group of enzymes that predominately reside within the nucleus. According to their sequence and structure homology, A-type HATs can be further classified into three distinct families: General control non-repressible 5 (GCN5)-related N-acetyltransferases (GNATs), MYST (named after the first-identified four members MOZ, Ybf2, Sas2, and Tip60), and cAMP response element binding protein (CREB)-binding protein (CBP)/p300 proteins (Hodawadekar and Marmorstein, 2007).

The functions of A-type HATs in inflammation and cancer have been universally reported. For instance, GCN5 and its homologous partner PCAF (also known as KAT2A and KAT2B, respectively) are two well-studied GNAT family proteins, which are characterized by the presence of an acetyltransferase domain and a C-terminal bromodomain (Marmorstein, 2001). They globally acetylate core histones to upregulate gene transcription (Herrera et al., 1997; Nagy and Tora, 2007). Histone H3 lysine 9 acetylation (H3K9ac) has been highlighted as their signature target, as loss of GCN5 and PCAF in cells specifically causes H3K9ac reduction (Jin et al., 2011). Importantly, genetic deletion or pharmacological inhibition of PCAF results in a significant reduction of H3K5ac and H3K9ac levels at the promoter region of the cytokine gene IL-6, leading to its transcriptional downregulation (Xia et al., 2021). Upon treatment with the proinflammatory stimulus LPS, PCAF displays a positive correlation with H3K18ac expression, which activates the transcription levels of inflammatory genes (Huang et al., 2015). PCAF deficiency in macrophages and leukocytes leads to a remarkable decrease in the expression of inflammatory cytokines such as TNFα, CCL2 and IL-6 (de Jong et al., 2017). Additionally, degrading GCN5/PCAF by GCN5/PCAF proteolysis targeting chimera (PROTAC) downregulates inflammatory mediators in macrophages and dendritic cells (Bassi et al., 2018). Aside from histone acetylation, GCN5/PCAF can also exert non-histone acetylation functions, which play an integral role in regulating inflammation as well. For example, PCAF acetylates the KLF4 TF to facilitate its transactivation effect on IL-6 (Xia et al., 2021).

CBP/p300 proteins are conserved paralogous factors that are well known transcriptional coactivators for promoting gene transcription. Their typical substrate, histone H3 lysine 27 acetylation (H3K27ac), is widely regarded as a marker of accessible chromatin and active genes (Pasini et al., 2010; Jin et al., 2011). Inhibition of CBP/p300 has been reported to decrease H3K27ac intensity at the promoters of pivotal inflammatory response genes in macrophages, thereby regulating inflammation-related signaling networks (Peng et al., 2019). In $CD4^+$ T-cells of patients suffering from the autoimmune disease systemic lupus erythematosus (SLE), CBP/p300 is recruited by the STAT family of TF proteins to confer accumulation of another active histone mark, H3K18ac, on the promoter and enhancer domains of the immunomodulatory cytokine gene IL-10, resulting in its upregulation that positively correlates with disease severity (Hedrich et al., 2014). Additionally, lower amount of H3K18ac at the promoter of another cytokine gene IL-2 in SLE patients, relative to healthy individuals, is partly attributed to the interaction between HDAC1 and CREMα (cAMP-responsive element modulator α), which contributes to histone modification changes and is induced at elevated levels in the patients' T-cells. Similar to GCN5/PCAF, CBP/p300 can also directly interact with and acetylate non-histone proteins such as NF-κB, a key regulator of inflammatory responses (Bhatt and Ghosh, 2014). Specifically, CBP/p300 acetylates p65, a core subunit of NF-κB, at lysine 211, 218 and 310 (Chen et al., 2002). The acetylation of p65 enhances its DNA-binding ability, activates NF-κB transactivation activity and triggers expression of downstream inflammatory genes (Chen et al., 2002; Mukherjee et al., 2013).

With regard to MYST family members, Tip60 (also known as KAT5) has been shown to catalyze the deposition of H3K27ac on the promoter regions of IL-6 and IL-8 to activate pro-inflammatory signaling cascades (Wang et al., 2020). In addition, another MYST protein, MOF, which specifically acetylates histone H4 at lysine 16 (H4K16ac), regulates inflammation signaling pathways involving TNFα and IL-33 (denDekker et al., 2020; Liu et al., 2021). Taken together, type-A HATs facilitate the production of inflammatory responsive gene transcripts and modulate key mediators of inflammation by both histone and non-histone acetylation functions.

In contrast to HATs, HDACs remove acetyl groups from histones, and hence mediate histone acetylation states dynamically with HATs to regulate gene expression. Substantial evidence reveal the role of HDACs in regulating the inflammatory gene program of immune cells. For example, HDAC3 disruption causes genomic hyperacetylation, leading to the upregulation of interferon-associated genes in LPS-stimulated macrophages (Chen et al., 2012). Treatment with HDAC inhibitors (HDACi) enhances the immunomodulatory effects of T cells and natural killer (NK) cells to activate cancer immunosurveillance. A case in point is the HDACi depsipeptide (FK228) that was reported to bolster tumor antigen expression through the enrichment of H3 acetylation, which facilitates T cell cytotoxicity against melanoma (Murakami et al., 2008). Pan-HDACi, panobinostat and vorionstate, modulate the expression of the cancer-testis antigen NY-ESO-1 and enhance tumor cell recognition by NY-ESO-1-specific T-cells, thereby benefiting adoptive T cell therapy in soft tissue sarcoma (Gong et al., 2022).

NK cell-mediated tumor recognition relies on the expression of several ligands on the cell surface of tumor cells, such as UL16-binding proteins (ULBPs). Prior studies showed that HDACi treatment increases expression of ULBPs in cancer cells, which

subsequently activates NK cell-mediated cytotoxicity (Lopez-Soto et al., 2009). In addition to tumor antigens, HDACi also increases the expression of NKG2D, a receptor of ULBPs and an activating cell surface receptor expressed on NK cells, triggering NK cell cytotoxic activities (Poggi et al., 2009; Yamanegi et al., 2010). Collectively, HDAC inhibition contributes to antigen processing and tumor cell recognition, which in turn activates immune cell cytotoxicity and serves as a potential pre-treatment approach for adoptive immune cell therapy to efficiently eliminate cancer cells.

Histone Phosphorylation

Post-translational phosphorylation of histones is a fundamental epigenetic event implicated in multiple biological processes, such as DNA damage repair and carcinogenesis. It predominantly occurs in tyrosine, serine, and threonine residues on the N-terminal histone tail, which is dynamically modulated by a myriad of protein kinases and phosphatases (Nowak and Corces, 2004). In histone phosphorylation, a phosphate group from ATP is transferred to the hydroxyl group of the target amino acid, leading to a build-up of negative charge on histones, which in turn weakens histone-DNA interaction and facilitates the establishment of a transcriptionally permissive chromatin landscape (Bannister and Kouzarides, 2011).

Phosphorylation has been reported for the following histone H3 residues: serine 10, 28, threonine 3, 6, 11, 45, and tyrosine 41, as well as serine 32 of histone H2B (Shanmugam et al., 2018). Importantly, histone phosphorylation has been linked to inflammation-dependent tumorigenesis. For instance, stress-activated protein kinase 1 (MSK1) mediates phosphorylation of histone H3 at serine 10 (H3S10ph) on the promoter of NAFTC2 to activate the expression of the proinflammatory cytokines IL-6 and IL-11 in gastric cancer (Qi et al., 2020). Moreover, high levels of H3S10ph are positively associated with *Helicobacter pylori* infection-induced gastric carcinogenesis and neoplastic cellular transformation in nasopharyngeal carcinoma (Li B. et al., 2013; Yang et al., 2018). Expression of the immune regulatory cytokines IL-10 and its homolog IL-19 in macrophages is also influenced by histone H3 phosphorylation (Zhang et al., 2006), with crucial repercussions to the regulation of inflammation, as diminished expression of IL-10 and IL-19 triggers inflammatory signaling via the upregulation of inflammasome components, thereby enhancing the assembly of the inflammasome complex that promotes secretion of the proinflammatory cytokine IL-1β (Hofmann et al., 2015; Brandt et al., 2018).

Nonetheless, histone phosphorylation often does not act in isolation, but partners with other histone modifications to control gene regulatory processes. An *in vitro* study illustrated that the histone acetyltransferase GCN5 exhibits a preference for histones decorated with H3S10ph, compared to non-phosphorylated histones (Cheung et al., 2000). H3S10ph can also stabilize histone H4 acetylation, while dephosphorylation of H3S10 collaborates with HDAC1, 2 and 3-induced deacetylation of histone H4 under stress conditions (Hu et al., 2014). It has also been reported that H3S10ph assists in expanding genomic domains harboring H3K4 methylation, a marker of accessible chromatin, and restricts the propagation of heterochromatin enriched with H3K9me2 and DNA methylation (Komar and Juszczynski, 2020). Therefore, extensive crosstalk takes place between histone phosphorylation and other post-translational histone modifications to dynamically regulate gene expression patterns, especially in the context of inflammation and cancer.

Histone Ubiquitination

Histone ubiquitination is a less well-studied post-translational modification that exerts roles in chromatin compaction and transcription regulation. It is mediated by the sequential interactions among E1, E2 and E3 enzymes: E2 is the conjugating enzyme, which transfers ubiquitin from the ubiquitin-activating enzyme E1, while E3 ligases act as protein binding platforms to catalyze the ubiquitination of substrate proteins' lysine residues by directly transferring ubiquitin from their E2 enzymes (Berndsen and Wolberger, 2014). The function of ubiquitination primarily involves regulating the cellular localization, stability and activity of its target proteins, which include all core histone subunits. Among them, mono-ubiquitination on lysine 118 or 119 of histone H2A (H2AK118/119ub) and lysine 120 of histone H2B (H2BK120ub) are the most abundant forms of histone ubiquitination, accounting for 5–15% of H2A and 1% of H2B, respectively (Mattiroli and Penengo, 2021). H2AK118/119ub is correlated with transcriptional repression by Polycomb Repressive Complex 1 (PRC1), whereas H2BK120ub plays an important role in transcriptional elongation by the E3 enzymes RNF20 and RNF40 (Mattiroli and Penengo, 2021), both of which are associated with the DNA damage response.

H2BK120ub has been highlighted for its role in inflammation-related colorectal cancer. Specifically, the reduced levels of H2BK120ub and its E3 ligase RNF20 activate colonic inflammation and tumorigenesis by recruiting NF-kB, a master TF regulating inflammation signaling, in both mice and humans (Tarcic et al., 2016). Other studies also demonstrated that dysregulated H2BK120ub causes genomic instability, as well as promotes tumorigenesis and cancer progression in breast and lung tumors (Jeusset and McManus, 2021). Like histone phosphorylation, histone ubiquitination can also interact with and influence other histone modifications. For instance, H2BK120ub contributes to histone H3K79 and H3K4 methylation at promoter regions to induce gene transcription (Worden and Wolberger, 2019; Worden et al., 2020). Taken together, histone ubiquitination possesses roles in both transcription regulation and inflammation-induced tumorigenesis.

HISTONE VARIANTS

Further to the plethora of covalent histone modifications as described above, an under-appreciated aspect of epigenetic alteration pertinent to histones is the inclusion of non-canonical forms of these DNA-scaffolding proteins, which are commonly referred to as histone variants. Differences in these variants from the core H2A, H2B, H3 and H4 histones can be in the form of changes to the primary amino acid sequence or the

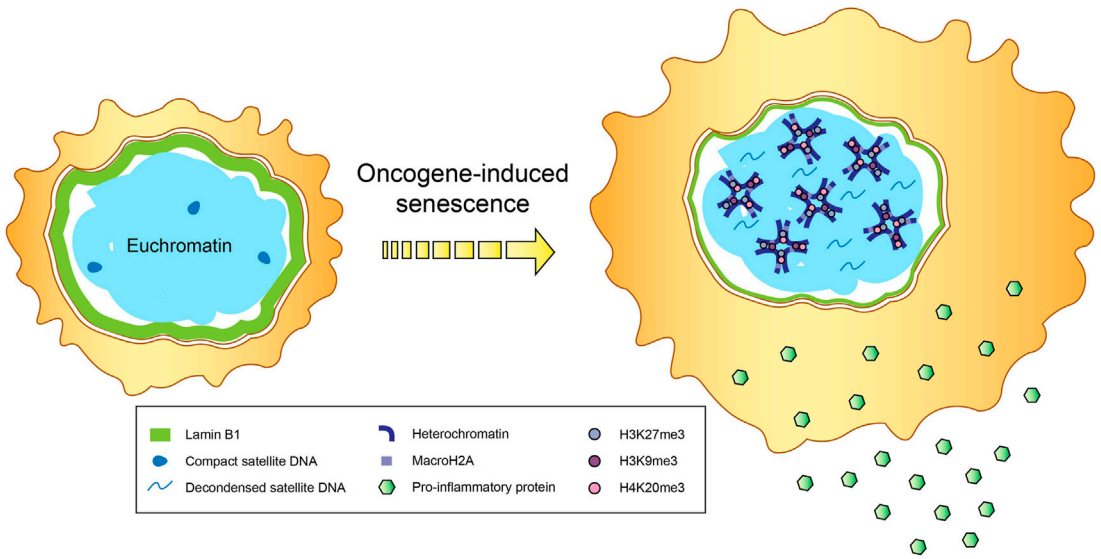

FIGURE 1 | Epigenetic changes in senescence. Secretion of proinflammatory cytokines and immunomodulatory proteins that constitute the senescence-associated secretory phenotype (SASP), accompanied by the formation of senescence-associated heterochromatin domains (SAHDs) and compact senescence-associated heterochromatic foci (SAHF), occur in cells undergoing oncogene-induced senescence (OIS). In addition to the enrichment of repressive histone modifications (e.g., trimethylation of H3K9, H3K27 and H4K20) and the histone variant macroH2A within SAHFs, OIS-induced cells also tend to exhibit a reduction in lamin B1 levels and decondensation of satellite DNA, in a process called senescence-associated distension of satellites (SADS).

incorporation of extra domains (Ghiraldini et al., 2021), thereby permitting variant-specific histone modifications that collectively influence the biochemical and physical characteristics of the nucleosome (Bonisch and Hake, 2012). For instance, even though only five amino acid residues distinguish the histone variant H3.3 from its canonical counterpart H3, euchromatic histone modifications like H3K9ac and H3K4me1 are found to accumulate selectively on H3.3 relative to H3, resulting in the elevated transcriptional activity of H3.3 enriched loci (Talbert and Henikoff, 2010).

Perhaps the most prominent biological process that showcases the increased abundance of histone variants at the expense of canonical histones is senescence, which takes place in cells undergoing irreversible proliferative arrest due to extensive stress-induced genomic damage (Hernandez-Segura et al., 2018). The accumulation of senescent cells over time triggers the inflammatory response due to the secretion of numerous signaling proteins, immune modulators, cytokines, extracellular matrix factors and proteases that make up the senescence-associated secretory phenotype (SASP) (Coppe et al., 2008; Childs et al., 2017). This in turn establishes a proinflammatory milieu that leads to chronic inflammation and induces neighboring cells to enter senescence as well, ultimately culminating in tissue dysfunction and tumorigenesis (Coppe et al., 2010; Lopez-Otin et al., 2013; Franceschi and Campisi, 2014; Lecot et al., 2016).

Examples of the loss of canonical histone proteins include the decreased expression of the core histones H3 and H4 during replicative senescence (RS) (O'Sullivan et al., 2010), which occurs in cells that experience stress induced by prolonged telomere shortening following numerous cellular divisions (Campisi and d'Adda di Fagagna, 2007). Lower levels of the linker histone H1, along with the dearth of *de novo* histone H1 synthesis from its post-translational silencing, have also been observed in cells undergoing oncogene-induced senescence (OIS) (Funayama et al., 2006), which is another type of senescence caused by induction of oncogenes and/or repression of tumor suppressor genes (Serrano et al., 1997; Sarkisian et al., 2007; Courtois-Cox et al., 2008). The reduced amount of histones adversely disrupts the global chromatin architecture, and hence exacerbates genomic damage to a greater extent (O'Sullivan et al., 2010).

On the other hand, histone variants such as histone H3.3 accumulates during cellular senescence, and its ablation resulted in cell cycle arrest *via* the repression of key cell cycle regulators (Duarte et al., 2014). Histone H2A.J, a relatively uncommon variant of H2A that exists only in mammals, is found to be enriched in DNA damage-induced senescence, and it plays a critical role in increasing the expression of inflammatory and immune-related genes during chronic inflammation, especially those implicating the SASP (Contrepois et al., 2017). Moreover, the gene encoding histone H2A.J has been documented to be aberrantly expressed in breast cancer (Colotta et al., 2009; Cornen et al., 2014; Rube et al., 2021), though its role in oncogenesis remains to be defined.

One of the major hallmarks of senescence is the establishment of senescence-associated heterochromatin domains (SAHDs) that are abundantly marked with H3K9me3 (**Figure 1**). These domains subsequently develop into senescence-associated heterochromatic foci (SAHF), which depict hotspots of compact heterochromatin decorated by a myriad of repressive epigenetic modifications like H3K27me3 and H4K20me3, and are found mainly in OIS (Narita et al., 2003; Chandra et al., 2012;

Nelson et al., 2016). MacroH2A variants, the biggest known histone variants with gene repressive roles, are shown to accumulate within SAHF (Zhang et al., 2005). In particular, one of the macroH2A family isoforms, macroH2A1, is repositioned away from SASP genes to promote their expression (Chen et al., 2015), a process aided by the ATM protein kinase that is vital for regulating the cellular response to double strand breaks (DSBs), including those induced by OIS (Mallette et al., 2007). ATM also catalyzes the phosphorylation of another histone variant H2AX (commonly referred to as γH2AX) (Burma et al., 2001), which is thought to stabilize the ends of DSBs within spatial proximity for supporting DNA repair (Bassing and Alt, 2004). Notably, elevated levels of γH2AX have been documented in both cancer and inflammation-associated pathways like NF-κB signaling (Mah et al., 2010; Matsuya et al., 2022).

Nevertheless, histone variants are not always expressed at elevated levels in senescent cells. A case in point is the histone H3 variant CENP-A, which is the epigenetic marker of chromosomal centromeres that are extensively heterochromatinized and exhibit substantial changes in structure during senescence (Swanson et al., 2015). Protein levels of CENP-A are reduced in human senescent primary fibroblasts, as well as in old, compared to young, human islet cells. Accordingly, shRNA-mediated depletion of CENP-A led to premature senescence in fibroblast cells (Lee et al., 2010; Maehara et al., 2010).

CHROMATIN STRUCTURE MODULATION AND ENHANCER-BASED REGULATION

In order to facilitate chromatin accessibility for establishing a transcriptionally competent environment, chromatin structure can be modulated by post-translational histone alterations, such as the incorporation of methyl, acetyl or phosphate moieties, as described above. Alternatively, nucleosomes can be physically displaced by chromatin remodelers to expose the underlying genetic material for binding by RNA Polymerase II (RNAPII) and other components of the transcription machinery (Smith and Peterson, 2005).

Chromatin Remodeling

Genes that respond to inflammatory signals can be grouped into two classes based on their requirement for chromatin remodeling: "remodeling-dependent" genes are typically characterized by the lack of promoter CpG content, with low levels of RNAPII and active histone modifications, as exemplified by the tetratricopeptide repeats-containing gene family encoding interferon-activated proteins (Ramirez-Carrozzi et al., 2009; Bhatt et al., 2012). Another example is the chromatin remodeling by oncogenic RAS of select enhancer domains that enables deposition of the active histone mark H3K27ac and recruitment of the transcriptional coactivator BRD4 *via* the pioneer TF activity of GATA4 (Nabet et al., 2015). In contrast, "remodeling-independent" genes e.g., TNF (encodes tumor necrosis factor), FOS and JUN (encode the AP1 transcription factor) often harbor RNAPII-enriched promoters with high CpG content, such that P-TEFb and other transcription elongation factors can easily bind with high accessibility for rapid gene induction (Kininis et al., 2009; Ramirez-Carrozzi et al., 2009; Xu et al., 2009).

A recent study by Alizada et al. (2021) offered key insights into the expression dynamics of both these classes of genes that are triggered by NF-kB, a master TF implicated in various inflammatory signaling pathways (Natoli, 2009). Upon its translocation into the nucleus, NF-kB binds to promoters and enhancers of proinflammatory genes to stimulate their transcription (Pierce et al., 1988). In particular, NF-kB can engage enhancers by adopting a chromatin conformation that features distal enhancer domains within three-dimensional (3D) spatial proximity to target genes (Jin et al., 2013). The most well-studied way by which NF-kB interacts with DNA is its recruitment to "remodeling-independent" genomic loci that are made transcriptionally open by the prior occupancy of other TFs (Heinz et al., 2013; Hogan et al., 2017; Link et al., 2018). These loci are often linked to the rapid expression of inflammatory genes and suppression of cell fate determination genes (Schmidt et al., 2015). Additionally, NF-kB can gain access to "remodeling-dependent" regions with the aid of transcriptional coactivators, lineage-specifying or signal-mediated TFs (Natoli, 2009; Ghisletti et al., 2010; Natoli, 2012; Freaney et al., 2013; Kaikkonen et al., 2013). Genes residing within these regions are mostly associated with dampening the inflammatory response, and they exhibit reduced activation kinetics (Natoli, 2009).

Intriguingly, NF-kB has also been demonstrated to utilize a third mode of chromatin interaction, by binding to nucleosome-occluded domains in a manner that is reminiscent of pioneer TFs, although its functional importance remains controversial (Steger and Workman, 1997; Angelov et al., 2003; Angelov et al., 2004; Lone et al., 2013; Cieslik and Bekiranov, 2015). Through comparative epigenomic investigation of the genome-wide localization dynamics of NF-kB in human, murine and bovine cells stimulated with the proinflammatory cytokine TNFα, Alizada et al. (2021) showed a substantial proportion of conserved orthologous NF-kB binding not only to accessible, but also nucleosome-bound chromatin regions. In fact, NF-kB occupancy within the latter context is likely an integral aspect of the NF-kB-induced acute inflammatory response, as reproducible results were obtained with ChIP-seq using different NF-kB subunits in diverse cell types, and these regions were significantly enriched within super-enhancer (SE) domains, which constitute about a third of all NF-kB SE binding peaks (Alizada et al., 2021).

Another notable discovery pertaining to NF-kB occupancy dynamics is that a small minority of loci with considerable NF-kB binding before TNFα treatment were the most highly expressed less than an hour after TNFα stimulation. Importantly, these NF-kB pre-bound domains were conserved across different species and cell types, harbored numerous NF-kB motifs, overlapped human non-coding inflammatory disease mutations, and belonged to several inflammation-associated SEs located in close proximity to NF-kB target genes (Alizada et al., 2021).

Thus, the efficient recruitment of NF-kB to a low number of these conserved pre-bound sites bears a disproportionately robust effect on the transcriptional regulation of inflammatory genes.

The mechanistic basis of action of NF-kB involves key chromatin regulatory players like the histone acetyltransferase CBP/p300 and the epigenetic factor BRD4 (Ashburner et al., 2001; Zhong et al., 2002; Huang et al., 2009). BRD4 is part of the bromodomain and extraterminal (BET) family of transcriptional coactivators (Dey et al., 2000; LeRoy et al., 2008) that interacts with the positive transcription elongation factor P-TEFb and the SWI/SNF chromatin remodelers at active genomic loci (Jang et al., 2005; Yang et al., 2005; Shi et al., 2013). Specifically, CBP/p300 mediates NF-kB acetylation upon treatment with the proinflammatory stimuli TNFα or LPS, thereby enhancing BRD4 binding *via* its acetyl lysine-recognizing bromodomains (Greene and Chen, 2004). This interaction is essential for the productive activation of NF-kB, and heralds a key function of BRD4 in inflammatory gene transcription (Huang et al., 2009).

Super-Enhancers

Super-enhancers (SEs) are active transcriptional hubs that consist of multiple enhancer elements densely bound by TFs and coactivators, especially the Mediator complex, and they exert crucial functions during cell fate specification and oncogenesis (Hnisz et al., 2013; Loven et al., 2013; Whyte et al., 2013). The molecular partnership between NF-kB and BRD4 is particularly evident on SE loci, where both factors are found to accumulate at significantly higher densities relative to typical enhancers and active transcription start sites. Strikingly, NF-kB cooperates with BRD4 to set up novel SE networks that govern the expression of nearby proinflammatory genes, and this is accompanied by the unexpected displacement of BRD4 from other pre-existing SE sites, such as those that regulate non-inflammatory and cell identity genes (Brown et al., 2014). These newly formed proinflammatory SEs are enriched with the p65 (canonical subunit of NF-kB) motif, indicating that direct binding of NF-kB to the new SEs is likely causal in the distribution changes of BRD4 SE occupancy in inflammation (Brown et al., 2014).

Importantly, BET bromodomain-mediated inhibition of BRD4 ablated *de novo* NF-kB-induced SE formation, which culminated in the reduction of proinflammatory gene expression, thereby illuminating the critical role of BET bromodomains in regulating global, dynamic changes in inflammatory gene transcription. Brown et al. further highlighted the physiological consequences of BRD4 inhibition *in vivo* through the disrupted responses of NF-kB-activated endothelial cells, which drive the initiation and maintenance of inflammatory phenotypes (Gimbrone et al., 1990; Ley et al., 2007), as well as the loss of inflammatory cells and atherogenesis (an inflammatory disorder) in a well-established mouse model of atherosclerosis (Brown et al., 2014).

In a separate study pertaining to SEs, Hah et al. (2015) demonstrated that following LPS treatment, upregulated genes harboring increased SE activity tend to be associated with proinflammatory transcription and immune-related processes, while downregulated genes containing decommissioned SEs are linked to chromatin organization and cell metabolism. Moreover, NF-kB and BRD4-induced SE formation is vital for proinflammatory microRNA gene activation, which is yet another epigenetic mechanism known to influence inflammation and cancer pathogenesis (Duan et al., 2016). Interestingly, inflammatory disease-specific SEs can be further differentiated from the archetypal NF-kB-mediated SEs. For instance, the RUNX1 and ETS1 TFs showed elevated binding levels within SE loci of synovial-fluid derived CD4 T lymphocytes in patients with the autoimmune disorder juvenile idiopathic arthritis (JIA), leading to a greater expression of inflammatory genes regulated by these JIA-associated SEs including interleukins and chemokine receptors (Peeters et al., 2015). Collectively, these findings reveal SEs as potential therapeutic targets for controlling inflammation and immune-related gene regulatory networks by perturbing inflammatory SE architecture and function.

From an evolutionary standpoint, the origin of numerous enhancers can be traced back to endogenous retroviruses (ERVs), such that gene regulatory programs driving inflammatory phenotypes have gradually gained enhancer elements by co-opting genomic sequences from ERVs (Chuong et al., 2013; Chuong et al., 2016). Additionally, enhancer-encoded RNA and its chromatin milieu often undergo post-translational alterations (Li et al., 2016). Therefore, certain enhancers are able to establish a specific epigenetic memory of the initial inflammatory signal in a phenomenon called enhancer bookmarking, which contributes to innate "trained" immunity and promotes a quicker response to future stimulatory cues (Ostuni et al., 2013).

HIGHER-ORDER SPATIAL GENOME ORGANIZATION

Beyond the epigenetic regulation of inflammatory gene transcription by histone modifications, chromatin remodeling and SE dynamics, as discussed in the previous sections, higher-order genome topology of varying hierarchical levels, ranging from long range chromatin looping within the same and across different chromosomes to topologically associating domains (TADs) that make up A (euchromatin) and B (heterochromatin) compartments, also undergird the multi-faceted nature of chromatin-dependent inflammatory responses. A case in point is highlighted by the increased appreciation of promoters from different genes aggregating in close spatial proximity to facilitate their co-regulation (Li et al., 2012), to the extent that some promoters appear to possess enhancer capabilities, dubbed "ePromoters," which were found to come together in 3D space to regulate the interferon-α response (Dao et al., 2017).

Transcription Factories and Chromatin Loops

The advent of chromosome conformation capture (3C) techniques led to the understanding that transcription regulation is not confined to a linear segment of chromatin,

but occurs within defined nuclear regions called transcription factories, in which RNAPII and members of the transcriptional apparatus that are far apart in 3D space can colocalize with one another during gene activation (Dekker et al., 2002; Osborne et al., 2004; Papantonis et al., 2010; Larkin et al., 2012; Papantonis et al., 2012; Sharaf et al., 2014). Inflammatory genes are generally not found in transcription factories prior to stimulation, but swiftly localize to these specialized domains upon activation by proinflammatory signals (Papantonis et al., 2010; Larkin et al., 2012; Papantonis et al., 2012). For example, LPS treatment resulted in the close spatial assembly of the regulatory elements of IL-1A, IL-1B and IL-37 cytokine genes in human monocytes, suggesting co-regulation within a specific transcription factory (Sharaf et al., 2014). Papantonis et al. (2012) uncovered the crucial role of active NF-kB-mediated transcription factories in coordinating select nascent mRNA and non-coding miRNA production, following TNFα-induced stimulation.

Notably, transcriptional dynamics within transcription factories operate in a hierarchical fashion involving both cis and trans chromosomal interactions (Fanucchi et al., 2013). Such changes in chromatin spatial configurations have been elegantly illustrated in the context of antigen stimulation of naïve T lymphocytes, which differentiate into Th1, Th2 and Th17 cells that express distinct cytokine genes located on different chromosomes. The Th2 cytokine locus is instrumental for establishing long-range chromatin contacts with three promoters that regulate the genes specifying IL-4, IL-5 and IL-13 interleukins across hundreds of kilobases on the same chromosome (Spilianakis and Flavell, 2004; Lee et al., 2005). Additionally, this highly accessible Th2 locus can associate with the IL-17 and IFN-γ gene promoters located on different chromosomes. Intriguingly, such inter-chromosomal crosstalk is abrogated in favor of intra-chromosomal interactions upon cytokine gene activation, which is a unique approach harnessed by naïve T cells to alter its developmental trajectory for counter-balancing chronic inflammation (Spilianakis et al., 2005; Kim et al., 2014).

NF-kB, the master regulator of multiple inflammatory signaling pathways, also leverages on higher-order genome organization to discharge its gene regulatory roles (Kolovos et al., 2016). For instance, activation of NF-kB upon a viral infection provokes long range chromatin re-wiring between the IFN-β gene locus and three distant NF-kB bound loci on separate chromosomes, which is characterized by a diminution of these inter-chromosomal contacts at the onset of transcriptional initiation and elongation, relative to its inactive state (Apostolou and Thanos, 2008). In another study, NF-kB occupancy on the microRNA gene loci of miR-155 and miR-146a, located on different chromosomes, led to their colocalization and concomitant gene suppression during the induction of endotoxin tolerance in activated naïve macrophages (Doxaki et al., 2015).

Calandrelli et al. (2020) recently dissected the global changes in 3D spatial chromatin dynamics in stress-induced transcriptional dysregulation of endothelial cells, which feature prominently in several diseases. Treatment with TNFα and high glucose levels that mimic the inflammatory response in diabetic patients not only resulted in the loss of the repressive histone modifications H3K9me3 and H3K27me3, thereby activating inflammatory NF-kB target genes, but also significantly enhanced genome-wide inter-chromosomal RNA-chromatin interactions, particularly at sites harboring super-enhancer loci that drive proinflammatory gene expression and endothelial-mesenchymal transition (Calandrelli et al., 2020).

CTCF, a well-known architectural insulator protein that plays integral roles in both intra- and inter-chromosomal genome organization (Ong and Corces, 2014), has also been implicated in the inflammatory response modulation by TNFα and LPS stimuli. For example, treatment with TNFα induced the formation of enhancer-promoter loops at the human cytokine genes lymphotoxin-α (LTα) and TNFα, as well as the promoter region of another NF-kB-responsive gene LTβ, but loss of CTCF diminished TNF expression while promoting LTβ activation (Watanabe et al., 2012). Nikolic et al. (2014) also reported a drastic decrease in the production of TNFα and the IL-10 family of cytokines in activated macrophages lacking CTCF. LPS treatment was found to trigger CTCF detachment, accompanied by non-coding RNA expression at the chicken lysozyme genomic locus in macrophages (Lefevre et al., 2008; Witham et al., 2013).

Topologically Associating Domains (TADs) and A/B Compartments

The classic role of CTCF in regulating 3D genome architecture is attributed to its insulator function at the boundary between TADs, which are sub-megabase chromatin regions that can self-associate by forming loops with cis-regulatory elements and their target genes within the domain, while restricting interactions outside the domain (Dixon et al., 2012; Dixon et al., 2016). At the next genomic layer, chromatin is broadly partitioned to two large-scale compartments: transcriptionally open euchromatic (A) versus compact heterochromatic (B) compartments (Kempfer and Pombo, 2020). Inflammatory challenges can impinge on 3D chromatin topology at both the TAD and A/B compartment levels, thereby altering gene expression profiles and cell fates, as discussed in this section.

One of the essential processes to quell inflammation is the production of IL-4 cytokines, which induce macrophage polarization to the anti-inflammatory M2 population (Mills et al., 2000). Phanstiel et al. (2017) uncovered distinct differences in the chromatin landscape of naïve macrophages before IL-4 stimulation, compared to those treated with IL-4 and then rested for a day. In addition, differentiation of human monocytes to macrophages initiates spatial chromatin modifications at the TAD level, with enrichment of the stress-associated and cell type-specific TF AP-1 on active enhancer-bound loops at key macrophage genes, as opposed to undifferentiated monocytes (Phanstiel et al., 2017).

Viruses have been demonstrated to hijack and re-wire the 3D chromatin organization of the host cell for subverting its immune defense system and exerting long-term inflammatory and other gene regulatory effects (Heinz et al., 2018; Liu et al., 2020). In light

of the ongoing COVID-19 pandemic, Wang et al. (2021) recently reported that SARS-CoV-2 infected cells showed a significant ablation of cohesin, another architectural protein complex that collaborates with CTCF to mediate DNA looping (Dekker and Mirny, 2016), within TADs, causing a widespread weakening of intra-TAD chromatin interactions. Furthermore, A/B compartmentalization manifested a drastic perturbation in the form of A-to-B switching, resulting in erosion of the euchromatic A compartment that is coupled with a global decrease in the active histone H3K27ac mark. The physiological ramifications of these epigenetic disruptions and higher-order chromatin reconfigurations included downregulation of antiviral interferon response genes and upregulation of proinflammatory genes, shedding important insights into the inflammatory phenotypes observed in COVID-19 patients (Carvalho et al., 2021).

Importantly, 3D genome organization is a key driver of cellular senescence, which enacts chromatin restructuring at multiple levels, ranging from an increase in local chromatin interactions to a global shortening of chromosomal arms (Criscione et al., 2016). Zirkel et al. (2018) revealed one example of such chromatin reconfiguration stemming from the loss of HMGB2 at several TAD borders in senescent cells. HMGB2 belongs to the family of high-mobility group (HMG) proteins, which are ubiquitous non-histone regulatory factors that bind to and influence chromatin architecture (Reeves, 2001; Bianchi and Agresti, 2005). Senescence-mediated abolishment of HMGB2 led to the anomalous assembly of CTCF clusters, and in line with the insulating function of HMGB2, novel long range CTCF-based loops were established at genomic sites where HMGB2 previously occupied (Zirkel et al., 2018).

Another recent report addressed chromatin looping aberrations during OIS. Specifically, enhancer-promoter contacts at the IL-1 cytokine gene cluster, where key cell cycle and SASP-related genes reside, were disrupted, resulting in the increased expression of proinflammatory genes and silencing of cell cycle genes (Olan et al., 2020). These alterations are partially due to the transcription-mediated redistribution of cohesin, forming "cohesin islands" that arise from the accumulation of the cohesin complex at the 3' ends of active genes caused by the inefficient removal of cohesin, which in turn generates new cohesin-induced DNA loops (Busslinger et al., 2017). Nonetheless, TAD boundaries and A/B compartmentalization remain largely unaffected in OIS (Chandra et al., 2015; Olan et al., 2020).

Both OIS and RS forms of senescence exhibit a dampening of short-range chromatin contacts, but an increase in long range genomic interactions (Sati et al., 2020). Moreover, A/B compartment transitions are highly conserved in both types of senescence, which correspond to downstream transcriptional outcomes in the form of gene activation for B-to-A compartment switches and gene repression for A-to-B compartment changes. However, A/B compartmentalization differences are also evident, as OIS features elevated B-B and diminished A-B interactions, while RS displays diminished A-A and elevated A-B interactions (Sati et al., 2020). Importantly, the architectural protein condensin plays a critical role in sustaining the senescent phenotype, as it functions in B-to-A compartment switching and stabilizes the A compartment, thereby enabling senescence-associated gene induction (Iwasaki et al., 2019). Additionally, genes within the vicinity of SAHF are expectedly downregulated (Iwasaki et al., 2019), yet Sati et al. (2020) reported that SAHF can serve as hubs for the aggregation of select gene loci to facilitate their expression, especially genes pertaining to inflammation and oncogenesis.

OIS is widely believed to hinder oncogenesis, owing to its role in restraining cellular proliferation, but it can also promote cancer development through the effect of certain SASP molecules on the cells' immune system, such as the recruitment of anti-inflammatory M2 macrophages by CCL2 cytokines that sets up an immunosuppressive environment for supporting cancer progression (Allavena et al., 2008), as well as the secretion of proinflammatory SASP factors IL-6 and IL-8 by senescent fibroblasts that stimulates prostate cancer development in mice (Laberge et al., 2015). Interestingly, in colorectal cancer, Johnstone et al. (2020) recently highlighted a weakening of A/B compartmentalization, along with the establishment of a novel intermediate compartment that features long range chromatin interactions with both A and B compartments. However, the silencing histone H3K27me3 modification is found to accumulate in this intermediate compartment specifically in tumor cells, accompanied by the repression of genes residing within it, yet some genes encoding cancer-testis antigens (CTAs) and ERVs become unexpectedly upregulated (Johnstone et al., 2020), a phenomenon that has previously been observed in colon tumors and associated with pro-immunity and viral mimicry roles (Rooney et al., 2015; Roulois et al., 2015; Gibbs and Whitehurst, 2018).

Nuclear Substructures and Chromosome Territories

On top of genomic macro-domains like TADs and A/B compartments, heterochromatinization engenders the 3D nuclear structure (Falk et al., 2019), which consists of regions associated with the nucleolus and nuclear lamina, including pericentric heterochromatin (Guenatri et al., 2004; Nemeth et al., 2010; van Steensel and Belmont, 2017). Chromatin localization to various substructures within the nucleus is important for regulating its transcriptional status, as active genes tend to be found within the nuclear interior and/or in proximity to nuclear speckles that abound with splicing factors (Lamond and Spector, 2003; Kim et al., 2020), while inactive genes typically border the lamina in regions termed as lamina-associated domains (LADs) and/or nucleolar peripheries (Nemeth et al., 2010; Kind et al., 2015; van Steensel and Belmont, 2017). Finally, individual chromosomes are preferentially arranged within defined areas of the nuclear space to form chromosome territories that represent the apex global level of chromatin organization (Meaburn and Misteli, 2007; Fritz et al., 2019).

At the level of LADs, cells undergoing OIS exhibited a heterochromatic lamina-specific reduction of chromatin contacts, whereby these GC-poor domains were

transcriptionally closed and adorned with H3K9me3 (Chandra et al., 2015). Despite the loss of LAD-mediated interactions, these regions could still coalesce in spatial proximity with one another that is reminiscent of SAHF establishment (Chandra et al., 2015). Analysis by polymer modeling lent further support to the roles of LAD detachment and SAHD decompaction in the development of OIS-induced SAHF (Sati et al., 2020). A different study using senescent human lung fibroblasts illustrated the physical condensation of individual chromosomes that accounts for the generation of SAHF (Funayama et al., 2006). Nonetheless, even though SAHF domains are replete with repressive proteins and histone modifications, they are not found within constitutive heterochromatic domains like centromeres and telomeres (Narita et al., 2003; Funayama et al., 2006; Zhang et al., 2007). There is hitherto no report involving alterations to chromosome territories as a result of inflammatory signaling or inflammation-induced senescence.

REPETITIVE ELEMENTS

A central epigenetic theme in cellular senescence is the genome-wide chromatin remodeling of repetitive sequences, which encompass up to two-thirds of the entire human genome (de Koning et al., 2011). This is usually manifested in the transcriptional relaxation of transposable elements such as Alu, SINE-VNTR-Alus and LINE-1, thereby facilitating non-coding RNA (ncRNA) expression from these loci and their mobilization, which activates several inflammatory/immunological gene networks including the cGAS-STING signaling pathway, type-1 interferon (IFN-1) response and the SASP (De Cecco et al., 2013; Criscione et al., 2016; De Cecco et al., 2019). Specifically, silencing of retrotransposons is performed by multiple heterochromatic players like HP1, DNMT1 and SIRT6. Hence, cells lacking the SIRT6 histone deacetylase showed an increase in LINE-1 transcripts that induced a robust IFN-1 output by activating cGAS (Simon et al., 2019).

De Cecco et al. (2019) recently delineated the mechanistic basis underpinning the aberrant activation of LINE-1 retrotransposons during senescence, which entailed depletion of the RB1 tumor suppressor protein by relieving the silencing histone H3K9me3 and H3K27me3 marks [RB1 has been reported to occupy LINE-1 and other repetitive loci to aid in their repression (Ishak et al., 2016)], increased binding of the pioneer TF FOXA1 to the LINE-1 promoter region for its activation [senescent cells show upregulation of FOXA1 (Li Q. et al., 2013)], and loss of the 3' exonuclease TREX1 that removes foreign invading DNA species (Thomas et al., 2017), causing the accumulation of LINE-1 cDNA (De Cecco et al., 2019). Despite the delayed onset of LINE-1 reactivation and its accompanying IFN-1 response, they are crucial contributors to the proinflammatory outcome and maturation to the full-fledged SASP, including the expression of key cytokines IL-6, CCL2 and MMP3. Notably, the establishment of innate immune signatures following senescence-mediated LINE-1 induction takes place *via* the interferon-stimulatory DNA route, and treatment with nucleoside reverse transcriptase inhibitors (NRTIs) that target the reverse transcriptase function of LINE-1 (Dai et al., 2011) can significantly ameliorate both the IFN-1 response and chronic inflammation in diverse tissue types (De Cecco et al., 2019).

In another study, mouse embryonic fibroblasts transfected with LINE-1 expression plasmids demonstrated a heightened IFN-β immune response that requires the ORF2 endonuclease function of LINE-1, implying the necessity of LINE-1's transposase activity in IFN-β activation (Yu et al., 2015). Interestingly, the interplay between LINE-1 and IFN-β sets up a negative feedback loop, as exogenous or induced IFN-β can in turn hinder LINE-1 transposition (Yu et al., 2015).

Besides transposable elements, the deleterious reconfiguration and reactivation of repetitive elements in senescent cells can also affect non-mobile centromeric and satellite DNA, leading to substantial structural changes in a process called senescence-associated distension of satellites (SADS), during which these typically constitutively silenced genomic sequences become decondensed and gain transcriptional accessibility (Swanson et al., 2013; Criscione et al., 2016) (**Figure 1**). These elements are also hypomethylated, in line with their distension and derepression (Cruickshanks et al., 2013). The occurrence of SADS precedes SAHF formation, and marks one of the initial alterations to the epigenetic landscape in cellular senescence (Swanson et al., 2013; Criscione et al., 2016), but the requirement of SADS in triggering and/or sustaining the senescent state remains unknown. Importantly, the loss of linker histone H1, which is a common chromatin modification observed during senescence (Funayama et al., 2006), is not causal of SADS, as most SADS-containing cells still possess high amounts of H1 protein (Swanson et al., 2013). Swanson et al. (2013) postulated that SADS may instead be attributed to the depletion of lamin B1, as almost all cells harboring normal endogenous levels of lamin B1 maintained compact heterochromatinized satellite sequences, compared to about a quarter of cells with decreased lamin B1 showcasing satellite distension.

In a similar vein, human lung fibroblasts exposed to X-ray-induced senescence elicited a dramatic increase in ncRNA expression from pericentromeric repetitive loci known as human satellite II (hSATII), which are usually repressed in healthy cells (Miyata et al., 2021). Mechanistically, these chromatin-associated hSATII RNA bind and sequester CTCF, which in turn impedes CTCF function by changing its genomic occupancy and rewiring 3D chromatin conformation particularly at SASP gene loci, leading to an increase in chromatin accessibility of these genes' regulatory elements that induces SASP proinflammatory gene transcription (Miyata et al., 2021). In fact, lower levels of CTCF in aged cells can promote pericentromeric satellite RNA transcription and further abrogate CTCF function through a positive feedback cycle, which consequently enhances SASP-mediated inflammation and oncogenesis during the aging process (Miyata et al., 2021). This may partly explain the appearance of transformed foci in embryonic fibroblast-derived cells of CTCF-haploinsufficient (Ctcf+/-) mice, which become exceptionally prone to developing cancer (Kemp et al., 2014), and Ctcf-null mice are inviable beyond early embryogenesis (Moore et al., 2012).

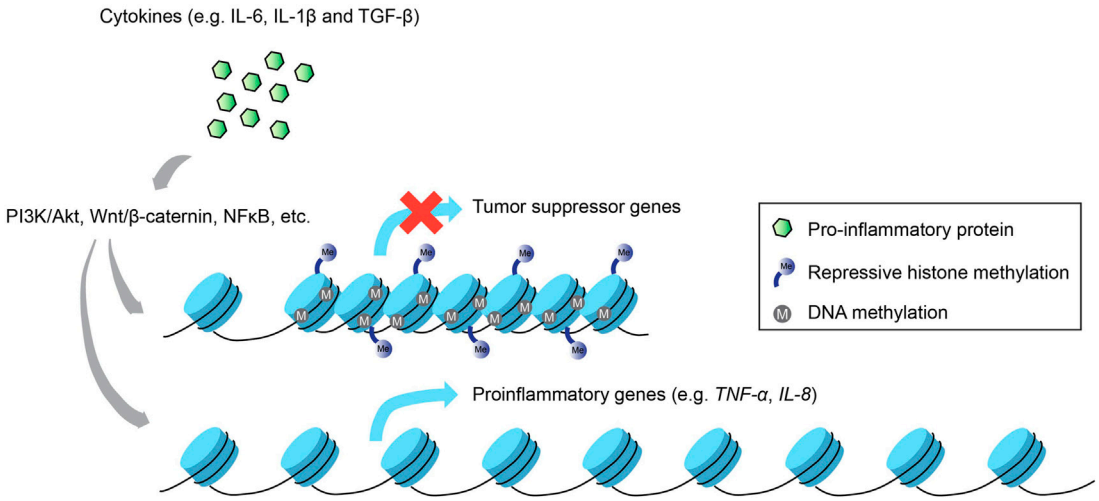

FIGURE 2 | Inflammation-induced epigenetic alterations. Not only do epigenetic modifications regulate inflammatory gene expression, the activation of inflammatory signaling pathways via proinflammatory cytokines can also enact changes to the epigenetic landscape that result in the silencing of tumor suppressor genes and the increased activation of proinflammatory genes that promote carcinogenesis.

INFLAMMATION-INDUCED EPIGENETIC ALTERATIONS

Thus far, we have described how epigenetic changes at different hierarchical levels of the eukaryotic genome regulate the expression of inflammatory and immunological genes, translating to downstream physiological consequences that control cell function and disease state. Nevertheless, the reverse relationship, i.e., how inflammatory signals impinge on the chromatin landscape, also bears significant relevance to fully appreciate the crosstalk that exists between these two molecular entities, especially in the context of cancer (**Figure 2**).

One of the most well-studied diseases associated with chronic inflammation that subsequently re-wires the host epigenome is gastric cancer caused by the bacterium *Helicobacter pylori*, which activates proinflammatory gene transcription via multiple signaling pathways such as PI3K/Akt, Wnt/β-catenin and NF-kB (Yamaoka et al., 2004; Lu et al., 2005; Tabassam et al., 2009). Inflammation-induced epigenetic perturbations that ensued from gastric mucosa cells infected by *H. pylori* included upregulation of proinflammatory genes, e.g., TNFα and IL-1β caused by aberrant modifications in DNA methylation of their promoter regions (Maeda et al., 2017). These alterations are believed to be linked to infection-induced inflammation and not the infection per se, since methylome changes directly influenced the expression profiles of various inflammation-associated genes in a gerbil model, and treatment with an immunosuppressant drug mitigated these methylation changes with negligible effects on bacterial colonization (Kurkjian et al., 2008; Katayama et al., 2009; Niwa et al., 2010). Furthermore, inflammation-induced DNA methylation dysregulation precipitated by infection with *H. pylori* or Epstein-Barr virus in the gastric mucosa drives gene expression changes that bolster oncogenesis, including tumor suppressor genes like LOX and p16Ink4a, and proinflammatory genes like IL-8 and TNFα (Matsusaka et al., 2014).

Katayama et al. (2009) reported that the DNA methylation alterations were largely attributed to macrophage production of nitric oxide in response to *H. pylori* infection. In cervical cancer, nitric oxide-induced inflammation is also culpable for affecting the promoter methylation levels of multiple genes, including cancer-related genes, e.g., protein tyrosine phosphatase receptor type R (PTPRR), and genes with immune functions, e.g., T-lymphocyte maturation-associated protein (MAL) (Su et al., 2017; Holubekova et al., 2020), thereby establishing the causal connection between infection-driven inflammatory signaling and its downstream epigenetic changes.

Inflammation has typically been associated with bacterial or viral infections, but it can also be induced by exposure to allergens and particulates like dust, chemicals and inhalable fibers that mimic proinflammatory stimuli, and can link inflammation to tumorigenesis. Smoking exemplifies such a non-infection, lifestyle-based inflammation, in which global epigenetic alterations, ranging from dysregulated histone and DNA methylation to aberrant microRNA expression patterns, can promote lung carcinogenesis (Sharma et al., 2010). Seiler et al. (2020) recently revealed that inflammation-induced modifications upset the balance of DNA methylation and demethylation in the lungs of nicotine-addicted mice, resulting in changes to histone acetylation levels and concomitant gene expression profiles that facilitate the development of lung cancer. Epigenetic modulations can also be actuated by hormonal treatments like sex steroids, which were demonstrated to change methylation levels and gene expression of various inflammatory signaling factors in prostate cancer patients (Wang et al., 2016).

IL-6

Numerous inflammatory molecules can incite epigenetic disruptions, particularly in DNA methylation, which in turn promote various facets of cancer development in diverse cell

types. IL-6 is one such example of a proinflammatory cytokine that orchestrates chronic inflammation, and has been connected to poor patient survival in different cancers (De Vita et al., 1998). NF-kB-mediated secretion of IL-6 from immune cells in cancer originating from colon inflammation appears to activate NF-kB and STAT3-dependent signaling in epithelial cells of the gastric mucosa, such as upregulation of DNA methyltransferase activity and associated methylome changes (Hartnett and Egan, 2012). Specifically, IL-6-directed increase in DNMT1 expression led to the hypermethylation and consequent repression of adhesion, apoptosis and tumor suppressor genes, thereby contributing to inflammation-linked colon tumorigenesis (Foran et al., 2010). In another study, inflammation caused by IL-6 in oral squamous cell carcinoma was responsible for reducing global methylation levels of LINE-1 retrotransposons, while increasing promoter methylation and concomitant silencing of select tumor suppressor genes (Gasche et al., 2011). Prior reports have also provided critical insights into the epigenetic mechanisms that govern the IL-6-induced generation of cancer stem cells (Drost and Agami, 2009; Iliopoulos et al., 2009; Iliopoulos et al., 2010), which are a subset of chemo-resistant tumor cells that drive cancer metastasis (Yu et al., 2012).

A well-established gene regulatory network that links IL-6-mediated chronic inflammation with cancer consists of two distinct but complementary feedback loops, one involving IL-6, NF-kB, Lin28 and let-7 miRNA, and the other comprising IL-6, NF-kB, STAT3, miR-181b-1, miR-21, CYLD and PTEN (Iliopoulos et al., 2009; Iliopoulos et al., 2010). In the former loop, activation of the Src oncogene *via* IL-6 secretion induces a proinflammatory output that is mediated by NF-kB, which leads to the increased expression of Lin28, an RNA binding factor that interacts with and impedes the expression of let-7 miRNA (Kumar et al., 2008). Loss of let-7, which usually targets IL-6, causes IL-6 accumulation, which then induces NF-kB, thereby creating a positive feedback circuit that sustains human breast cancer cells in a transformed state (Drost and Agami, 2009; Iliopoulos et al., 2009). As for the latter loop involving the STAT3 TF, which is induced by IL-6 that supports NF-kB in its active form, STAT3 triggers miR-181b-1 and miR-21 expression, which target the CYLD and PTEN tumor suppressor genes, respectively, resulting in the activation of NF-kB (Iliopoulos et al., 2010). Therefore, IL-6 works synergistically with the TFs NF-kB and STAT3, as well as multiple miRNAs, to set up dynamic regulatory feedback loops for perpetuating inflammatory cues that promote chronic inflammation and cancer.

IL-1β

IL-1β is another potent proinflammatory cytokine that is not only abundantly expressed within the tumor microenvironment of several cancers, but is also a key contributor to various aspects of cancer development, including tumor growth, angiogenesis and metastasis (Elaraj et al., 2006; Voronov et al., 2007). In gastric cancer, IL-1β promotes DNA methyltransferase function *via* the synthesis of nitric oxide, resulting in promoter CpG island methylation-induced gene repression (Hmadcha et al., 1999). Similarly, IL-1β-mediated inflammatory signaling accounted for the promoter hypermethylation and gene silencing of E-cadherin, which is important for impeding cell migration and metastasis, based on a mouse model of gastric cancer (Huang et al., 2016). IL-1β has also been demonstrated to re-wire the DNA methylome of colon cancer cells by increasing DNMT3a and ablating DNMT3b expression, with minimal changes to DNMT1, leading to reduced CpG island methylation at the promoter regions of the IL-6 and IL-8 proinflammatory cytokine genes (Caradonna et al., 2018).

Further to the inflammation-mediated epigenetic changes at the primary tumor location, the interplay between inflammatory signaling and epigenetic mechanisms is also pertinent to cancer metastasis, especially during epithelial-to-mesenchymal transition (EMT), a trans-differentiation process by which transformed epithelial cells are reprogrammed to acquire mesenchymal features for invading and spreading to other sites of the body (Lopez-Novoa and Nieto, 2009; Suarez-Carmona et al., 2017). A case in point is the activation of the EMT program in IL-1β-induced non-small cell lung cancer (NSCLC) that facilitates epigenetic alterations at the E-cadherin gene promoter (Li R. et al., 2020). Mechanistically, acute exposure to IL-1β raises the expression level of a key EMT TF, SLUG, causing a decrease in active histone marks like H3K9ac and H3K4me3, while increasing inactive histone marks like H3K27me3. Chronic IL-1β exposure engenders greater accumulation of SLUG that induces *de novo* deposition of H3K9me2/3 and further enriches H3K27me3, collectively reinforcing E-cadherin gene repression during EMT memory (Li R. et al., 2020). Another related study revealed that IL-1β triggers oncogenic Lin28B expression by repressing miR-101, thereby dysregulating cellular proliferation and migration in inflammation-induced NSCLC (Wang et al., 2014).

TGF-β

TGF-β is an anti-inflammatory cytokine that can activate the gene expression of DNA methyltransferases, which in turn alters the methylome of ovarian cancer cells during EMT (Cardenas et al., 2014). A similar function of TGF-β is recapitulated in breast cancer, whereby TGF-β robustly induces a suite of oncogenic EMT TFs like SNAIL, SLUG and TWIST1 to engage the EMT transcriptional program by upregulating mesenchymal cell-specific genes and antagonizing the expression of epithelial cell markers (Dong et al., 2012; Dong et al., 2013). Mechanistically, SNAIL-dependent repression consists of its interaction with the histone methyltransferases SUV39H1 and EHMT2 that collaborate to catalyze the deposition of the transcriptionally repressive histone modification H3K9me3, which is essential for recruiting DNA methyltransferases to carry out promoter methylation and stable silencing of target genes such as E-cadherin (Dong et al., 2012; Dong et al., 2013; Tam and Weinberg, 2013). TGF-β-induced EMT in breast cancer *via* the action of DNA and histone methyltransferases is also instrumental for the generation of cancer stem cells (Dong et al., 2012; Dong et al., 2013; David and Massague, 2018).

TGF-β signaling can trigger the expression of another epigenetic player, KDM6B, a histone demethylase that erases the silencing H3K27me3 mark to promote gene transcription, and this is crucial for the activation of SNAIL-induced EMT in

both human and mouse mammary epithelial cells (Ramadoss et al., 2012). In support of this, Ramadoss et al. reported a dramatic elevation of KDM6B expression in metastatic breast cancer relative to healthy breast cells (Ramadoss et al., 2012). Additionally, stimulation of the EMT program by TGF-β in mammary epithelial cells leads to an increase in SIRT1 expression, which induces histone deacetylation and represses miR-200a expression (Eades et al., 2011). Because miR-200a targets SIRT1, both these epigenetic factors regulate each other via a negative feedback loop (Eades et al., 2011), and similar reciprocal feedback circuits have also been demonstrated in other studies between the ZEB family of EMT TFs and members of the miR-200 family that mutually regulate one another's expression, thereby dynamically controlling the EMT transcriptional network (Shimono et al., 2009; Wellner et al., 2009).

Other noteworthy examples of epigenomic re-wiring driven by TGF-β-induced EMT include a widespread diminution of the silencing histone mark H3K9me2, and increase in the transcriptionally competent marks H3K4 and H3K36 trimethylation. These chromatin alterations rely on the LSD1 demethylase, as LSD1 depletion exerts adverse impacts on EMT-linked cancer cell metastasis and chemoresistance (McDonald et al., 2011). In addition to its role in TGF-β signaling, LSD1 can also participate in the NF-kB-mediated inflammatory pathway, as nuclear PKCα phosphorylates LSD1 to enable the binding and stabilization of NF-kB, suggesting that the PKCα-LSD1-NF-kB regulatory axis is important in the epigenetic control of EMT and its associated inflammatory phenotypes (Kim et al., 2018).

Finally, the dual crosstalk between inflammatory signaling and epigenetic modulations can set up a self-regulatory feedback circuit as a homeostatic mechanism to finetune the expression of inflammatory genes. This is elegantly illustrated in a seminal study by Foster et al. (2007), who showed that the robust activation of proinflammatory genes at the onset of LPS treatment was significantly muted upon subsequent stimulations. This was attributed in part to the maintenance of low histone H4 acetylation levels at the promoter regions of proinflammatory genes after the second round of LPS challenge, which reflects the dynamics of inflammatory gene activation and explains why macrophages appeared to lack sensitivity toward subsequent rounds of LPS induction (Foster et al., 2007).

In a different study, Cheng et al. (2013) discovered that canonical inflammatory genes like chemokines and adhesion factors were rapidly upregulated upon initial treatment with the proinflammatory cytokine TNFα, but their expression reduced over time despite continuous TNFα treatment. Yet, miR-146α/β expression displayed the opposite trend—higher induction at later compared to earlier timepoints of TNFα stimulation, which accounts for miR-146α/β activation coinciding with the downregulation of genes encoding adhesion factors, and that miR-146α/β served as a negative regulator of inflammatory signaling by targeting IRAK1, IRAK2 and TRAF6, thereby intricately controlling the ideal level of inflammatory output (Cheng et al., 2013). Taken together, inflammation-induced changes to the epigenome can efficiently feedback onto subsequent waves of inflammatory challenge to refine the overall kinetics of the inflammatory gene regulatory network, so as to avoid the deleterious outcome of excessive and uncontrolled inflammation.

EPIGENETIC AND ANTI-INFLAMMATORY THERAPIES IN CANCER

Given the closely intertwined nature between inflammatory signaling and epigenetic alterations, and how their dynamic bidirectional interaction augments oncogenesis, it is therefore not surprising that the administration of drug therapeutics targeting either or both pathways hold significant value in combating cancer. For instance, the immunosuppressive drug tocilizumab not only antagonizes IL-6-STAT3 inflammatory signaling, but also restrains the IL-6-STAT3-NF-kB epigenetic feedback axis, which heralds an exciting therapeutic prospect for triple-negative breast cancer patients (Alraouji et al., 2020).

Importantly, certain anti-inflammatory drugs are capable of functioning at the epigenetic level as well, such as non-steroidal anti-inflammatory drugs (NSAIDs) that can alleviate cancer progression by regulating the expression of HDACs. For example, the application of a commonly utilized NSAID, aspirin, in a mouse model of colitis-linked colon cancer, led to a decrease in the active histone H3K27ac levels and accompanying repression of the proinflammatory genes TNFα, IL-6 and inducible nitric oxide synthase (iNOS) (Guo et al., 2016). Aspirin also heightened the efficacy of another HDAC inhibitor drug, romidepsin, by boosting p21 expression, thereby hindering tumorigenesis in COX-1-positive ovarian cancer (Son et al., 2010). Additional support for NSAIDs in epigenetically mitigating cancer oncogenesis is documented in a recent report that long term treatment with ibuprofen correlates with a lower propensity to develop certain cancers (Shen et al., 2020). Specifically, ibuprofen not only suppressed numerous inflammation-associated stemness genes in breast, liver and lung cancer cells, but also decreased cancer cell metastasis and chemoresistance *via* the downregulation of HDAC and histone demethylase KDM6A/B both *in vitro* and *in vivo* (Shen et al., 2020).

Similarly, several epigenetic drugs possess the ability to counter inflammation. For instance, treatment with resveratrol and MS-275, a SIRT1 activator and a HDAC inhibitor, respectively, elicited anti-inflammatory properties by impeding microglia-macrophage activation in a mouse model of permanent brain ischemia (Mota et al., 2020). Another study revealed that administration of 5-azacytidine, a DNA methyltransferase inhibitor, and trichostatin A, another HDAC inhibitor, abrogated inflammation-dependent pyroptosis and apoptosis in acute lung injury *via* the repression of IL-1 and select caspase activities in bone-marrow-derived macrophages (Samanta et al., 2018). DNA methyltransferase inhibitors were similarly touted as a promising class of therapeutic candidates for tackling pancreatic cancer, as induction of NF-kB inflammatory signaling in pancreatic cancer stem cells requires DNA methylation of the promoter region of SOX9, a critical gene for cancer metastasis (Sun et al., 2013).

In the past decade, BET inhibitors, a prominent category of epigenetic drugs targeting the BET domain, which are bromodomain-containing proteins with well-established roles in gene regulation via histone modification and chromatin remodeling (Fujisawa and Filippakopoulos, 2017), have been successfully developed for various cancer therapies, including hematological tumors and the comparatively uncommon nuclear protein in testis (NUT) midline carcinomas (Filippakopoulos et al., 2010; Gallenkamp et al., 2014). Nicodeme et al. (2010) manufactured a synthetic histone mimic named I-BET that interferes with the binding of BET proteins to acetylated histones, resulting in the inhibition of chromatin complex formation necessary for inflammatory gene transcription in activated macrophages. This highlights the anti-inflammatory potential of synthetic compounds that specifically target proteins recognizing epigenetically modified histones in modulating physiological and pathological cell states.

Other documented examples of BET inhibitors include ABBV-075 and I-BET151, which were shown to exude apoptotic functions in multiple blood disorders like acute myeloid leukemia and non-Hodgkin's lymphoma (Dawson et al., 2011; Bui et al., 2017). Notably, these epigenetic drugs also harbor anti-inflammatory characteristics, e.g., I-BET151 hampers the expression of the proinflammatory genes IL-1β and TNFα in rheumatoid arthritis synovial fibroblasts, leading to a decreased ability in recruiting immune cells and their lowered proliferative capacity (Klein et al., 2016). A recent report by Ullmann et al. (2021) demonstrated that treatment with the BET inhibitors I-BET151 and Ro 11–1,464 in cultured macrophages not only increases endogenous levels of the tumor suppressor protein CEBPD, but also downregulates key cytokine genes like CCL2 and IL-6, buttressing their anti-inflammatory functions. Furthermore, beyond the realm of drug therapeutics, natural dietary supplements like Vitamins C, D and E can also enact both anti-inflammatory and epigenetic effects (Saccone et al., 2015; Gerecke et al., 2018; Zappe et al., 2018; Yang et al., 2019).

CONCLUSION

Epigenetic processes at various hierarchical levels of the genome take place in response to environmental stimuli, especially during infections and other inflammatory challenges, thereby modulating gene expression networks that govern cell identity and disease states. The aforementioned studies described here clearly illustrate the intimate connection between epigenetics and inflammation, and how they interact with each other through various feedback loops and regulatory axes, especially in the context of cancer. Owing to the reversible nature of epigenetic alterations and their susceptibility to inflammatory signaling from both internal and external environments, it is of paramount importance to decipher how these molecular mechanisms drive cancer initiation and progression. For example, prior studies have pinpointed the fundamental role of deleterious epigenetic modifications, particularly in DNA methylation profiles, in promoting inflammation-induced tumorigenesis (Chan et al., 2003; Maekita et al., 2006).

Importantly, the reversibility of epigenetics enables them to be harnessed as ideal cancer therapeutics to target the epigenetic changes within both the tumor core and microenvironment. High-throughput epigenomic and metabolomic approaches can be leveraged to elucidate a more thorough understanding of the repertoire of epigenetic and inflammation-related alterations in patient-derived cancer tissues, so that the appropriate treatments can be tailored to each patient. The combination of epigenetic drugs with anti-inflammatory roles, and vice versa, promise to offer much propitious prospects in long term palliative care and cancer therapy.

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Mitochondrial Epigenetics Regulating Inflammation in Cancer and Aging

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Inflammation is a defining factor in disease progression; epigenetic modifications of this first line of defence pathway can affect many physiological and pathological conditions, like aging and tumorigenesis. Inflammageing, one of the hallmarks of aging, represents a chronic, low key but a persistent inflammatory state. Oxidative stress, alterations in mitochondrial DNA (mtDNA) copy number and mis-localized extra-mitochondrial mtDNA are suggested to directly induce various immune response pathways. This could ultimately perturb cellular homeostasis and lead to pathological consequences. Epigenetic remodelling of mtDNA by DNA methylation, post-translational modifications of mtDNA binding proteins and regulation of mitochondrial gene expression by nuclear DNA or mtDNA encoded non-coding RNAs, are suggested to directly correlate with the onset and progression of various types of cancer. Mitochondria are also capable of regulating immune response to various infections and tissue damage by producing pro- or anti-inflammatory signals. This occurs by altering the levels of mitochondrial metabolites and reactive oxygen species (ROS) levels. Since mitochondria are known as the guardians of the inflammatory response, it is plausible that mitochondrial epigenetics might play a pivotal role in inflammation. Hence, this review focuses on the intricate dynamics of epigenetic alterations of inflammation, with emphasis on mitochondria in cancer and aging.

Keywords: mitochondria, epigenetic modifications, inflammation, aging, cancer

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INTRODUCTION

Inflammation, one of the first lines of defence is frequently repurposed from its fundamental role in immune surveillance to a pro-tumorigenic role. Recent studies report that inflammation can aid proliferation of cancer cells and promote tumor microenvironment by selectively blocking anti-tumor immunity (Greten and Grivennikov, 2019). Acute inflammation might be initiated due to several factors, like bacterial or viral infection, autoimmune diseases, obesity, tobacco smoking, asbestos exposure, and excessive alcohol consumption. On the other hand, chronic inflammation has been suggested to be involved in almost all the stages of tumorigenesis. This can further aggravate the phenotype, by generating a pro-tumorigenic inflammatory microenvironment (Grivennikov et al., 2010). Inflammation is hence, considered one of the pivotal factors responsible for predisposition to cancer development (Greten and Grivennikov, 2019). Apart from cancers, chronic or acute inflammation is also strongly associated with age related disorders including atherosclerosis, diabetes, Alzheimer's disease, rheumatoid arthritis, and aging (Rea et al., 2018). Aging related low grade persistent inflammation is known as 'senoinflammation'. This is affected by factors, like proinflammatory senescence-associated secretome, inflammasome, ER stress, Toll like Receptors (TLRs), and microRNAs (Chung et al., 2019). Inflammageing, is described as a condition

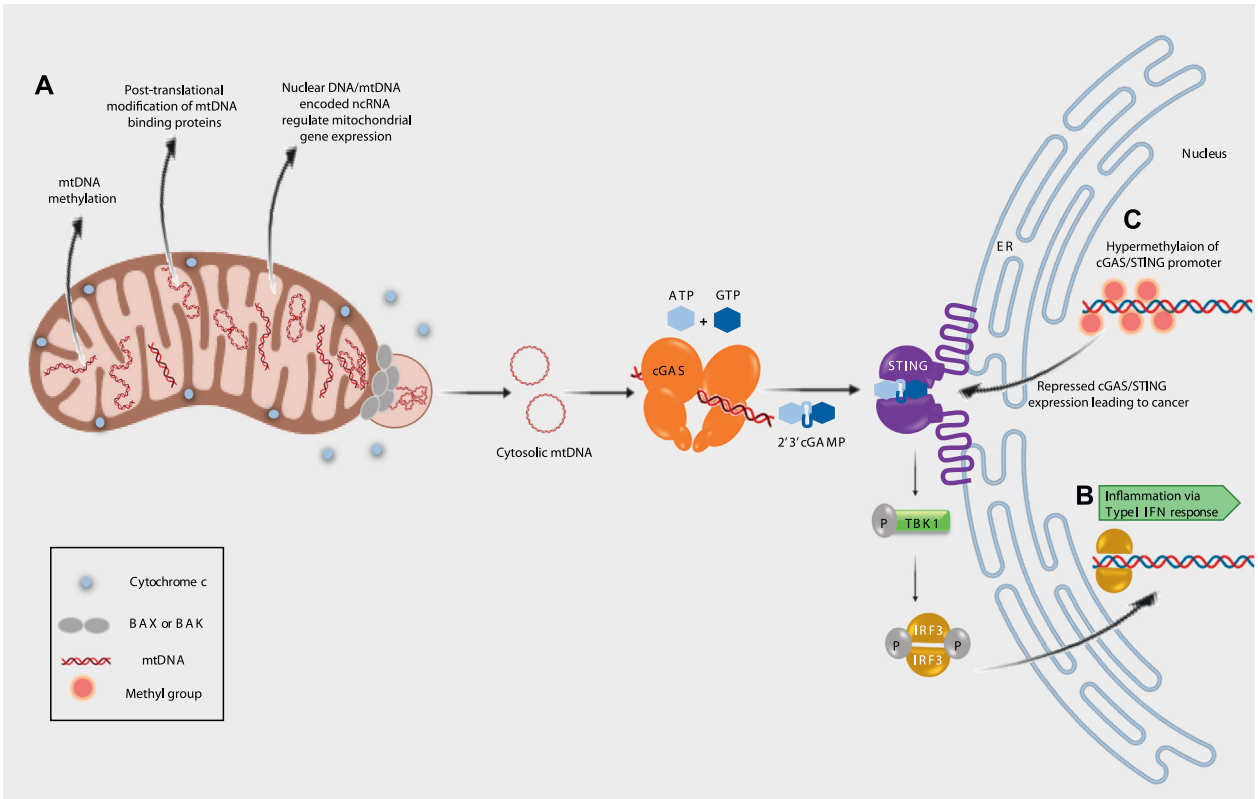

FIGURE 1 | Implications of mitochondrial epigenetics on inflammation, cancer and aging. **(A)**. Three major pathways regulating mitochondrial epigenetics. **(B)**. Extrusion of mtDNA into the cytosol induces inflammation (Type I IFN response) *via* activated cGAS/STING pathway. **(C)**. Epigenetic silencing of cGAS/STING promoter region correlates with cancer prognosis.

characterized by elevated levels of blood inflammatory markers that signify high susceptibility to chronic morbidity, disability, frailty, and premature death. Some of the plausible etiologies of inflammageing are obesity, altered gut permeability and microbiota composition, cellular senescence, NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation, mitochondrial oxidative stress, immune cell dysregulation, genetic predisposition, and chronic infections. Inflammageing can lead to multiple pathological conditions including chronic kidney disease, diabetes mellitus, sarcopenia, depression, dementia as well as cancer (Ferrucci and Fabbri, 2018). Mitochondria, besides being the powerhouse of a cell perform a wide array of functions, like maintenance of calcium homeostasis, orchestration of apoptosis and differentiation (Missiroli et al., 2020). Recent scientific advances reveal that mitochondria actively participate in evoking innate immune and inflammatory responses. Mitochondrial dysfunctions can lead to severe chronic inflammatory disorders (Missiroli et al., 2020).

It is suggested that epigenetic changes in mitochondria, termed as 'mitoepigenetics', are progressively being implicated as heritable changes that can be at the crossroads of several age-related diseases like cardiovascular diseases, osteoarthritis, neurodegenerative diseases and cancers (Coppedè and Stoccoro, 2019). These epigenetic changes include, but are not limited to, alteration in the mitochondrial DNA (mtDNA). Covalent modifications, such as methylation and hydroxymethylation, play a crucial role in altered mtDNA replication and transcription. Post-translational modification of proteins like the mitochondrial transcription factor A (TFAM) is suggested as an essential epigenetic modulator of mtDNA replication and transcription. Post-transcriptional modifications of mitochondrial RNAs (mtRNAs) (like mt-rRNAs, mt-tRNAs and mt-mRNAs) are important epigenetic modulations that affect cellular physiology. mtDNA or nuclear DNA (n-DNA) derived non-coding RNAs (ncRNAs) also play significant roles in the regulation of translation and function of mitochondrial genes (Dong et al., 2020) (**Figure 1A**). Thus, in this review, we attempt to delineate mitochondrial epigenetic signatures, direct or indirect, which affect inflammation and alter the immune-surveillance mechanism leading to inflammageing, cancer and aging.

Inflammation and Aging

Mitochondrial dynamics, cellular differentiation and glucose oxidation processes regulate local and systemic inflammation. Mitochondria adapt to oxidative stress by regulating the processes of fission/fusion, optimizing mitochondrial biogenesis, and

altering the integrity and copy number of mtDNA (Lee and Wei, 2005; Chen et al., 2018). These mitochondrial processes are similarly affected during oxidative stress associated aging. The activity of the pivotal regulators of mitochondrial biogenesis, like peroxisome proliferator-activated gamma coactivator (PGC)-1α, TFAM, and nuclear respiratory factor 1 (NRF-1), is controlled by post-translational modifications. These modifications are also implicated in regulating mitochondrial metabolism (Skuratovskaia et al., 2021). An increase in Interleukin 6 (IL-6), tumor necrosis factor (TNF)-α, and their receptor levels are detected in aged tissues and cells. IL-6 family cytokines and its receptor complex (with gp130 subunits) have been found to regulate mitochondrial dynamics by decreasing TFAM protein production in liver biopsies of obese patients with and without Type 2 diabetes (Skuratovskaia et al., 2021). Cellular senescence elicits senescence associated secretory phenotype (SASP). This evokes several inflammatory cytokines, chemokines as well as matrix metalloproteases. Aging leads to impaired clearance of senescent cells, thus leading to elevated SASP and chronic inflammation. The mitochondrial dysfunction-associated senescence (MiDAS), can lead to the release and accumulation of mitochondrial components which are recognized as damage-associated molecular patterns (DAMPs). NLRP3 inflammasome identifies DAMPs and promotes its self-oligomerization, leading to the secretion of activated Caspase-1. Activated Caspase-1 further promotes the release of proinflammatory cytokines, including IL-1β and IL-18. Viral infections induce the accumulation and aggregation of signature molecules known as mitochondrial antiviral-signaling proteins (MAVS) on the mitochondrial outer membrane (OMM). This leads to chronic inflammation by the activation of interferon regulatory factor 3 (IRF3) and the NF-κB pathway (Thoudam et al., 2016). Elevated blood serum levels of IL-1 and IL-18 are associated with aging. This indicates that increased secretion of pro-inflammatory cytokines is an early event in aging associated inflammation (Dinarello, 2006). On the contrary, blockade of NLRP3 has been shown to greatly reduce multiple aging associated degenerative changes like insulin resistance, thymic involution, T cell senescence, and bone loss as well as physical and cognitive function decline (Zhu et al., 2021). ROS produced by dysfunctional mitochondria can also trigger an inflammatory response by activating the NF-κB signalling pathway (Ferucci and Fabbri, 2018). Further, the association of cytosolic oxidized mtDNA with NLRP3 has emerged as an essential prerequisite for activation of the inflammasome complex; this results in uncontrolled inflammation as evidenced in several diseases. Furthermore, recent studies have implicated that the increased systemic inflammation observed in aging individuals is associated with increased circulating cytosolic mtDNA. All these, indirectly point towards a role for mitoepigenetics in inflammation and inflammageing (Picca et al., 2018).

Inflammation and Cancer

Almost 90% of cancers are caused by somatic mutations and environmental factors, barring a few that are associated with germline mutations. These environmental causes and cancer risk factors are mostly associated with some form of chronic inflammation (Multhoff et al., 2012). Viral or bacterial infection induced cancers transform the protective immune inflammation response triggered as the first line of immune defence, into a persistent, low-grade chronic inflammation. This generates a beneficial microenvironment for the tumor to sustain and proliferate. A low mtDNA copy number is associated with a heightened inflammatory response; it triggers elevated levels of hs-CRP, IL-6, fibrinogen, and increases white blood cell count (Wu et al., 2017). Many cellular responses involved in cancer have been implicated to interact with the signal transducer and activator of transcription 3 (STAT3) protein, a transcription factor known to mediate cytokine signalling. This, in turn, induces sustained autophosphorylation, maintenance of enhanced proliferation and upregulation of antiapoptotic BCL-xL and Cyclin-D. Inflammation in general is a self-restricting phenomenon with a balance between the anti-inflammatory and proinflammatory cytokines. However, in presence of tumorigenic insults, the proinflammatory cytokines over-ride the anti-inflammatory cascade and lead to a chronic inflammatory state, comprising cytokines that propagate tumorigenic growth. Interestingly, the inflammatory signalling pathway comprising IL-6 and STAT3 molecules have been implicated in stomach, colorectal, bladder and lung cancers (Coussens and Werb, 2002). Inflammatory factors, like cytokines, chemokines, growth factors, inflammasomes and inflammatory metabolites have emerged as regulators of tumorigenicity. They do so by modulating multiple signalling pathways, such as nuclear factor kappa B (NF-kB), Janus kinase/signal transducers and activators of transcription (JAK-STAT), toll-like receptor (TLR) pathways, cGAS/STING, and mitogen-activated protein kinase (MAPK) pathways (Zhao et al., 2011).

Inflammation and Epigenetics

Innate immune responses, elicited during tissue damage or microbial infection are known to induce inflammation (Akira et al., 2006; Brennan et al., 2015). The presence of cytosolic DNA, like microbial DNA or part of nuclear DNA (that has escaped from the nucleus), can trigger innate immunity. Under such conditions, two proteins play essential roles in eliciting innate immune responses—these are, 1) cyclic GMP-AMP synthase (cGAS), a cytosolic DNA sensor and 2) stimulator of interferon genes (STING), an ER resident protein (Sun et al., 2013). It is now, well established, that mtDNA released into the cytosol can bind cGAS (McArthur et al., 2018; Riley et al., 2018; Kim et al., 2019). The concerted activity of cGAS and activated STING initiates a signalling cascade that culminates in the transcription of Interferon stimulated genes (ISGs) (McArthur et al., 2018; Riley et al., 2018; Kim et al., 2019; Zhang et al., 2019). Thus, the presence of cytosolic mtDNA can elicit inflammation via an innate immune response (**Figure 1B**).

Interestingly, it has been observed that cGAS and/or STING expression is decreased in various cancers, like—colon cancer and melanoma (Xia et al., 2016a; Xia et al., 2016b). Reduced cGAS/STING expression corresponds with poor survival in lung and gastric cancer patients (Song et al., 2017; Yang et al., 2017). One of the reasons, for the loss of cGAS-STING signalling is suggested to be the epigenetic silencing of cGAS/STING promoter regions

(Konno et al., 2018). Hypermethylation of cGAS/STING promoters contributes to the transcriptional silencing and perturbed STING signalling function is implicated in various cancers (Konno et al., 2018; Falahat et al., 2021) (**Figure 1C**). Hence, the interconnectivity between all these factors opens up new and important avenues for future research as this would help establish their therapeutic potential.

Mitochondrial Epigenetics

Mitochondrial epigenetics remains less understood primarily due to the lack of classical epigenetic regulators and substrates for mtDNA. However, epigenetic regulation of mtDNA may be potentiated by post-translational modification on mtDNA interacting proteins. Mitochondrial metabolites can also serve as substrates for epigenetic modifications (Weise and Bannister, 2020). Circular mtDNA (16,569 base pairs) comprises one purine rich heavy strand and the complementary light strand is pyrimidine rich (Asin-Cayuela and Gustafsson, 2007). There is another linear strand, 7S DNA that forms the displacement loop or D-loop; however, its presence is not ubiquitous through all cell types and organisms (Nicholls and Minczuk, 2014). mtDNA is maternally inherited and intron-less (Iacobazzi et al., 2013); it also lacks histone protein. Thus, unlike nuclear DNA, epigenetic regulation of mtDNA is methylation-dependent.

Classically, during DNA methylation, a methyl group is added from S-adenosyl-methionine (SAM) to DNA bases cytosine (C) or adenine (A) by DNA methyltransferase (DNMT) enzymes. DNA methylation is usually observed at the CpG islands in the promoter region. mtDNA methylation has been a debatable subject. It was believed that mtDNA would not get methylated as mitochondria are inaccessible to methylase and mtDNA is not complexed with histones (Iacobazzi et al., 2013). However, reports suggest that mtDNA methylation does occur in a non-random manner. Due to its small size, CpG islands are absent, but 3%–5% CpG dinucleotides of mtDNA are found to be methylated (Pollack et al., 1984). The presence of DNMT1, targeted to mitochondria (mtDNMT1) further, emphasizes the methylation event of mtDNA. mtDNMT1 is a nuclear encoded protein, which consists of a mitochondrial targeted sequence (MTS) upstream of the translation start site (Shock et al., 2011). mtDNMT1 is detected on the outer mitochondrial membrane in adult neurological tissues, heart and skeletal muscles (Wong et al., 2013). Varying expression levels of mtDNMT1 in cells is shown to affect gene expression pattern -like when mtDNMT1 is overexpressed, the protein coding gene from the light strand promoter (LSP) MT-ND6, gets significantly downregulated (Shock et al., 2011). However, in the same condition, MT-ND1 is upregulated from heavy strand promoter (HSP) without affecting MT-ATP6 or MT-CO1 (Shock et al., 2011). DNA methylation, is mostly observed in D- loop region of mtDNA comprising both HSP and LSP promoter elements. However, the exact mechanism by which mtDNA gets methylated or demethylated remains potentially elusive, till date. Identifying all the participating enzymes would be the first step in that direction. ALKBH1, a demethylase is reported to affect oxidative phosphorylation in mitochondria (Koh et al., 2018). The presence of ten-eleven translocation (TET) 1 and 2 suggests oxygen mediated demethylation in mtDNA (Dzitoyeva et al., 2012). Demethylation of cytosine residues can also be achieved by deamination. Identification of APOBEC3 (Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3) in mitochondria further suggests epigenetic regulation of mtDNA (Wakae et al., 2018).

In the nucleus, the epigenetic function is controlled through post-translational modifications of histone proteins. As mentioned earlier, mtDNA lacks histone proteins, however, the DNA binding proteins could serve as targets for post-translational modifications. mtDNA is present in the nucleoids, which are membrane-less pseudo-compartments in mitochondrial matrix comprising nucleoprotein complexes. It is reported that, 63% of all proteins localized within the mitochondria consist of lysine acetylation sites. Numerous phosphorylation sites are also suggested to be present in those proteins (Zhao et al., 2011). One of the most studied nucleoid associated proteins, TFAM, is involved in mtDNA compaction and transcription. TFAM can be post-translationally modified by acetylation, O-linked glycosylation and phosphorylation (Suarez et al., 2008; Lu et al., 2013; King et al., 2018). Being a member of the high mobility group (HMG) protein, TFAM binds mtDNA co-operatively as a homodimer (Kaufman and Van Houten, 2017). Alteration in the binding affinity of TFAM, affects the mtDNA replication and transcription rates. When the dimer/monomer ratio of TFAM increases, heavy strand replication is stopped, and transcription starts. Also, mtDNA transcription is halted when TFAM/mtDNA ratio is high (Audano et al., 2014). Phosphorylation of HMG1 inhibits the binding of TFAM to mtDNA, preventing activation of transcription (Lu et al., 2013). Other nucleoid associated proteins also have phosphorylation sites, like mtSSB (mitochondrial single strand binding protein) and POLG (DNA polymerase gamma) (Matsuoka et al., 2007; Zhou et al., 2013). But the accurate mechanism of epigenetic control through post- translational modification is yet to be completely unravelled.

Further, nuclear DNA and mtDNA encoded lncRNAs can regulate mitochondrial gene expression. mtDNA encodes for three such lncRNAs — ND5, ND6, and CYB. Nuclear DNA encoded RNaseP complex can control the expression of these lncRNAs. These three lncRNAs, are capable of forming intermolecular duplexes with their functional counterparts, and thus can regulate their expression (Rackham et al., 2011). Another example of mtlncRNAs containing MDL1 (mitochondrial D-loop 1), which spans the anti-sense region of tRNApro and mitochondrial D-loop. The functional importance of mtlncRNAs, however, remains elusive. D-loop is slowly emerging as one of the most essential components of mtDNA for epigenetic regulation. MDL1 and its anti-sense could also participate in epigenetic regulation of mtDNA significantly (Gao et al., 2018). RNA processing endoribonuclease (RMRP) is a lncRNA encoded in nucleus, but transported to mitochondria. It can modify mtDNA replication and transcription (Wang et al., 2010; Noh et al., 2016).

Besides lncRNAs, small non coding RNAs also play a crucial role in the epigenetic regulation mechanism. Mitochondrial microRNAs or mito-microRNAs (mitomiRs) are single

stranded 17–25 bp long RNA molecules, either encoded by nuclear DNA and transported to mitochondria or transcribed from mtDNA (Bandiera et al., 2011; Sripada et al., 2012; Ro et al., 2013). Complementary base pairing between miR-2392 and mtDNA in an argonaute-2 (AGO-2) dependent manner prevents mtDNA transcription partially and affects OXPHOS protein expression. miR-181C targets the 3' end of MT-CO1 mRNA to repress its expression (Das et al., 2012; Das et al., 2014). Translocation of miR-1 and miR1a-3p causes upregulation of MT-CO1 and MT-ND1 (He et al., 2012; Zhang et al., 2014).

Mitochondrial Epigenetics in Inflammation

As already indicated, mitochondrial epigenetics to date is rather less explored. Hence, it's implication in various signalling pathways contributing to varied disease phenotypes are being investigated only recently. Phosphorylation of TFAM by cAMP-dependent protein kinase in mitochondria, within its HMG box 1 leads to impaired ability of binding of TFAM to DNA and hence decreased transcription of mtDNA (Lu et al., 2013). Alteration of mtDNA copy number directly regulates inflammatory response. Hence, it is plausible to hypothesize that TFAM/mtDNA/interleukin axis plays a pivotal role in diseases like osteoarthritis and neurodegeneration (Kang et al., 2018; Zhan et al., 2020). Among the post-translational modifications, ubiquitination of TFAM has been implicated in the disease prognosis of diabetic retinopathy (Santos et al., 2014). Alterations in the activity of the mtDNMT1 have been indicated in modulating methylation profiles and transcription efficiency of various signalling pathways including inflammation and angiogenesis. These are also important in several common age-related pathologies and cancer (Shock et al., 2011; Atilano et al., 2015). Hypoxia is known to turn on the hypoxia-responsive transcription factors including peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) and nuclear respiratory factor 1 (NRF1). These further upregulate mtDNMT1 activity and cause hypermethylation of mtDNA. This leads to repressing gene expression from the light strand promoter during vascular oxidative stress. Recently mtDNA methylation has emerged as a novel non-invasive epigenetic biomarker and is implicated in the etiology of cardiovascular diseases, where increased mtDNA methylation of genes encoding for cytochrome c oxidases, tRNA leucine 1 as well as genes involved in ATP synthesis have been reported (Mohammed et al., 2020). Hypermethylation of mtDNA ND-6 has been implicated in non-alcoholic fatty liver disease and is suggested to be strongly associated with steatohepatitic condition. Steatohepatitis is an aggressive form of liver disease characterized by liver inflammation that ultimately progresses to cirrhosis and liver failure. Hence, the association of epigenetically modified mtND-6 in steatohepatitis could highlight the importance of mitoepigenetics in inflammation and prognosis of certain diseases (Pirola et al., 2013). Further, mitomiRs, a subset of miRNAs, are potential epigenetic regulators of the mitochondria. They affect some of the major mitochondrial functions, like maintenance of membrane potential and electron transport chain (ETC). miR-107 is known to affect the oxidative pathway of mitochondria and its reduction leads to a decrease in mitochondrial volume and altered cristae. It causes mitochondrial dysregulation due to a reduction in mitochondrial membrane potential and ETC activity by decreasing the protein levels of complexes 1,3,4, and 5 (John et al., 2020). miR-125b is implicated in neural cell apoptosis by switching the balance between BAX and BCL-2 towards an apoptotic fate. BCL-2 and BAX can, in turn, regulate mitochondrial membrane permeability by inducing transition pore formation and release of Cytochrome c. This suggests an antitumorigenic effect of mitoepigenetics brought about by enhancing apoptosis. Furthermore, miR-125b is known to negatively regulate IL1β-induced inflammatory genes by targeting the TRAF6-mediated MAPKs and NF-κB signalling in human osteoarthritic chondrocytes (Rasheed et al., 2019).

Inflammation is indirectly regulated by mitochondrial epigenetics *via* altered ROS production and mitochondrial metabolism. These, in turn, affect the known direct players of mitoepigenetics like methylation of DNA, mtDNMT1 activity, release of mtDNA, and TFAM expression. Mitochondrial ROS levels affect DNA methylation (Kietzmann et al., 2017). ROS can directly convert 5-methylcytosine to 5-hydroxymethylcytosine, thereby, blocking the activity of DNMT1. This leads to global hypomethylation. ROS can also oxidize guanosine to 8-oxo-20-deoxyguanosine (8-oxodG) and inhibit the methylation of adjacent cytosine. This can further contribute to the global hypomethylation of DNA. Evidence shows that the formation of 8-oxodG promotes the transcription of TNF-α responsive pro-inflammatory genes. 8-oxodG is also capable of interacting with HIF1α and negatively modulates its binding with the VEGF promoter. This results in impaired angiogenesis. In line with these observations, two recent meta-analyses have shown that high levels of 8-oxodG are associated with atherosclerotic vascular disease and predicts the eventual disease prognosis (Hooten et al., 2012; Carracedo et al., 2020). High ROS levels also influence both repressive (H3K9me2/3 and H3K27me3) and active histone marks (H3K4me2/3). Hence, it may as well be proposed that mitochondrial metabolism and DNA methylation go hand-in-hand (Audia and Campbell, 2016; Lopes, 2020).

Mitoepigenetics in Cancer

Silencing the key regulator of mtDNA, TFAM, leads to a pro-tumorigenic microenvironment (Araujo et al., 2018). This favours metabolic reprogramming towards aerobic glycolysis—as is suggested by decreased respiratory capacity coupled with increased lactate production. Secondly, enhanced ERK1/2-Akt-mTORC-S6 signalling activity leads to enhanced cell growth, metastasis and chemoresistance. On the other hand, increased TFAM expression leads to a significant reversal of these phenotypic changes (Hsieh et al., 2021). Cell lines like those derived from gynaecological origin (ovarian cancer) are known to have upregulated TFAM; this positively correlates with cell proliferation, colony formation, migration, and invasion. It supports a protumorigenic phenotype (Hu et al., 2020). MitomiRs have been implicated to regulate various important tumorigenic phenotypes like, alteration of mitochondrial bioenergetics, invasion, and angiogenesis. miR-126 is known to alter mitochondrial energy metabolism by reducing

mitochondrial respiration and promoting glycolysis. This is executed *via* IRS1 associated modulation of ATP-citrate lyase deregulation; this leads to suppression of the malignant mesothelioma tumor phenotype. An increase in ATP and citrate production leads to reduced Akt signalling and cytosolic sequestration of Forkhead box O1 (FoxO1). This leads to reduced expression of downstream genes involved in gluconeogenesis and defence against oxidative stress. miR-126 is suggested to play an important regulatory role in multiple human cancers, like breast, lung, gastric cancers, melanoma and acute leukaemia (Tomasetti et al., 2012).

Among the several oncogenic stimuli, hypoxia has been reported to alter mitomiR expression (Giuliani et al., 2018). Under conditions of hypoxia, both normal and transformed cells have elevated levels of miR-210 expression, suggesting its role in an adaptive response to this stress (Puisségur et al., 2011). It is now believed that elevated miR-210 expression represents hypoxia gene signatures in tumor tissues like those of breast, head and neck cancers. miR-210 can regulate various signalling mechanisms, like those involved in the cell cycle, survival, differentiation, angiogenesis, and metabolism. Over-expression of miR-210 is further reported in lung cancer derived cell line, A549; thus, suggesting the role of mitomiRs in lung cancer (Grosso et al., 2013; Qin et al., 2014). Another important mitomiR identified to be involved in tumor progression is miR-200 (Korpal and Kang, 2008). One of the prime miR-200 targets is TFAM, which has been implicated both in regulating mitochondrial biogenesis and inflammation. TFAM has been described as a functional target of miR-200 in breast cancer cells. Since TFAM is a transcription factor, its activity is required for mtDNA replication, transcription and maintenance. An alteration in the quality control of mtDNA severely affects the inflammation process. TFAM has also been implicated as a primary architectural protein of the mitochondrial genome by packaging mtDNA. In addition, TFAM expression has been reported to be involved in tumor progression, cancer cell growth, and chemoresistance (Rencelj et al., 2021). Further, the reduced mtDNA copy number is associated with several aggressive phenotypes, like the onset of apoptosis, metabolic shift towards glycolysis, and increased invasiveness in various human cancers (Wu et al., 2017). All these taken together suggest that mitochondria and their epigenetic modifications are closely associated with the tumorigenic phenotypes of invasion, metastasis and chemoresistance in many types of cancers.

Mitoepigenetics in Aging

Mitochondrial dysfunction is implicated at the core of the aging process; this mainly comprises mtDNA mutations, impaired respiratory chain functions and elevated ROS production (Trifunovic and Larsson, 2008). Altered mtDNA methylation can lead to enhanced ROS production. ROS is a known messenger of the inflammatory cytokine signalling pathway. Taken together, it is plausible to hypothesize a complex inter relationship between the three processes of mitoepigenetics, inflammation and aging. Experiments on mtDNA methylation within the 12S ribosomal RNA gene has shown that hypomethylation of two CpG sites (M1215 and M1313) have a direct correlation with age. This suggests that mtDNA methylation could be an epigenetic marker of aging (Mawlood et al., 2016). Further, decreased levels of 5-hydroxymethylcytosine on mtDNA, but not 5-methylcytosine, is detected in the frontal cortex of aging mice (Dzitoyeva et al., 2012). Reduced 5-hydroxymethylcytosine correlates with increased mRNA levels of ND2, ND4, ND4L, ND5 and ND6 regions of the mitochondrial D-loop. This could in turn be due to the downregulation of DNMT1 and upregulation of TET2 in the mitochondria of the frontal cortex of aging mice (Dzitoyeva et al., 2012). Higher mitochondrial 12S rRNA gene (RNR1) methylation corresponds with increased mortality risk—this hence suggests the importance of mitochondrial epigenetics in aging and survival (D'Aquila et al., 2015). Recently, decreased global methylation level of both mtDNA strands is suggested to be associated with aging (Dou et al., 2019). mtDNA methylation is implicated to play a pivotal role in aging *via* the regulation of mitochondrial gene expression (Cao et al., 2021). Again, methylation profiling studies of humans over a wide age range have revealed that missense mutations in the six-transmembrane epithelial antigen of the prostate-2 (STEAP2) gene are associated with the maintenance of homeostasis of metal ions. These metal ions (iron and copper) are known to play a role in the proper functioning of the ETC. This would lead to further complications like ROS mediated anomalies and impaired DNA damage—all ultimately culminating in an aggravated 'senile' state (Hannum et al., 2013). The conserved histone lysine demethylases, jmjd-1.2/PHF8 and jmjd-3.1/JMJD3 are reported to be positive regulators of lifespan. Their presence across species suggests an evolutionarily conserved mitoepigenetic mechanism (Merkwirth et al., 2016). Since aging and mitochondrial dysfunction are interdependent, it is rational to hypothesize that mitochondrial stress induced methylation marks and associated downstream signalling mechanism might potentially contribute to the aging process. It has been found that mitochondrial stress response activation is associated with the di-methylation of histone H3K9 through the activity of the histone methyltransferase met-2 and the nuclear co-factor lin-6. This leads to global gene silencing, though there are portions of the chromatin which open up due to the binding of canonical stress responsive factors, like DVE-1. A metabolic stress response specific gene expression signature negatively modulates the aging phenotype, ultimately leading to an extension of lifespan (Tian et al., 2016).

In some age-related neurodegenerative diseases, mtDNA methylation is found to be critically important. Evidence shows increased detection of 5-methylcytosine levels in the mtDNA D-loop region in Alzheimer disease-related pathology. Further, lower 5-methylcytosine levels in mtDNA D-loop region are also detected in patient samples positive for Parkinson's disease (Blanch et al., 2016). Experiments in transgenic mice have shown decreased D-loop methylation and elevated RNR1 methylation in the hippocampus region (Xu et al., 2019). Patients with Down syndrome are reported to have decreased levels of the

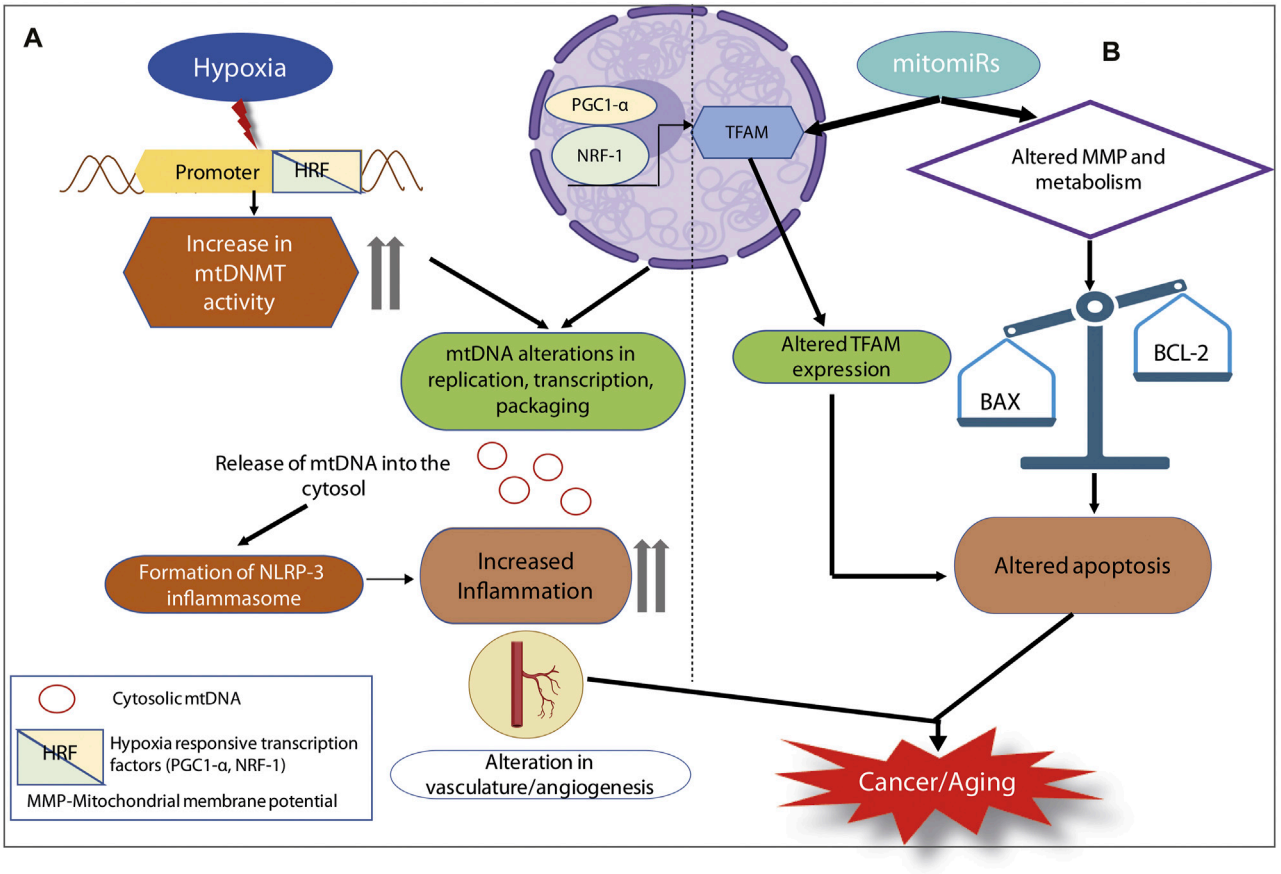

FIGURE 2 | Mitoepigenetic regulation of inflammation in cancer or aging. **(A)** Hypoxia as an oncogenic stimulus, it turns on the HRFs. HRFs alter mtDNMT activity as well as the expression of TFAM. They can combinatorially lead to several mtDNA alterations and the release of mtDNA into the cytosol. Extrusion of cytosolic mtDNA can trigger the formation of NLRP3 inflammosome, elevated inflammation culminating in cancer or aging. **(B)** mitomiRs are capable of altering TFAM expression as well as mitochondrial membrane potential and metabolic status. These two pathways can converge on the apoptotic fate of cell, thereby leading to cancer or aging.

methyl group donor SAM (S-Adenosyl Methionine), correlating with hypomethylation of mtDNA (Infantino et al., 2011). All these taken together suggest a close correlation between mitoepigenetics and the process of aging.

CONCLUSION

Inflammation is regulated by several factors. Mitochondria have now emerged as central in innate immunity, inflammatory responses, aging and cancer. Likewise, mitochondrial epigenetics, though less understood is fast gaining significance as a potential regulator of inflammation and an important contributing factor for physiological and pathological conditions, like aging, neurodegenerative diseases and cancer (Liu et al., 2016; Iske et al., 2020). It is now well understood that mitochondrial epigenetics reaches beyond the confines of classical epigenetic signatures as these organelles lack histones and the conventional CpG islands. Studies have reported that methylation and demethylation of mtDNA could bring about the repression of downstream genes like mtND-6, mt-ATP6 and mt-CO1 (Stoccoro and Coppedè, 2021). Altered expression of these genes leads to differences in mitochondrial metabolism (like glucose metabolism). This would ultimately regulate mitochondrial antiviral signalling protein (MAVS) and result in cGAS/STING mediated immune dysfunction (Zou et al., 2021). Further, the presence of cytosolic mtDNA can trigger inflammation *via* cGAS/STING pathway (Bahat et al., 2021). mtDNA dysfunction due to changes in the copy number, altered compaction, deregulated transcription, or extrusion into the cytosol, is the leading driver for NLRP3 mediated inflammosome formation (Zhong et al., 2018). Post-translational modifications of TFAM, mtDNMT1 activity and hypoxia contribute to changes in mtDNA. This culminates in mtDNA induced inflammation, as is reported in diseases like lung cancer, osteoarthritis, neurodegeneration etc (Nakayama and Otsu, 2018; Iske et al., 2020). Altered mitochondrial DNA methylation, alongwith the deregulated balance between methylases and demethylases is fast emerging as epigenetic markers of aging (Mawlood et al., 2016). mtDNA methylation that affects the expression of certain genes responsible for the maintenance of metal ion (iron, copper) homeostasis affects

"senoinflammation" and hence could also regulate aging. Also, altered mtDNA methylation is detected in multiple age-related neurological disorders. Hypoxia, hypoxia responsive factors like PGC1α or NRF1, can alter the mtDNMT1 activity, bring out changes in methylation of mtDNA, the interleukin axis (that releases proinflammatory cytokines like TNF-α), and result in inflammation. All these can eventually have pathological outcomes. Differential expression of mitomiRs might regulate TFAM expression and alter the mitochondrial membrane potential and metabolism. These lead to cellular changes, like skewing the balance between pro-apoptotic and anti-apoptotic proteins, and altered interleukin signalling. Mitoepigenetic regulation of inflammation, tissue remodelling, cellular differentiation, enhanced vasculature and angiogenesis would culminate in a pro-cancerous phenotype (**Figure 2**). Mitoepigenetics in its many forms is at the crossroad of immune signalling and inflammation; this modulates the physiological process of aging and affects the pathology of various cancers. Thus, strategies aimed at compensating for changes brought about by mitoepigenetics like restoration of dysfunctional mtDNA or TFAM activity might emerge as promising preventive and therapeutic interventions for pathological conditions occurring due to exacerbated inflammation.

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DC, PD, and OC conceived the idea and wrote the paper.

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High enhancer activity is an epigenetic feature of HPV negative atypical head and neck squamous cell carcinoma

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Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease with significant mortality and frequent recurrence. Prior efforts to transcriptionally classify HNSCC into groups of varying prognoses have identified four accepted molecular subtypes of the disease: Atypical (AT), Basal (BA), Classical (CL), and Mesenchymal (MS). Here, we investigate the active enhancer landscapes of these subtypes using representative HNSCC cell lines and identify samples belonging to the AT subtype as having increased enhancer activity compared to the other 3 HNSCC subtypes. Cell lines belonging to the AT subtype are more resistant to enhancer-blocking bromodomain inhibitors (BETi). Examination of nascent transcripts reveals that both AT TCGA tumors and cell lines express higher levels of enhancer RNA (eRNA) transcripts for enhancers controlling BETi resistance pathways, such as lipid metabolism and MAPK signaling. Additionally, investigation of higher-order chromatin structure suggests more enhancer-promoter (E-P) contacts in the AT subtype, including on genes identified in the eRNA analysis. Consistently, known BETi resistance pathways are upregulated upon exposure to these inhibitors. Together, our results identify that the AT subtype of HNSCC is associated with higher enhancer activity, resistance to enhancer blockade, and increased signaling through pathways that could serve as future targets for sensitizing HNSCC to BET inhibition.

KEYWORDS

epigenome analyses, head and neck cancer, enhancer regulation, BET inhibitors, drug resistance

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and the predominant form of head and neck cancer (Marur et al., 2010; Zhou et al., 2016). In the United States, over 60,000 new HNSCC cases and more than 13,000 HNSCC deaths are reported per year (Zhou et al., 2016). HNSCC covers a wide variety of anatomical sites, including the oral cavity, oropharynx, hypopharynx, and larynx (Forastiere et al., 2001). The prognosis for HNSCC is overall poor with a 5-year survival of approximately 50%, which has remained relatively unchanged for decades (Marur et al., 2010). This is largely attributed to factors such as late stage at initial presentation and high rates of primary tumor recurrence (Bonner et al., 2006; Pickering et al., 2013). Treatment for HNSCC involves combinations of surgery, chemotherapy, and radiotherapy, with exact treatment plans depending on tumor location and TNM stage (Marur et al., 2010; Zhou et al., 2016).

To date, studies on HNSCC have focused largely on genomic characterizations such as exome sequencing and copy number alterations. The most common alterations, such as mutations in *TP53* at 17p13 and alterations in *p16* at 9p21, have been known for decades (Forastiere et al., 2001; Zhou et al., 2016). More recent comprehensive analyses of HNSCC tumors have supported these previous findings, in addition to identifying common alterations in the Notch1 pathway and cell cycle genes (Agrawal et al., 2011; Pickering et al., 2013; Cancer Genome Atlas, 2015). Unfortunately, very few of these studies have resulted in clinically actionable findings. There are, however, some disputed exceptions, such as the EGFR inhibitor cetuximab, which showed benefit when combined with radiotherapy (Bonner et al., 2006).

One interesting result of these and other studies is the notion of molecular subtypes of disease. Inspired by similar studies in other tumors such as breast, lung, and brain, transcriptomic data from patient samples was used to classify head and neck tumors into 4 subtypes: Atypical (AT), Basal (BA), Classical (CL), and Mesenchymal (MS) (Chung et al., 2004; Walter et al., 2013; Cancer Genome Atlas, 2015). These studies have largely focused on the relationship of these subtypes to genomic alterations, such as mutation patterns, copy number changes, or alterations in key transcription factor expression, and clinical features, such as progression free survival and lymph node metastasis at time of diagnosis. However, there have been very few studies describing the epigenomic features of the subtypes. The importance of chromatin modification states in HNSCC is further evidenced by the finding that global levels of certain histone tail modifications correlate with clinical measurements such as tumor stage, cancer-specific survival, and disease-free survival in oral squamous cell carcinoma (Chen et al., 2013). Because there are currently only a sparse number of HNSCC epigenomics datasets, particularly in the realm of histone modifications and chromatin regulation, there remains an unmet need to investigate these aspects of gene regulation and leverage newly discovered biology to better define the disease and develop new therapeutic approaches (Castilho et al., 2017; Serafini et al., 2020).

Since HNSCC subtypes are defined by their transcriptomic signatures, it stands to reason they would also have unique epigenomic features, such as enhancer landscapes, that may, in part, be driving the defining transcriptomic signatures. Through mapping of H3K27ac-marked active enhancers in 28 HPV-negative HNSCC cell lines, we demonstrate that the AT subtype is characterized by high enhancer activity. Consistently, the AT subtype was associated with resistance to enhancer-blocking bromodomain inhibitors (BETi). BETi resistance pathways specifically showed high enhancer activity as measured by nascent enhancer RNAs (eRNAs) and enhancer-promoter contacts, providing mechanistic insights into the aggressive nature of the AT subtype. Overall, our data suggests high enhancer activity as an epigenetic feature of atypical HNSCCs.

Methods

Cell culture

Human HNSCC cell lines were acquired and characterized as previously described (Zhao et al., 2011). Briefly, cell lines were cultured in DMEM supplemented with 10% FBS, L-glutamine, sodium pyruvate, nonessential amino acids, vitamins, and 1% penicillin-streptomycin. All cell lines were cultured at 37°C in an atmosphere of 5% CO_2.

RNA-Sequencing processing and analysis

RNA-seq data for cell line subtype assignments were obtained as raw counts, processed as previously described (data available at GEO accession GSE122512) (Kalu et al., 2017; Gleber-Netto et al., 2019). To assign HNSCC cell lines to their representative subtypes, we used the HNSCC gene list templates generated by Yu et al. (Yu et al., 2019) and utilized the CMScaller workflow and implementation of the NTP algorithm to find the closest matching subtype for each cell line (FDR <0.1) based on their transcriptomic profiles (Hoshida, 2010; Eide et al., 2017). Upregulated genes for each subtype were computed using CMScaller in a one-vs-rest fashion.

For the PLX51107 treatment RNA-seq experiments, representative cell lines were selected for the AT subtype (HN4) and a non-AT subtype (MDA1186, CL subtype) and treated with DMSO, GR_{50} of the MDA1186, or GR_{50} of HN4 for 24 h prior to RNA isolation. RNA extraction was performed using an RNeasy Mini Kit per manufacturer's instructions (Qiagen). Isolation of mRNA was performed using NEBNext

Poly(A) mRNA Magnetic Isolation Module and libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep kit (New England BioLabs). Library quality was checked on an Agilent TapeStation 4150 and quantified by Qubit 2000 fluorometer (Invitrogen). Libraries were pooled in equimolar ratios and sequenced on Illumina NovaSeq6000 SP runs with paired-end 100-bp reads at The Advanced Technology Genomics Core (ATGC) at MD Anderson Cancer Center.

PLX51107 treatment RNA-seq raw reads were processed using the provided pipeline: https://github.com/sccallahan/QUACKERS_RNAseq-pipeline. In brief, raw reads were aligned to the hg19 genome using STAR v2.7.2b (Dobin et al., 2013) and quality checked using FastQC v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Counts were generated using featureCounts from subread v1.6.3 80 (Liao et al., 2013). Downstream normalization and differential expression analysis were performed using DESeq2, with size factors being calculated using data-driven housekeeping gene method as implemented in the CustomSelection R package (Love et al., 2014; Dos Santos et al., 2020). Pathway enrichment analyses were performed using GSEA's pre-ranked list option (Subramanian et al., 2005). Overlaps of HN4 and MDA1186 low dose PLX51107 differentially expressed genes were performed using the VennDiagram package in R, and the HN4 uniquely upregulated gene list was subjected to pathway enrichment analysis using the gsea-msigdb online tool (http://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp).

CCLE RNA-seq data were downloaded as raw counts from the DepMap download portal (https://depmap.org/portal/download/). Subtype assignment and downstream analysis were carried out as above.

Whole exome sequencing processing and analysis

Whole exome sequencing (WES) data was processed as previously described and obtained as a MAF file from the authors (Kalu et al., 2017; Gleber-Netto et al., 2019). To cluster the cell lines based on mutation background, all mutation calls were binarized to 1 or 0 to represent "mutated" or "not mutated," respectively. The Jaccard distance matrix was then computed, and the resulting matrix was clustered using Ward's minimum variance method. Total mutational burden was calculated by summing the number of mutations per sample, then grouping the samples based on their assigned molecular subtype. Data for cell line tissue of origin and "source" were obtained as previously described (Zhao et al., 2011).

ChIP-Sequencing processing and analysis

ChIP assays were performed as described previously (Terranova et al., 2018). Briefly, approximately 2×10^7 cells were harvested by scraping. Samples were cross-linked with 1% (wt/vol) formaldehyde for 10 min at 37°C with shaking. After quenching with 150 mM glycine for 5 min at 37°C with shaking, cells were washed twice with ice-cold PBS and frozen at −80°C for further processing. Cross-linked pellets were thawed and lysed on ice for 30 min in ChIP harvest buffer (12 mM Tris-Cl, 1 × PBS, 6 mM EDTA, 0.5% SDS) with protease inhibitors (Sigma). Lysed cells were sonicated with a Bioruptor (Diagenode) to obtain chromatin fragments (~200–500 bp) and centrifuged at 15,000 × g for 15 min to obtain a soluble chromatin fraction. In parallel with cellular lysis and sonication, antibodies (5 µg/3 × 10^6 cells) were coupled with 30 µL of magnetic protein G beads in binding/blocking buffer (PBS +0.1% Tween +0.2% BSA) for 2 h at 4°C with rotation. The antibody used for ChIP was anti-H3K27ac (Abcam; ab4729). Soluble chromatin was diluted five times using ChIP dilution buffer (10 mM Tris-Cl, 140 mM NaCl, 0.1% DOC, 1% Triton X, 1 mM EDTA) with protease inhibitors and added to the antibody-coupled beads with rotation at 4°C overnight. After washing, samples were treated with elution buffer (10 mM Tris-Cl, pH 8.0, 5 mM EDTA, 300 mM NaCl, 0.5% SDS), RNase A, and Proteinase K, and cross-links were reversed overnight at 37°C. Immune complexes were then washed five times with cold RIPA buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% DOC), twice with cold high-salt RIPA buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% DOC), and twice with cold LiCl buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% DOC). ChIP DNA was purified using SPRI beads (Beckman Coulter) and quantified using the Qubit 2000 (Invitrogen) and TapeStation 4150 (Agilent). Libraries for Illumina sequencing were generated following the New England BioLabs (NEB) Next Ultra DNA Library Prep Kit protocol. Amplified ChIP DNA was purified using double-sided SPRI to retain fragments (~200–500 bp) and quantified using the Qubit 2000 and TapeStation 4150 before multiplexing.

Raw fastq reads for all ChIP-seq experiments were processed using a Snakemake based pipeline https://github.com/crazyhottommy/pyflow-ChIPseq. Briefly, raw reads were first processed using FastQC and uniquely mapped reads were aligned to the hg19 reference genome using Bowtie version 1.1.2 (Langmead et al., 2009). Duplicate reads were removed using SAMBLASTER (Faust and Hall, 2014) before compression to bam files. To directly compare ChIP-seq samples, uniquely mapped reads for each mark were downsampled per condition to 15 million, sorted, and indexed using samtools version 1.5 (Li et al., 2009). To visualize ChIP-seq libraries on the IGV genome browser, we used deepTools version 2.4.0 (Ramirez et al., 2016) to generate bigWig files by scaling the bam files to reads per kilobase per million (RPKM) and WiggleTools (Zerbino et al.,

2014) to create average profile plots for each molecular subtype.

Peak overlaps were performed by first generating consensus peak files for each subtype, defined as any peak found in at least 2 samples from the subtype. The resulting 4 peaksets (one per subtype) were then used as input for intervene's (Khan and Mathelier, 2017) upset module to generate upset plots and common/unique peaksets for further analysis. Gene linkage was performed using previously published enhancer-promoter linkage data from Cao et al. (Cao et al., 2017), and the resulting gene list was used as input for pathway enrichment analysis using the gsea-msigdb online tool. Enrichment plots were generated using two definitions of common peaks. The first method uses DiffBind (Stark and Brown, 2020) (https://bioconductor.org/packages/release/bioc/html/DiffBind.html) to define a peakset using any peak found in at least 2 samples, irrespective of subtype. The second uses the peaks found in all subtypes when overlapped using the intervene package mentioned previously. Both resulting peaksets were used as input for ngs.plot (Shen et al., 2014) to generate figures.

Drug response assays

Cell confluence and proliferation were measured using the IncuCyte ZOOM system (Essen Biosciences). For each cell line, seeding density was optimized such that the cells would be in their exponential growth phase for the duration of drug treatment. On day 0, cells were seeded into 96 well plates and left in the incubator overnight. On day 1, media containing either drug (PLX51107 or OTX015) or DMSO was added to the wells. Plates were then left in the IncuCyte ZOOM with treated media for 72 h, at which point cell confluence was measured. Each assay was performed in biological duplicate with technical triplicate wells. Drug response metrics were calculated using GR metrics (Hafner et al., 2016) using cell confluence as a proxy for growth rate, and GR_{AOC} values from each cell line were combined based on their molecular subtype for statistical analysis.

CCLE drug response data were downloaded and processed using the PharmacoGx "auc_recomputed" dataset (Smirnov et al., 2016). Compounds which were missing data for more than 25% of samples were excluded. For the JQ1 analysis, 1 sample was missing data and was imputed using predictive mean matching [as implemented in the mice package (Buuren and Groothuis-Oudshoorn, 2011) (https://www.jstatsoft.org/article/view/v045i03)] on the complete, filtered drug response matrix.

PRO-Seq processing and eRNA analysis

Extraction of nuclei and precision run-on reaction was carried out as described previously (Mahat et al., 2016). Nuclei were isolated from approximately 10 million cells after treating with 12 ml of ice-cold swelling buffer (10 mM Tris-HCl pH 7.5, 2 mM MgCl2, 3 mM CaCl2) for 10 min and scraping out the cells. After spinning at 600 × g for 10 min at 4°C, the supernatant was removed and the cells were lysed in 10 ml of lysis buffer (10 mM Tris-HCl pH 7.5, 2 mM MgCl2, 3 mM CaCl2, 10% glycerol, 0.5% NP-40, 4 U/ml SUPERase inhibitor) on ice for 5 min. The lysate was spun at 600 × g for 8 min and the nuclei were collected. The nuclei were then resuspended in 1 ml of freezing buffer (50 mM Tris-HCl pH 8.0, 5 mM MgCl2, 0.1 mM EDTA, 40% glycerol) and spun at 900 × g for 10 min. For performing precision nuclear run-on reaction, nuclei were resuspended in 100 µL of freezing buffer and added to 100 µL of NRO-reaction mix - NRO-reaction buffer (10 mM Tris-HCl pH 8.0, 5 mM MgCl2, 300 mM KCl), 1 mM DTT, 100 U/ml SUPERase-In, 1% Sarkosyl, 250 M ATP, 250 M GTP, 50 M biotin-11-UTP, 50 M biotin-11-CTP. Reaction was carried out at 29°C for 4 min. RNA was extracted using TRIzol. Base hydrolysis was carried out by heat denaturing briefly at 65°C for 40 s following by cooling on ice and treatment with 1N NaOH for 6 min on ice. The sample with fragmented RNA was neutralized with 1 M Tris-HCl pH 6.8 and isolated by passing through P-30 column (Biorad, #732-6250). The NRO-reaction products containing biotinylated RNA was purified using Streptavidin C1 beads which were washed thrice with wash buffer (0.1 N NaOH, 50 mM NaCl) and twice with 100 mM NaCl. The washed beads were resuspended in binding buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and added to the sample and incubated at room temperature for 30 min on a rotator. After removing the supernatant using a magnetic stand, beads were washed twice with high salt wash buffer (50 mM Tris-HCl pH 7.4, 2 M NaCl, 0.5% Triton X-100), once with low salt wash buffer (10 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.1% Triton X-100) and twice with no salt wash buffer (5 mM Tris-HCl pH 7.4, 0.1% Triton X-100). Beads were then resuspended in TRIzol and RNA was extracted. The bead purification of biotinylated RNA was performed once more, and RNA was extracted using TRIzol to improve the purity of the sample.

Libraries were generated based on previously described protocol (Van Nostrand et al., 2016). Isolated RNA samples were dephosphorylated by FastAP (ThermoFisher) and T4 Polynucleotide kinase (NEB). Samples were cleaned up using MyONE Silane beads and RNA was isolated with RLT buffer (Qiagen). To the eluted RNA, a barcoded RNA adapter (RiL19) was ligated to the 3′ end using T4 RNA ligase (NEB). The 3′ adaptor ligated RNA was again cleaned up as mentioned above. RNA was then reverse transcribed with AR17 primer and AffinityScript reverse transcriptase (Agilent). cDNA was then cleaned up by treating with ExoSAP-IT (Affymetrix) to remove excess oligonucleotides. Excess RNA was removed from cDNA by treating with 1 M NaOH at 70°C for 12 min and neutralizing with 1 M HCl. cDNA was then cleaned up with MyONE Silane

beads and RLT buffer and eluted in 5 mM Tris-Cl, pH 7.5. A second 5′adaptor (rand3Tr3) was ligated to cDNA with T4 RNA ligase in an overnight reaction at room temperature. The adaptor ligated cDNA was then cleaned up with MyONE Silane beads and RLT buffer and eluted in 10 mM Tris-Cl, pH 7.5. cDNA samples were then PCR amplified using NEBNext® Ultra™ II Q5® Master Mix multiplexing was done with D50X and D70X primers. Libraries were size selected and purified using SPRI beads. Final libraries were quantified using D1000 tapestation and Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) and sequenced using NovaSeq6000 with 100 nt paired-end format.

Fastq files from precision nuclear run-on sequencing (PRO-seq) experiments were processed using the previously described PEPPRO pipeline (Smith et al., 2021). Briefly, fastq files first undergo pre-processing steps of adapter removal, read deduplication, read trimming, and reverse complementation. The resulting files are then "pre-aligned" to the human rDNA genome to siphon off these unwanted reads. The rDNA-removed files are then aligned to the human hg19 genome using bowtie2 (Langmead and Salzberg, 2012). After quality control assessment, 5 samples from the AT subtype (representing the 3 unique cell lines used in the drug response assays) and 3 samples from the CL subtype (representing the 2 unique cell lines used in the drug response assays) were carried forward for further analysis. The aligned, sorted bam files for these samples were used as input for downstream analysis using the previously described NRSA downstream analysis pipeline (Wang et al., 2018). In brief, NRSA uses bidirectional transcription in intergenic regions to identify and call enhancers/eRNAs. A raw counts table for these eRNAs is then generated and fed into the DESeq2 tool for differential expression analysis. Identified enhancers are assigned to their nearest genes to generate a list of genes with upregulated eRNA expression in the AT subtype, which was then used as input for pathway enrichment analysis.

TCGA RPKM expression levels of numerous eRNAs from typical enhancers were downloaded from publicly available datasets (https://bioinformatics.mdanderson.org/Supplements/Super_Enhancer/TCEA_website/parts/3_eRNA_quantification.html) based on previously published work (Chen et al., 2018; Chen and Liang, 2020). TCGA mRNA-seq for subtype assignment was downloaded from FireBrowse (http://firebrowse.org/). TCGA samples were assigned to molecular subtypes by first generating templates based on previously assigned molecular subtypes from the initial HNSCC TCGA cohort (Cancer Genome Atlas, 2015). These assigned subtypes were then expanded to the current cohort of samples by using the CMSCaller functionality described above. As before, samples were only retained for further analysis if they possessed an assignment FDR <0.1. Samples were then grouped into "Atypical" or "Other" based on their molecular subtype, and significant differential expression of eRNAs was determined by > 1.5 fold-change in expression and FDR <0.05. As with the PRO-seq data, these eRNAs were linked to their nearest gene using bedtools *via* the bedr R package (Quinlan and Hall, 2010), and the resulting gene list was used as input for pathway enrichment analysis. Intersections of the TCGA eRNA enriched pathways and PRO-seq eRNA enriched pathways were performed using the Venn Diagram package in R and significance was calculated using the hypergeometric overlap method, with a Universe size set to the number of unique pathways in a particular gene set. Only pathways with a p.adjust <0.25 with a maximum of 20 enriched pathways per gene set were included in the analysis.

HiChIP protocol, processing, and analysis

HiChIP was performed as described (Mumbach et al., 2016). Briefly, 1×10^7 cells for each HNSCC cell line (1 unique cell line per HNSCC subtype) were crosslinked. *In situ* contacts were generated in isolated and pelleted nuclei by DNA digestion with MboI restriction enzyme, followed by biotinylation of digested DNA fragments with biotin–dATP, dCTP, dGTP, and dTTP. DNA was then sheared with Bioruptor (Diagenode); chromatin immunoprecipitation was done for H3K27Ac with use of anti-H3K27ac antibody. After reverse-crosslinking, 150 ng of eluted DNA was taken for biotin capture with Streptavidin C1 beads followed by transposition with Tn5. In addition, transposed DNA was used for library preparation with Nextera Ad1_noMX, Nextera Ad2.X primers, and Phusion HF 2XPCR master mix. The following PCR program was performed: 72°C for 5 min, 98°C for 1 min, then 11 cycles at 98°C for 15 s, 63°C for 30 s, and 72°C for 1 min. Afterward, libraries were two-sided size selected with AMPure XP beads. Libraries were paired-end sequenced with reading lengths of 76 nucleotides.

Using HiC-Pro (Servant et al., 2015), HiChIP paired-end reads were aligned to the hg19 genome with duplicate reads removed, assigned to MboI restriction fragments, and filtered for valid interactions. Interaction matrices were then generated with the same software. To generate anchor points for downstream looping analysis, outputs from HiC-Pro were used as inputs for peak calling in HiChIP-Peaks (Shi et al., 2020). To ensure loops were called from similar background enhancers, peaks from HiChIP-Peaks were concatenated into a single file and used as anchor point inputs for loop calling via hichipper (Lareau and Aryee, 2018). HiChIP loop visualization was performed using DNAlandscapeR (https://molpath.shinyapps.io/dnalandscaper/).

Statistical analysis

Statistical analyses, including generation of graphs and plots, were performed using R versions 3.4.4 and 3.6.0. Significance levels are * = $p < 0.05$, ** = $p < 0.01$, and ***$p < 0.005$ unless otherwise indicated in figure legends.

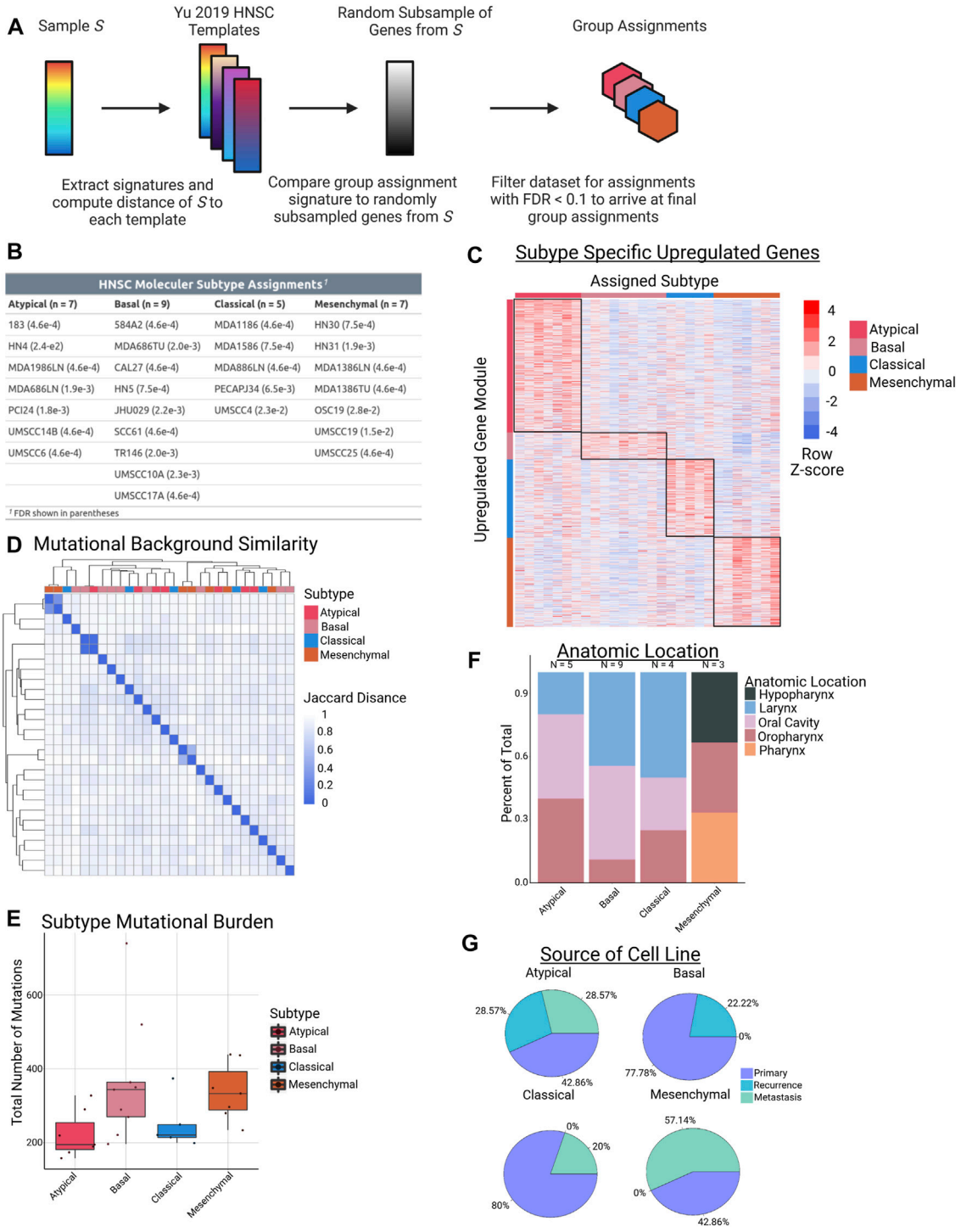

FIGURE 1
Cell line subtype assignments and characteristics. **(A)** Schematic of workflow used to assign HNSCC cell lines to their respective subtypes using RNA-seq data. **(B)** Table of subtype assignments for each of the 28 cell lines used in this study. **(C)** Heatmap of gene expression modules in each molecular subtype, defined as FC > 3 in a one-vs-rest comparison. **(D)** Hierarchical clustering of the 28 cell lines based on Jaccard distance metrics obtained from binarized mutation counts from WES data. **(E)** Boxplots demonstrating total number of mutations in each sample, grouped by molecular subtype (p = NS for each comparison). **(F)** Stacked barplot showing distribution of cell line anatomic location for each molecular subtype. **(G)** Pie chart showing percentage of samples in each molecular subtype that came from primary, recurrent, or metastatic lesions.

Statistical tests utilized are as indicated in respective text and figure legends.

Results

Head and neck squamous cell carcinoma cell lines assigned to known molecular subtypes have similar mutation profiles and tissue origins

Identifying inter-tumor heterogeneity can help better understand the diversity of biological mechanisms driving the neoplastic phenotypes within pathology-based tumor types (e.g., breast cancer, colon cancer, etc.) and discover targeted therapies for specific patient populations (Guinney et al., 2015; Fragomeni et al., 2018; Collisson et al., 2019; Vasaikar et al., 2019). We sought to define heterogeneity within HNSCC patients, especially at the epigenetic level. To this end, we first leveraged published work by Yu et al., which extensively studied CCLE cancer cell lines and their appropriateness as models of human cancer by comparing them to corresponding TCGA tumors (Yu et al., 2019). As part of this work, the group generated "templates" of gene expression values for numerous subtypes in 9 different tumor types. Using the HNSCC templates from this study (one per molecular subtype) and RNA-seq data from the panel of cell lines available to us (Supplementary Table S1), we utilized the nearest template prediction method, as implemented in the CMScaller R package, to assign our cell lines to their most representative molecular subtype (Figure 1A) (Hoshida, 2010; Eide et al., 2017). After selecting only samples with an assignment FDR <0.1, twenty-eight HPV-negative HNSCC cell lines were successfully matched to a molecular subtype, resulting in 7 AT samples, 9 BA samples, 5 CL samples, and 7 MS samples (Figure 1B). Analysis of RNA-seq data in one-versus-rest comparisons demonstrated varying levels of differential expression based on subtype, with a large number of upregulated genes (FC > 3, n = 756) in the AT subtype (Figure 1C).

To further investigate a potential genomic basis that could be driving the transcriptomic partitioning into molecular subtypes, we investigated WES data on our panel of cell lines. We first clustered our lines based on their mutational background, and, interestingly, we did not observe any clustering of samples from the same molecular subtype. In fact, the only examples of tight clusters in the data came from 3 matched pairs of cell lines in which the samples were either from a primary or metastatic lesion of the same patient (Figure 1D). Similarly, we did not observe any significant differences between subtypes based on total mutational burden (Figure 1E). This finding is largely consistent with HPV-negative TCGA data, with the singular exception of the AT vs. MS comparison showing a significant difference in mutation number in TCGA (p.adj = 0.031) (Supplementary Figure S1) (Cancer Genome Atlas, 2015). Importantly, observed clustering was neither associated with the anatomic site of origin of the primary tumor from which each the cell lines were derived, nor with the type of tumor the sample was from (e.g., primary vs. recurrence). With the possible exception of the MS subtype, all of the HNSCC subtypes had a fairly equal distribution of samples from the oral cavity, oropharynx, and larynx (Figure 1F). We note this varies from the findings in the TCGA data, where subtype was correlated with anatomic location (Cancer Genome Atlas, 2015). With respect to cell line source, the AT subtype was the only one to contain cell lines from all 3 groups of samples (primary, recurrence, and metastasis), while BA contained only primary and recurrence, and CL and MS contained only primary and metastasis (Figure 1G). Taken together, these results demonstrate that HNSCC molecular subtypes can be successfully assigned to cell lines using RNA-seq data, and that despite their transcriptomic differences, the unique HNSCC subtypes do not have significantly different mutational backgrounds, overall mutation burden, or tissues of origin from one another in our cell line models.

Head and neck squamous cell carcinoma molecular subtypes are associated with distinct enhancer landscapes

Transcription of a gene is regulated by concerted action of multiple complexes on specific epigenetic elements located in *cis* or *trans* the gene promoter. Enhancers are a major component of the gene regulation circuits and known to be deregulated in cancers (Lee and Young, 2013; Herz et al., 2014). They act as binding platforms for transcription factors that, upon various environmental cues relayed by the cell surface signaling pathways, cooperate with chromatin modifying and remodeling machinery to activate target genes (Lee and Young, 2013). We hypothesized that these transcriptional subtypes could have underlying differences in gene regulatory landscapes that could partly explain the observed transcriptomic differences. To investigate these differences, we generated enhancer profiles for each cell line by performing ChIP-seq for the H3K27ac histone mark, which is widely used as a marker of active enhancers (Creyghton et al., 2010; Rada-Iglesias et al., 2011). We next generated consensus peak sets for each subtype by overlapping the enhancer regions of all cell lines within a subtype and taking the set of enhancers that occurred in 2 or more samples of the subtype. This resulted in 4 total consensus peak sets, each representing a unique subtype (i.e., one consensus peak set per subtype). We observed distinct enhancer peak enrichment among the four molecular subtypes (Figure 2A) (Lex et al., 2014; Khan and Mathelier, 2017). Notably, we discovered the AT subtype has a much larger set of consensus enhancers than any of the other subtypes, and the most common

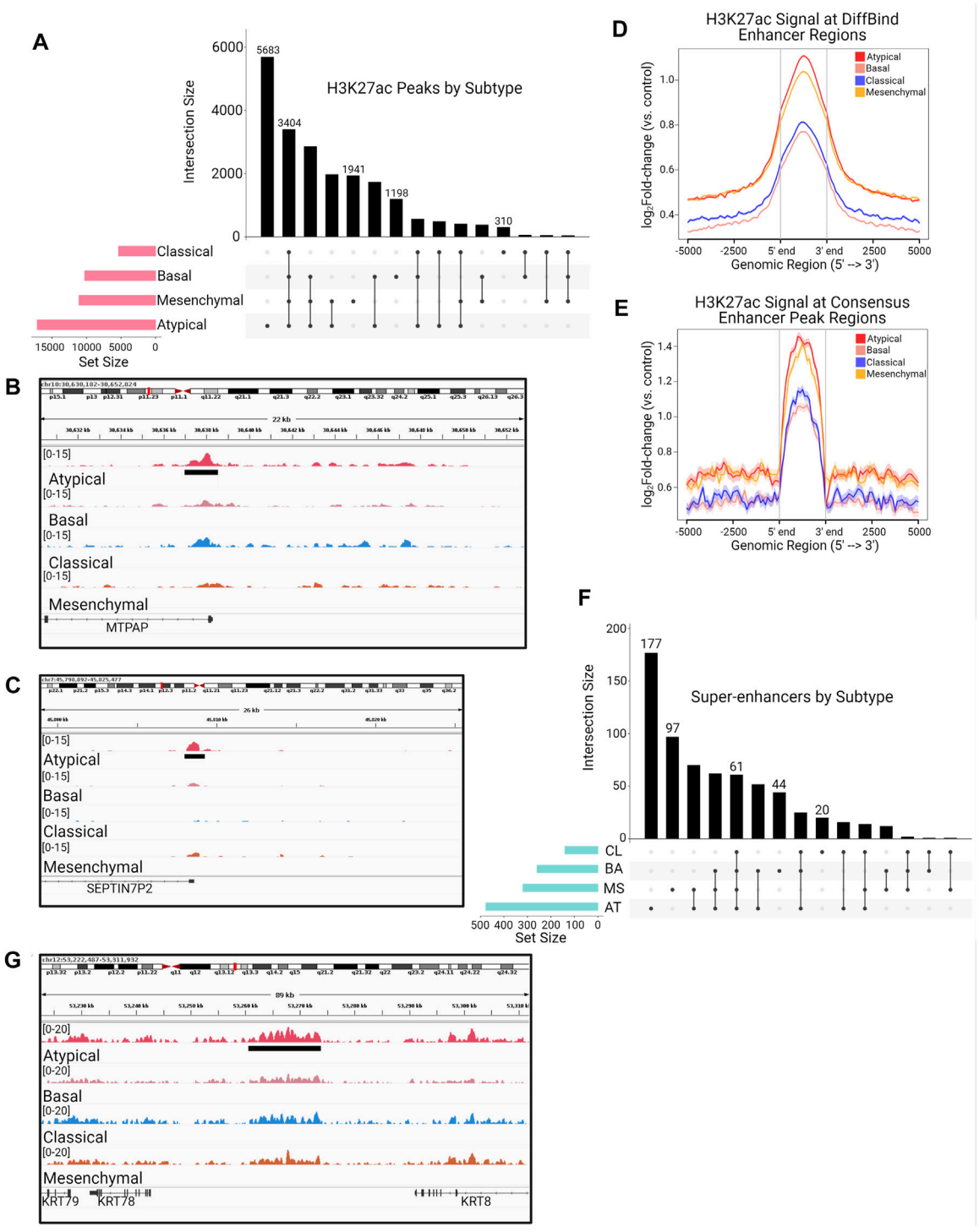

FIGURE 2
The Atypical subtype is associated with unique enhancer peaks regulating genes related to lipid metabolism and MAPK signaling. **(A)** UpSet plot showing the total number of H3K27ac typical enhancer peaks in each molecular subtype (pink horizontal barplot), as well as the number of peaks in each possible intersection of peaksets (black bars and dot plot). **(B,C)** Visualization of mean bigWig signal for each subtype at **(B)** MAP3K8 and **(C)** IGFBP3 enhancer loci containing H3K27ac peaks unique to the AT subtype (green bar/grey shading). **(D,E)** H3K27ac ChIP-seq enrichment plots of enhancer loci common to all HNSCC molecular subtypes, defined as **(D)** any peak contained within 2 or more individual samples or **(E)** the 3,404 peaks shared among all consensus peaksets in **(A)**, demonstrating the strongest signal in the AT subtype. **(F)** UpSet plot showing the total number of super enhancer peaks in each molecular subtype (blue horizontal barplot), as well as the number of super enhancer peaks in each possible intersection of peaksets (black bars and dot plot). **(G)** Visualization of mean bigWig signal for each subtype at a MAP3K12 super enhancer containing peaks unique to the AT subtype (green bar/grey shading).

subset of enhancers in our analysis is the set that is unique to the AT subtype (Figure 2A). To investigate the function of the 5,683 enhancers unique to the AT subtype, we utilized data from Cao et al. (Cao et al., 2017), which constructed enhancer-target networks across multiple cancer and sample types, to assign each of these enhancers to their target genes (Figures 2B,C). These genes were then used for pathway enrichment analysis, which revealed enrichment for pathways involved in lipid metabolism, MYC signaling, and MAPK signaling (Figures 2B,C, Supplementary Table S2).

In addition to identifying unique enhancers, we investigated the total H3K27ac signal enrichment across enhancers shared by all 4 subtypes to determine if, in addition to the largest number of H3K27ac peaks, the AT subtype also had greater signal enrichment overall. Indeed, using two separate methods to arrive at a "shared" H3K27ac peak set, we observed that the AT subtype had more enrichment of H3K27ac signal across enhancers shared among all HNSCC subtypes (Figures 2D,E). In agreement with our typical enhancer analysis, we found that the AT subtype also harbored the largest number of called super-enhancers (Figures 2F,G). Further, linking of these super-enhancers to their gene targets not only displayed enrichment for MAPK signaling and lipid metabolism, but also identified enrichment for PI3K and WNT-β-catenin signaling (Supplementary Table S3). These results demonstrate that the AT subtype of HNSCC is enriched for H3K27ac-marked typical enhancers and super-enhancers compared to other HNSCC, and these enhancer regions may activate important cell signaling pathways that are associated with aggressive HNSCCs.

The Atypical subtype is more resistant to bromodomain inhibition

Recently, the realm of "epigenetic" therapies for cancer has been of major interest for both research and in clinical applications (Castilho et al., 2017; Cheng et al., 2019; Bates, 2020). Targeting epigenetic modifications and the proteins that regulate their placement and/or removal is a particularly attractive approach to cancer therapeutics since these modifications are generally considered to be reversible, particularly when compared to more "permanent" changes such as mutations and copy number alterations. One class of compounds with numerous clinical trials for a variety tumor types is BRD and extraterminal domain (BET) inhibitors, which function by inhibiting the "reader" proteins responsible for recognizing and propagating the signal of acetylated histone residues. These inhibitors have been used in prior studies as enhancer-blocking agents, and the pathways we found to be activated by AT-specific enhancers and super-enhancers (e.g., MAPK and PI3K signaling) are well-characterized mechanisms of BET inhibitor resistance (Supplementary Tables S2, S3) (Rathert et al., 2015; Kurimchak et al., 2016; Iniguez et al., 2018; Cochran et al., 2019; Loganathan et al., 2019; Tonini et al., 2020; Yan et al., 2020). Hence, we hypothesized that the AT subtype may be differentially responsive to BET inhibition.

We first investigated HNSCC cell line response to JQ1 using the publicly available CCLE drug response data (Basu et al., 2013; Seashore-Ludlow et al., 2015; Rees et al., 2016). We used the available CCLE RNA-seq data to assign samples to their respective HNSCC molecular subtype, then compared their response to BET inhibition in this dataset. Interestingly, we found that the AT samples have a lower JQ1 AOC (Area Over the Curve, where lower values indicate resistance) than those in the non-AT group (p = 0.0503, Welch's t-test) (Figure 3A, Supplementary Figure S2A), indicating the AT subtype is more resistant to JQ1 treatment. To extend this analysis, we selected 2 compounds currently being evaluated in clinical trials, OTX015 (Birabresib) and PLX51107, and performed drug response assays in our HNSCC cell lines (Bates, 2020). For each compound, we selected representative cell lines for each molecular subtype (3 AT, 3 BA, 2 CL, and 3 MS), treated with the respective compound for 72 h, then computed the GR_{AOC} of each cell line using cell confluence as a proxy for cell number. We elected to use the GR_{AOC} metric for drug response since GR metrics have been demonstrated to be more reproducible than traditional metrics, such as Area Under the Curve (AUC) and IC_{50}, when measuring drug sensitivity in cancer cell lines (Hafner et al., 2016). As we anticipated based on our previous analysis, the BET inhibitor PLX51107 demonstrated significantly lower GR_{AOC} values in the AT subtype compared to any other HNSCC subtype, indicating an increased resistance to treatment in that group (Figure 3B). The inhibitor OTX015 also displayed a similar trend towards increased resistance in the AT subtype that was similar to, but more pronounced than, the JQ1 response data in the CCLE database (Supplementary Figures S2A,B). Given that BRD proteins are responsible for mediating gene transcription and are the main targets of BET inhibitors, we investigated the expression of this family of proteins both in our cell line panel and in the HNSCC TCGA data (Rathert et al., 2015; Kurimchak et al., 2016; Iniguez et al., 2018; Cheng et al., 2019; Cochran et al., 2019). This analysis demonstrated a significant elevation in BRD7 in the AT subtype compared to other subtypes in our HNSCC cell lines (Supplementary Figures S2C,D), and a significant elevation in BRD4 expression in AT vs. BA and AT vs. MS comparisons in the HNSCC TCGA data (Supplementary Figures S2E,F).

To better understand the mechanisms behind this observed resistance to treatment, we selected one representative cell line from the AT subtype (HN4) and

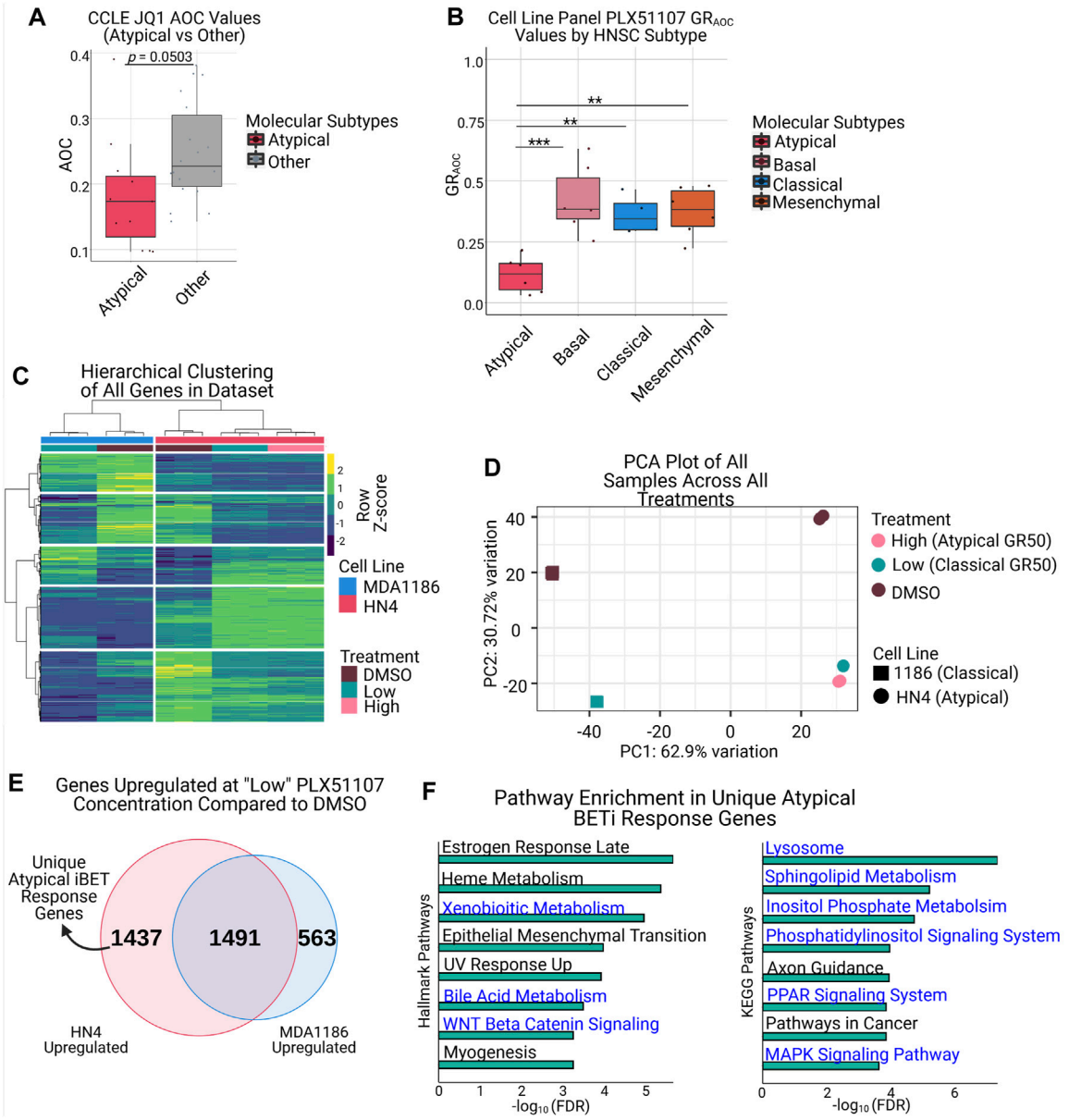

FIGURE 3
Atypical HNSCC shows increased resistance to BET inhibition and uniquely upregulates genes associated with resistance pathways upon treatment. **(A)** Atypical samples in the HNSCC CCLE dataset demonstrate lower JQ1 AOC values than non-Atypical samples (p = 0.0503, Welch's t-test). **(B)** Drug response assays with the BET inhibitor PLX51107 demonstrate the Atypical subtype is significantly more resistant to BET inhibition than other molecular subtypes (*** $adj.p < 0.001$, ** $adj.p < 0.01$). **(C)** Hierarchical clustering of all genes from HN4 and MDA1186 samples treated with DMSO, PLX51107 at GR_{50} MDA1186 (low), or GR_{50} HN4 (high). **(D)** PCA plot of samples as described in **(C)**, displaying separation on the basis of cell line (PC1) and treatment status (PC2). **(E)** Overlap of genes upregulated ($|\log_2$fold-change$| > 1.5$ & FDR <0.05) in HN4 and MDA1186 at PLX51107 low concentration; numbers in Venn diagram represent size of set. **(F)** Horizontal barplots of Hallmark (left) and KEGG (right) pathway enrichment results from the 1,437 genes uniquely upregulated by HN4 in **(E)**; pathways highlighted in blue are associated with known mechanisms of BET inhibitor resistance.

one cell line from the non-AT subtypes (MDA1186, CL subtype), treated with PLX51107 or DMSO, and performed gene expression analysis using mRNA-seq profiling in each condition. MDA1186 was treated with PLX51107 at its own GR_{50} value (hereafter referred to as "low" concentration), and HN4 was treated at its own GR_{50} value (hereafter referred to as "high"), as well as the low concentration. To ensure PLX51107 behaved similarly to other published BET

inhibitors, we created BET inhibitor response signatures using publicly available data and, using GSEA, confirmed that the response of the AT and non-AT cell lines to PLX51107 was consistent with previously documented BET inhibitor response signatures (Supplementary Figures S3C,D) (Puissant et al., 2013; Picaud et al., 2016). Hierarchical clustering of all genes in the RNA-seq dataset demonstrated 2 major clusters, one per cell line, as well as 2 sub-clusters, either DMSO or PLX51107-treated, per major cluster (Figure 3C). To further examine the differences in response to drug treatment, we performed a principal component analysis (PCA), which revealed a first component driven by the cell line identity, and a second component driven by treatment status (Figure 3D). Examining the results from the hierarchical clustering and PCA analysis together, we noted that the majority of transcriptional response to BET inhibition in the AT cell line occurs at the lower drug concentration, and only a minority of gene expression changes occur between the low and high concentrations (Figures 3C,D). Closer inspection of the gene expression heatmap indicates that the genes that are specifically upregulated in the AT subtype after PLX51107 treatment, but not in the non-AT subtypes, may be responsible for mediating resistance to BET inhibition (Figure 3C).

To investigate these uniquely upregulated genes, we overlapped the set of genes upregulated by the AT subtype and by the non-AT subtype at the low PLX51107 concentration. As we expected, we discovered a set of 1437 genes uniquely upregulated in the AT subtype after BET inhibition (Figures 3C,E). Pathway analysis of these 1437 genes using the Hallmarks and KEGG gene sets reveals enrichment for multiple pathways previously demonstrated to convey resistance to BET inhibition and identified by our previous enhancer-based analysis, including MAPK signaling, WNT-β-catenin signaling, phosphatidylinositol signaling, and lipid metabolism pathways (Figure 3F) (Rathert et al., 2015; Kurimchak et al., 2016; Iniguez et al., 2018; Cochran et al., 2019; Loganathan et al., 2019; Tonini et al., 2020; Yan et al., 2020).

These results support our previous hypothesis that the AT subtype is more resistant to BET inhibition than other HNSCC subtypes and suggest the enrichment of H3K27ac-marked enhancers involved in these pathways is a contributing factor to the observed resistance.

Bromodomain inhibitor resistance is mediated by baseline enhancer activity and chromatin structure

Our previous analyses have indicated that the AT subtype of HNSCC has two intriguing properties with respect to BET inhibition: first, H3K27ac-marked enhancers unique to the AT subtype regulate genes enriched for known BET inhibitor resistance pathways, and, second, the AT subtype is able to uniquely upregulate genes enriched for BET inhibitor resistance pathways after treatment with BET inhibitor (Figures 2B,C,G; Supplementary Tables S2, S3; Figures 3E,F). Because of these observations, we suspected the AT subtype may have a stronger baseline enhancer activity at genes involved in resistance pathways and that these genes may have higher numbers of enhancers-promoter contacts, enabling a more robust response to BET inhibitor treatment.

To investigate the activity of enhancers involved in regulating baseline resistance gene expression, we performed PRO-seq to investigate the eRNA landscape of the AT and non-AT subtypes (Supplementary Table S4) (Mahat et al., 2016). Enhancer RNAs are a recently discovered class of non-coding transcripts found at active enhancers that arise from the transcription of enhancer elements themselves and are involved in functions such as regulating gene transcription and controlling enhancer-promoter looping (Arnold et al., 2019; Zhang et al., 2019; Sartorelli and Lauberth, 2020). We used PRO-seq, with a particular focus on eRNAs, to investigate differential enhancer activity in our AT subtype. For this experiment, we expanded our AT group to include the 3 cell lines from our drug assay, and we expanded the non-AT group to include the 2 cell lines from the CL subtype used in our drug assay. Differential expression analysis of PRO-seq-defined eRNAs revealed 321 differentially expressed eRNAs, with 207 being upregulated and 114 being downregulated (Figure 4A).

To assess the likely functional output of these eRNAs, we assigned each eRNA to its nearest gene and performed pathway enrichment analysis, which demonstrated an enrichment in multiple metabolic pathways, including lipid metabolism and cholesterol homeostasis, and hedgehog signaling (Figure 4B). These findings are largely in agreement with our previous enhancer-based analysis of H3K27ac-linked genes, which displayed enrichment for similar BET inhibitor resistance associated pathways (Supplementary Table S2). To extend this finding to human tumors, we leveraged data from recent publications investigating eRNA expression in TCGA tumors (Cheng et al., 2019; Chen and Liang, 2020). After assigning all the HNSCC TCGA tumor samples to their molecular subtype, we examined eRNA expression in the AT subtype compared to non-AT samples and found the AT subtype upregulated 1,867 eRNAs. After linking these eRNAs to their nearest gene, we found that, in agreement with our PRO-seq data from HNSCC cell lines, these genes were enriched for cholesterol homeostasis, hedgehog signaling, and MAPK signaling function (Figure 4C). Next, we overlapped both our HNSCC cell line PRO-seq data and the TCGA HNSCC eRNA data with the predicted enhancers for Cao et al. (Cao et al., 2017) and found that 52% (3766/7246) of the cell line

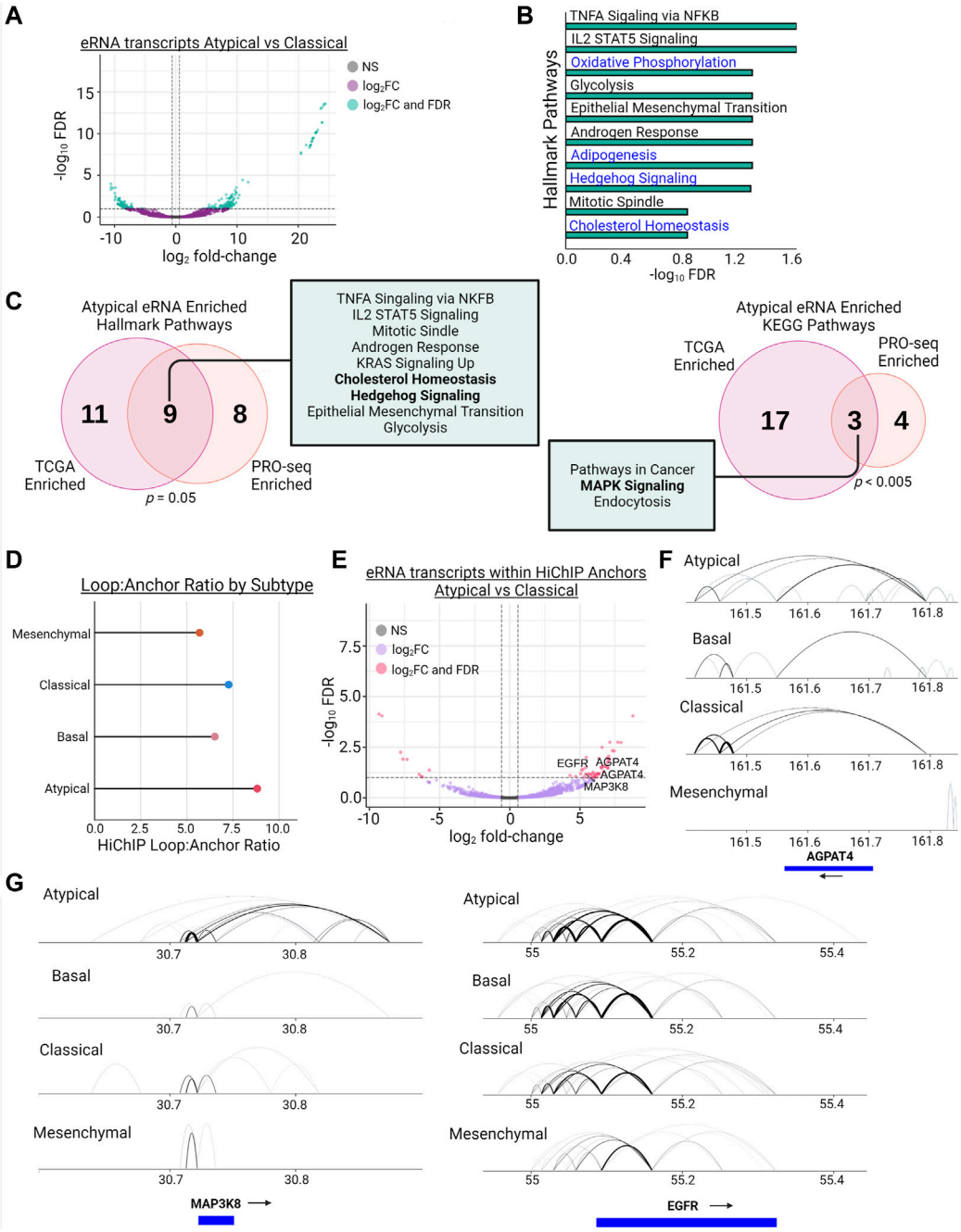

FIGURE 4
Enhancers of MAPK signaling, WNT signaling, and Cholesterol Homeostasis genes display increased eRNA transcription and enhancer-promoter looping in Atypical HNSCC. **(A)** Differential transcription (|log_2fold-change| > 1.5 & FDR <0.1) of eRNAs between the Atypical and Classical subtypes as measured by PRO-seq (green dots meet fold-change and FDR thresholds, purple dots meet fold-change threshold only). **(B)** Hallmark pathway enrichment analysis of genes linked to eRNAs with significantly increased transcription from **(A)**; pathways in blue have been previously associated with BET inhibitor resistance. **(C)** Overlap of hallmark (left) and KEGG (right) pathway enrichment results between PRO-seq-determined significantly enriched eRNAs from **(A)** and **(B)** and TCGA-measured differentially expressed eRNAs between Atypical and non-Atypical samples; *p* values represent hypergeometric tests of gene set enrichment result overlaps; bolded terms represent shared pathways associated with BET inhibitor resistance. **(D)** Lollipop plot demonstrating the loop count:anchor count ratio of H3K27ac HiChIP data for each molecular subtype. **(E)** Volcano plot of differentially transcribed (|log_2fold-change| > 1.5 & FDR <0.1) eRNAs between the Atypical and Classical subtypes after filtering transcripts for only those contained within H3K27ac HiChIP anchors (pink dots meet fold-change and FDR thresholds, purple dots meet fold-change threshold only). **(F)** Joined Hallmark and KEGG pathway enrichment analysis of genes linked to differentially transcribed eRNAs in **(E)**; pathways in blue have been previously associated with BET inhibitor resistance. **(G)** Visualization of H3K27ac HiChIP loops at the MAP3K8 locus (left) and EGFR locus (right) in all 4 HNSCC molecular subtypes.

PRO-seq enhancers and 67% of the TCGA eRNA enhancers (42391/63479) matched a predicted enhancer (Supplementary Figures S4). These results support the hypothesis that the AT subtype has active enhancers, as measured by eRNA expression, enriched for signaling pathways that have been demonstrated to confer resistance to BET inhibition across cancer types.

To assess the enhancer-promoter contacts in our HNSCC cell lines, we performed HiChIP for H3K27ac-marked histones to capture the E-P looping involving this enhancer mark (Supplementary Figure S5) (Mumbach et al., 2016). Consistent with the previous enhancer analyses, we discovered that the AT subtype has the highest ratio of H3K27ac-mediated loops to H3K27ac anchors across all 4 HNSCC subtypes, indicating the AT subtype may have more redundancy in its enhancer architecture than the other subtypes (Figure 4D). To assess if these loops are related to enhancer function, we overlapped our PRO-seq called eRNA enhancer regions with the H3K27ac HiChIP anchor data and performed differential expression analysis of this subset of eRNAs. We identified 48 of 57 differentially transcribed eRNAs as upregulated, and these eRNAs were associated with genes involved in MAPK signaling and lipid metabolism, such as MAP3K8, EGFR, and AGPAT4 (Figure 4E). Inspection of genes identified by this integrative analysis revealed increased contact formation between respective gene promoters and H3K27ac-marked enhancers, supporting the association of eRNA expression with active enhancers and enhancer-promoter loop formation (Figures 4F,G). Further, we compared our HiChIP data for MAP3K8, EGFR, and AGPAT4 to the Cao et al. (Cao et al., 2017) predicted enhancers, which, while demonstrating variable numbers of overlaps depending on the gene queried, maintained the enrichment of enhancer looping in the AT subtype (Supplementary Tables S5, S6). Increasing the loop call stringency by increasing the number of required paired-end tags (PETs) further exaggerated the enrichment of E-P looping in the AT subtype (Supplementary Table S6).

Overall, insights from the eRNA expression and HiChIP data support a model in which the AT subtype has more active enhancers regulating genes associated with lipid metabolism and MAPK signaling, and AT enhancers have, on average, a higher level of redundancy in their control of gene expression than non-AT enhancers by forming larger numbers of enhancer-promoter contacts.

Discussion

Here, we have demonstrated that HNSCC cell line molecular subtypes have largely similar mutational backgrounds, mutational burden, and anatomic sites of origin. In contrast, the enhancer landscapes, marked by histone H3K27 acetylation, are distinct among subtypes. In particular, we discovered the AT subtype has the highest number of enhancers and super-enhancers, as well as the most enhancer signal at common enhancer regions and a global increase in enhancer-promoter loop formation. We also demonstrate that the AT subtype is more resistant to BET inhibition and that, upon treatment with BET inhibitors, the AT subtype is able to uniquely upregulate genes associated with cell growth and BET inhibitor resistance pathways (MAPK signaling, WNT signaling, and lipid metabolism) (Rathert et al., 2015; Kurimchak et al., 2016; Iniguez et al., 2018; Cochran et al., 2019; Loganathan et al., 2019; Tonini et al., 2020; Yan et al., 2020). Further, we demonstrate a significant baseline upregulation of eRNA transcription from the enhancers of genes involved in BET inhibitor resistance pathways such as lipid metabolism and hedgehog signaling in the AT subtype (Cochran et al., 2019). Interestingly, many of these genes with increased eRNA expression in their enhancers were also found to have baseline increased enhancer-promoter looping. Together, our findings suggest that the AT subtype of HNSCC is characterized by high enhancer activity, which likely drives the expression of pathways known to confer resistance to BET inhibition.

Delineation of HNSCC into 4 subtypes was originally proposed by Walter et al. and the TCGA HNSCCC study (Walter et al., 2013; Perez Sayans et al., 2019). These two manuscripts largely focus on genomic alterations, such as copy number alterations and somatic mutations, and only one epigenetic element, in the form of DNA methylation, was assessed in the TCGA paper. As such, limited epigenomic data for HNSCC is available (Serafini et al., 2020). Despite these limitations, interest in therapies that target the epigenome continues to grow, indicating a need for more studies that focus on the epigenome of HNSCC (Alsahafi et al., 2019; Bates, 2020). The work presented here is, to our knowledge, the first to characterize the enhancer landscape of HNSCC based on the Walter/TCGA molecular subtypes. Interestingly, we identified HPV-negative samples belonging to AT subtype, which has traditionally been associated with HPV-positive or “HPV-like” samples, have increased enhancer activity compared to the non-AT subtypes. This activity is measured by increased H3K27ac peak counts, increased H3K27ac signal at common enhancer peaks, global increases in enhancer-promoter looping, and significant upregulation of eRNA expression compared to non-AT samples. This finding suggests that defining features of the AT subtype are enhancer architecture and activity - two key epigenomic aspects of HNSCC subtypes that were not previously explored. Clinical and translational significance of enhancer-based classification was shown by our recent studies in other tumor types like colorectal cancer (Orouji et al., 2021) and MPNST (Kochat et al., 2021).

Unfortunately, BET inhibitors have shown limited promise in clinical studies in solid tumors (Shorstova et al., 2021). However, in specific solid tumor contexts, such as BRD4-NUT midline carcinoma, BET inhibitors have had very encouraging

results in clinical trials (Stathis et al., 2016; Piha-Paul et al., 2020; Shorstova et al., 2021). Considering these findings, identifying subsets of patients with tumor biology favorable or unfavorable to BET inhibitor response could improve the clinical utility of these compounds. Since BET inhibitors inherently rely on modulating the reader protein of H3K27ac-marked enhancers in target cells, understanding enhancer landscapes and their role in BET inhibitor response becomes an important first step in sorting patients into "favorable" or "unfavorable" groups (Stathis and Bertoni, 2018; Cheng et al., 2019). In our work, we discovered that the AT subtype is significantly more resistant to BET inhibition than other HNSCC subtypes, and that this resistance seems, at least in part, mediated by increased enhancer activity on pathways associated with lipid and cholesterol metabolism, MAPK signaling, and WNT-β-catenin signaling. Accordingly, we expect that including compounds that target these pathways in combination with BET inhibitors may sensitize otherwise resistant tumors to BET inhibition and expand the current chemotherapeutic repertoire for HNSCC treatment. As such, other enhancer/transcription blocking inhibitors, such as those against CDK9 (Zhang et al., 2018), could be tested in such enhancer-based subtypes.

We acknowledge that a limitation of our work is the focus on cell line models of HNSCC, which has certain limitations compared to studying human tumors directly. In particular, we noticed differences in BRD expression patterns between our cell line RNA-seq data and the HNSCC TCGA dataset, and the possibility of this being at least partially the result of the sample sources cannot be excluded and warrants further investigation in subsequent studies. However, given the relative sparsity of data in the HNSCC enhancer regulation space, our data can serve as a valuable resource as this field continues to grow. The work presented in this manuscript also serves as an early investigation into the enhancer regulatory landscape of HNSCC using multiple methods that can be technically challenging to perform in human tissue because of the amount of sample required and the associated difficulty of acquiring sufficient numbers of human samples. Moving forward, it will be important to perform similar studies in human tumor samples and animal models to corroborate the findings from our work in an *in vivo* setting.

While our work focused on HPV-negative HNSCC, our findings suggest increased enhancer activity on genes involved in lipid and cholesterol metabolism, MAPK signaling, and WNT-β-catenin signaling may serve as a general mechanism of baseline resistance to BET inhibition. Since enhancer architecture is a critical component of cell identity, it is possible that, moving forward, an assessment of a tumor's baseline enhancer activity could serve as a potential epigenomic biomarker of response to BET inhibition and aid in tailoring treatment in a patient-specific manner (Hnisz et al., 2013; Kron et al., 2014). This could be especially useful in the case of HNSCC, where subtype-specific and tumor-specific treatments are generally lacking (Alsahafi et al., 2019).

Data availability statement

Processed data is available from the Gene Expression Omnibus under repositories GSE185531 (ChIP-seq), GSE185532 (HiChIP), GSE185533 (PRO-seq), and GSE185534 (mRNA-seq). Please note that the raw sequence data cannot be provided due to these being old cell lines without proper consents. Please contact KR for the raw sequence data. Relevant code for the manuscript can be found at: https://gitlab.com/railab/hnscc_subtypes.

Author contributions

SCC conceived, conceptualized and designed the study, planned and carried out experiments, performed data analysis, prepared figures, and wrote the manuscript. VK planned and carried out PRO-seq experiments. ZL planned and carried out ChIP-seq experiments. ATR aided in study design and analysis. MD assisted with manuscript preparation and formatting. JS, CJT, AKG, and MT provided technical help. FMJ, JW, HDS, and CRP contributed to study design and provided reagents and datasets. JNM and KR conceived, conceptualized and designed the study and wrote the manuscript. All authors edited the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.936168/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Boxplots demonstrating total number of mutations in each sample, grouped by molecular subtype (*p.adj* = .031 for AT vs. MS, *p.adj* = NS for all other comparisons).

SUPPLEMENTARY FIGURE S2
(A) CCLE-derived JQ1 AOC values grouped by individual HNSC molecular subtype. **(B)** Response of HNSC cell lines to the BET inhibitor OTX015, reported as GR_{AOC} to adjust for cell line growth rates and grouped by molecular subtype (** *adj.p* < 0.01). **(C)** Box and whisker plot of BRD4 expression in HNSCC cell lines (* = *p.adj* < 0.05, NS = not significant). **(D)** Box and whisker plot of BRD7 expression in HNSCC cell lines (* = *p.adj* < 0.05, ** = *p.adj* < 0.01, and *** *p.adj* < 0.005). **(E)** Box and whisker plots of BRD gene expression in TCGA HNSCC samples. **(F)** Box and whisker plot of BRD4 expression in HNSCC cell lines (* = *p.adj* < 0.05, NS = not significant).

SUPPLEMENTARY FIGURE S3
GSEA analysis of **(A)** MDA1186 and **(B)** HN4 response signatures to 24 h of PLX51107 treatment at their respective GR_{50} values; gene sets for enrichment calculations were generated from previously published BET inhibitor response signatures.

SUPPLEMENTARY FIGURE S4
(A) Venn diagram of overlaps between HNSCC cell line PRO-seq eRNA-based enhancers (orange) and Cao et al. (Cao et al., 2017) predicted enhancers (blue). **(B)** Venn diagram TCGA HNSCC eRNA-based enhancers (magenta), and Cao et al. (Cao et al., 2017) predicted enhancers (blue).

SUPPLEMENTARY FIGURE S5
(A–D) HiC-Pro valid interaction and contact range metrics for HN4, CAL27, MDA1186, and UMSCC25 HNSCC cell lines, respectively. **(E)** Number of intrachromosomal paired-end tags (PETs) and distribution in distance bins per sample as reported by hichipper.

SUPPLEMENTARY TABLE S1
List of cell lines used for H3K27ac ChIP-seq, their total H3K27ac peak counts, and their total uniquely mapped read counts.

SUPPLEMENTARY TABLE S2
List of top 20 enriched Hallmark and KEGG pathways and their respective FDRs from genes lists generated by linking AT-unique typical enhancers to their target genes.

SUPPLEMENTARY TABLE S3
List of top 20 enriched Hallmark and KEGG pathways and their respective FDRs from genes lists generated by linking AT-unique super enhancers (sheet 2) to their target genes.

SUPPLEMENTARY TABLE S4
PRO-seq quality metrics for each HNSCC cell line sample as reported by the PEPPRO pipeline.

SUPPLEMENTARY TABLE S5
Table showing relationship between Cao et al. (Cao et al., 2017) predicted enhancers and HiChIP anchors for AGPAT4, MAP3K8, and EGFR.

SUPPLEMENTARY TABLE S6
Table showing relationship between Cao et al. (Cao et al., 2017) predicted enhancers and HiChIP H3K27ac-based loops. The "threshold" value is the number of required PET interactions used for generating Figures 4F,G (10 for AGPAT4, 5 for MAP3K8 and EGFR).

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Quaking but not parkin is the major tumor suppressor in 6q deleted region in glioblastoma

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Glioblastoma (GBM) is a high-grade, aggressive brain tumor with dismal median survival time of 15 months. Chromosome 6q (Ch6q) is a hotspot of genomic alterations, which is commonly deleted or hyper-methylated in GBM. Two neighboring genes in this region, *QKI* and *PRKN* have been appointed as tumor suppressors in GBM. While a genetically modified mouse model (GEMM) of GBM has been successfully generated with *Qk* deletion in the central nervous system (CNS), *in vivo* genetic evidence supporting the tumor suppressor function of *Prkn* has not been established. In the present study, we generated a mouse model with *Prkn*-null allele and conditional *Trp53* and *Pten* deletions in the neural stem cells (NSCs) and compared the tumorigenicity of this model to our previous GBM model with *Qk* deletion within the same system. We find that *Qk* but not *Prkn* is the potent tumor suppressor in the frequently altered Ch6q region in GBM.

KEYWORDS

GBM, parkin, QKI, glioma, glioblastoma

Introduction

Gliomas are primary tumors that arise from the supporting glial cells or progenitor cells of the brain and the spinal cord (Cohen and Colman, 2015; Lapointe et al., 2018). The most common and deadliest type of glioma is glioblastoma (GBM), which is a highly aggressive primary brain tumor that has been a therapeutic challenge (Ostrom et al., 2015; Lapointe et al., 2018; Louis et al., 2021). The current standard of care for GBM consists of surgical resection followed by radiotherapy and chemotherapy, upon which the current median survival rate after diagnosis remains at about 14 months (Stupp et al., 2009; Tan et al., 2020; Louis et al., 2021). Molecular mechanisms contributing to tumorigenesis and tumor progression in GBM have long been exploited to identify potential targets for targeted therapies. While various genomic alterations have been associated with GBM, a particular genomic locus that has been deregulated in and associated with GBM is chromosome 6q, particularly 6q25-27 (Ichimura et al., 2006; Cancer Genome Atlas Research Network, 2008; Parsons et al., 2008; Veeriah et al.,

2010; Ma et al., 2012; Gao and Smith, 2014). 6q25-27 is a fragile region that is susceptible to instability, evidenced by its highly frequent deletion or methylation in various cancers such as melanoma, colon cancer, gastric cancer, and gliomas (Veeriah et al., 2010; Ma et al., 2012; Gao and Smith, 2014). Moreover, congenital deletion of the 6q27 region leads to a neurological condition named 6q terminal deletion syndrome, which is characterized by mental disability and brain abnormalities (Striano et al., 2006; Backx et al., 2010; Peddibhotla et al., 2015; Bhatta et al., 2020). Besides deleted in over 37% of GBM, chromosome 6q25-27 is also heavily hyper-methylated in ~20% of GBM, strongly suggesting that potential tumor suppressor(s) resides in this locus (Brennan et al., 2013; Chaligne et al., 2021; Chen et al., 2012; Ichimura et al., 2006; Miyakawa et al., 2000; Mulholland et al., 2006; Veeriah et al., 2010; Yin et al., 2009).

Three neighboring genes residing this locus are *PRKN* (PARKIN), *PACRG* (Parkin Coregulated Gene), and *QKI* (QUAKING), and both PRKN and QKI have been shown to be tumor suppressors in GBM (Gilbert, 2002; Brennan et al., 2013; Darbelli and Richard, 2016; de Castro et al., 2021). QKI is a KH-domain single-stranded nucleic acid-binding protein that modulates various cellular pathways through transcriptional and/or post-transcriptional regulation (Chenard and Richard, 2008; Darbelli and Richard, 2016). We have previously demonstrated that depletion of *Qk* (mouse gene encoding Quaking) along with tumor suppressors *Trp53* and *Pten* in neural precursor cells (NSCs) using Nestin-CreER^{T2} system (QPP) led to GBM formation in mice with a penetrance of over 90%, providing a novel and reliable system to study GBM (Shingu et al., 2017). PARKIN is an E3-ubiquitin ligase that has been named upon the discovery that it is mutated in autosomal recessive juvenile Parkinson Disease (ARJP) (Kitada et al., 1998; Lucking et al., 2000). Located in the 6q25-27 chromosomal region, PRKN is commonly lost/deleted in GBM similar as QKI, and PARKIN protein expression was shown to be downregulated during glioma progression (Cesari et al., 2003; Freije et al., 2004; Veeriah et al., 2010; Xu et al., 2014; Lin et al., 2015; de Castro et al., 2021). However, there is a lack of GBM GEMM models with *Prkn* deletion to provide genetic evidence reinforcing the tumor suppressive role of PARKIN in GBM (Chen et al., 2013). In the current study, we sought to compare the tumor suppressive functions of *Prkn* and *Qk* by deleting them on the same background of *Trp53/Pten* double knockout in NSCs using Nestin-Cre-LoxP system (Tronche et al., 1999).

Materials and methods

Mice

Previously we have established Nestin-CreER^{T2} *Pten*$^{L/L}$ *Trp53*$^{L/L}$ (PP) mice and Nestin-CreER^{T2} *Qki*$^{L/L}$ *Pten*$^{L/L}$ *Trp53*$^{L/L}$ (QPP) mice (Shingu et al., 2017). Parkin knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME) (Stock Number: 006582, Strain name: B6.1294-Park2tm1shn/J) (Goldberg et al., 2003). These mice were crossed with PP mice to obtain Nestin-CreER^{T2} *Pten*$^{L/L}$ *Trp53*$^{L/L}$ *Prkn*$^{-/-}$ (PPP) mice. Mice were subcutaneously injected with tamoxifen (200 mg/mouse, postnatal days 7 and 8) to activate Cre-recombinase and induce deletion of *Pten* and *Trp53* in *Nestin*-expressing cells. The mice were housed according to the Association for Assessment and Accreditation of Laboratory Animal Care and NIH standards. The mice were monitored for signs of illness every other day and euthanized and/or harvested when found moribund.

Brain and tumor harvest and sample preparation

Mice were euthanized with the use of anesthetic or carbon dioxide, followed by cervical dislocation. Brains were removed with or without transcardial perfusion using 4% paraformaldehyde (PFA), followed by post-fixation with formalin at room temperature. Serial sections of 5 µm thickness for paraffin sections were used for subsequent staining applications.

Antibodies

Antibodies for immunofluorescence (IF) and immunohistochemistry (IHC) were obtained and used as described in the following paragraph. Anti-GFAP (Z0334, rabbit, 1:1,000 for IHC) from DAKO, Agilent Technologies (Carpinteria, CA), anti-CD31 (77699, rabbit, 1:100 for IHC) from CST, Cell Signaling Technology, anti-Ki67 (ab15580, rabbit, 1:200 for IHC) from Abcam, anti-Iba1 (019-19741 rabbit, 1: 200 for IHC and 1:250 for IF) from Wako Chemicals United States, anti-Olig2 (EMD rabbit, 1: 200 for IHC) from EMD Millipore. Anti-CD8 (ab209775, rabbit, 1:200 for IF) from Abcam, anti-GrB (AF 1865, goat, 1:100 for IF) from R&D Systems, anti-Tmem119 (ab209064, rabbit, 1:200 for IF) from Abcam, and anti-F4/80 (30325T, rabbit, 1:400 for IF) from Cell Signaling Technology.

Immunohistochemistry

Formalin-fixed-paraffin-embedded (FFPE) brain tumor sections were deparaffinized at 60°, rehydrated through triple washes with Xylene, 100% EtOH, 95% EtOH, 70% EtOH, 50% EtOH, and ddH2O. After heat-mediated antigen retrieval in 5% citrate-buffer, 3% hydrogen peroxidase was used to

quench endogenous peroxidase prior to blocking with 3% bovine serum albumin (BSA) and 1% horse serum (HS). Following blocking, the tumor sections were incubated with primary antibodies overnight at 4° or 2 h at room temperature. The sections were then incubated with horseradish peroxidase (HRP)-conjugated polymer (Biocare Medical, Concord, CA) for 45 min and then with diaminobenzidine using the Ultravision DAB Plus Substrate Detection System (Thermo Fischer Scientific, Waltham, MA) for 5 min at room temperature, followed by hematoxylin staining for 1 min. The tumor sections were then washed, dehydrated, and mounted with coverslips. The light microscopy images were taken with Leica DFC295 Bright Field microscope.

Immunofluorescence

FFPE brain sections generated from PP, PPP, or QPP animals 4–6 weeks post tamoxifen injection were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval in 5% citrate buffer. Slides were then blocked with 1% horse serum (HS) and 3% bovine serum albumin (BSA) and incubated with primary antibodies overnight at 4°. The sections were incubated with secondary antibodies coupled to AlexaFluor dyes (488 or 594, Thermo Fischer Scientific) for 1–2 h at room temperature at a 1: 1,000 dilution. Vectashield with DAPI (Vector Laboratories) was used as the mounting medium and cover slips were applied to the stained and mounted sections. The fluorescence images were taken with a Nikon Upright Eclipse Ni-E microscope and cell counting analyses were performed using Fiji/Image J software. Immunofluorescence images were taken from brains harvested from $n = 3$ pairs of mice to be used in quantitative analyses, wherein each data point represents an individual image quantified for antibody-positive cellular signal. Cell numbers per area each represent cell counts in an area of 0.08 mm^2 within the subventricular zone.

Statistical analyses

For survival analyses, pairs of Kaplan-Meier survival curves were compared by the log-rank Mantel-Cox test using GraphPad Prism software. For the cell number count statistical analyses of immunofluorescence images, Image J was used to filter the background staining, enhance, and quantify the cellular signal whereas GraphPad Prism software was used to conduct Two-way ANOVA, testing for differences between the three groups/columns. Differences were considered statistically significant when provided p-value was less than 0.05.

Results

Qk deletion but not *Prkn* deletion leads to GBM development on the backdrop of *Pten* and *Trp53* double knockout

We have previously established Nestin-CreER^{T2} $Qk^{L/L}$ $Pten^{L/L}$ $Trp53^{L/L}$ (QPP) cohort and demonstrated that QPP mice injected with tamoxifen at postnatal day 7 (P7) developed GBM with a penetrance of over 90% and died with a median survival time of ~105 days, whereas Nestin-CreER^{T2} $Pten^{L/L}$ $Trp53^{L/L}$ (PP) cohort did not develop GBM (Shingu et al., 2017). To test whether *Prkn* deletion could also promote GBM development in the backdrop of *Pten/Trp53* double knockout, we crossed *Prkn*-null allele to Nestin-CreER^{T2} $Pten^{L/L}$ $Trp53^{L/L}$ (PP) mice to generate Nestin-CreER^{T2} $Pten^{L/L}$ $Trp53^{L/L}$ $Prkn^{-/-}$ (PPP) cohort (Figure 1A). Contrary to the QPP mice, neither PP mice nor PPP mice injected tamoxifen at P7 developed GBM, although 4/89 (4.5%) PP mice and 1/15 (6.7%) PPP mice did develop lower grade brain tumors (Figure 1B). In line with this, the glioma-free survival rate of the QPP cohort was significantly lower compared to both PP and PPP cohorts (Figure 1C). Together, these data suggest that, unlike *Qk*, *Prkn* is not a major tumor suppressor in GBM. Of note, total survival rate of the PPP cohort appeared lower than that of the PP cohort, suggesting that Parkin may play an important role in tissue homeostasis (Figure 1D).

Early premalignant lesions of the QPP mice demonstrated a tumor-permissive microenvironment compared to those of the PP and PPP mice

The tumor microenvironment (TME) has been studied for its critical role in modulating GBM progression, whereas the role of the premalignant brain microenvironment remained elusive (Quail and Joyce, 2017; Huang et al., 2020). Herein, we identified distinct populations of immune cells in the SVZ (subventricular zone) of our PP, PPP, and QPP mice at 6–8 weeks post-injection, before any microscopic tumors could be detected, and explored potential implications of premalignant immune microenvironment profiles on the differential tumorigenic abilities observed in our models.

Tumor-associated macrophages and microglia (TAMs) represent the majority of the immune population within GBM tumors and have been shown to act as immune-suppressors and facilitators of tumor growth (Zhai et al., 2011; Kennedy et al., 2013; Wei et al., 2020). Therefore, we first stained for microglia/macrophage marker Iba1 and found that Iba1$^+$ cells were concentrated alongside the SVZ region in all samples (Figure 2A). Iba1$^+$ cell numbers were significantly higher in the premalignant SVZ regions of the QPP mice, compared to both PP and PPP brains (Two-way ANOVA, $p < 0.01$) (Figures

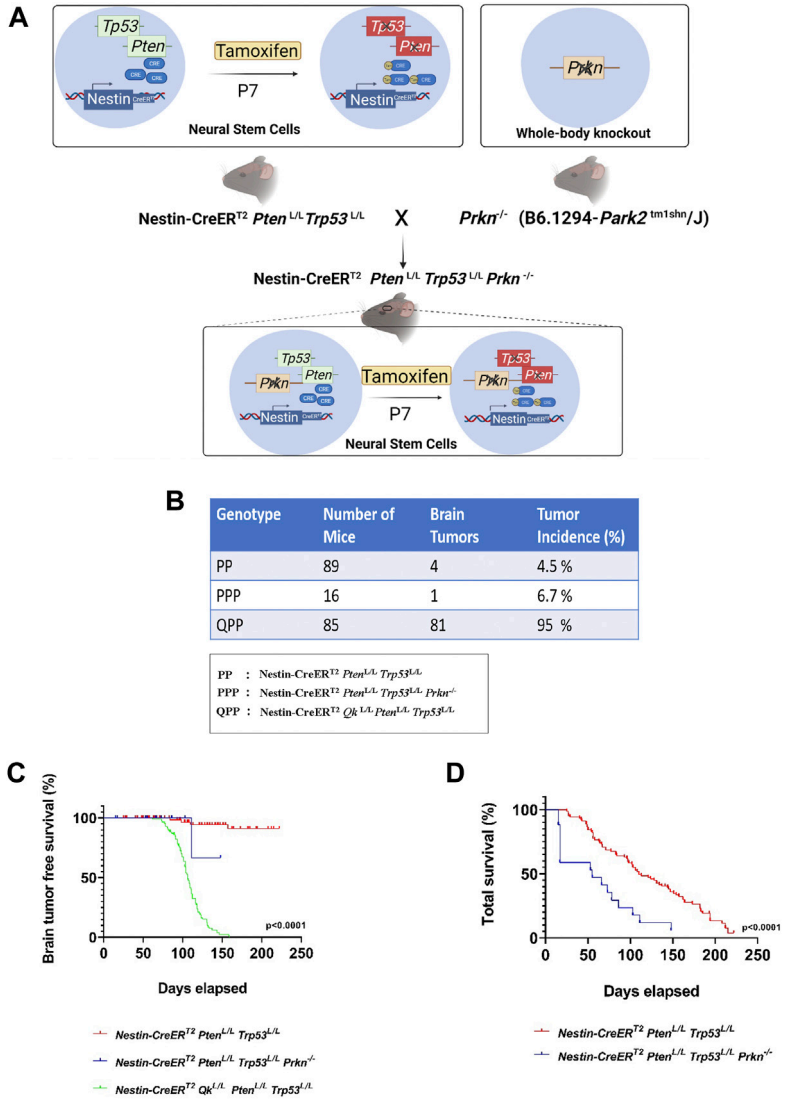

FIGURE 1
Prkn deletion does not lead to GBM development on the backdrop of Pten and Trp53 double knockout. **(A)**. Schematic describing the generation of the PPP genetic model. The illustrations were made using BioRender. **(B)**. The cohort sizes and brain tumor incidences tabulated for PPP, QPP, and PP models. **(C)**. Kaplan-Meier survival curves (long rank test) for PPP, QPP, and PP mice treated with tamoxifen (at P7-P10) demonstrating a significantly ($p < 0.0001$) reduced brain tumor free survival rate for QPP and not for the PP and PPP. **(D)**. Kaplan-Meier survival curves (long rank test) for PPP, and PP mice treated with tamoxifen (at P7-P8) demonstrating a significantly ($p < 0.0001$) reduced total survival rate for the PPP mice compared to the PP mice.

2A,B). Moreover, the PPP SVZ regions also appeared to have significantly higher Iba1$^+$ cell numbers compared to those of the PP mice (Figures 2A,B). Tissue-resident microglia were also assessed with Tmem119 staining, which demonstrated significantly higher coverage in the QPP premalignant SVZ regions, compared to both PP and PPP (Figures 2C,D).

We also compared murine macrophage marker F4/80$^+$ cell numbers in the premalignant SVZ between three models. F4/80$^+$ cell numbers were significantly higher in the QPP model compared to PP and PPP, with notably lower rates of infiltration by the peripheral macrophages in the PPP model (Two-way ANOVA, $p < 0.001$) (Figures 2E,F).

In order to assess the infiltration of peripheral lymphocytes, we co-stained pre-malignant SVZ regions of PP, PPP, and QPP with anti-CD8 and anti-Granzyme B antibodies. We detected overall considerably small numbers of CD8$^+$ T lymphocytes at this stage in the brains (< 5 cells per 0.08 mm^2 area). While the CD8-positive cell numbers appeared to be significantly higher in the SVZ of QPP compared to the PP brains, the *Prkn*-deficient PPP pre-malignant SVZ demonstrated comparable numbers (Figures 2G,H). We did not detect any CD8$^+$ GrB$^+$ double-

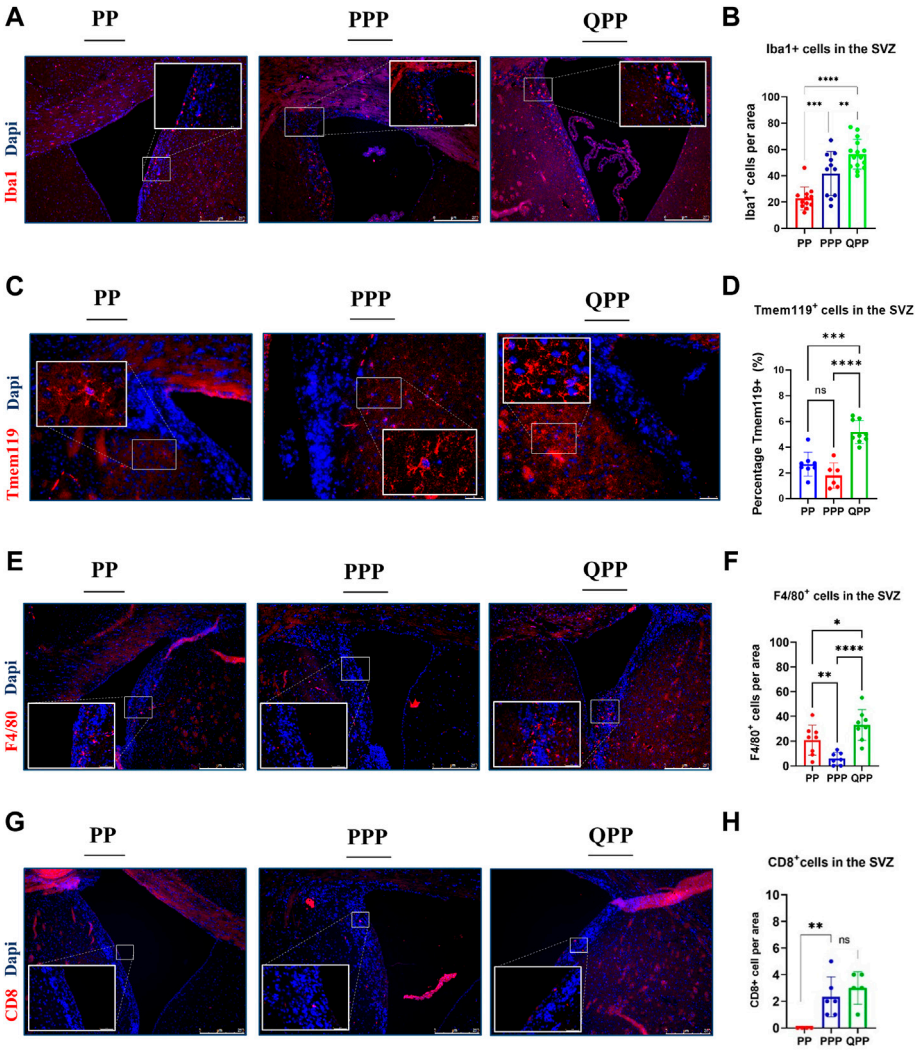

FIGURE 2
Early premalignant lesions of the QPP mice demonstrated a tumor-permissive microenvironment compared to those of the PP and PPP mice **(A)**. Immunofluorescence staining images of IBA1-positive myeloid cells in the SVZ regions of PP, PPP, and QPP brains, respectively. Scale bars represent 250 μm. **(B)**. Quantification and comparison of IBA1-positive myeloid cell numbers between PP, PPP, and QPP premalignant SVZ regions. **(C)**. Immunofluorescence staining images of TMEM119-positive microglia in the SVZ regions of PP, PPP, and QPP brains, respectively. Scale bars represent 50 μm. **(D)**. Quantification and comparison of TMEM119-positive area percentages between PP, PPP, and QPP premalignant SVZ regions. **(E)**. Immunofluorescence staining images of F4/80-positive macrophages in the SVZ regions of PP, PPP, and QPP brains, respectively. Scale bars represent 250 μm. **(F)**. Quantification and comparison of F4/80-positive macrophage numbers between PP, PPP, and QPP premalignant SVZ regions. **(G)**. Immunofluorescence staining images of CD8-positive lymphocytes in the SVZ regions of PP, PPP, and QPP brains, respectively. Scale bars represent 250 μm. **(H)**. Quantification and comparison of CD8-positive lymphocyte numbers between PP, PPP, and QPP premalignant SVZ regions. (Two-way ANOVA, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$).

positive cytotoxic/activated T lymphocytes in any of the pre-malignant samples, in line with the absence of cancerous lesions at this time point. Of note, we also did not detect any $CD4^+$ "helper" T lymphocytes or $CD4^+$ $Foxp3^+$ "regulatory" T-cells in the pre-malignant SVZ regions of our models, accurately representing the low density of these populations in the scRNA-seq analyses of established GBM tumors we recently reported (Zamler et al., 2022).

Together, these findings suggested that the premalignant microenvironment profiles of PP and PPP models appeared to be notably similar to each other when compared to that of the more tumorigenic QPP model. The QPP brain demonstrated an enriched immune suppressive microenvironment prior to tumor formation, characterized by tumor-associated macrophages (TAM), in addition to the potent cell-autonomous tumorigenicity of Qki-deletion detailed in our previous reports.

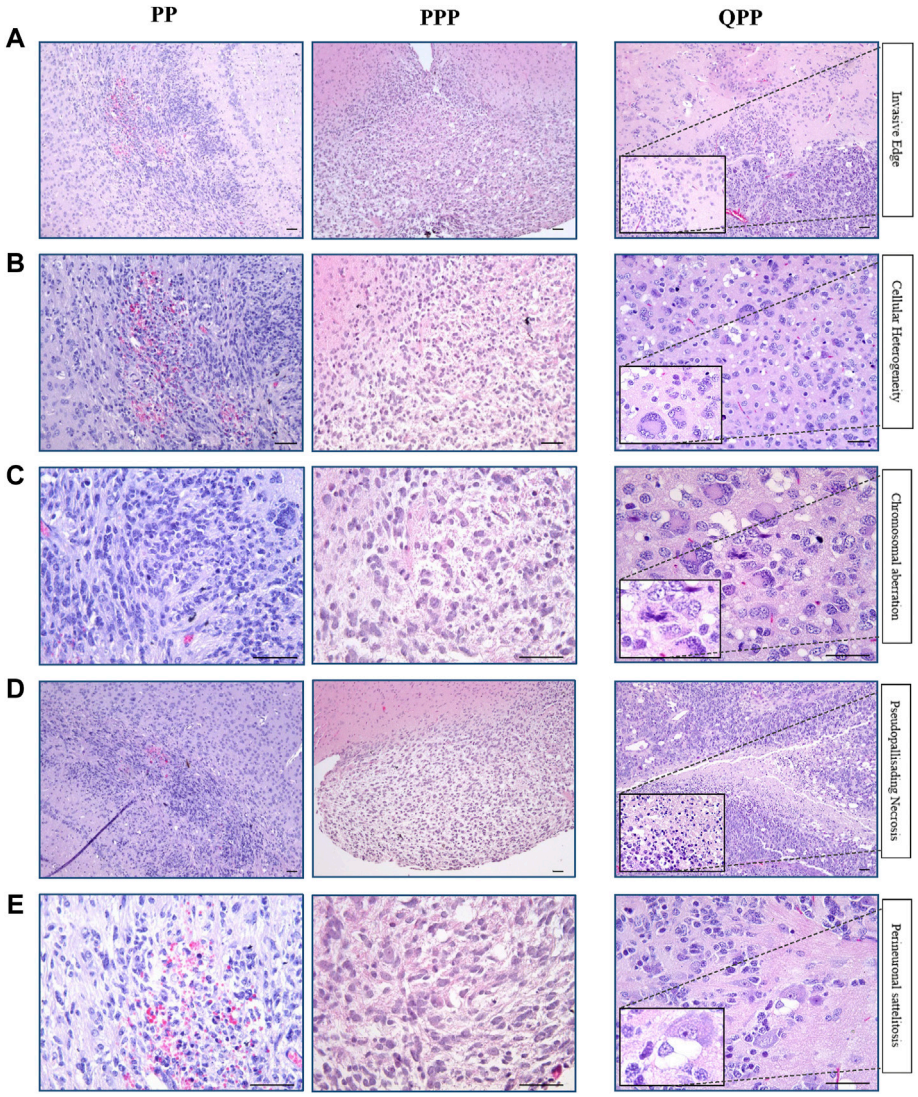

FIGURE 3
Histopathological analyses identified the brain tumor isolated from the PPP cohort as low-grade glioma. **(A)**. Representative H&E images of tumors harvested from QPP, PPP, and PP cohorts demonstrating invasive edges. **(B)**. Representative H&E images of tumors harvested from QPP, PPP, and PP cohorts indicating intra-tumor cellular heterogeneity. **(C)**. H&E images of tumors harvested from QPP, PPP, and PP cohorts representative of chromosomal aberrations. **(D)**. Representative H&E images of tumors harvested from QPP, PPP, and PP cohorts displaying intra-tumor necrosis. **(E)**. Representative H&E images of tumors harvested from QPP, PPP, and PP cohorts exemplifying perineuronal satellitosis. Scale bars represent 50 μm.

Histopathological analyses identified the brain tumor isolated from the PPP cohort as low-grade glioma

As noted above, our PPP cohort has only produced one brain tumor, of which we performed histopathological assessments using H&E staining and immune-histochemistry (IHC). Tumors harvested from our established cohorts PP and QPP were also assessed in comparison, with QPP tumors serving as an established representative for high-grade glioma.

As described in our previous report, QPP tumors exhibit invasive edges, high cellular heterogeneity, frequent chromosomal aberrations, necrosis, and perineuronal satellitosis, all of which suggested that they are high-grade gliomas (grade IV or GBM) (Figures 3A–E) (Shingu et al., 2017). In contrast, the PPP tumor appeared histologically more similar to the low-grade gliomas occasionally isolated from our PP cohort, and lacked the aforementioned characteristics exemplified in the QPP GBM tumors (Figures 3A–D).

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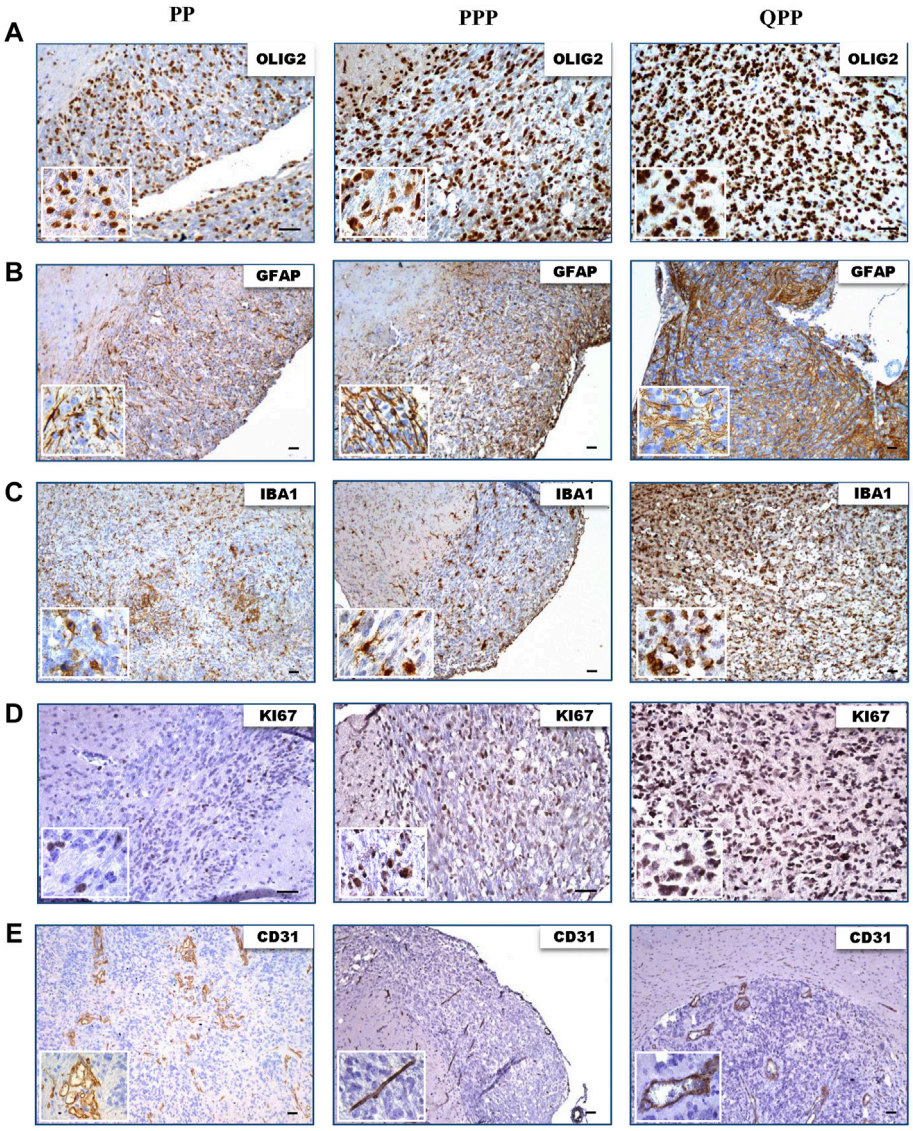

FIGURE 4
Tumors harvested from all three models express elevated levels of glioma biomarkers. **(A)**. IHC images of tumors harvested from QPP, PPP, and PP cohorts demonstrating OLIG2 expression. **(B)**. IHC images of tumors harvested from QPP, PPP, and PP cohorts demonstrating GFAP expression. **(C)**. IHC images of tumors harvested from QPP, PPP, and PP cohorts demonstrating IBA1 expression. **(D)**. IHC images of tumors harvested from QPP, PPP, and PP cohorts demonstrating KI67 expression. **(E)**. IHC images of tumors harvested from QPP, PPP, and PP cohorts demonstrating CD31 expression. Scale bars represent 50 μm.

We next performed IHC staining to assess the protein expression levels of various glioma markers. All three tumors showed high protein levels for oligodendrocyte lineage marker Olig2, astrocyte lineage marker Gfap, and macrophage/microglia marker Iba1 (Figures 4A–C). All tumors demonstrated proliferation and hyper-vascularity as marked by KI67 and CD31 staining, respectively (Figures 4D,E).

In summary, histopathological analyses of tumor sections obtained from the brains of PP, QPP, and PPP mouse models supported the tumorigenicity and brain-tumor-free survival data. QPP tumors demonstrated a trend of increased staining densities for GBM-indicative protein markers such as Olig2, Gfap, and Iba1, while the PP and PPP tumors displayed histological characteristics similar to the lower-grade gliomas. Nonetheless, a statistical analysis remained out of scope for this study as we could obtain fewer than three brain tumors from the PP and PPP cohorts given their extremely low penetrance.

Discussion

Chromosome 6q is a fragile region and a genomic alteration hotspot that has been implicated in both neurological diseases and cancer (Miyakawa et al., 2000; Denison et al., 2003; Ichimura et al., 2006; Striano et al., 2006; Weir et al., 2007; Mitsui et al., 2010; Morris et al., 2010; Ma et al., 2012; Bhatta et al., 2020). 95% of the allelic losses in gliomas were found to be affecting chromosome 6q arm, and the alteration rate appeared to be highest in GBM (37%) (Miyakawa et al., 2000). Two prominent genes in Ch6q 25-27 region, *PRKN*, and *QKI*, have both been lost or downregulated in GBM (Cesari et al., 2003; Brennan et al., 2013; Darbelli and Richard, 2016). In this study, we investigated the tumor suppressor role of *Prkn*, on the backdrop of a previously established GEMM system targeting premalignant (PM) NSCs to deplete major tumor suppressors *Trp53* and *Pten* (Shingu et al., 2017). Nestin-CreER^{T2} *Pten*$^{L/L}$ *Trp53*$^{L/L}$ *Prkn*$^{-/-}$ (PPP) mice injected at P7 did not form GBM tumors, and the brain tumor-free survival rates appeared similar to the PP animals with only *Trp53* and *Pten* deletions in the same system. Similar to what has been observed in the PP model, the PPP model was also inadequate for high rates of brain tumor formation.

The examination of the immune microenvironment bolstered these findings when we compared the SVZ regions of pre-malignant brains. We found that the QPP model inhabited the highest microglia/macrophage levels, as indicated by Tmem119 and Iba1 staining in the SVZ. This observation was followed by other macrophage markers such as F4/80, which demonstrated a sharp difference between the QPP and PPP SVZ regions, alluding to a scenario that the QPP mice had higher rates of infiltration by the peripheral macrophages compared to the PPP mice, well before the tumorigenesis took place. Lymphocyte infiltration appeared to be noticeably weaker compared to the myeloid lineage, as we have not detected any NK cells (NK1.1^{+}) and a very small number of CD8^{+} T cells. These findings demonstrated a clear trend where myeloid immune infiltration into the pre-tumor microenvironment is significantly enriched in QPP mice compared to PPP mice, potentially establishing an environment more susceptible to tumorigenesis.

QKI has long been associated with neurological diseases and cancers, modulating various pathways through both transcriptional and post-transcriptional regulation (Ebersole et al., 1996; Feng and Bankston, 2010; Darbelli and Richard, 2016). Previous TCGA analyses have appointed *QKI* as the common gene shared among the 6q26 chromosome alterations in GBM, alluding to its dominance as the tumor suppressor effector housed in this region (Brennan et al., 2013). Our QPP model demonstrated that loss of Qki leads to the downregulation of the endolysosomal pathway and subsequent receptor recycling, which then enables malignant glioma stem cells to maintain their dedifferentiated state outside their niches for subsequent tumorigenesis (Shingu et al., 2017).

The majority (82%) of chromosome 6q alterations have been found to affect PARKIN expression levels in GBM (Cesari et al., 2003; Veeriah et al., 2010; Xu et al., 2014; de Castro et al., 2021). The tumor suppressor role of Parkin has been implicated with the expression correlation studies where low Parkin expression was associated with poor GBM prognosis (Freije et al., 2004; Wang et al., 2017; de Castro et al., 2021). In the present study, we interrogated the functional role of Parkin as a tumor suppressor and demonstrated a significant difference in tumorigenicity between PPP and QPP models. A double knockout of Qki and Parkin besides Trp53 and Pten deletion using the same system warrants further exploration to inquire about a potential compound effect in GBM pathology. Interestingly, despite a low penetrance for brain tumor formation, the total survival rate of the PPP cohort nonetheless appeared to be lower than the PP cohort. This unprecedented premature lethality phenotype of our Parkin-null animals could be explained by the breeder mouse strain/background differences or Nestin-CreER^{T2} expression and consequent loss of Pten and p53 outside of the brain that could have exacerbated the original Parkin knockout phenotype (Noda et al., 2020).

While *PRKN* gene has been reported to be frequently mutated/lost in GBM, one possible scenario is that deletions on the *PRKN* gene exert indirect effects on QKI, owing to disruption of regulatory regions and long-range chromatin interactions that modulate QKI expression levels. One such example has already been well established in *qk*^{v} (quaking viable) mice, where a >1 Mb deletion on chromosome 17 encompasses *Prkn* coding sequence as well as ~1 kb upstream of the *Qk* gene (Ebersole et al., 1996; Lockhart et al., 2004; Sidman et al., 1964). The deletion of a putative tissue-specific enhancer in this region leads to a significant reduction of Qki expression in oligodendrocytes, leading to severe hypomyelination in the CNS. The phenotype in mutant mice was later confirmed to be solely caused by Qki loss and was not recapitulated by *Prkn*-null animals (Wolf and Billings-Gagliardi, 1984; Ebersole et al., 1996; Mata et al., 2004; Perez and Palmiter, 2005; Darbelli et al., 2016; Shingu et al., 2017). Similarly, somatic deletions within the *PRKN* gene sequence could potentially disrupt the regulatory sequences and tissue-specific enhancers acting on *QKI* gene expression, leading to an underestimation of *QKI* alterations in GBM while overestimating the tumor suppressor function of *PRKN*. Mapping of long-range chromatin interactions and identification of putative regulatory regions within Ch6q using functional and genetic assays will provide critical insights on this matter.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Adipocyte-mediated epigenomic instability in human T-ALL cells is cytotoxic and phenocopied by epigenetic-modifying drugs

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The world's population with obesity is reaching pandemic levels. If current trends continue, it is predicted that there will be 1.5 billion people with obesity by 2030. This projection is alarming due to the association of obesity with numerous diseases including cancer, with recent studies demonstrating a positive association with acute myeloid leukemia (AML) and B cell acute lymphoblastic leukemia (B-ALL). Interestingly, several epidemiological studies suggest the converse relationship may exist in patients with T cell acute lymphoblastic leukemia (T-ALL). To determine the relationship between obesity and T-ALL development, we employed the diet-induced obesity (DIO) murine model and cultured human T-ALL cells in adipocyte-conditioned media (ACM), bone marrow stromal cell-conditioned media, stromal conditioned media (SCM), and unconditioned media to determine the functional impact of increased adiposity on leukemia progression. Whereas only 20% of lean mice transplanted with T-ALL cells survived longer than 3 months post-inoculation, 50%–80% of obese mice with leukemia survived over this same period. Furthermore, culturing human T-ALL cells in ACM resulted in increased histone H3 acetylation (K9/K14/K18/K23/K27) and methylation (K4me3 and K27me3) posttranslational modifications (PTMs), which preceded accelerated cell cycle progression, DNA damage, and cell death. Adipocyte-mediated epigenetic changes in human T-ALL cells were recapitulated with the H3K27 demethylase inhibitor GSK-J4 and the pan-HDAC inhibitor vorinostat. These drugs were also highly cytotoxic to human T-ALL cells at low micromolar concentrations. In summary, our data support epidemiological studies demonstrating that adiposity suppresses T-ALL pathogenesis. We present data demonstrating that T-ALL cell death in adipose-rich microenvironments is induced by epigenetic modifications, which are not tolerated by leukemia cells. Similarly, GSK-J4 and vorinostat treatment induced epigenomic instability and cytotoxicity profiles that phenocopied the responses of human T-ALL cells to ACM,

which provides additional support for the use of epigenetic modifying drugs as a treatment option for T-ALL.

KEYWORDS

obesity, leukemia, epigenetics, cell cycle, genotoxic stress

Introduction

Among American adults with obesity, defined by body mass index (BMI) greater than 30, the incidence has increased from 30.5% in 1999–2000 to 42.4% in 2017–2018 and is predicted to impact 50% of the population by 2030 (Andolfi and Fisichella 2018). Given that obesity propagates various diseases (Bianchini et al., 2002; Hruby and Hu 2015; Ellulu et al., 2017), this increase has created huge burdens for the health care system (Tsai et al., 2011). A hallmark of obesity is the accumulation of adipocytes, which chronically secrete adipokines and metabolites (Lee and Pratley 2005; Saltiel and Olefsky 2017). Recent studies have demonstrated that these factors promote the growth of cancer cells of varying etiologies directly by providing "fuel" to cancer cells in the form of amino acids (e.g., glutamine) or lipids for β-oxidation. Furthermore, adipocyte-secreted factors can promote tumor growth by attenuating antitumor immunity (Nieman et al., 1999; Marti et al., 2001). The relationship between obesity and solid tumorigenesis is well established; whereas our understanding of how increased adiposity impacts the development of hematological malignancies is still in its earliest stages. Published studies largely demonstrate that mortality rates are higher in persons with obesity diagnosed with B cell acute lymphoblastic leukemia (B-ALL) and acute myeloid leukemia (AML) (Butturini et al., 2007; Ethier et al., 2012; Inaba et al., 2012; Orgel et al., 2014). Despite being controversial (Liu et al., 2021), emerging epidemiological studies suggest that obesity might be protective in cases of T cell acute lymphoblastic leukemia (T-ALL) (Heiblig et al., 2015); however, a mechanistic understanding of how adipocytes impact T-ALL pathogenesis is unknown.

T-ALL, which accounts for roughly 20% of leukemia cases in adult and pediatric populations, is characterized by the rapid proliferation of early lymphoid cells with immature T cell surface markers (Van Vlierberghe and Ferrando 2012; Belver and Ferrando 2016). This cancer is driven by mutations in genes or transcription factors involved in T cell development (Van Vlierberghe and Ferrando 2012; Belver and Ferrando 2016). Although long-term survival of T-ALL can approach 50% in adults (Rowe et al., 2005; Dores et al., 2012) and 90% in pediatric patients (Vora et al., 2013; Place et al., 2015; Moricke et al., 2016), the survival rates of the patients with relapsed or refractory disease are dismal, with a reported 5-year overall survival outcome of less than 30% (Goldberg et al., 2003; Oudot et al., 2008). Unfortunately, our best treatment options have failed to improve survival outcomes in high-risk patients over the past decade, which has prompted the need to define drivers of T-ALL pathogenesis and identify ways to therapeutically exploit novel dependencies.

In cancer, epigenetic modifications mediated by hypermethylation of CpG islands (Esteller 2005), oncohistones (Nacev et al., 2019), IDH1 mutations (Lu et al., 2012), EZH2 expression (Eich et al., 2020), and the SWI/SNF complex (Cenik and Shilatifard 2021) are major drivers of tumorigenesis and disease progression. Sequencing data have revealed that 25% of T-ALL samples analyzed at diagnosis contain genetic lesions in epigenetic modifying enzymes (Ntziachristos et al., 2012; Greenblatt and Nimer 2014; Ntziachristos et al., 2014; Peirs, Van der Meulen et al., 2015). Of these, the polycomb repressor complex 2 (PRC2) is frequently mutated in T-ALL cells (Ntziachristos et al., 2012). This complex is comprised of three protein complexes, EZH2, EED, and SUZ12, which methylate primarily promoter-localized histone H3 at lysine 27 (H3K27), resulting in transcriptional repression (Plath et al., 2003). The methyltransferase activity of this complex is mediated by EZH2, which is responsible for the addition of mono-, di-, and tri-methylation modifications on H3K27. Although EZH2 is overexpressed in many solid cancers, including breast, lung, and liver cancer (Eich et al., 2020), loss-of-function mutations of EZH2, inactivating mutations, or deletion of this epigenetic modifier is commonly present in human T-ALL cells (Ntziachristos et al., 2012; Girardi et al., 2017).

Despite emerging evidence demonstrate that mutations associated with altered epigenomes are common in T-ALL, epigenetic modifying drugs are not traditionally used to treat this disease; whereas, inhibitors targeting histone methyltransferases and histone demethylases are currently being tested in clinical trials as therapies for diffuse large B cell lymphoma (DLBCL) and AML (Morera et al., 2016). In this report, we aimed to determine how obesity impacts T-ALL pathogenesis with an emphasis on defining epigenomic modifications in T-ALL cells, which regulate disease progression. To this end, we found that T-ALL development was suppressed in obese murine models, which supported epidemiological studies reporting the protective effects of obesity in patients with T-ALL (Heiblig et al., 2015). Furthermore, we found that adipocyte-secreted factors directly induced increased transcription and cycle

progression with accompanying genotoxic stress and cell death in human T-ALL cells. The increased adipocyte-induced transcription observed in human T-ALL cells was accompanied by alterations in epigenetic states including increases in total H3 protein levels and increased H3 acetylation (K9/K14/K18/K23/K27) and methylation (K4me3 and K27me3). Acetylation of H3 is associated with gene transcription (Di Cerbo et al., 2014), which we observed in our RNA-sequencing studies; however, increased H3 tri-methylation at K4 and K27 is associated with transcriptional activation and repression, respectively (Howe et al., 2017; Guo et al., 2021). Recently, it was noted that H3K4me3 and H3K27me3 also appear at sites of transcription replication conflicts and DNA damage to slow down replication; thus, protecting the genome from DNA damage and instability (Chong et al., 2020). Our results demonstrate that adipocyte-mediated epigenetic changes on H3 are activating, and the increased methylation of H3 at K4 and K27 may mark regions of genomic instability. We also found that the epigenetic modifying drugs GSK-J4 and vorinostat were highly cytotoxic to human T-ALL cells, and epigenetic changes observed in leukemia cells after drug treatment phenocopied changes induced in human T-ALL cells exposed to the adipocyte secretome. Overall, our results demonstrate that adipocytes and epigenetic modifying drugs which increase acetylation and methylation on H3 in human T-ALL cells induce genomic instability and cell death.

Materials and methods

Cell lines

T cell acute lymphoblastic leukemia (T-ALL) cells lines were kindly provided from Dr. Christopher Porter and Dr. Douglas Graham laboratories (Department of Pediatrics; Emory University School of Medicine). DND-41, HSB2, Loucy, Molt4, and Peer cells were grown in 20% FBS-supplemented RPMI1640 (Cat#10-041-CV, Corning, NY, United States), and Jurkat cells were grown in 10% FBS-supplemented RPMI1640. The OP-9 mouse bone marrow stromal cell line was grown in 20% FBS-supplemented alpha-MEM (cat#15-012-CV, Corning, NY, United States) and differentiated into adipocytes following previously published protocols (Wolins et al., 2005; Wolins et al., 2006).

Epigenetic modifying drugs

Inhibitors of epigenetic modifications used in these studies were as follows: GSK-343 (S7164), an EZH2 inhibitor; GSK-J4 (S7070), a H3K27 histone demethylase JMJD3/UTX inhibitor; C646 (S7152), a histone acetyltransferase p300 inhibitor; and vorinostat (SAHA, S1047), a histone deacetylase (HDAC1 and HDAC3) inhibitor. Each drug was purchased from Selleckchem (Houston, TX, United States) and reconstituted in DMSO (cat#D2650, Sigma, St. Louis) as per the manufacturer's instructions prior to use.

Cell death assays

Cell death was assessed by using Annexin-V-FITC/PI (cat#BMS500FI-300, ThermoFisher, Waltham, MA, United States) staining, following the manufacturer's protocol. Briefly, T-ALL cells were conditioned in 10% FBS-supplemented RPMI1640 (control condition), stromal conditioned media (SCM), or adipocyte-conditioned media (ACM) for various time points or treated with epigenetic drugs for 72 h. The cells were harvested, washed with 1X PBS, and stained by Annexin-V-FITC in 1X binding buffer for 15–20 min at room temperature. T-ALL cells were then washed with 1X binding buffers and stained with 10 μg/ml propidium iodide (PI) (J66584, Alfa Aesar, Tewksbury, MA, United States) to assess apoptosis.

Cell cycle analysis

T-ALL cell cycles were measured using the Click-iT EdU Alexa-Fluor 488 Flow Cytometry Assay Kit (Cat#c10420, ThermoFisher Scientific, Waltham, MA, United States), following the manufacturer's protocol. Briefly, after conditioning in RPMI, SCM, and ACM or treated with epigenetic drugs, the cells were pulsed with EdU (15 μM) for 2 h at 37°C, followed by fixation in 4% paraformaldehyde in 1X PBS overnight. After washing with 1X PBS, the cells were permeabilized in 1X fixation/permeabilization solution for 15 min at room temperature. T-ALL cells were then stained with anti-EdU-FITC antibody for 30 min at room temperature, followed by PI staining for DNA contents. Results were acquired using a Cytoflex flow cytometer (Beckman Coulter, Indianapolis, United States), and data were analyzed using FlowJo version 10 software (BD, Ashland, United States).

RNA-sequencing and pathway analysis

Three human T-ALL cell lines (Jurkat, Loucy, and Peer) were cultured for 24 h in RPMI, SCM, or ACM (n = 9 samples with one condition/cell line). Total RNA was isolated from leukemia cells using the RNeasy Plus kit (cat#74034, Qiagen, Germantown, United States) and 200–500 ng of total RNA was used as input for the Stranded mRNA-Seq kit with PolyA capture beads (cat# KK8420, KAPA Biosystems) to generate RNA-seq libraries, according to the manufacturer's instructions. Final libraries

were quality checked using a Bioanalyzer (Agilent) and sequenced on a NextSeq500 using PE75 chemistry at the NYU Genome Technology Center.

Analysis of RNA-sequencing data was performed using Reactome, Microsoft Excel, and GraphPad Prism platforms. Normalized data in RPKM uploaded to Reactome and gene expression were analyzed using the camera method. Datasets were annotated according to the cell type (Jurkat, Loucy, and Peer) and media (ACM, SCM, and RPMI); differences between two media types at a time were directly contrasted with cell lines marked as covariates. Output was saved as an expression network diagram that was later cropped to focus on nodes of interest, with the entire diagram included in supplemental data. A total of 23 specific genes of interest for DNA damage and epigenetic modifiers were selected from the global RNA-seq workbook, and the percentage difference in gene expression between media type was calculated for each cell line individually in Excel. Percentage output was added to GraphPad Prism and graphed as percentage change in gene expression from baseline, with a baseline of RPMI media at zero. Raw count expression data were obtained, and count data were normalized and transformed using DESeq2. Genes with no expression across sample groups are filtered out prior to normalization. Furthermore, only genes with greater than zero coverage or read depth in all sample groups were used for normalization and downstream analysis.

Western blot studies

Human T-ALL cell lines, either treated in conditioned media as previously described or with epigenetic modifiers, were harvested, washed with 1X PBS, and resuspended in 1X RIPA (radio-immunoprecipitation buffer, 150 mM sodium chloride, 50 mM Tris-HCl (pH8.0), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate), containing protease (cat#11836153001) and phosphatase (cat#4906845001) inhibitors (MilliporeSigma, St. Louis, MO, United States) to extract proteins. Protein concentration was quantified using the Pierce BCA protein assay kit (cat#23227, ThermoFisher, Rockford, IL, United States), and 20 μg of protein was analyzed to detect proteins of interest. The protein levels assessed were that of γH2AX (cat#2577s, Cell Signaling Technology, Boston, MA, United States), ERK (p42/44, cat#9102s, Cell Signaling Technology, Boston, MA, United States), pErk [p42/44 (T202/T204), cat#9101s, Cell Signaling Technology, Boston, MA, United States], CHK1 (cat#2360s, Cell Signaling Technology, Boston, MA, United States), pCHK1Ser345 (cat#2348s, Cell Signaling Technology, Boston, MA, United States), CHK2 (cat#6334s, Cell Signaling Technology, Boston, MA, United States), pCHK2Thr68 (cat#2197s, Cell Signaling Technology, Boston, MA, United States), and EZH2 (cat#5246s, Cell Signaling Technology, Boston, MA, United States). For epigenetic assays, we determined the protein levels of EED (cat#PA5-92427, Thermo Fisher Scientific, Waltham, MA, United States), SUZ12 (cat#3737, Cell Signaling Technology, Boston, MA, United States), RING1A (cat#13069s, Cell Signaling Technology, Boston, MA, United States), BMI1 (cat#6964s, Cell Signaling Technology, Boston, MA, United States), H3K27me3 (cat#9733s, Cell Signaling Technology, Boston, MA, United States), H3K4me3 (cat#ab8580, Abcam, Waltham, MA, United States), H3 acetylated on K9/14/18/23/27 (ab47915, Abcam, Waltham, MA, United States), and H3 (cat#9715s, Cell Signaling Technology, Boston, MA, United States). For DNA damage and apoptosis responses, we performed Western blot assays for ATR (cat#2790s, Cell Signaling Technology, Boston, MA, United States), pATR (Ser428) (cat#2853s, Cell Signaling Technology, Boston, MA, United States), p53 (cat#sc-126, Santa Cruz Biotechnology, Dallas, TX, United States), and pp53 (Ser15) (cat#9284s, Cell Signaling Technology, Boston, MA, United States). Either β-actin (cat#4967s, Cell Signaling Technology, Boston, MA, United States) or α-tubulin (cat#2144s, Cell Signaling Technology, Boston, MA, United States) was used as an internal loading control and fluorescent-tagged secondary antibodies, IR Dye 800CW (goat anti-rabbit, cat#926-32211) or 680RD (goat anti-mouse, cat#926–6807, LI-COR Bioscience, Lincoln, United States) were used to detect signals. Signals were visualized using Odyssey (Odyssey CLx, LI-COR Bioscience, Lincoln, United States).

Histone acetyltransferase and histone deacetylase enzymatic activity assays

Jurkat, Loucy, and Peer T-ALL cells were cultured in RPMI (control), SCM, or ACM for 24 h, after which, leukemia cells were harvested and washed in 1X PBS. Nuclear extracts were prepared, following the manufacturer's protocol (Cat#OP-0002-1, Epigentek, Farmingdale, NY, United States), and 5 μg was used to measure either HAT (Cat#P-4003, Epigentek, Farmingdale, NY, United States) or HDAC (Cat#P-4034, Epigentek, Farmingdale, NY, United States) enzymatic activity as per the manufacturer's instructions. Each sample was assayed in duplicate. Enzymatic activities of HAT and HDAC were normalized to responses observed under the RPMI control conditions.

Chromatin stability assays

To measure the degree of chromatin fragmentation, human T-ALL cell lines were cultured in RPMI, SCM, or ACM for 48 h, and genomic DNA was isolated using DNeasy Blood and Tissue Kit (cat#69504, QIAGEN, Germantown,

MD, United States). Total genomic DNA (1 μg) was digested with the Dpn II (cat#R0543, NEB, Ipswich, MA, United States) restriction enzyme, analyzed on 0.8% agarose gel, and imaged using a Gel Doc XR imaging system (Bio-Rad, Hercules, CA, United States) to observe the degree of genomic DNA fragmentation.

To observe abnormal nuclei, we performed immunofluorescence. Briefly, Jurkat cells were plated on 0.01% poly-L-lysine (cat#8920, MiliporeSigma, St. Louis, MO, United States) coated coverslips and cultured in RPMI, SCM, or ACM or treated with GSK-J4 or vorinostat in RPMI for 48 h. The cells were fixed with 4% paraformaldehyde (Cat#J61899, Alfa Aesar, Haverhill, MA, United States) for 15 min at room temperature, followed by three washes with 1X PBS (5 min/wash). The cells were then permeabilized with 0.1% NP-40 in 1X PBS for 15 min at room temperature, washed three times with 1X PBS, followed by blocking for 1 h at room temperature using 10% normal goat serum (Cat#50062Z, Thermo Fisher Scientific, Waltham, MA, United States). The cells were incubated with 1:100 diluted laminin antibody (Cat#Nb300-144ss, Novus Biologicals, Centennial, CO, United States) at 4°C overnight and washed three times with 1X PBS. Alexa-Fluor 568 goat–rabbit IgG (H+L) (1:500 in 10% goat serum, cat#A11011, Invitrogen, Waltham, MA, United States) was used as the secondary antibody and incubated at room temperature for 1 h. T-ALL cells were then washed three times with 1X PBS, followed by one wash with 1X TBS, before mounting with one drop of Prolong Gold Antifade Reagent with DAPI (Cat#p36941, Invitrogen, Waltham, MA, United States). Immunofluorescence images were acquired with the Olympus FV1000 (Center Valley, PA, United States) confocal microscope, and data were analyzed using Fiji–ImageJ software.

T-ALL murine experiments

Two-month old C57BL/6 mice (The Jackson Laboratory) or NOG mice [Taconic; (genotype) sp/sp;ko/ko (nomenclature) NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac and (genotype) sp/sp;ko/y (nomenclature) NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac] were fed control (10% fat calories) or high-fat (60% fat calories) diets for 2–4 months prior to experimentation. The onset of obesity was verified in mice prior to experimentation based on the following criteria: significant weight gain, the chronic production of cytokines/chemokines (IL-6; Invitrogen, cat# 88-7064-22 and TNF-α; Invitrogen, cat# 88-7324-22), and elevated insulin levels (RayBioTech, CODE: ELM-Insulin-1). The chow was purchased from Bio-Serv (cat# F4031 for control diets and cat# S3282 for high-fat diets) and sterilized by irradiation prior to usage. Male and female mice were used for these experiments.

For syngeneic experiments, 2-month old C57BL/6 mice (n = 10 mice/diet) were treated with busulfan as previously described as a method of mild myeloablation (Henry et al., 2015), followed by intravenous transplantation with 5×10^5 NOTCH-1-GFP expressing c-kit + cells [created using lentiviral transduction methodology from young (2 month old) hematopoietic stem and progenitor cells as previously described (Henry et al., 2015)]. For xenograft experiments, 10^5 Peer cells (human T-ALL cell line) were injected intravenously, without conditioning, into NOG mice (n = 5 mice/diet). For all experiments, mice were monitored daily for signs of morbidity including hind limb paralysis, labored breathing, abnormal gait, ruffled fur, reduced responsiveness to tactile stimulation, and removed from the study at the first sign of distress per our approved IACUC protocol. Death was not an endpoint for these studies. All murine experiments received ethical approval from the Emory University School of Medicine Institutional Animal Care and Use Committee (IACUC) under the approved protocol number DAR-3000013.

Real-time PCR gene expression analyses

Human T-ALL cell lines (Jurkat, Loucy, and Peer) were treated in RPMI, SCM, and ACM for 48 h, and RNA was isolated using RNeasy mini kit (Cat#74104, Qiagen, Germantown, MD, United States). Once isolated, 0.8–1 μg of total RNA was used to make cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Cat#04 379 012 001, Roche, Indianapolis, IN, United States). cDNA was diluted 1:5 with H_2O and 2 μl of diluted cDNA was used to perform real-time PCR using iTaq Universal SYBR Green Supermix (Cat#1725121, Bio-Rad, Hercules, CA, United States). Primers were ordered from Integrated DNA Technologies (IDT, Coralville, IA, United States), and the sequences used for these experiments are listed in Table 1. GAPDH was used as an internal control, and gene expression levels were normalized to expression values found in human T-ALL cells cultured in RPMI.

Murine and human T cell epigenetic drug treatment assays

Healthy human peripheral blood mononuclear cells (PBMCs; n = 3) were a generous gift from Dr. Sunil Raikar's laboratory (Emory University, Department of Pediatrics, Aflac Cancer and Blood Disorders Center). Naïve $CD4^+$ and $CD8^+$ T cells were purified using the MiniMACS separator, following the manufacturer's protocol. Briefly, PBMCs were washed in 1X MACS buffer (2 mM EDTA, pH 8.0, 1% fetal bovine serum in 1X PBS), and the cells were incubated with CD4 (Miltenyi Biotec, Cat#130-045-101) or CD8 (Miltenyi Biotec, Cat#130-045-201) microbeads. T cells were purified using an LS column (Miltenyi Biotec, Cat#130-042-401).

TABLE 1 Real-time PCR primer sequences.

Genes	Sequences	PCR Product Size	References
ATM	Forward: 5′-TGGATCCAGCTATTTGGTTTGA-3′	82bp	PMID: 19661131
	Reverse: 5′-CCAAGTATGTAACCAACAATAGAAGAAGTAG-3′		
ATR	Forward: 5′-TGAAAGGGCATTCCAAAGCG-3′	144bp	PMID: 22319212
	Reverse: 5′-CAATAGATAACGGCAGTCCTGTCAC-3′		
CHEK1	Forward: 5′-CAGGTCTTTCCTTATGGGATACCAG-3′	122bp	PMID: 22319212
	Reverse: 5′-TGGGGTGCCAAGTAACTGACTATTC-3′		
CHEK2	Forward: 5′-AGTGGTGGGGAATAAACGCC-3′	117bp	PMID: 28944848
	Reverse: 5′-TCTGGCTTTAAGTCACGGTGTA-3′		
CDKN1A	Forward: 5′-CCTCATCCCGTGTTCTCCTTT-3′	97 bp	PMID: 27572311
	Reverse: 5′-GTACCACCCAGCGGACAAGT-3′		
TP53BP1	Forward: 5′-TGGCAACCCCGTGAAAATC-3′	178 bp	PMID: 22319212
	Reverse: 5′-CCACCACATCAAATACCCCTAAAG-3′		
BMI1	Forward: 5′-GGTACTTCATTGATGCCACAACC-3′	124 bp	Origene
	Reverse: 5′-CTGGTCTTGTGAACTTGGACATC-3′		
EED	Forward: 5′-GACGAGAACAGCAATCCAGACC-3′	121 bp	Origene
	Reverse: 5′-TCCTTCCAGGTGCATTTGGCGT-3′		
EZH2	Forward: 5′-GACCTCTGTCTTACTTGTGGAGC-3′	115 bp	Origene
	Reverse: 5′-CGTCAGATGGTGCCAGCAATAG-3′		
RING1A	Forward: 5′-CCTATCTGCCTGGACATGCTGA-3′	127 bp	Origene
	Reverse: 5′-GCTTCTTTCGGCAGGTAGGACA-3′		
SUZ12	Forward: 5′-CCATGCAGGAAATGGAAGAATGTC-3′	135 bp	Origene
	Reverse: 5′-CTGTCCAACGAAGAGTGAACTGC-3′		
GAPDH	Forward: 5′-AGGGCTGCTTTTAACTCTGGTAAA-3′	91 bp	PMID: 15153541
	Reverse: 5′-CATATTGGAACATGTAAACCATGTAGTTG-3′		

To isolate murine T cells, spleens were harvested from 4-month-old C57BL/6 mice (n = 3), and naïve $CD4^+$ and $CD8^+$ T cells were purified as described earlier using αCD4 (Miltenyi Biotec, Cat#130-117-043) and αCD8 (Miltenyi Biotec, Cat#130-117-044) microbeads, respectively, using MiniMACS magnet separation kit (Cat#130-090-312, Miltenyi Biotech., Gaithersburg, MD, United States).

Human T cells were plated in 10% FBS in RPMI (Cat#10-041-CV, Corning, NY, United States) media supplemented with human IL-7 (50 ng/ml, Cat#200-07, Peprotech, Rocky Hill, NJ, United States) and treated with the epigenetic drugs, GSK-J4, vorinostat, and C646 at concentrations of 25% of the IC_{50}, 50% of the IC_{50}, and IC_{50} for 72 h. Murine T cells were plated in 10% FBS in RPMI (Cat#10-041-CV, Corning, NY, United States) media supplemented with murine IL-7 (50 ng/ml, Cat#217-17, Peprotech, Rocky Hill, NJ, United States) and treated for 24 h as described previously. In both experiments, the percentage of dead or dying T cells was determined using Annexin-V/PI staining (Cat#BMS500FI-300, Thermo Fisher Scientific, Waltham, MA, United States) followed by flow cytometric analysis. The samples were acquired on the Cytoflex flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Ashland, Oregon).

Ex vivo human T-ALL cell analysis of DNA damage and apoptosis from lean and obese mice

Lean and obese immunocompromised (NOG) mice were generated as described earlier. Mice fed control or high-fat diet for 2 months were injected intravenously (i.v.) with 10^5 Peer cells (human T-ALL cell line), without conditioning (n = 5 mice/diet). Mice were sacrificed at 20 days post human T-ALL cell injection, prior to the onset of visible signs of morbidity. Spleens were harvested from euthanized mice, and transplanted human T-ALL cells were sorted to greater than 97% purity using αCD45 (Biolegend; Cat#304011) and αCD3 (Biolegend; Cat#300405) antibodies using the Benton Dickison FACS Aria II Cell Sorter. Intracellular staining was performed on purified human T-ALL cells to ascertain the percentage of phospho-γH2AX (Thermo Fisher Scientific; Cat#12-9865-42) and cleaved caspase 3 (Cell Signaling Technology; Cat#12768S) positive T-ALL cells. The samples were acquired using the Cytoflex flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Ashland, Oregon). All murine experiments

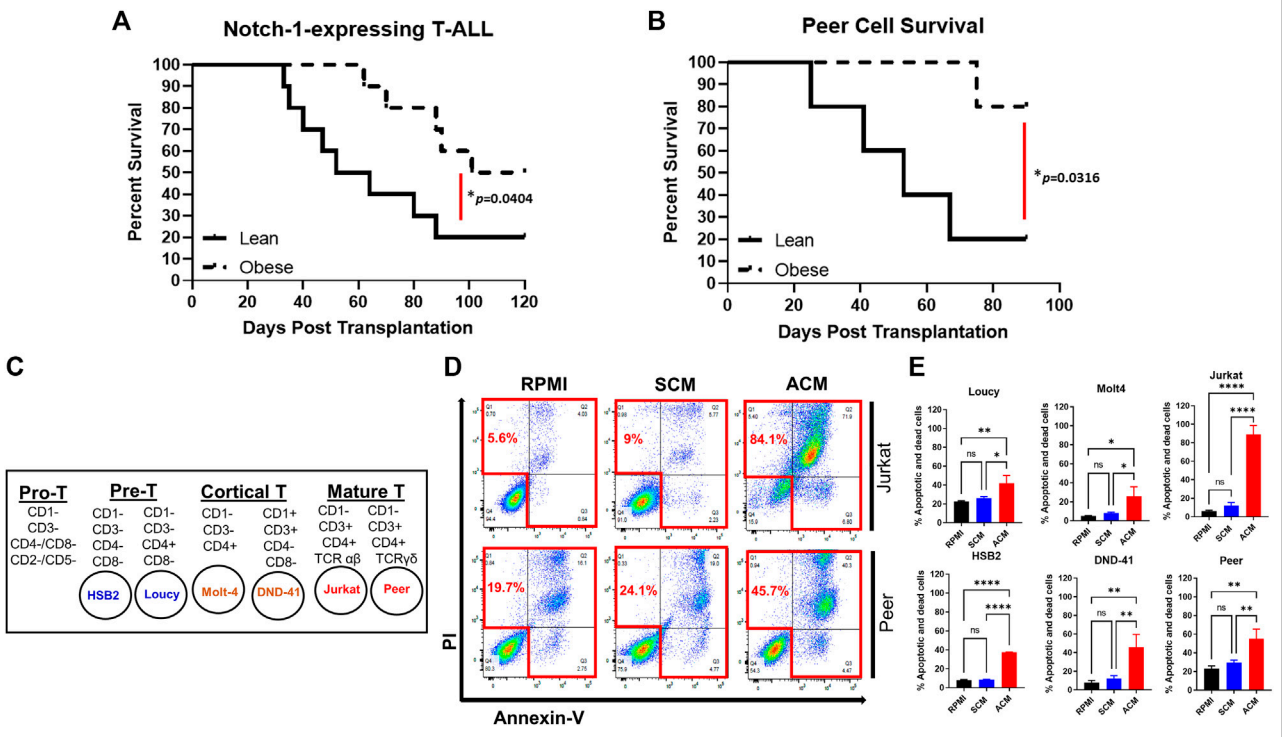


FIGURE 1
Obesity protects against T-ALL pathogenesis with adipocyte-secreted factors being cytotoxic to human T-ALL cells. **(A)** Murine *c-kit* + cells were isolated from the spleens of C57BL/6 mice and transduced with lentivirus expressing NOTCH1. NOTCH1-expressing *c-kit* cells were injected intravenously *via* tail vein delivery (5 x 10^5 cells/mouse) into recipient mice (n = 10/group) and their survival was monitored. **(B)** Human T-ALL cells (Peer cells) were injected (10^5 cell/mouse) into lean or obese immunocompromised (NOG) mice as described earlier and survival was monitored. **(C)** List of human T-ALL cell lines used in this study depicting their maturation state as determined by surface immunophenotyping. This figure was adapted from Burger et al. (1999). **(D)** Human T-ALL cells, Jurkat, and Peer, were cultured in RPMI (control condition), stromal cell-conditioned media (SCM), or adipocyte-conditioned media (ACM) for 72 h and cell death was assessed using Annexin-V/PI staining as determined *via* flow cytometric analysis. Primary data from a representative experiment is shown. **(E)** Quantitative data from six independent experiments are shown. Statistical significance in **(A,B)** was determined using a log-rank test and in **(E)** using a one-way ANOVA followed by Tukey's multiple comparison post-test. *p < 0.05, **p < 0.01, and ****p < 0.0001.

received ethical approval from the Emory University School of Medicine Institutional Animal Care and Use Committee (IACUC) under the approved protocol number DAR-3000013.

Results

Obesity is protective against T-ALL pathogenesis

Obesity is associated with poor prognosis in multiple solid and hematological cancers (Butturini et al., 2007; Sheng and Mittelman 2014). The tumor promoting property of the obese microenvironment is multifactorial and results in altered pharmacokinetics/pharmacodynamics of chemotherapies (Sheng et al., 2017), compromised cancer immune surveillance (Calle and Kaaks 2004; Iyengar et al., 2016), and the chronic secretion of cytokines, chemokines, and metabolites from adipocytes, which promotes oncogenesis (Park et al., 2014; Jiramongkol and Lam 2020). Although obesity is a well-established risk factor for many cancers, T cell acute lymphoblastic leukemia (T-ALL) is an exception in which, although controversial, significant increases in survival are documented for patients with overweight and obesity (Heiblig et al., 2015; Liu et al., 2021).

Given the unclear relationship between obesity and T-ALL pathogenesis, we determined how obesity impacted the survival of mice transplanted with murine or human T-ALL cells. For syngeneic experiments, lean and obese C57BL/6 mice were transplanted with hematopoietic stem and progenitor cells (c-kit$^+$ cells) expressing activated *NOTCH1*, which is a potent driver of T-ALL development found to be mutated in 60% of childhood T-ALL cases (Lee et al., 2005; Garcia-Peydro et al., 2018). Using this approach, we found that while 20% of lean mice transplanted with *NOTCH1*-expressing cells survived over

TABLE 2 Human T-ALL cell line characteristics.

	Origin	NOTCH1	CDKN2A/2B	p53
HSB2	PB of 11 year boy	wt HD and PEST	mut (del)	wt
Loucy	PB of 38 year women	wt HD and PEST	mut (del)	mut (hom, pm)
Molt4	PB of 19 year boy	mut HD (het, pm) PEST (het, del)	mut (hom, del)	mut (het, pm)
DND-41	PB of 13 year boy	mut HD (het, pm) PEST (het, ins/del)	mut (het, del/pm)	mut (hom, pm)
Jurkat	PB of 14 year boy	mut (het, ins)	mut (hom, del)	mut (het, pm)
Peer	PB of 4 year boy	mut (pm)	mut (het, del)	mut (pm)

Wt, wild-type; Mut, mutant; HD, homodomain; PEST, PEST sequence, proline (P), glutamic acid (E), serine (S), and threonine (T); PM, point mutation; Ins, insertion; Del, deletion; Hom, homozygous; Het, heterozygous.

3 months post-transplantation, this percentage significantly increased to 50% of obese mice over this period (Figure 1A). Similarly, in xenograft experiments where lean and obese immunocompromised mice were transplanted with human T-ALL cells, the survival advantage of obesity was more dramatic with 20% of lean and 80% of obese mice surviving over 2 months post-transplantation with *NOTCH1*-expressing cells (Figure 1B). In xenograft experiments, the increased survival of obese was consistent with increased DNA damage (Supplementary Figure S1A) and caspase 3 activation (Supplementary Figure S1B) observed in transplanted human T-ALL cells at 20 days post-transplantation, prior to signs of morbidity manifesting in mice. These results support epidemiological studies demonstrating superior survival outcomes in obese patients with T-ALL.

The adipocyte secretome is cytotoxic to human T-ALL cells

To interrogate the mechanism of obesity-mediated suppression of T-ALL pathogenesis, we used a high-throughput *in vitro* system of adiposity to generate large quantities of adipocytes and conditioned media (**CM**) from differentiated bone marrow stromal cells. For these experiments, we determined how the survival of human T-ALL cell lines, derived from both sexes of varying ages, harboring diverse mutations, and presenting at different maturation states (n = 6; Table 2; Figure 1C) (Squiban et al., 2017), was impacted after 3 days of culture in unconditioned media, bone marrow stromal cell-conditioned media (**SCM**), and adipocyte-conditioned media (**ACM**). Similar to the obesity-induced suppression of T-ALL pathogenesis *in vivo*, ACM was highly cytotoxic to human T-ALL cells over 3 days of culture (45%–84% cell death) and occurred independently of wild-type or mutant p53 expression (Table 2; Supplementary Figure S2A; Figures 1D,E). In contrast, apoptosis observed in human T-ALL cells cultured in RPMI and SCM was minimal (<25% cell death; Supplementary Figure S2A; Figures 1D,E). A closer analysis of the data revealed that the degree of T-ALL cell death correlated with their differentiation status. The more differentiated human T-ALL cells (Jurkat and Peer T-ALL cell lines) exhibited 45%–84% cell death compared to Loucy [an early T cell progenitor (ETP) cell line] and H2B2 cells, where cell death ranged from 25 to 65% over 3 days of culture in ACM. Despite this observation, cytotoxicity levels in all human T-ALL cells cultured in ACM reached 85%–99%, regardless of the maturation state, with longer (5 day) cultures (Supplementary Figure S2B). In all, these results demonstrate that obesity-induced protection of T-ALL development might result from increased adiposity and the direct cytotoxic effects of the adipocyte secretome on malignant T cells.

The adipocyte secretome induces gene expression programs in human T-ALL cells indicative of cell cycle progression, DNA damage, and altered epigenetics

To delineate how the adipocyte secretome modulated gene expression profiles in ACM-exposed human T-ALL cell lines relative to those cultured in unconditioned media and SCM, we performed RNA-sequencing analyses. Principal component analysis (PCA) revealed that a 24-h culture in ACM-induced distinct gene expression changes in the more phenotypically mature Jurkat and Peer T-ALL cell lines; whereas profiles observed in unconditioned and SCM cultured leukemia cells were similar (Figure 2A). In contrast, the ETP ALL cell line, Loucy, exhibited similar gene expression programs when cultured in SCM and ACM relative to unconditioned media, which varied from observations with Jurkat and Peer cells (Figure 2A). In addition to PCA, we performed pathway analysis on the human T-ALL cells cultured under each condition using the Reactome pathway database. To identify pathways commonly up or downregulated in human T-ALL cells cultured in ACM relative to the other conditions tested, gene expression profiles from all three cell lines were combined for each condition. When responses after 24 h of culturing human T-ALL cells in ACM were compared with programs active in leukemia cells cultured in unconditioned media, we observed that

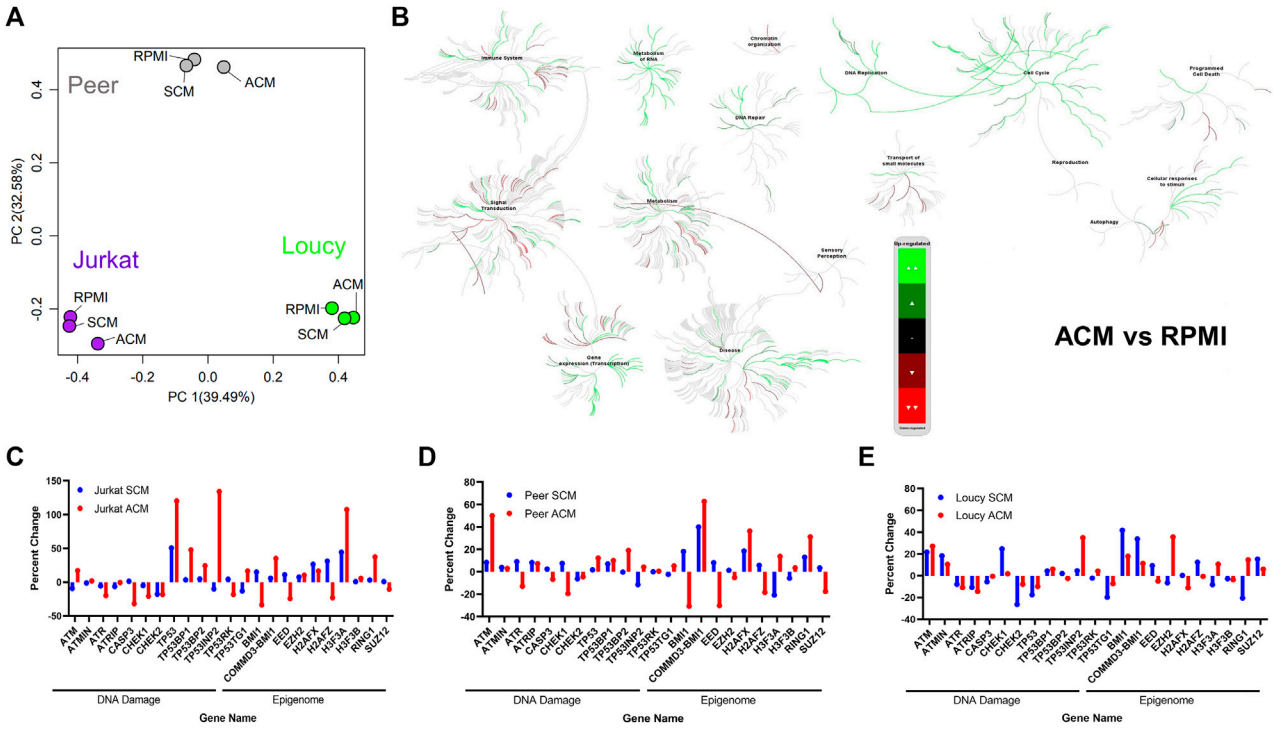


FIGURE 2
Adipocyte secretome induces gene expression programs in human T-ALL cells indicative of cell cycle progression, DNA damage, and altered epigenetics. RNA-sequencing was performed on human T-ALL cell lines (Jurkat, Loucy, and Peer) after 24 h of culture in unconditioned media RPMI, SCM, and ACM. **(A)** Principal component analysis (PCA) was performed to determine gene expression relationships between leukemia cell lines and culture conditions. **(B)** Data generated from the RNA-sequencing experiments were processed for pathway analysis using Reactome software. Pathways modulated by ACM relative to unconditioned media are shown. **(C–E)** Targeted analysis was performed for genes involved in the DNA damage response (DDR) and epigenetic stability. The fpkm value for each gene in T cells cultured in unconditioned media was used as the control value, and the data are graphed as the percentage increase or decrease relative to control values for each cell line under each condition.

ACM upregulated gene expression programs in T-ALL, which were involved in cellular responses to stimuli, cell cycle, DNA replication, DNA repair, metabolism of RNA, and transcription (Figure 2B). Downregulated pathways in ACM-cultured human T-ALL cells include those involved in signal transduction, transport of small molecules, and chromatin organization (Figure 2B). Similar activation and inhibition profiles were observed in ACM-cultured human T-ALL cells relative to SCM-exposed leukemia cells (Supplementary Figure S3). In contrast, culturing human T-ALL cells in SCM relative to unconditioned media did not induce extensive changes in many of the pathways analyzed, with the exception being the modest upregulation of genes involved in the cell cycle, metabolism, and immune response; whereas, those involved in chromatin organization, transcription, and DNA repair were downregulated (Supplementary Figure S4). A list of the most significantly altered pathways in human T-ALL cells cultured in ACM vs. unconditioned media, ACM vs. SCM, and SCM vs. unconditioned media can be found in Tables 3–5, respectively, and increased transcriptional profiles in ACM-stimulated human T-ALL cells was confirmed using quantitative PCR (Supplementary Figures S5A,B).

Given the extensive increase in DNA replication and transcription with accompanying decreases in chromatin organization pathways in human T-ALL cells exposed to the ACM relative to SCM or unconditioned media, we mined our RNA-sequencing data to query the expression of genes, which regulate DNA repair, cell cycle progression, apoptosis, and epigenetic states. The gene expression levels observed in human T-ALL cells cultured in RPMI were used as the baseline (0% level). In support of the induction of DNA damage programs in human T-ALL cells cultured in ACM, we observed increased gene expression levels of *ATM*, which responds primarily to double-stranded breaks (DSBs), in leukemia cells exposed to the adipocyte secretome; whereas, this gene was downregulated in T-ALL cells cultured in SCM (with the exception of Loucy cells; Figures 2C–E). Furthermore, the gene encoding ATR, which is serine/threonine-specific kinase involved in sensing DNA damage and activating the DNA damage checkpoint, was more extensively suppressed in ACM-cultured human T-ALL cells (Figures 2C–E). In support of the hypothesis that human T-ALL cells cultured in ACM may not effectively activate cell cycle checkpoints when experiencing

TABLE 3 RNAseq pathway analysis of ACM vs. RPMI (all cell lines combined).

Pathway name	Entities found	*p*-value	FDR	Direction of alteration
Mitochondrial translation	94/102	4.25e-18	1.01e-14	Increased
Mitochondrial translation termination	88/94	1.20e-17	1.18e-14	Increased
Mitochondrial translation elongation	88/94	1.49e-17	1.18e-14	Increased
Mitochondrial translation initiation	88/96	3.92e-17	2.32e-14	Increased
Activation of APC/C and APC/C:Cdc20 mediated degradation of mitotic proteins	77/77	3.20e-16	1.52e-13	Increased
APC/C:Cdc20 mediated degradation of mitotic proteins	76/76	4.28e-16	1.69e-13	Increased
APC:Cdc20 mediated degradation of cell cycle proteins prior to satisfaction of the cell cycle checkpoint	74/74	7.20e-16	2.44e-13	Increased
Regulation of mitotic cell cycle	88/92	1.52e-15	4.01e-13	Increased
APC/C mediated degradation of cell cycle proteins	88/92	1.52e-15	4.01e-13	Increased
Cdc20:Phospho-APC/C mediated degradation of cyclin A	73/73	1.92e-15	4.56e-13	Increased
Regulation of APC/C activators between G1/s and early anaphase	81/83	2.16e-15	4.66e-13	Increased
rRNA modification in the nucleus and cytosol	60/71	7.28e-15	1.44e-12	Increased
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins	112/153	1.04e-14	1.89e-12	Increased
APC/C:Cdc20 mediated degradation of securin	68/68	1.18e-14	1.99e-12	Increased
APC/C:Cdh1 mediated degradation of Cdc20 and other APC/C:Cdh1 targeted proteins in late mitosis/early G1	74/74	1.52e-14	2.41e-12	Increased
Switching of origins to a post-replicative state	91/93	1.93e-14	2.86e-12	Increased
Synthesis of DNA	120/133	2.15e-14	3.01e-12	Increased
Regulation of ornithine decarboxylase (ODC)	50/51	2.53e-14	3.33e-12	Increased
Autodegradation of Cdh1 by Cdh1:APC/C	64/64	4.78e-14	5.86e-12	Increased
DNA replication	128/142	4.93e-14	5.86e-12	Increased
The citric acid (TCA) cycle and respiratory electron transport	157/235	7.56e-14	8.42e-12	Increased
CDK-mediated phosphorylation and removal of Cdc6	73/75	7.92e-14	8.42e-12	Increased
FBXL7 downregulates AURKA during mitotic entry and in early mitosis	54/55	8.16e-14	8.42e-12	Increased
Vif-mediated degradation of APOBEC3G	55/56	9.19e-14	9.09e-12	Increased
Orc1 removal from chromatin	71/73	1.37e-13	1.30e-11	Increased

DNA damage, *CHEK1* and *CHEK2* gene expressions were lower in human T-ALL cells when cultured in ACM relative to RPMI (and in some cases, SCM), with responses being more apparent in the more differentiated human T-ALL cells (Jurkat and Peer; Figures 2C–E). In addition, genes coding for TP53, or its binding partners, were largely upregulated in human T-ALL cells cultured

TABLE 4 RNAseq pathway analysis of ACM vs. SCM (all cell lines combined).

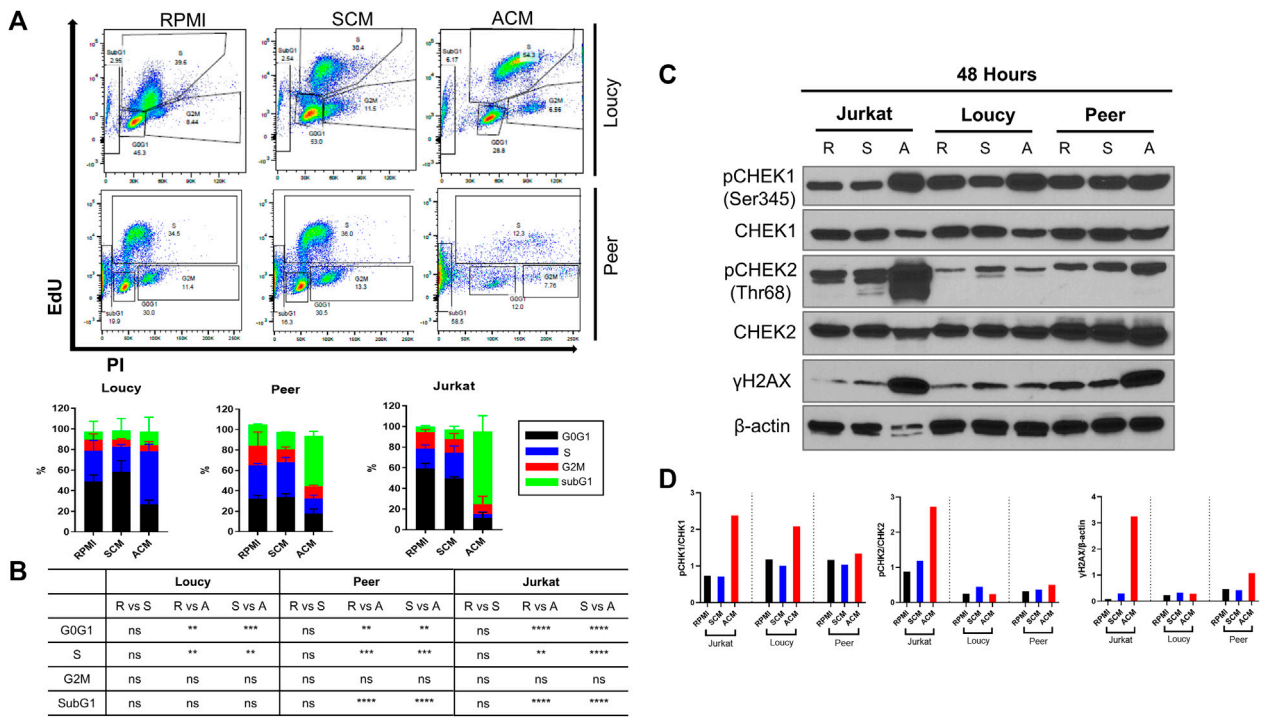
Pathway name	Entities found	*p*-value	FDR	Direction of alteration
Mitochondrial translation	94/102	4.92e-17	1.17e-13	Increased
Mitochondrial translation elongation	88/94	1.11e-16	1.32e-13	Increased
Mitochondrial translation termination	88/94	2.41e-16	1.91e-13	Increased
Mitochondrial translation initiation	88/96	4.25e-16	2.53e-13	Increased
rRNA modification in the nucleus and cytosol	60/71	7.55e-15	3.59e-12	Increased
mRNA splicing—major pathway	177/185	5.10e-12	2.02e-09	Increased
mRNA splicing	185/196	8.92e-12	2.74e-09	Increased
Processing of capped intron-containing pre-mRNA	240/256	9.24e-12	2.74e-09	Increased
Synthesis of DNA	120/133	3.41e-11	8.29e-09	Increased
DNA replication	128/142	3.49e-11	8.29e-09	Increased
Switching of origins to a post-replicative sate	91/93	1.24e-10	2.67e-08	Increased
DNA replication pre-initiation	85/88	1.71e-10	3.25e-08	Increased
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins	112/153	1.78e019	3.25e-08	Increased
APC:Cdc20 mediated degradation of cell cycle proteins prior to satisfaction of the cell cycle checkpoint	74/74	2.00e-10	3.39e-08	Increased
Activation of APC/C and APC/C:Cdc20 mediated degradation of mitotic proteins	77/77	2.92e-10	4.63e-08	Increased
Regulation of mitotic cell cycle	88/92	3.33e-10	4.66e-08	Increased
APC/C mediated degradation of cell cycle proteins	88/92	3.33e-10	4.66e-08	Increased
S phase	162/180	4.29e-10	5.16e-08	Increased
APC/C:Cdc20 mediated degradation of mitotic proteins	76/76	4.42e-10	5.16e-08	Increased
Cdc20:Phospho-APC/C mediated degradation of Cyclin A	73/73	4.45e-10	5.16e-08	Increased
Orc1 removal from chromatin	71/73	4.56e-10	5.16e-08	Increased
G1/S transition	145/150	4.99e-10	5.21e-08	Increased
Regulation of APC/C activators between G1/S and early anaphase	81/83	5.04e-10	5.21e-08	Increased
Eukaryotic translation elongation	94/102	1.03e-09	1.02e-07	Decreased
The citric acid (TCA) cycle and respiratory electron transport	157/235	1.08e-09	1.03e-07	Increased

in ACM relative to the other conditions tested (Figures 2C–E). Notable changes in genes regulating the epigenome were also observed. The gene expression levels of *BMI1*, which is rapidly recruited to sites of double-stranded DNA breaks (Ismail et al., 2010), were extensively downregulated in more differentiated human T-ALL cells cultured in ACM; whereas, the converse was

TABLE 5 RNAseq pathway analysis of SCM vs. RPMI (All cell lines combined).

Pathway name	Entities found	*p*-value	FDR	Direction of alteration
Eukaryotic translation elongation	94/102	4.14e-20	7.10e-17	Increased
Peptide chain elongation	90/97	5.97e-20	7.10e-17	Increased
Viral mRNA translation	91/114	2.15e-19	1.71e-16	Increased
Eukaryotic translation termination	94/106	2.34e-18	1.39e-15	Increased
Nonsense mediated decay (NMD) independent of the exon junction complex (EJC)	96/101	6.77e-18	3.22e-15	Increased
Formation of a pool of free 40S subunits	102/106	1.63e-17	6.46e-15	Increased
Selenocysteine synthesis	94/112	9.87e-17	3.35e-14	Increased
L13a-mediated translational silencing of ceruloplasmin expression	112/120	3.14e-16	9.33e-14	Increased
GTP hydrolysis and joining of the 60S ribosomal subunit	113/120	1.54e-15	4.08e-13	Increased
Eukaryotic translation initiation	120/130	2.58e-14	5.57e-12	Increased
Cap-dependent translation initiation	120/130	2.58e-14	5.57e-12	Increased
SRP-dependent cotranslational protein targeting to membrane	113/119	3.67e-14	7.27e-12	Increased
Response of EIF2AK4 (GCN2) to amino acid deficiency	105/115	2.78e-13	5.08e-11	Increased
Regulation of expression of SLITs and ROBOs	164/183	6.17e-13	1.05e-10	Increased
Nonsense mediated decay (NMD)	117/124	2.11e-12	3.13e-10	Increased
Nonsense mediated decay (NMD) enhanced by the exon junction complex (EJC)	117/124	2.11e-12	3.13e-10	Increased
Influenza viral RNA transcription and replication	141/176	2.27e-12	3.18e-10	Increased
Selenoamino acid metabolism	113/180	3.92e-11	5.17e-09	Increased
Influenza infection	160/200	1.01e-09	1.26e-07	Increased
Signaling by ROBO receptors	202/235	1.08e-09	1.29e-07	Increased
Translation initiation complex formation	59/62	3.39e-09	3.84e-07	Increased
Formation of the ternary complex, and subsequently, the 43S complex	52/54	4.36e-09	4.71e-07	Increased
Ribosomal scanning and start codon recognition	59/64	1.24e-08	1.25e-06	Increased
Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S	60/66	1.26e-08	1.25e-06	Increased
Metabolism of amino acids and derivatives	297/661	5.45e-06	5.18e-04	Increased

true in the Loucy ETP ALL cell line (Figures 2C–E). Furthermore, the gene encoding the polycomb protein EED, which is involved in maintaining the transcriptional repressive state of genes (Obier et al., 2015), was suppressed in all human T-ALL cell lines tested when cultured in ACM relative to the other conditions tested; whereas, *RING1*, which is also part of the



	Loucy			Peer			Jurkat		
	R vs S	R vs A	S vs A	R vs S	R vs A	S vs A	R vs S	R vs A	S vs A
G0G1	ns	**	***	ns	**	**	ns	****	****
S	ns	**	**	ns	***	***	ns	**	****
G2M	ns	ns	ns	ns	ns	ns	ns	ns	ns
SubG1	ns	ns	ns	ns	****	****	ns	****	****

FIGURE 3
Adipocyte secretome induces cell progression in human T-ALL cells with concomitant DNA damage. **(A)** Human T-ALL cells (Loucy and Peer) were cultured in unconditioned media RPMI, SCM, or ACM for 72 h, and cell cycle progression was determined using EdU analysis *via* flow cytometry. Representative flow cytometry data are shown. **(B)** Quantitative results are shown for three independent experiments. **(C)** Human T-ALL cells (Jurkat, Loucy, and Peer) were cultured for 48 h as described in **(A),** and Western blot analysis was performed to detect the protein levels of the indicated cell cycle checkpoint regulators (CHEK1/CHEK2) and indicators of genotoxic stress (γH2AX). Primary data from a representative experiment is shown. **(D)** Protein expression levels from the Western blot experiments were determined using ImageJ analysis and a representative blot from two independent experiments is shown. R = RPMI, S = SCM, and A = ACM in **(B,C)**.

polycomb complex (Stock et al., 2007), was upregulated when human T-ALL cells were exposed to the adipocyte secretome (Figures 2C–E). In addition to ACM-mediated changes in gene expression regulators in human T-ALL cells, genes coding for histones were drastically altered in human T-ALL cultured in ACM relative to the other conditions tested (Figures 2C–E). Specifically, the *H2AFZ* gene (which encodes H2A.Z.1) was downregulated in ACM-cultured T-ALL cells (Figures 2C–E), and high expression levels of this gene are associated with more aggressive solid cancers such as hepatocellular carcinoma (Dong et al., 2021). In contrast, the *H3F3A* gene (which encodes H3.3) was upregulated in ACM-exposed T-ALL cells (Figures 2C–E). This observation is consistent with its role in increasing transcription (Park et al., 2016) and our pathway analysis results of human T-ALL cells cultured in the adipocyte secretome (Figure 2B; Supplementary Figure S3). Furthermore, H3.3 histones are deposited after DNA damage (Pinto et al., 2021), and the upregulation of *H3F3A* may result from the increased genomic instability observed in human T-ALL cells cultured in ACM (Figure 2B; Supplementary Figure S3). In addition, we observed that the gene expression levels of *H2AFX* were variable among ACM-cultured human T-ALL cells; whereas the *H3F3B* gene was expressed at similar levels under all conditions tested. This analysis identified adipocyte-induced transcriptional changes in human T-ALL cells, which precede cell death (Figure 1; Supplementary Figure S2).

The adipocyte secretome induces cell cycle progression in human T-ALL cells with concomitant DNA damage

Based on our RNA-sequencing analysis, which revealed substantial alterations in genes regulating the cell cycle and checkpoint responses to double-strand DNA breaks (*ATM*, *ATR*, *CHEK1*, and *CHEK2*) when human T-ALL cells were exposed to the adipocyte secretome, we next assessed cell cycle progression in leukemia cells cultured in unconditioned media, SCM, and ACM. After 3 days of culture, every human T-ALL cell line cultured in ACM exhibited increased cell cycle progression relative to responses observed in leukemia cells cultured in unconditioned or stromal cell-conditioned media

(Supplementary Figure S6A; Figures 3A,B). Notably, the percentage of leukemia cells in the SubG1 population was higher in more differentiated human T-ALL cells (Jurkat and Peer) cultured in ACM (Figures 3A,B), which is indicative of increased cell death (Supplementary Figure S2; Figure 1). Similar to the slower apoptotic responses observed in the ETP ALL cell line (Loucy), a higher percentage of cells were observed in S phase, compared to SubG1, when cultured in ACM relative to the other conditions tested (Figures 3A,B).

Given that cell cycle progression was induced in every human T-ALL cell line cultured in ACM, and our gene expression profiles demonstrating adipocyte-induced alterations in *CHEK1* and *CHEK2* levels in malignant T cells, we next determined total and activated CHEK1 and CHEK2 protein levels in leukemia cells cultured under each condition. The protein levels of CHEK1 were similar in all human T-ALL cell lines tested after 24 h of culture in unconditioned or conditioned media (Supplementary Figure S4B); whereas, paralleling the reduced gene expression levels observed, there were lower CHEK1 protein levels in Jurkat and Loucy T-ALL cells cultured for 48 h in ACM (Figure 3C). Unlike CHEK1 and CHEK2 total protein levels were equivalent in all T-ALL cells cultured in unconditioned and conditioned media at 24 and 48 h (Supplementary Figure S6B; Figure 3C). In support of ACM-induced DNA damage altering the cell cycle of human T-ALL cells, we observed rapid (Jurkat cells at 24 h) and increased (Jurkat and Peer cells at 48 h) γH2AX protein expression in human T-ALL cells cultured in ACM relative to the other conditions tested (Supplementary Figure S6B; Figures 3C,D). This response occurred concomitantly with increased activation of CHEK1 and to a lesser extent CHEK2 (Supplementary Figure S6B; Figures 3C,D). These observations suggested that malignant T-ALL cells were unsuccessfully attempting to repair damaged DNA due to increased cell cycle progression when cultured in ACM. In support of this hypothesis, we observed decreased activation of ATR (reduced pATR protein levels) in all human T-ALL cell lines cultured in ACM for 48 h relative to the other conditions tested while total protein levels remained equivalent (Supplementary Figure S6C). The significant increase in ACM-induced apoptosis observed in human T-ALL cells (Supplementary Figure S2; Figure 1) could not be explained by altered p53 protein levels or increased p53 activation, which could potentially be attributed to the mutated status of this tumor suppressor in the cell lines tested (Table 2). The DNA damage response (DDR) is activated in cells accumulating single-stranded breaks/replication stress or double-stranded breaks with the goal of repairing DNA lesions (Rouse and Jackson 2002; Harrison and Haber 2006; Harper and Elledge 2007). The DDR is mediated by ATR/CHEK1 (Cimprich and Cortez 2008) for single-stranded breaks or ATM/CHEK2 (Shiloh 2003) for double-stranded breaks, which results in DNA repair, cell cycle arrest, or apoptosis if lesions are not repaired as the cell cycle progresses (Jackson and Bartek 2009). Collectively, these results demonstrate that the ACM-induced T-ALL cell death is preceded by deregulation of the cell cycle with accompanying DNA lesions, which occurs concurrently with reductions in phospho-ATR activation.

The adipocyte secretome alters the epigenome of human T-ALL cells

Given the extensive gene expression changes associated with transcription, alterations in epigenetic modifiers, and histones in human T-ALL cells cultured in ACM relative to the other conditions tested (Supplementary Figure S3; Figure 2), we next determined how the adipocyte secretome impacted the nuclear chromatin landscape of human T-ALL cells. Adipocyte-mediated changes occurred very quickly in human T-ALL cells cultured in ACM relative to the other conditions tested. Culturing human T-ALL cells in ACM for 24 and 48 h led to a modest increase in the total H3 protein levels compared to the SCM and untreated conditions (Figures 4A–E; Supplementary Figure S7A). This result is consistent with replication-dependent histone biosynthesis (Armstrong and Spencer 2021). In addition, we observed 2- to 4-fold increases in transcriptional activation-associated histone posttranslational modifications (Hyun et al., 2017) on H3 (Figures 4A–E; Supplementary Figure S7A). These included increased acetylation (K9ac/K14ac/K18ac/K23ac/K27ac) and methylation (K4me3 and K27me3) PTMs on H3 in ACM-treated T-ALL cells compared to the control conditions. Histone H3 methylated at lysine 27 is associated with bivalent chromatin and transitional gene expression states (Kinkley et al., 2016) and gene silencing (Pan et al., 2018). Despite multiple cellular functions attributed to increased methylation on H3 at K4 and K27, our results demonstrate that adipocyte-secreted factors induce an epigenetic transition toward increased gene transcription (Supplementary Figure S3; Figure 2; Tables 3, 4).

To determine the mechanism of the adipocyte-mediated epigenetic fluctuations in human T-ALL cells, we investigated whether epigenetic machinery, chromatin-modifying enzymes, and structural proteins were altered in malignant T cells cultured in ACM. To this end, we tested the hypothesis that the increase in histone acetylation was accompanied by increased histone acetyltransferase (HAT) activity and/or lower histone deacetylation (HDAC). However, we observed no significant differences in HAT and HDAC activity in any of the cell lines, except increased HDAC activity in Jurkat T-ALL cells (Supplementary Figures S7C,D). To investigate the cause of increased H3K27me3, we measured changes in the histone methyltransferase complex polycomb repressive complex 2 (PRC2) *via* Western blot of EED, EZH2, and SUZ12 (Lehmann et al., 2012). We neither saw significant differences in PRC2 levels nor did we observe any changes in the chromatin proteins from the gene silencing complex PRC1 (BMI1 and

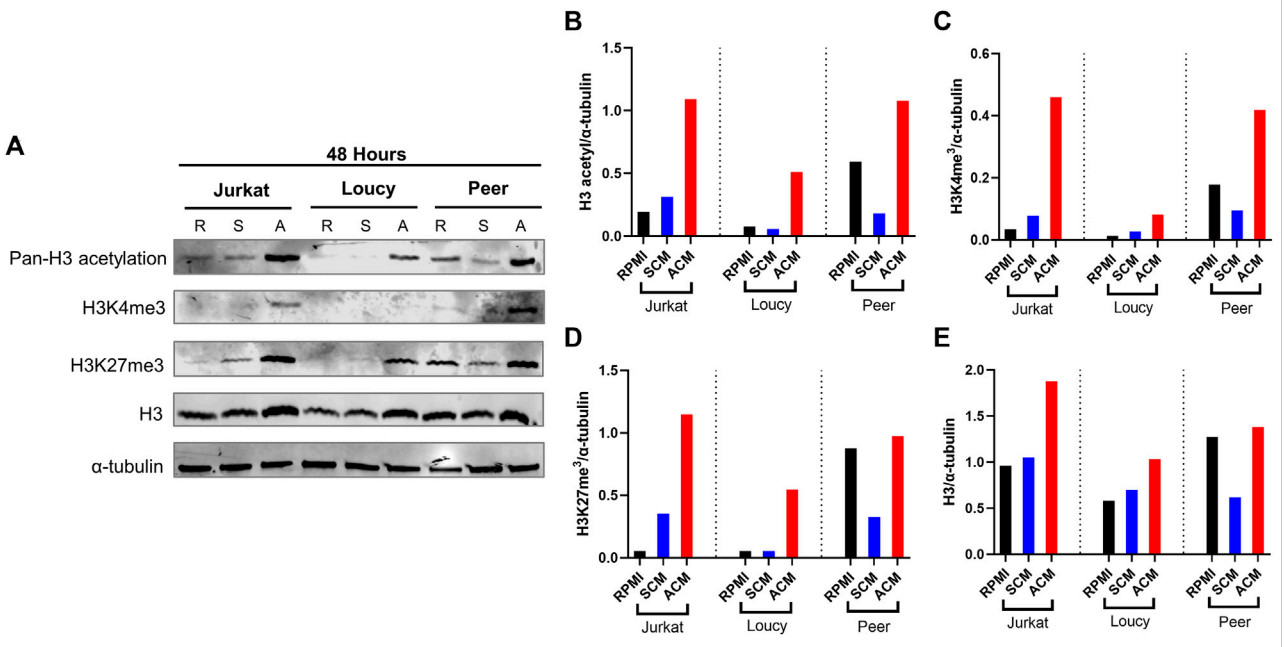


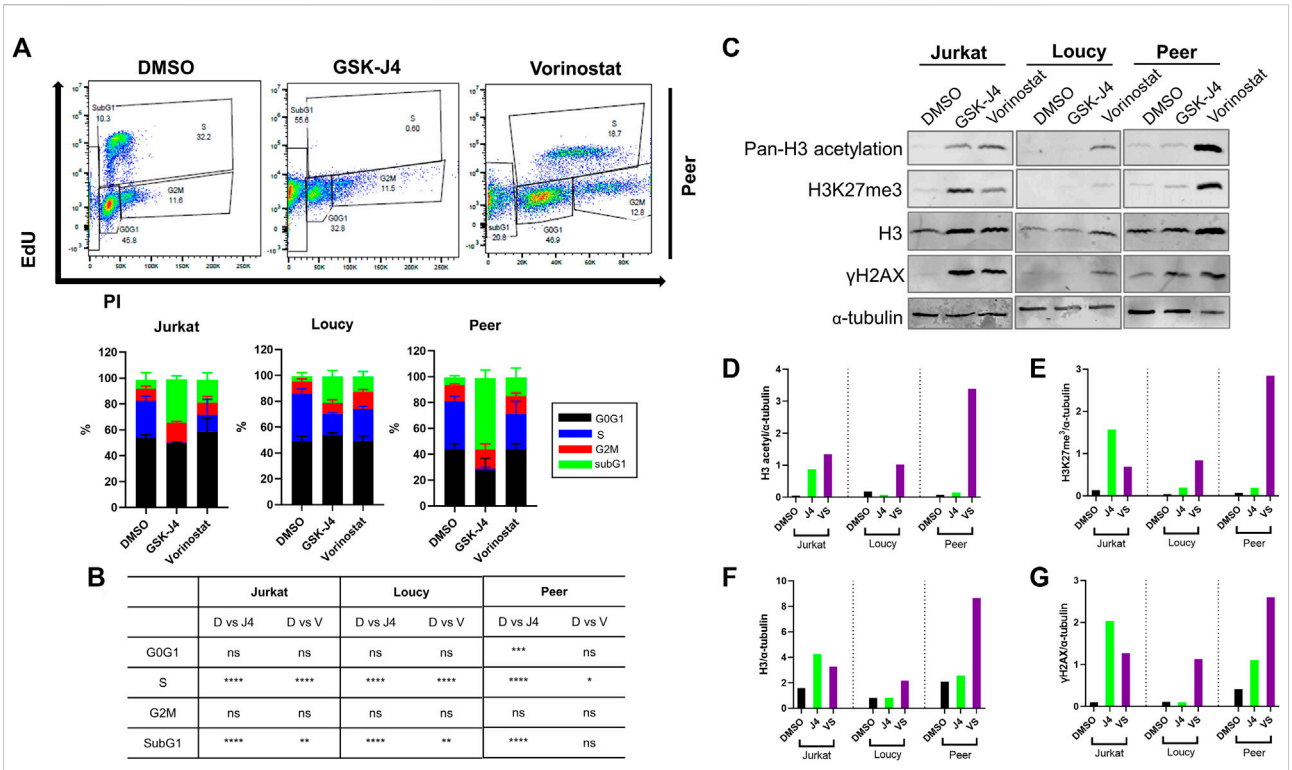
FIGURE 4
Adipocyte secretome alters the epigenome of human T-ALL cells. Human T-ALL cells (Jurkat, Loucy, and Peer) were cultured in unconditioned media, SCM, or ACM for 48 h, and Western blot analysis was conducted to determine the levels of modifications on histone 3 (H3) and total H3 levels. **(A)** Primary data from a Western blot experiment are shown. **(B–E)** Protein expression levels from Western blot experiments were determined using ImageJ analysis, and a representative blot from two independent experiments is shown.

RING1A) (Supplementary Figure S7B). These results demonstrate that increased H3K27me3 in adipocyte-exposed human T-ALL cells is not driven by changes in total PRC complex proteins; however, it is possible that adipocyte change the enzymatic activity of the PRC or its association with chromatin. Given the lack of detectable changes in the chromatin-modifying machinery, it is also possible that changes in the availability of epigenetic substrates (e.g., acetyl-CoA and SAM) might underlie ACM-induced epigenetic changes in human T-ALL cells (Phan et al., 2017). In all, these results demonstrate that the adipocyte secretome increases acetylation and methylation on H3, despite the induction of the inhibitory H3K27me3 PTM, augments transcription in malignant T cells.

Epigenetic modifying drugs phenocopy cell cycle and epigenetic changes induced by adipocyte-conditioned media in human T-ALL cells

To determine whether the relationship between changes in histone modifications and genomic stability in human T-ALL was causal, we sought to determine if treating human T-ALL cells with epigenetic modifying drugs phenocopied ACM-induced genotoxic stress in malignant T cells. For these experiments, we tested how epigenetic modifying drugs, which increase acetylation and methylation (Supplementary Figure S8A) altered T-ALL cell cycle progression, PTMs on H3, and the induction of DNA damage. Upon determining the IC_{50} of GSK-343 (a histone methyltransferase inhibitor), GSK-J4 (a histone demethylase inhibitor), C646 (a histone acetyltransferase inhibitor), and vorinostat (a histone deacetylase or pan-HDAC inhibitor), we decided to move forward with testing the effects of GSK-J4 and vorinostat due to their low IC_{50} values (Supplementary Figures S8B–F). Similar to the impact of ACM on human T-ALL cells, GSK-J4 and vorinostat augmented cell cycle progression leading to a significant increase in cells in the SubG1 phase of the cell cycle (Supplementary Figure S9A; Figures 5A,B), which is indicative of cell death (Supplementary Figures S8B–F, S9B).

Given that treating human T-ALL cells with GSK-J4 and vorinostat promoted cell cycle progression similar to responses observed when malignant T cells were cultured in ACM, we next assessed the impact of these treatments on H3 acetylation and methylation by determining their on-target effects. Both drugs performed as expected when human T-ALL cells were treated; GSK-J4 increased methylation and vorinostat augmented acetylation in human malignant T cells (Supplementary Figure S9C; Figures 5C–E). Interestingly, similar to responses observed



	Jurkat		Loucy		Peer	
	D vs J4	D vs V	D vs J4	D vs V	D vs J4	D vs V
G0G1	ns	ns	ns	ns	***	ns
S	****	****	****	****	****	*
G2M	ns	ns	ns	ns	ns	ns
SubG1	****	**	****	**	****	ns

FIGURE 5
Epigenetic modifying drugs phenocopy cell cycle and epigenetic changes induced by ACM in human T-ALL cells. Human T-ALL cells (Jurkat, Loucy, and Peer) were treated with DMSO (control), GSK-J4 (a histone demethylase inhibitor), or vorinostat (a histone deacetylase inhibitor) for 72 h, and cell cycles were assessed using EdU analysis and flow cytometry. **(A)** Representative primary data from three independent experiments (top panel) and **(B)** cell cycle distribution data (bottom panel) from the combined experiments are shown. **(C)** Cells treated in **(A)** were processed for Western blot analysis to determine the levels of modifications on histone 3 (H3), total H3 levels, and indicators of genotoxic stress (γH2AX). **(D–G)** Protein expression levels from the Western blot experiments were determined using ImageJ analysis and a representative blot from two independent experiments is shown.

in ACM-treated T-ALL cells, both drugs also increased H3 protein levels and promoted DNA damage (γH2AX) in human T-ALL cells (Supplementary Figure S9C; Figures 5C,F,G). Taken together, these results reveal that genotoxic stress induced in human T-ALL cells after treatment with GSK-J4 and vorinostat results from increased acetylation or methylation on H3, which mimics epigenetic alterations observed in ACM-treated T-ALL cells.

The adipocyte secretome augments the cytotoxic effects of epigenetic modifying drugs

Given that the adipocyte secretome and epigenetic modifying drugs promoted similar epigenetic changes and genotoxic stress in human T-ALL cells, we hypothesize that the adipocyte secretome would sensitize malignant T cells to the cytotoxic effects of epigenetic drugs.

To further assess genomic alterations induced by the adipocyte secretome and epigenetic modifying drugs, we performed a nucleosome protection assay, in which DNA was isolated from human T-ALL cells cultured in unconditioned media, SCM, ACM, or cells cultured with the histone demethylase inhibitor GSK-J4 or HDAC inhibitor vorinostat, treated with nuclease, and run on an agarose gel to elevate chromatin fragmentation in malignant T cells. These experiments revealed that ACM induced the most significant amount of DNA fragmentation relative to cells cultured in unconditioned media or SCM (Supplementary Figure S10A). Albeit to a lesser extent, treating human T-ALL cells with GSK-J4 and vorinostat resulted in a similar response, further demonstrating that both an adipose-rich microenvironment and epigenetic modifying drugs induce genomic alterations in human T-ALL cells (Supplementary Figure S10A). In further support of this conclusion, confocal analyses of DNA integrity in human T-ALL cells under each condition revealed that ACM, GSK-J4, and vorinostat induced similar amounts of nuclear

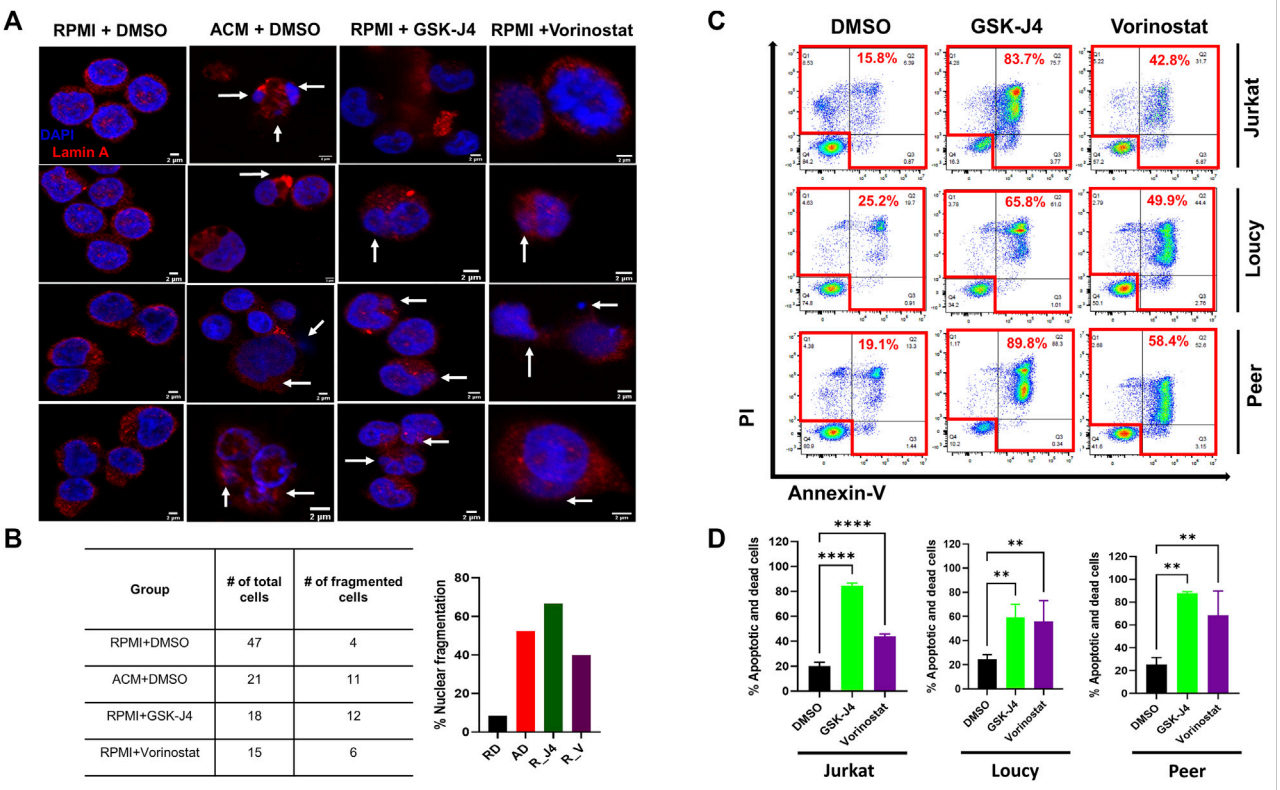


FIGURE 6
Treating human T-ALL cells with GSK-J4 and vorinostat phenocopy ACM-induced DNA damage and cytotoxicity in leukemia cells. Jurkat T cells were cultured in RPMI + DMSO, ACM + DMSO, or with RPMI + epigenetic modifying drugs (GSK-J4 or Vorinostat) for 48 h. The cells were then stained with lamin A with DAPI to visualize nuclei. **(A)** Representative images are shown with white arrows indicating nuclei spillage or fragmented nuclei. The percentage of cells harboring fragment nuclei, calculated by dividing the # of cells containing fragmented nuclei/total # of cell counted, is shown in **(B)**. **(C,D)** Human T-ALL cells (Jurkat, Loucy, and Peer) were treated with DMSO (control), GSK-J4 (a histone demethylase inhibitor), or vorinostat (a histone deacetylase inhibitor) for 72 h. The percentage of dead cells after 3 days of treatment was determined using Annexin-V/PI staining flow by flow cytometry. Representative primary data from one of three independent experiments are shown in **(C)** with quantitative data from combined experiments presented in **(D)**. Statistical significance was calculated using a one-way ANOVA followed by Tukey's multiple comparison post-test. $^{**}p < 0.01$ and $^{****}p < 0.0001$.

fragmentation (abnormal nuclei as determined by dysmorphic DAPI staining, nuclei shrinkage/condensation, or spillage of the nuclear protein Lamin A) in malignant T cells (Figures 6A,B).

Given our results demonstrating that epigenetic instability and genotoxic stress induce cell death in human T-ALL cells treated with ACM, we next determined the extent of cytotoxicity induced by single-agent treatments with GSK-J4 and vorinostat in malignant T cells. After 3 days of culture, we found that GSK-J4 treatment induced between 45 and 85% cell death in human T-ALL cells (Supplementary Figures S8B–D, S9B; Figures 6C,D) and treatment with vorinostat induced between 20 and 90% cell death in human T-ALL cells (Supplementary Figure S8B,E,F, S9B; Figures 6C,D). The amount of T-ALL cell death over 3 days of culture was largely dependent on the differentiation state of the leukemia cell. Notably, the cytotoxic effects of ACM, and single-agent GSK-J4, vorinostat, and C646 treatment was specific to human T-ALL cells, given that we did not observe cell death in non-malignant murine (Supplementary Figure S11) or human (Supplementary Figure S12) T cells when exposed under either condition.

We next determined if the adipocyte secretome sensitized human T-ALL cells to the cytotoxic effects of epigenetic modifying drugs. In most cases we observed that leukemia cells cultured in ACM were sensitized to the cytotoxic effects of epigenetic modifying drugs within 24–48 h (Figures 7A–G). Notably, this effect was more pronounced in the more differentiated Jurkat and Peer T-ALL cell lines, where cytotoxicity ranged from 40 to 95% over 2 days of culture for GSK-J4 and vorinostat (Figures 7A–G). Furthermore, the ACM-mediated effect was more pronounced in Jurkat T cells after 3 days of culture as cytotoxicity exceeded 90% in all cases (Supplementary Figure S10B). We are currently in the process of identifying the adipocyte-secreted factors, which induce

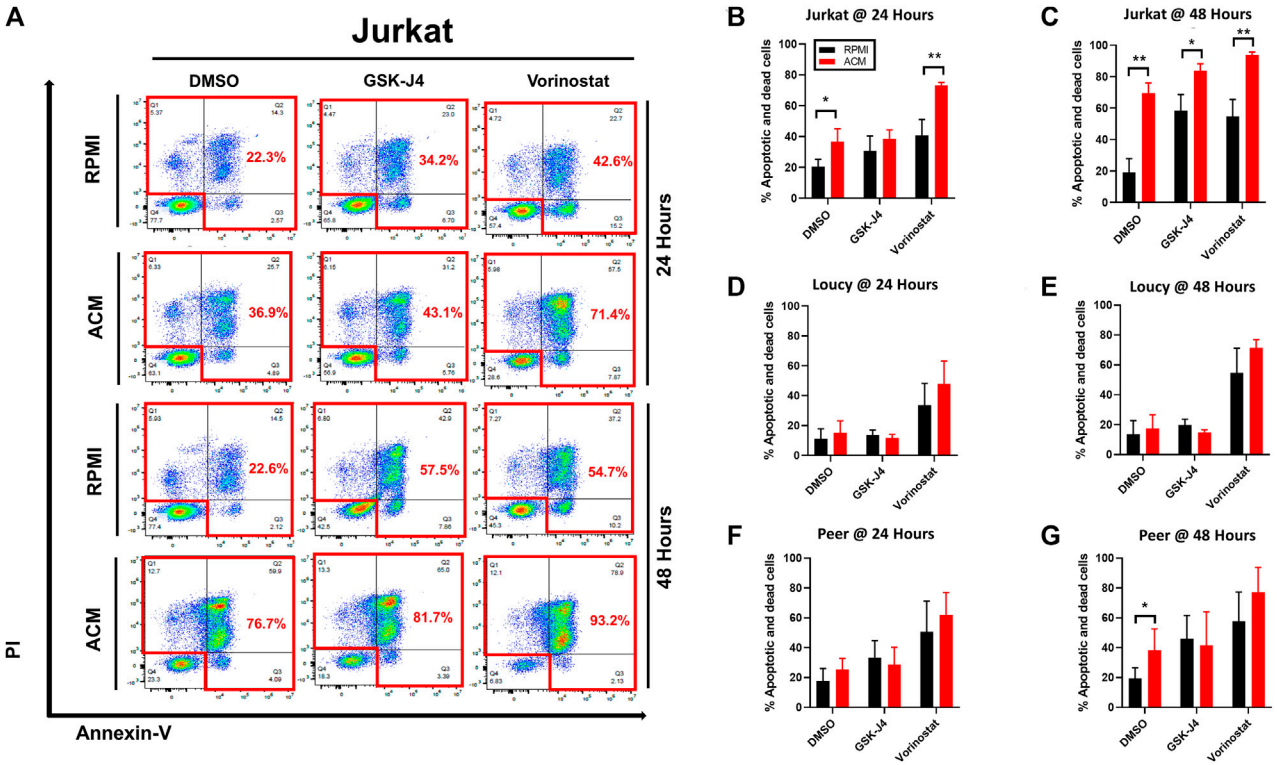


FIGURE 7
Adipocyte secretome augments the cytotoxic effects of epigenetic modifying drugs. Human T-ALL cells (Jurkat, Loucy, and Peer) were cultured in either RPMI or ACM with or without the epigenetic modifying drugs GSK-J4 or vorinostat for 24 and 48 h. T-ALL cell death was determined using Annexin-V/PI staining followed by flow cytometric analysis at both time points. **(A)** Annexin-V/PI flow cytometry data are shown for a representative of three independent experiments. **(B–G)** Quantitative data from combined experiments ($n \geq 3$) are presented. A Student's *t*-test was performed to determine statistical significance in **(B–G)**. $*p < 0.05$ and $**p < 0.01$.

human T-ALL cell death. We have performed mass spectrometry analysis of the adipocyte secretome and have identified candidates such as vascular non-inflammatory molecule 3 (VNN3), an ectoenzyme which induces inflammation (Wang N et al., 2018), as a potential contributor to apoptosis (data not shown).

Discussion

Obesity rates are increasing globally, with the United States being particularly affected by this pandemic (Finkelstein et al., 2012). In the United States, people with obesity are increasing in both adult (>19 years old) and pediatric (ages 2–19 years old) populations (Ogden et al., 2014; Andolfi and Fisichella 2018). In spite of the fact that obesity is a risk factor for developing and succumbing to many cancers (Butturini et al., 2007), these associations are still controversial for some cancer types such as T-ALL (Heiblig et al., 2015).

Using murine models of obesity, we present data in support of previously published epidemiological studies demonstrating that obesity is protective against T-ALL pathogenesis in pediatric populations (Heiblig et al., 2015). In both syngeneic and xenograft studies, obese mice exhibited a 20%–60% greater chance of surviving over 3 months post-leukemia cell transplantation relative to their lean counterparts. Furthermore, we demonstrate that the adipocyte secretome is highly cytotoxic to human T-ALL cells, which was not dependent on the p53 mutation status. Interestingly, the kinetics of apoptosis varied between more and less differentiated T-ALL cells, with extensive cell death being observed rapidly (2–3 days) for the more differentiated T-ALL cells, while the phenotypically immature T-ALL cells reached similar levels of cytotoxicity after 5 days of culture.

Mechanistically, we found that the adipocyte secretome promoted extensive gene expression changes in human T-ALL cells, which was highlighted by the activation of cell cycle, gene transcription, DNA replication, and DNA repair pathways. These alterations were associated with increased cell cycle progression with accompanying DNA damage, which could be partially explained by decreases in gene and protein levels of cell cycle regulators (notably *CHEK1* and *CHEK2*), although the

activation of these mediators was elevated in human T-ALL cells cultured in adipocyte-secreted factors. In addition to reducing *CHEK1* and *CHEK2* gene expression levels, the adipocyte secretome also suppressed *BMI1* transcription in more differentiated T-ALL cells as demonstrated by rapid death of human T-ALL cells when cultured in the adipocyte secretome. This is notable due to BMI1-mediated suppression of the *INK4A/ARF* locus and warrants further investigation into the regulation of this tumor suppressor in T-ALL cells exposed to adipocyte-secreted factors.

In addition, the phenotypic and functional changes in human T-ALL cells were accompanied by increases in H3 protein levels and selective posttranslational modifications of H3 (acetylation and tri-methylation of K4 and K27), which were indicative of gene activation and repression occurring in ACM-cultured human T-ALL cells. From our RNA-sequencing studies, we concluded that the modifications resulting in activation dominated in human T-ALL cells cultured in ACM, given our pathway analysis data demonstrating enhanced gene transcription and cell cycle progression in malignant T cells cultured in the adipocyte secretome.

Despite the epigenetic flux observed in ACM-stimulated human T-ALL cells, we did not observe increases in the detection of proteins, which compromise PRC1 (BMI1 and RING1A) or PRC2 (EZH2, EED, and SUZ12), which can monomethylate, dimethylate, and trimethylate H3K7 (Dobrinic et al., 2021). Histone 3 lysine 4 methylation is regulated by several histone methyltransferases [KMT2A-F; (Shilatifard 2012)] and demethylases [six known enzymes; (Hyun et al., 2017)]. Histone acetylation is also a highly dynamic process, which is regulated by 19 histone acetyltransferases (Roth et al., 2001) and 18 histone deacetylases (Bolden et al., 2006). To narrow down candidate epigenetic modifiers, which may be differentially regulated in ACM-exposed human T-ALL cells, we determined histone deacetylase (HDAC) and histone acetyltransferase (HAT) activities in malignant T cells cultured under each condition. Only one of the three human T-ALL cell lines exhibited a significant increase in HAT activity when cultured in ACM relative to the other conditions tested, while HDAC activity was elevated, albeit insignificantly, in the more differentiated human T-ALL cell lines. These results demonstrate that adipocyte-mediated PTM alterations on H3 in human T-ALL cells are neither driven by changes in total PRC complex proteins nor HDAC or HAT activity. Therefore, it is possible that the adipocyte secretome alters PRC assembly, its association with chromatin, or the availability of epigenetic substrates (e.g., acetyl-CoA and SAM) to mediated PTMs on H3 in human T-ALL cells and these possibilities are currently under investigation.

Given our findings that adipocyte-induced alterations in the T-ALL epigenome were associated with increased DNA damage and cell death, we also determined if inhibitors of enzymes regulating PTMs on H3 promote cell death in a similar manner. Indeed, we observed that epigenetic modifying drugs worked as expected with GSK-J4 and vorinostat increasing methylation and acetylation PTMs on H3 in drug-treated human T-ALL cells similar to profiles observed in ACM-cultured leukemia cells. Interestingly, we observed that GSK-J4 is cytotoxic to human T-ALL cells at dosages much lower (0.5–3 μM) than previously reported (2–10 μM) (Ntziachristos et al., 2014; Benyoucef et al., 2016), and we achieved ≥80% *in vitro* cytotoxicity if experiments were carried out for greater than 3 days (data not shown). Our results highlight the potency of GSK-J4 against this leukemia subtype. Furthermore, inhibition of either HDACs or histone demethylases promoted DNA damage and were highly cytotoxic to human T-ALL cells. Given that both the adipocyte secretome and inhibition of chromatin-modifying enzymes promoted cytotoxicity in human T-ALL cells by similar epigenetic mechanisms, we hypothesized that we would observe enhanced killing of adipocyte-exposed human T-ALL cell treated with GSK-J4 or vorinostat. For these studies, we found that the more differentiated human T-ALL cells were killed faster by treatment with HDAC or histone demethylase inhibitors if cultured in the adipocyte secretome, which suggest that adipocyte-induced changes to the epigenome of human T-ALL cells can be exploited to enhance the potency of epigenetic modifying drugs.

The most common mutation in human T-ALL cells is a deletion of *CDKN2A,* which occurs in about 70% of cases (Hebert et al., 1994). A deletion in *CDKN2A* gene confers survival properties to malignant T cells due to the gene product promoting the expression of the tumor suppressors p16(INK4A) and p14(Arf), which maintain cells harboring DNA damage from growing and dividing very rapidly. Therefore, most human T-ALL cells present with elevated genotoxic stress due to deletions in CDKN2A. Gain-of-function mutations in *NOTCH1* are also commonly found in human T-ALL cells (Weng et al., 2004). These mutations promote increased CDK2 activity (Sarmento et al., 2005), and thus, cell cycle progression (Dohda et al., 2007). Due to increased proliferation and the metabolic demands associated with this phenotype, replicative and genotoxic stress is also a common feature of malignant T cells with this mutation. In addition, mutations in epigenetic modifiers are common in human T-ALL cells (Liu et al., 2017). Mutations in *EZH2,* the catalytic subunit of PRC2, are the most common epigenetic mutation found in T-ALL (Wang C et al., 2018). However, the impact of mutations in this complex is unknown in T-ALL, given that mutations in PRC2 are associated with transformation and it also has been identified as a tumor suppressor in certain cancers (Wang C et al., 2018).

Based on our data demonstrating that there is a delicate balance between increased proliferation and genotoxic stress in human T-ALL cells, and that these traits are commonly associated with mutations in epigenetic modifiers, it stands to

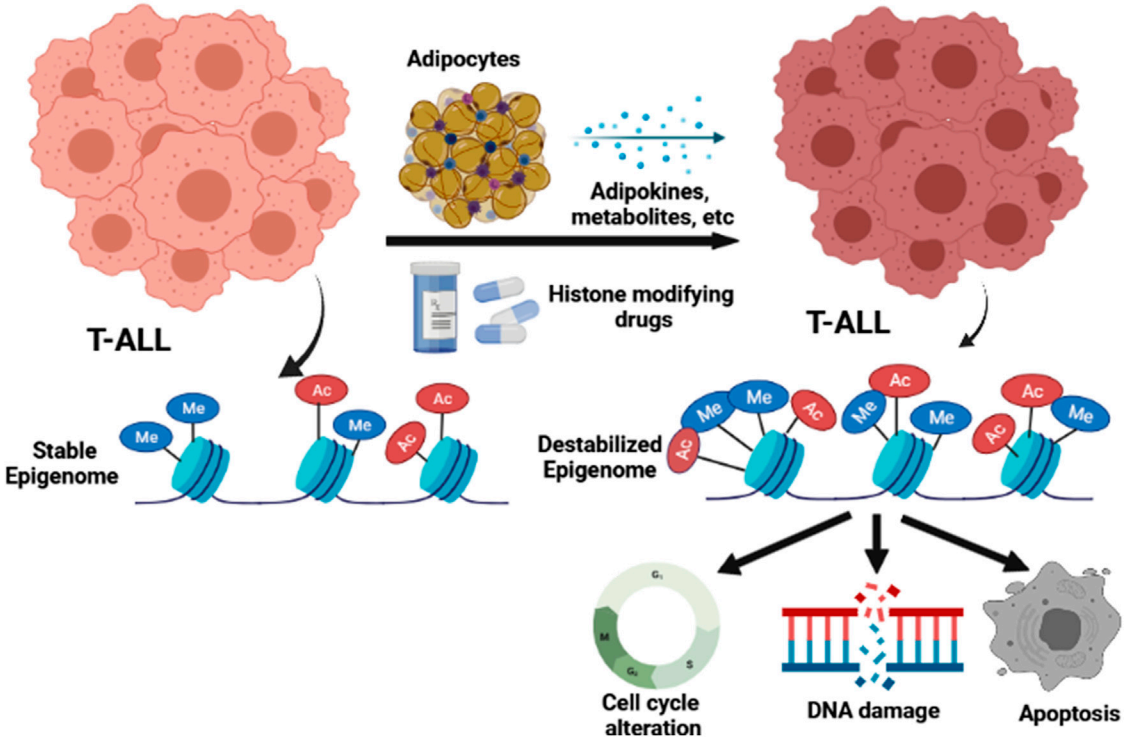


FIGURE 8
Model for how the adipocyte secretome and epigenetic modifying drugs impact the epigenome and function of human T-ALL cells.

reason that the latter property could be leveraged for therapeutic benefit. Indeed, vorinostat has shown preclinical efficacy in lean mice when combined with other treatment modalities for T-ALL (Gao et al., 2016; Jing et al., 2018) and was FDA-approved in 2006 for the treatment of cutaneous T cell lymphoma (CTCL) in patients with progressive, persistent, or recurrent disease (Chen et al., 2020). Despite the success of vorinostat in treating patients with CTCL, epigenetic modifying drugs are not FDA-approved to treat T-ALL. Encouragingly, preclinical assessment of the third-generation epigenetic modifying drugs ivosidenib (an isocitrate dehydrogenase 1 inhibitor) and tazemetostat (an EZH2 inhibitor) show enhanced potency and broader spectrum of cytotoxicity for hematological malignancies and solid cancers (Italiano et al., 2018).

Data presented in our study further support the continued exploration of inhibitors targeting chromatin-modifying enzymes as novel therapeutic strategies for T-ALL. In addition to vorinostat, we present data demonstrating that GSK-J4 and C646 induce significant cytotoxicity in human T-ALL cells at low micromolar concentrations. To our knowledge, data presented in our study represent the first reports of C646-mediated cytotoxicity of human T-ALL cells. This drug has also shown efficacy in models of AML, pancreatic, gastric, and cervical cancers at higher dosages (10–50 μM) than we observed in our study; however, preclinical dosages of 2 μM have been documented to translate to effective clinical responses (Gao et al., 2013; Ono et al., 2016; He et al., 2017; Wang et al., 2017; Ono et al., 2021). In addition to our results demonstrating that JMJD3/UTX and p300 inhibition may provide therapeutic benefit to patients with T-ALL, two additional manuscripts present preclinical evidence in support of this claim (Ntziachristos et al., 2014; Xia et al., 2019). Furthermore, to our knowledge, this study is the first to demonstrate that the small molecule inhibitor, C646, is capable of potently killing human T-ALL cells when used at clinically achievable doses. In contrast, GSK-343 required excessively high concentrations to kill human T-ALL cells; whereas, this small molecule inhibitor appears to be effective at killing solid cancers (Yu et al., 2017).

Conclusion

In summary, our studies reveal that the inherent epigenetic instability in human T-ALL cells can be usurped to promote extensive leukemia cell death, and we present data demonstrating that this mechanism explains adipocyte-mediated protection against T-ALL pathogenesis. Furthermore, our data reveal that

the adipocyte secretome and inhibitors that target the chromatin-modifying enzymes JMJD3/UTX and HDACs, induce similar epigenetic programs, which are highly cytotoxic to human T-ALL cells (Figure 8).

Data availability statement

The datasets generated during this study are available from the corresponding author upon request. The raw data and processed RNA-seq data used in this study are deposited in the GEO repository under accession number GSE202225 and can be accessed without restrictions (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE202225).

Ethics statement

The animal study was reviewed and approved, and all murine experiments received ethical approval from the Emory University School of Medicine Institutional Animal Care and Use Committee (IACUC) under the approved protocol number DAR-3000013.

Author contributions

ML contributed to the study design, collected/interpreted data, created manuscript figures, and drafted the manuscript. DG performed the RNA-sequencing pathway analyses using the Reactome software, created figures for these analyses, and drafted figure legends/materials/methods. JH performed the confocal microscopy studies in collaboration with ML and provided critiques on the drafting of the manuscript. CS, with the support of JB, performed the RNA-sequencing studies and deposited the primary data in the GEO repository. JS and KH provided epigenetic expertise and carefully reviewed the presented manuscript. CH conceived the study, provided overview for the study design, provided financial support for the study, analyzed data, assisted with figure creation, and drafted the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted without commercial or financial relationships, and thus, no conflicts of interest exist for the study presented.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.909557/full#supplementary-material

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Transcriptional regulation of INK4/ARF locus by cis and trans mechanisms

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9p21 locus is one of the most reproducible regions in genome-wide association studies (GWAS). The region harbors *CDKN2A/B* genes that code for $p16^{INK4a}$, $p15^{INK4b}$, and $p14^{ARF}$ proteins, and it also harbors a long gene desert adjacent to these genes. The polymorphisms that are associated with several diseases and cancers are present in these genes and the gene desert region. These proteins are critical cell cycle regulators whose transcriptional dysregulation is strongly linked with cellular regeneration, stemness, aging, and cancers. Given the importance of this locus, intense scientific efforts on understanding the regulation of these genes via promoter-driven mechanisms and recently, via the distal regulatory mechanism have provided major insights. In this review, we describe these mechanisms and propose the ways by which this locus can be targeted in pathologies and aging.

KEYWORDS

INK4/ARF, enhancer, 9p21, gene desert, $p15^{INK4b}$, $p16^{INK4a}$, ANRIL, CDKN2BAS

Introduction

The INK4/ARF locus functions are attributed to three distinct but related proteins, namely, $p14^{ARF}$, $p16^{INK4a}$, and $p15^{INK4b}$. These proteins are coded by two genes; *CDKN2A* and *CDKN2B*. $p14^{ARF}$ and $p16^{INK4a}$ are transcribed from the *CDKN2A* gene, whereas $p15^{INK4b}$ is transcribed from the *CDKN2B* gene (Figure 1). The initial exons of $p14^{ARF}$ (exon1β) and $p16^{INK4a}$ (exon1α) are different, but the second and third exons are identical. While the mRNA sequences of $p14^{ARF}$ and $p16^{INK4a}$ are relatively similar, the resultant proteins do not share any sequence similarity due to the alternative reading frames; thus, these proteins are not isoforms. On the other hand, $p15^{INK4b}$ and $p16^{INK4a}$ have a high degree of amino acid similarity (about 80%) and are thought to have emerged from a gene duplication event (Lopez et al., 2017). Additionally, there is a *CDKN2BAS* gene that transcribes a non-coding RNA known as ANRIL. Because ANRIL is transcribed in the antisense direction relative to *CDKN2B*, the gene is termed *CDKN2BAS*. Together, these proteins regulate the cell cycle progression and are known to operate as a barrier to the reprogramming of somatic cells. Inactivation of this locus due to homozygous deletions or epigenetic alterations such as transcriptional silencing by DNA methylation or polycomb-mediated suppression is a frequent event that occurs in a wide spectrum of

cancers. Furthermore, single nucleotide polymorphisms (SNPs) in this locus are associated with several aging-related disorders, including coronary artery disease (CAD), type 2 diabetes, and atherosclerosis. The majority of the SNPs in this locus are located within the genes and the ~0.3 Mb long adjacent gene desert region, but the mechanisms of their action are largely unknown. Thus, the identification of molecular pathways that regulate this locus in different diseases is of great therapeutic relevance.

Cell cycle regulation by INK4/ARF proteins

Several stress signals including oncogene overexpression, DNA damage, oxidative stress, etc., induce the expression of *INK4/ARF* genes (Romagosa et al., 2011). Once activated, these genes trigger a cascade of signaling events that effectively bring the cell cycle to a halt (Ivanchuk et al., 2001). Mechanistically, p53 (a well-studied tumor suppressor that blocks the cell cycle at the G1 phase) is a downstream effector of the $p14^{ARF}$ pathway (Sherr 2001). The interaction of MDM2 with p53 alters the stability and cellular localization of p53 (Kubbutat, Jones, and Vousden 1997). MDM2 acts as an E3 ubiquitin ligase and mediates the proteasomal degradation of p53 by ubiquitinating its C-terminal domain (Wade, Wang, and Wahl 2010). Multiple domains of p53 interact with MDM2, including the DNA binding domain (DBD), the transactivation domain (TAD), and the carboxy-terminal domain (CTD). MDM2, on the other hand, interacts with p53 via its N-terminal hydrophobic domain (HD) and acid domain (AD) (Chi et al., 2005; Yu et al., 2006; Poyurovsky et al., 2010). When expressed, $p14^{ARF}$ interacts with the acid domain of MDM2, preventing it from interacting with p53. This interaction alters the conformation of MDM2 that exposes its Nucleolar localization signal (NoLS) present in the RING domain (RD), leading to sequestration of the MDM2-$p14^{ARF}$ complex in the nucleolus (Weber et al., 1999; Maggi et al., 2014). Sequestration of MDM2 in the nucleolus prevents MDM2-mediated export of p53 to the cytoplasm, hence preventing its degradation (Maggi

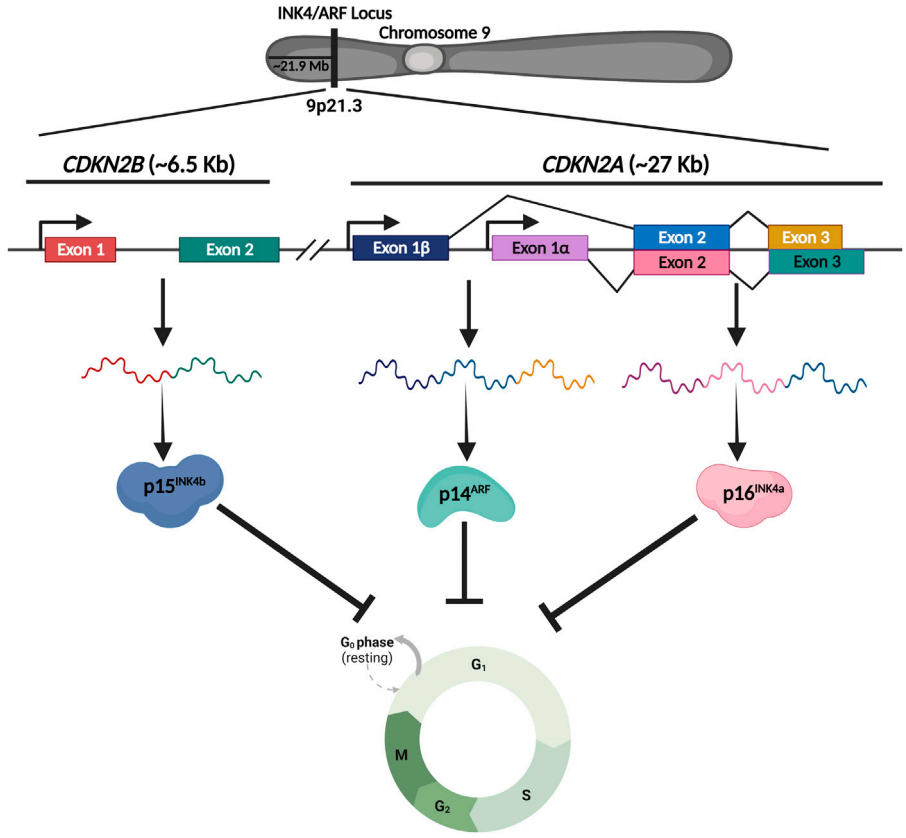

FIGURE 1
Schematic representing the genomic structure of INK4/ARF locus. INK4/ARF locus harbors two genes, *CDKN2A* and *CDKN2B*, that code for three critical cell cycle regulators. *CDKN2A* gene produces two proteins, $p16^{INK4a}$ and $p14^{ARF}$, whereas the *CDKN2B* gene produces $p15^{INK4b}$. Together these genes regulate the cell cycle under various conditions.

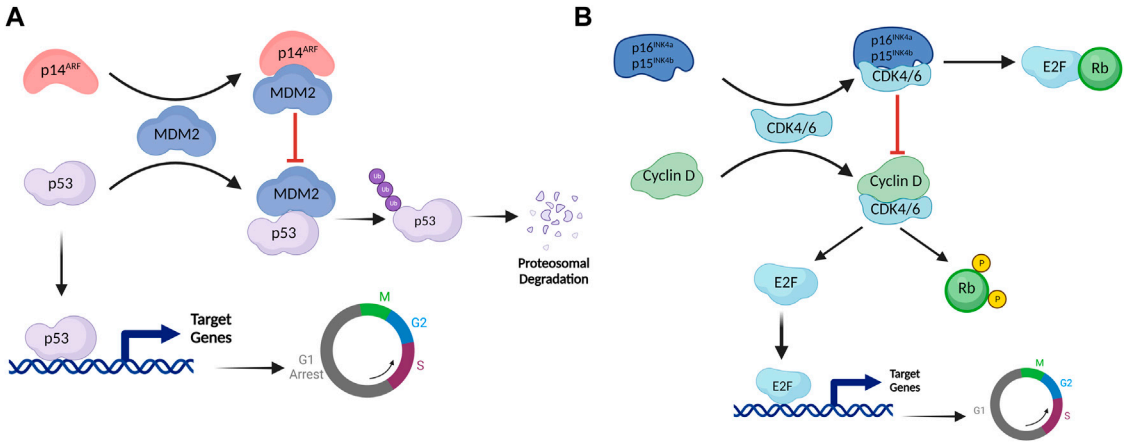

FIGURE 2
The INK4/ARF cell cycle regulatory network. **(A)** The p53-MDM2 pathway is controlled by the upstream effector protein $p14^{ARF}$. By establishing a complex with MDM2, $p14^{ARF}$ permits p53 to activate its transcriptional targets. MDM2 ubiquitinates p53, which mediates proteasomal degradation in normal conditions. However, when MDM2 interacts with $p14^{ARF}$, NoLS of MDM2 is exposed, resulting in MDM2 sequestration in the nucleolus. MDM2 sequestration prevents degradation of p53, allowing it to activate its transcriptional targets and arrest the cell cycle in the G1 phase. **(B)** The retinoblastoma pathway is regulated by $p16^{INK4a}$ and $p15^{INK4b}$. E2F is a transcription factor that activates genes involved in the transition from G1 to M phase. Rb inhibits this function of E2F by establishing a complex with it. Under normal conditions, the cyclin D-CDK4/6 complex phosphorylates Rb. Phosphorylated Rb doesn't engage with E2F, as a result, E2F binds to target genes to activate them. Once expressed, $p16^{INK4a}$/$p15^{INK4b}$ inhibits cyclin D-CDK4/6 complex formation, keeping Rb hypophosphorylated. Hypophosphorylated Rb forms a complex with E2F, inhibiting its transcriptional activity.

et al., 2014). These events lead to p53 translocation into the nucleus thereby, activating genes that cause the cell cycle to arrest at the G1 phase (Weber et al., 1999) (Figure 2A).

$p16^{INK4a}$ and $p15^{INK4b}$, on the other hand, regulate the retinoblastoma (Rb) pathway. These proteins activate Rb, a tumor suppressor protein that blocks the cell cycle at the G1 phase (Kim and Sharpless 2006). CDK4/6 typically forms an active complex with cyclin D that binds to and phosphorylates Rb (Cobrinik 2005). Rb loses its ability to interact with the E2F transcription factor in the phosphorylated state (Dimova and Dyson 2005). E2F activates genes involved in the cell cycle transition from G1 to S (Giacinti and Giordano 2006). When stress signals activate $p16^{INK4a}$/$p15^{INK4b}$, these proteins bind to CDK4/CDK6, causing an allosteric shift in the latter proteins, preventing them from forming the active complex with cyclin D, thereby maintaining Rb in a hypophosphorylated state (Hannon and Beach 1994; Russo et al., 1998). Hypophosphorylated Rb binds with the transactivation domain of E2; this complex subsequently recruits HDAC1 and SUV39H1 to the E2F target genes, thereby inhibiting them and preventing the G1 to S phase transition (Giacinti and Giordano 2006) (Figure 2B). These proteins being high in cellular senescence, permanently inhibit cell division. However, HPV-positive cancer cells express significant levels of $p16^{INK4a}$, $p14^{ARF}$, and $p15^{INK4b}$ without undergoing cell cycle arrest, attributed to two HPV-encoded oncoproteins, E6 and E7 (Kanao et al., 2004). These proteins inhibit the downstream effectors of $p14^{ARF}$ and $p16^{INK4a}$ genes, thereby preventing cell cycle arrest. E7 interacts with Rb, leading to its inactivation, whereas E6 induces the degradation of p53 protein (Munger et al., 1992).

Implication of INK4/ARF locus in aging, cancer, and regeneration

INK4/ARF locus in senescence/aging

Senescence is an innate cellular response in which normally proliferating cells cease to divide permanently in response to specific intrinsic and extrinsic stimuli. Senescent cells exhibit morphological and physiological changes, the formation of senescence-associated heterochromatin foci (SAHF), and the release of senescence-associated secretory phenotype (SASP), etc., (van Deursen 2014). This irreversible cell cycle halt is thought to be the first line of defense against cancer by preventing the division of abnormal cells (Prieto and Baker 2019). Senescence, on the other hand, plays a significant role in aging-related pathologies, as it impairs tissue repair and regeneration (McHugh and Gil 2018). Several recent studies have expanded our understanding of the role of senescence in other complex biological processes such as development, and

tissue repair, among others (Herranz and Gil 2018). $p16^{INK4a}$ is the fundamental driver and a well-established biomarker of senescence (Krishnamurthy et al., 2004; Rayess, Wang, and Srivatsan 2012). Studies have demonstrated that ectopic expression of oncogenes like Ras and Raf, increases $p16^{INK4a}$ expression, triggering premature senescence in various cell types (Lin et al., 1998; Zhu et al., 1998). For example, fibroblasts, epithelial cells, and T lymphocytes, express higher $p16^{INK4a}$ when they approach replicative senescence (Lin et al., 1998; Zhu et al., 1998; Mirzayans et al., 2012). In summary, the INK4/ARF locus regulates oncogene-induced and replicative senescence in several cell types (Mirzayans et al., 2012).

INK4/ARF locus in cancer

Cancer cells proliferate abnormally and do not respond to signals that regulate cell growth and division. Most frequently, cancer cells contain mutations in genes that regulate the cell cycle; once altered, these genes lose their ability to control the cell cycle (Papp and Plath 2011). As mentioned previously, *INK4/ARF* genes are cell cycle regulators that arrest the cell cycle at various stages in response to stress signals such as DNA damage. These tumor suppressor genes must be silenced for cancer to progress. Thus, INK4/ARF locus harbors homozygous deletions in several malignancies, silencing the expression of all three cell cycle regulator genes (Sherr 2012). Similarly, loss of $p16^{INK4a}$ expression through specific point mutations has been reported in several cancers (Forbes et al., 2006). The suppression of this locus by DNA hypermethylation at the promoters or through histone modifications mediated by the PRC2 complex is also prevalent in cancers. In animal studies, mice lacking either *INK4a* or *ARF* gene are more susceptible to certain tumors than mice lacking the *INK4b* gene. On the other hand, overexpression of the *INK4/ARF* genes results in a threefold reduction in tumor incidence in mice (Matheu et al., 2004).

INK4/ARF locus in cellular reprogramming

Cellular plasticity facilitates the reprogramming of somatic cells to a more pluripotent state. This reprogramming process considerably alters the epigenetic and chromatin landscapes of the cells (Papp and Plath 2011). A few critical transcription factors, like Oct4, Sox2, Klf4, Nanog, and others, can transform a somatic cell into a pluripotent cell (Papp and Plath 2011). However, the primary limitation of reprogramming is its significantly lower efficiency (approx. 1%). In the fast-dividing embryonic stem cells and induced pluripotent stem cells (iPSCs), the INK4/ARF locus is repressed. This locus, however, is activated during the reprogramming process as a result of highly mitogenic cell culture conditions (Sharpless 2005). As a result of the activation of this locus in somatic cells, reprogramming efficiency decreases significantly. Conversely, mouse embryonic fibroblasts (MEFs) lacking the INK4/ARF locus reprogram more efficiently with 15-fold higher efficiency (Li et al., 2009). While silencing *INK4a* or *ARF* alone improves reprogramming efficiency, double silencing results in increased efficiency, as seen in *INK4/ARF* null cell lines (Li et al., 2009). Not only is the efficiency increased, but the rate at which iPSC colonies develop is also increased in *INK4/ARF* defective cells. Interestingly, *ARF* is the primary regulator of cell reprogramming in murine cells, but *INK4a* is the dominant regulator in humans (Li et al., 2009).

Transcriptional regulation of INK4/ARF locus

Repression of INK4/ARF locus *via* PRC complexes

Polycomb group (PcG) proteins are epigenetic modifiers that play a crucial role in transcriptional repression and therefore regulate cell proliferation, differentiation, embryonic development, cellular memory, and other vital cellular functions (Wang et al., 2015). PcGs form two major protein complexes, the Polycomb repressive complex 1 (PRC1) and the Polycomb repressive complex 2 (PRC2). PRC2 exerts inhibition by adding trimethyl marks to lysine 27 of histone 3 (H3K27me3). The trimethyl mark serves as a docking site for PRC1, which recognizes this mark and monoubiquitinates Histone 2A at lysine 119 (H2AK119ub) (Chittock et al., 2017). The H2AK119ub further enhances H3K27me3 deposition by PRC2 and subsequent recruitment of PRC1 (Chittock et al., 2017). Both PRC1 and PRC2 are multimeric protein complexes with several core subunits and a few auxiliary subunits (Kerppola 2009). The PRC1 core consists of RING1A/B, PCGF2/4, CBX2/4/6/7/8, PHC1/2/3 subunits, while PRC2 contains Suz12, Ezh2, RbAp46/48, and Eed as core subunits (Chittock et al., 2017). Additionally, various auxiliary subunits aid or improve the activity of these complexes (Chittock et al., 2017). Ezh2, a SET domain-containing protein, is the enzymatic component of the PRC2 complex responsible for the trimethylation of H3K27. At the INK4/ARF locus, PcG inhibits the promoters by trimethylating H3K27 to increase cell proliferation (Bracken et al., 2007). PcGs have been demonstrated to repress all three *INK4a*, *ARF*, and *INK4b* genes in some instances, but only *INK4a* and *INK4b* in others (Bruggeman et al., 2005; Bracken et al., 2007; Kheradmand Kia et al., 2009). Ectopic expression of PcG subunits such as Bmi1, Ezh2, CBX7, and CBX8 has been shown to downregulate *INK4a* and *INK4b* expression to

bypass senescence (Jacobs et al., 1999; Gil et al., 2004; Dietrich et al., 2007). In contrast, depletion of the PcG subunits activates this locus, resulting in cell growth inhibition and senescence (Bracken et al., 2007; Dietrich et al., 2007).

Several transcription factors facilitate PcG binding to *INK4/ARF* promoters; for example, Zfp277, a zinc finger protein, interacts with the Bm1 subunit of PRC1 to recruit PRC1 to these promoters in MEFs (Negishi et al., 2010). Zfp277 depletion causes the PRC1 complex to displace from the promoters, activating *INK4a/ARF* gene and early senescence (Negishi et al., 2010). Similarly, Homeobox proteins such as HLX1 and HOXA9 play an essential role in suppressing *INK4a*. These proteins cooperate with PRC2 and HDACs at the *INK4a* promoter to mediate the repression (Martin et al., 2013). Haematopoietically expressed homeobox gene (Hhex) is vital in maintaining acute myeloid leukemia (AML), as its deletion causes upregulation of *INK4a* and *ARF*. Further, Hhex, like HLX1 and HOXA9, facilitates PRC2 binding to the promoters by interacting with the Suz12 subunit, thereby repressing the genes (Shields et al., 2016). In neonatal human diploid fibroblasts (HDFs), PRC2 binding to the *INK4a* promoter and the upstream region of the *INK4b* promoter induces a long-range interaction (repressive chromatin loop) between these promoters (Kheradmand Kia et al., 2009). Similar long-range interaction between the *INK4a* and *INK4b* promoters has been observed in hematopoietic progenitor cells and malignant rhabdoid tumors (MRTs) (Kheradmand Kia et al., 2009). In mature HDFs, however, the chromatin architecture of these genes is noticeably different where the looping between *INK4a* and *INK4b* is lost. Under such alterations, transcriptional activation and senescence induction occurs due to the concomitant loss of Ezh2 binding on promoters (Kheradmand Kia et al., 2009).

JMJD3-mediated transcriptional activation of INK4/ARF locus

Jumonji domain-containing D3 protein (JMJD3) is a lysine-specific histone demethylase. Its role in development, cancer progression, infectious diseases, immune disorders, and other conditions has been extensively studied (Xiang et al., 2007; Zhang X et al., 2019). JMJD3 belongs to the Jumonji (JmjC) domain-containing protein family, and this domain enzymatically catalyzes the removal of trimethyl marks from Histone 3 at lysine 27 (H3K27me3). Ubiquitously transcribed TPR protein on the X chromosome (UTX) is another demethylase that also demethylates H3K27me3 (Agger et al., 2007). While UTX is ubiquitously expressed, JMJD3 is induced in response to certain signaling events such as stress, etc (Swigut and Wysocka 2007). Due to its antagonistic role relative to PcG proteins, JMJD3 is a positive regulator of the *INK4/ARF* during the onset of cellular senescence (Agger et al., 2009; Barradas et al., 2009). Many cellular signals have been implicated in the induction of JMJD3 expression and subsequent activation of the *INK4/ARF* genes. For example, oncogene-mediated upregulation of JMJD3 causes activation of *INK4/ARF* genes in various cell types like fetal lung fibroblasts (IMR90), MEFs, etc., which results in INK4a-mediated growth arrest in these cells (Agger et al., 2009). By activating the INK4/ARF locus, JMJD3 prevents Schwann cells from proliferating uncontrollably in response to tumorigenic signals or following injury (Gomez-Sanchez et al., 2013). Under these conditions, JMJD3 binds to and demethylates the *INK4/ARF* promoters, activating these genes and initiating senescence. These cells lose the cell cycle control and continue to proliferate, resulting in neurofibromas when this pathway is disturbed (Gomez-Sanchez et al., 2013). As mentioned in previous section, INK4/ARF locus functions as a barrier to MEFs and keratinocyte reprogramming, and its silencing enhances reprogramming efficiency (Li et al., 2009). JMJD3 increases $p16^{INK4a}$ and $p14^{ARF}$ expression, limiting MEF reprogramming (Zhao et al., 2013). Therefore, JMJD3 silencing inhibits INK4/ARF-mediated cellular senescence, improving reprogramming efficiency. Moreover, double knockdown of JMJD3 and INK4a or ARF further enhances the reprogramming efficiency (Zhao et al., 2013).

KDM2B-mediated transcriptional repression of INK4/ARF locus

KDM2B is an epigenetic modifier that preferentially demethylates trimethylated lysine 4 (H3K4me3) and dimethylated lysine 36 of histone H3 (H3K36me2) (Frescas et al., 2007). It regulates numerous biological processes, including cellular senescence, differentiation, and stem cell self-renewal (He et al., 2008; Liang et al., 2012; He et al., 2013). Furthermore, it is highly expressed in various cancers and plays a crucial role in cancer progression, especially in leukemia (Yan et al., 2018). KDM2B associates with the promoters of the *INK4/ARF* genes and demethylates histones H3K36me2 and H3K4me3. Demethylation results in a decrease in PolII binding and an increase in H3K27me3. KDM2B suppresses this locus by epigenetic modifications of histones and also by preventing the downregulation of Ezh2 (Tzatsos et al., 2009). Consequently, KDM2B protects MEFs from replicative and oncogenic senescence, and its knockdown decreases proliferation and induces senescence. Another study showed that KDM2B functions as a proto-oncogene and inhibits senescence by negatively regulating *INK4b*. Similarly, KDM2B achieves repression of *INK4b* by removing the active H3K36me2 mark near the promoter and the gene body, whereas its knockdown causes increased expression of *INK4b* (He et al., 2008).

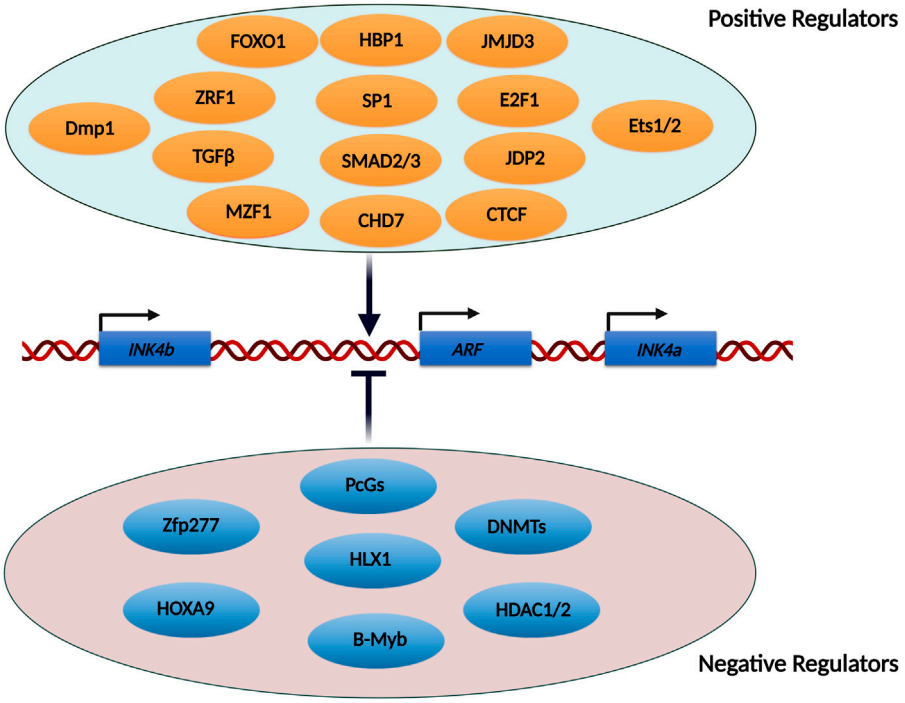

FIGURE 3
List of transcription factors and epigenetic modifiers known to regulate the INK4/ARF locus. The INK4/ARF locus is regulated by a number of transcription factors and epigenetic modifiers. Some of the factors stimulate transcription from this locus, whereas others repress it. Some of these factors act directly on the promoters, facilitating the binding of RNA polymerase, while others activate enhancers located upstream of the genes.

DNA methylation-mediated transcriptional repression of INK4/ARF locus

In addition to the aforementioned mechanisms, DNA methylation is another epigenetic mechanism to silence *INK4/ARF* genes. DNA methylation is catalyzed by DNA methyltransferases (Dnmts), which transfer the methyl group from S-adenosyl methionine (SAM) to carbon 5 of cytosine to generate 5-methylcytosine (5mC) (Lyko 2018). CpG islands in the promoters of tumor suppressor genes undergo abnormal hypermethylation in cancers (Robertson and Jones 1998). Notably, *INK4a* was one of the first genes discovered to be silenced in human cancers as a result of DNA methylation (Esteller et al., 2001). Numerous malignancies have been linked to aberrant CpG island methylation in the promoter region of the *INK4/ARF* genes. CpG islands are present near the promoter of *ARF* and exon1α of *INK4a* (Robertson and Jones 1998). Aberrant methylation of the *ARF* promoter is more prevalent than *INK4a* (Dominguez et al., 2003). Numerous types of cancer, including colon cancer, Merkel cell carcinoma, breast cancer, bladder tumors, and oligodendrogliomas, harbor abnormal DNA methylation of these genes (Watanabe et al., 2001; Tsujimoto et al., 2002; Lee et al., 2006; Lassacher et al., 2008).

Chromatin remodelling of INK4/ARF locus *via* SWI/SNF complex

SWI/SNF is a multi-subunit ATP-dependent complex. This complex is largely involved in chromatin remodelling, which facilitates gene transcription by allowing transcription factors to access their DNA binding sites (Wilson and Roberts 2011). Abnormal expression and mutations in the SWI/SNF components can cause cancer (Klochendler-Yeivin, Muchardt, and Yaniv 2002; Orlando et al., 2019). Malignant rhabdoid tumors (MRTs) exhibit the loss of the hSNF5 gene, which encodes one of the subunits of the SWI/SNF complex (Biegel et al., 1999; Sevenet et al., 1999; Roberts and Orkin 2004). On the other hand, ectopic expression of hSNF5 inhibits cell growth and induces cellular senescence (Oruetxebarria et al., 2004). It was found that hSNF5 exerts these effects via the $p16^{INK4a}$/Rb pathway, as the re-expression of hSNF5 in MRT cells activated *INK4b* and *INK4a*, but not *ARF* (Chai et al., 2005; Kia et al., 2008). hSNF5 activates *INK4a* in these cells by

recruiting the SWI/SNF complex to its promoter. As a result of its recruitment, the PRC1 and PRC2 complexes are displaced from the promoter (Kia et al., 2008).

Transcription factors involved in the regulation of INK4/ARF locus

Many transcription factors have been implicated in the regulation of the INK4/ARF locus. The majority of these transcription factors have been shown to act directly on the promoters of these genes. A few of them have been found to operate via upstream enhancer elements. While several transcription factors are required for the activation of the *INK4/ARF* genes, some have been demonstrated to inhibit their transcription (Figure 3). Due to the limited scope of this review, we have described only a few transcription factors involved in the activation of this locus.

FOXO1. FOXO1 is a tumor suppressor protein that inhibits Myc-induced lymphomagenesis in mice by activating the *ARF* gene. FOXO1 directly regulates *ARF* expression by binding to a motif located in the intron between exon1β and exon1α (Bouchard et al., 2007).

ZRF1. Zuotin-related factor 1 (ZRF1), a ubiquitin recognition domain-containing transcription factor, promotes the expression of PRC1-repressed genes during differentiation by competing for H2AK119Ub with PRC1 (Richly et al., 2010). ZRF1 expression is enhanced in MEFs and is recruited to the *INK4/ARF* promoters following hRas overexpression. Ectopic expression of ZRF1 activates *INK4a* and *INK4b* in IMR90, but not ZRF1delUBD, showing that ZRF1 binding to H2AK119Ub is necessary for its recruitment during senescence. (Ribeiro et al., 2013).

Dmp1. The deletion of Dmp1, a well-characterized tumor suppressor, accelerates tumor growth in mice. It acts as a link between Ras/Raf overexpression and *INK4/ARF* gene activation. Dmp1 expression is promoted by Ras overexpression, and it enhances *ARF* transcription by directly binding to Dmp1/ETS motif present in its promoter (Sreeramaneni et al., 2005).

JDP2. Jun dimerization protein 2 (JDP2) is a transcription factor that binds to JDP2 response regions and prevents histone acetylation and methylation (Huang et al., 2011). JDP2 is required for normal cell differentiation and proliferation, as MEFs lacking JDP2 do not undergo replicative senescence. Its overexpression inhibits MEF proliferation by increasing the expression of *INK4a* and *ARF* (Nakade et al., 2009).

CTCF. In U2OS cells, CTCF binds to a DNA sequence near the *ANRIL* promoter, and its silencing results in down-regulation of all three *INK4/ARF* genes. CTCF binding is lost when its DNA motif is methylated, resulting in the downregulation of these genes (Rodriguez et al., 2010).

CHD7. Chromodomain helicase DNA binding protein 7 (CHD7) is an ATP-dependent chromatin remodeler that plays a critical role in Ras-mediated senescence. It is essential for the activation of *INK4a* following Ras overexpression (Su et al., 2018). Transcription factors like c-Jun and Ets1 promote Myeloid zinc finger 1 (MZF1) expression during Ras-induced senescence, which in turn recruits CHD7 to the promoter of *INK4a* for its upregulation (Wu et al., 2022).

HBP1. HMG box-containing protein 1 (HBP1) transcription factor is a downstream effector protein in the Ras signaling pathway. *INK4a* promoter contains a putative binding motif for this transcription factor between positions −426 and −433. Its binding to this motif triggers cellular senescence (Li et al., 2010). HBP1 promotes acetylation of the *INK4a* promoter by assisting in the recruitment of histone acetyltransferase p300 and CREB-binding protein (CBP) (Wang et al., 2012). Furthermore, ectopic expression of HBP1 induces premature cellular senescence in normal fibroblasts via *INK4a*, while its knockdown delays senescence and senescence-associated phenotypes (Wang et al., 2012).

SP1. *INK4a* promoter has numerous GC-rich regions that are required for its induction upon senescence onset (Wu et al., 2007). SP1, a transcription factor, with a strong affinity for GC-rich motifs binds to these regions to enhance *INK4a* expression. In human fibroblasts, ectopic expression of SP1 upregulates the *INK4a* (Wu et al., 2007). Furthermore, SP1, like HBP1 physically interacts with p300/CBP to promote *INK4a* expression. (Wang et al., 2008).

Transcriptional regulation of INK4/ARF locus by distal regulatory elements

As stated earlier, the gene desert region upstream of the *CDKN2A/B* genes contains several SNPs that are strongly associated with the risk of CAD and type 2 diabetes in humans. In mice, deletion of this CAD (70 kb) interval resulted in a substantial decrease in the cardiac expression of *CDKN2A/B* genes, significantly increased mortality upon high cholesterol diet and ARF-dependent developmental abnormalities (Visel et al., 2010). Primary cells isolated from such mice showed increased proliferation compared to wild-type cells and exhibited no signs of senescence over subsequent passages (Visel et al., 2010). Furthermore, allele-specific expression analysis in heterozygous mice carrying a CAD interval deletion on one chromosome revealed that the *cdkn2b* gene was preferentially expressed from the allele with a wild-type CAD interval, but the expression of the allele bearing the CAD deletion was dramatically reduced in the heart and other organs, implying that CAD interval may regulate these genes through a distant-acting cis-regulatory mechanism (Visel et al., 2010). Further work indicated that the mice lacking the CAD interval developed primary vitreous hyperplasia at the E13.5 developmental stage. It is well established that TGFβ regulates *ARF* expression in developing eyes and MEFs

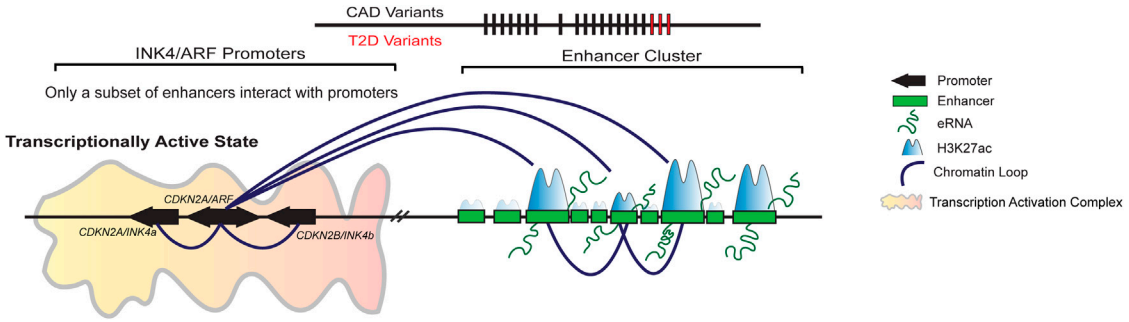

FIGURE 4
A subset of enhancers in the enhancer cluster upstream regulates INK4/ARF locus. Gene desert upstream of *INK4/ARF* genes contains 21 enhancers in HeLa cells. Only 15 of these enhancers are active, displaying both H3K27ac and H3K4me1 marks. Out of 15 active enhancers, only a subset of enhancers interacts with the promoters of *INK4/ARF* genes. The promoter interacting enhancers are critical for the regulation of these genes. The deletion of a single enhancer causes down regulation of gene transcription and EZH2 loading on the promoters. Furthermore, the deletion of interacting enhancers has an effect on the other enhancers in the cluster, indicating that the enhancers are interdependent.

(Freeman-Anderson et al., 2009). It leads to *ARF* induction in MEFs and HeLa cells (Zheng et al., 2010).

In pursuit of understanding how this interval regulates the expression of *INK4/ARF* genes and to biologically underpin the genetic variations in the interval seen in several diseases, the interval was tested for the presence of distal regulatory elements known as enhancers. Towards this, a landmark study established the presence of several enhancers in the gene desert region of this locus (Harismendy et al., 2011). A relationship between the CAD-associated genetic variations (rs10811656 and rs10757278) in one of the enhancer elements (ECAD9) where STAT1 binds upon IFNγ stimulation was established. STAT1 binding on the homozygous CAD risk allele was reduced in lymphoblastoid cells (LCL) therefore, the knockdown of STAT1 in LCLs that were homozygous for the non-risk CAD allele upregulated *CDKN2BAS* suggesting a repressive role of STAT1 on *CDKN2BAS* expression. However, HUVEC cells exhibited an activatory role of STAT1 on the expression of *CDKN2BAS* suggesting, the effects of CAD risk allele on *INK4/ARF* genes could be cell-type specific (Harismendy et al., 2011). Notably, the CAD risk interval contains a cis-acting enhancer that collaborates with TGFβ to promote *ARF* expression during development (Zheng et al., 2013), and mice lacking the CAD interval don't show such induction of *ARF*, implying that TGFβ works on *ARF* via the enhancers in CAD interval. Furthermore, TGFβ induces three H3K27ac peaks at the 110 kb distance from the *CDKN2A* promoter in HeLa cells and the deletion of a 20 kb area spanning all three peaks significantly lowers *ARF* and *INK4b* expression (Liu et al., 2019). These findings imply that TGFβ affects the transcription of these genes by activating the enhancers upstream of the genes (Liu et al., 2019). Macrophages derived from mice with an atherosclerosis susceptibility locus express significantly lower levels of *INK4a* and *ARF* (p19 in mice), but not *INK4b* (Kuo et al., 2011). Furthermore, individuals with the risk allele rs10757278, which has been related to an increased risk of atherosclerosis, have lower expression of all three *INK4/ARF* genes and even ANRIL in peripheral blood T-cells (Liu et al., 2009). Another study discovered a cis-regulatory region adjacent to the *ARF* promoter that represses *INK4a* gene expression. This element loops with the promoter of *INK4a* to repress its transcription. Perturbation of the element stimulated the transcription of the *INK4a* gene. (Zhang Y et al., 2019). All of these studies show a connection between disease-associated SNPs in the gene desert interval and *INK4/ARF* gene expression. Taken together, the risk alleles for CAD and atherosclerosis are primarily associated with lower expression of the *INK4/ARF* genes and these effects are cell-type specific.

Recently, we showed that the gene desert region upstream of the *INK4/ARF* genes contains 21 potential enhancer elements in the HeLa cells. Among these enhancers, 15 enhancers exhibited marks of active enhancers such as H3K27ac, PolII, and eRNA transcription. Out of these, only five active enhancers interacted with the *CDKN2A/B* gene promoters. However, disruption of any of these interacting enhancers but not non-interacting enhancer impacted the expression of *INK4a*, *ARF*, and *INK4b* at similar levels (Farooq et al., 2021). Interestingly, the interacting and non-interacting enhancers were indistinguishable at the levels of enhancer marks such as levels of H3K27ac, p300, and eRNA expression. This indicates that the bio-chemical marking of enhancers alone fails to predict enhancer activity (Farooq et al., 2021) (Figure 4). However, how SNPs in these enhancers regulate the locus in various diseases requires more efforts focused on functional studies to molecularly underpin the genetic variation and associated diseases in this locus. The

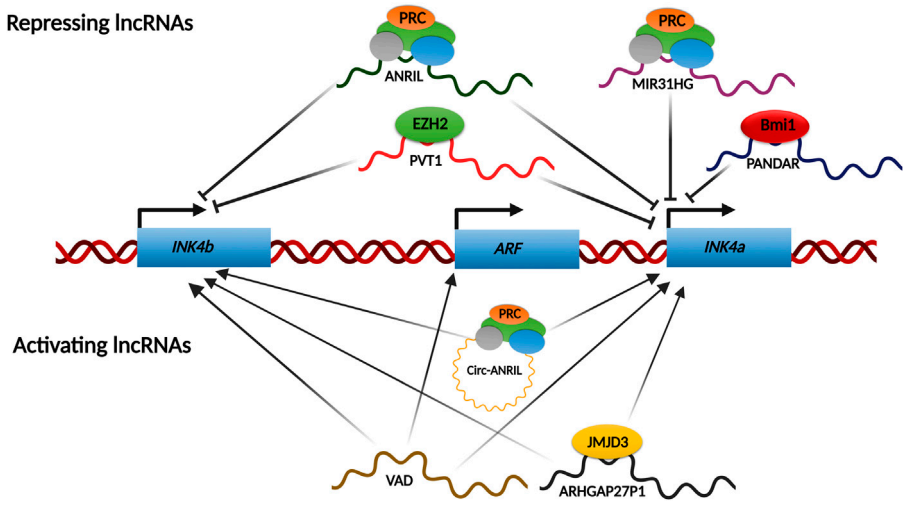

FIGURE 5
lncRNAs network regulating INK4/ARF locus. Various lncRNAs regulate the INK4a/ARF locus under certain conditions. Most of the known regulatory lncRNAs shut down the transcription from this locus by recruiting PcGs onto the promoters. However, some lncRNAs have been shown to activate this locus transcriptionally by either recruiting chromatin modifiers such as JMJD3 onto the promoters or by removing the repressive complexes like PcGs from the promoters.

resultant mechanistic understanding will pave the way for future therapeutic interventions.

LncRNAs as transcriptional regulators of the INK4/ARF locus

Long noncoding RNAs (lncRNAs) are a subclass of RNAs that are longer than 200 nucleotides and do not code for any protein product. They play critical roles in gene regulation, chromatin organization, translational regulation, etc. Several lncRNAs have been reported to influence the INK4/ARF locus expression (Puvvula 2019). Most of them are repressive and act by recruiting the PcG complexes onto the promoters of these genes. Recently, certain lncRNAs have been described to activate this locus (Figure 5).

LncRNAs-mediated repression of INK4/ARF locus

INK4/ARF locus contains a lncRNA, ANRIL, which is transcribed antisense to the genes. ANRIL is ~3.8 kb and has over 20 different splice variants. These splice variants of ANRIL play a differential protective role depending on the presence or absence of CAD risk interval (Lo Sardo et al., 2018). ANRIL is required for *INK4/ARF* silencing in growing cells, as its expression in these cells is inversely correlated to gene expression (Yap et al., 2010; Kotake et al., 2011). This repression is a result of PRC2 loading on the *INK4a* promoter by nascently transcribing ANRIL RNA (Yap et al., 2010). Another study demonstrated similar recruitment of PRC2 to the *INK4b* promoter (Kotake et al., 2011). Therefore, ANRIL expression decreases as senescence progresses for the activation of this locus. Through RNA binding experiments, ANRIL was shown to interact with the CBX7 component of PRC1 to enhance *INK4/ARF* gene silencing (Yap et al., 2010). Subsequently, ANRIL binding to SUZ12, a component of the PRC2 complex, was shown to enhance the silencing of *INK4b*, but not *INK4a* (Kotake et al., 2011). In contrast to these observations, ANRIL expression is positively linked with *INK4a/ARF* expression, in cervical cancers. In a recent study, we report that the PRC2 complex can bind to the *INK4/ARF* promoters independent of ANRIL levels in cervical cancer cell lines (Farooq et al., 2021). Another lncRNA, MIR31HG, which is transcribed from the short arm of chr9 itself, has been shown to recruit PRC complex on the *INK4a* promoter (Montes et al., 2015). Interestingly, during OIS (Oncogene induced senescence), MIR31HG localizes solely to the cytoplasm. This leads to the loss of the PRC complex from the *INK4a* promoter, resulting in transcriptional activation of *INK4a* (Montes et al., 2015). PANDAR (promoter of CDKN1A antisense DNA damage-activated RNA) is elevated in breast cancer tissues and cell lines. PANDAR interacts with Bmi1, a PRC1 subunit, inhibiting *INK4a* transcription by loading Bmi1 to its promoter. PANDAR silencing reduces cell proliferation and colony formation in MCF7 cells and causes G1/S arrest in a $p16^{INK4a}$-dependent manner (Sang et al., 2016). PVT1 is critical for gastric cancer progression. It accomplishes this in part by forming a complex with EZH2 and directing it to the promoters of *INK4b* and *INK4a*,

suppressing their expression in gastric cancer (Kong et al., 2015). ANROC, a recently discovered lncRNA, is found downstream of the *INK4a* gene, and its silencing results in overexpression of all three genes, indicating that ANROC is a repressive RNA (Kotake and Tsuruda 2020).

LncRNAs-mediated activation of INK4/ARF locus

LncRNA ARHGAP27P1 is downregulated in gastric cancer cells, and when overexpressed, it inhibits gastric cancer cell proliferation, migration, and other functions in a $p16^{INK4a}$ and $p15^{INK4b}$- dependent manner. This lncRNA regulates *INK4/ARF* expression by interacting with and directing the histone demethylase JMJD3 to the promoters for removal of the repressing H3K27me3 mark (Zhang G et al., 2019). AUF1 is an RNA-binding lncRNA that has been found to enhance the degradation of various RNAs. P14AS was identified using RNA capture sequencing as a novel RNA with its promoter located on the antisense strand of the fragment near *CDKN2A* exon1β. P14AS binds to AUF1, preventing ANRIL/INK4a RNA from interacting with AUF1. This competitive interaction between P14AS and AUF1 promotes *ARF*, *INK4b*, and *INK4a* gene expression (Ma et al., 2020). During OIS, VAD (Vlinc RNA Antisense to DDAH1) is highly upregulated and required to maintain senescence characteristics. VAD functions in trans on the INK4/ARF locus, and its depletion causes downregulation of *ARF*, *INK4b*, and *INK4a*. VAD promotes the expression of these genes by removing H2A.Z from their promoters. H2A.Z deposition represses these genes by promoting the recruitment of the PRC complex to the promoters (Lazorthes et al., 2015). Several circular ANRIL isoforms have been identified that activate the *INK4/ARF* genes rather than inhibiting them. They switch from repressors to activators of these genes during RAF1-mediated senescence. These circular isoforms engage with Polycomb subunits and displace EZH2 from the *INK4b* and *INK4a* promoters, stimulating transcription of these genes. As a result of the PRC2 dislocation, H3K27me3 levels at these promoters drop (Muniz et al., 2021). Similarly, TUBA4b is downregulated in CRC tissues and cells, and its overexpression inhibits CRC cell proliferation by upregulating *INK4a* and *INK4b* (Zhou, Sun, and Zhou 2020). The precise mechanisms by which TUBA4b long noncoding RNA activates these genes are unknown.

Discussion

Since the products of the *INK4/ARF* genes are implicated in a wide range of cancers and age-related diseases, they hold immense promise for treating or mitigating the consequences of these diseases. Regulation of the INK4/ARF locus is multi-layered, with a plethora of factors involved. As a result, the greatest challenge in harnessing this locus for therapeutic purposes is identifying the critical regulatory elements that can be targeted in a particular disease. Targeting transcription factors or epigenetic modifiers involved in its regulation has very broad effects, affecting not just this locus but others as well. We recently uncovered a few enhancers in the upstream enhancer cluster that regulate these genes. These DNA regulatory elements can be altered to provide a more precise and targeted effect. Since this enhancer cluster contains multiple enhancers, these enhancers may act in a tissue type-specific manner. Thus, determining which enhancers regulate these genes under various physiological conditions is critical for the therapeutic use of enhancers or enhancer products like eRNAs. Additionally, SNPs in upstream enhancer regions have been associated with several diseases. These SNPs can facilitate the identification of regulatory enhancers in various cell types. Thus, a functional genomics approach is required to decipher how these SNPs result in changes in gene regulation.

Author contributions

UF and DN have prepared the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Resetting the epigenome: Methylation dynamics in cancer stem cells

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The molecular mechanisms that regulate stem cell pluripotency and differentiation has shown the crucial role that methylation plays in this process. DNA methylation has been shown to be important in the context of developmental pathways, and the role of histone methylation in establishment of the bivalent state of genes is equally important. Recent studies have shed light on the role of RNA methylation changes in stem cell biology. The dynamicity of these methylation changes not only regulates the effective maintenance of pluripotency or differentiation, but also provides an amenable platform for perturbation by cellular stress pathways that are inherent in immune responses such as inflammation or oncogenic programs involving cancer stem cells. We summarize the recent research on the role of methylation dynamics and how it is reset during differentiation and de-differentiation.

KEYWORDS

chromatin, epigenetics, epitranscriptomic modification, cancer stem cells, RNA methylation, readers

Introduction

The field of 'Epigenetics' has led to a "paradigm shift" in several domains of biomedical research (Deichmann, 2016). Waddington proposed the "epigenetic landscape" (EL) model in 1940, depicting a series of developmental options that a differentiating cell in the embryo could choose from. Epigenetics is now defined as "mitotically and/or meiotically heritable alterations in gene function that cannot be explained by changes in the DNA sequence." The pluripotency of the undifferentiated cell and the eventual development of specific cell types is heavily reliant on the coordinated action of hundreds of transcription factors that bind to particular DNA regions to activate or repress cell lineage specific gene transcription (Srivastava and DeWitt, 2016). This establishment phase most closely reflects what is regarded as Conrad Waddington's description of epigenetics, namely the study of the mechanisms by which the genotype gives the developmental phenotype. The maintenance phase usually involves a plethora of non-DNA sequence-specific chromatin cofactors that accumulate and maintain

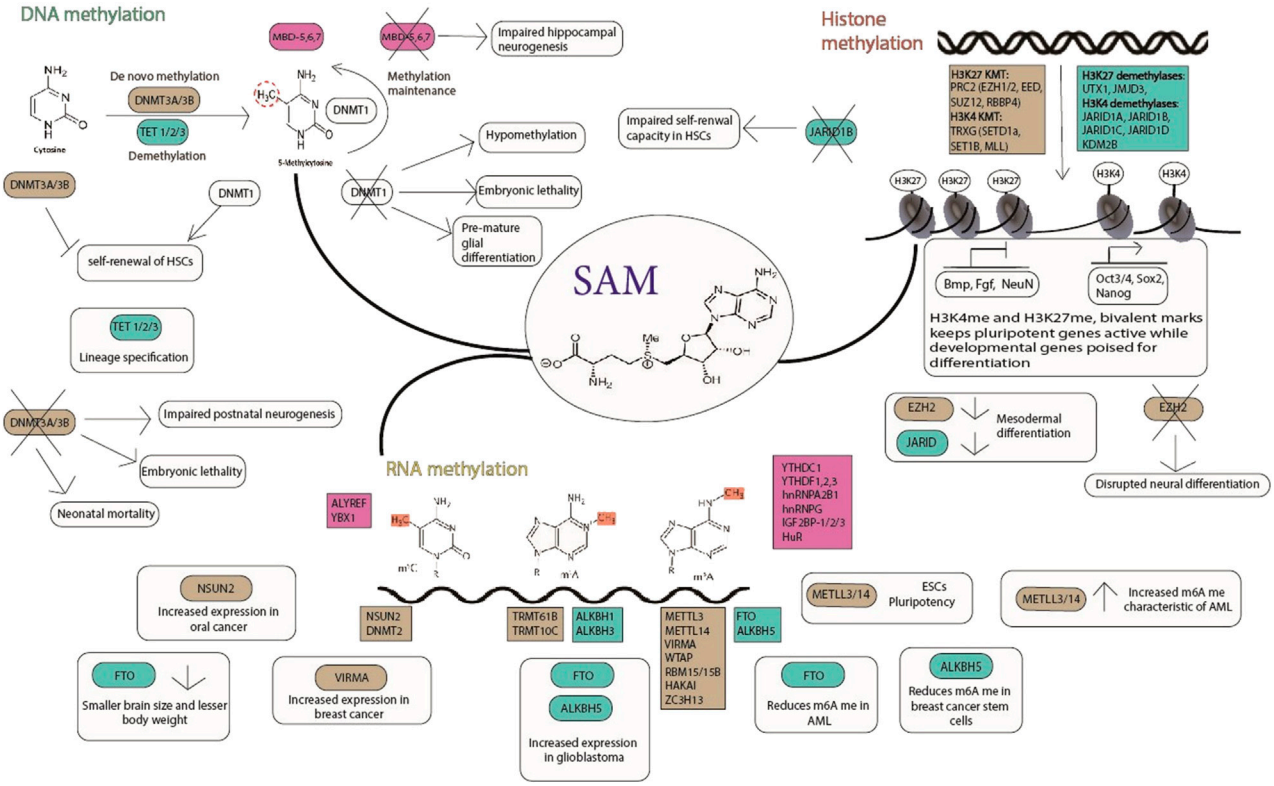

FIGURE 1
Methylation dynamics in stem cells; the readers (pink), writers (brown), and erasers (green) are indicated. SAM (S-adenosyl methionine) is the common methyl donor for histone, DNA and RNA methylation. The writers, erasers and readers play important roles in maintaining pluripotency and lineage commitment of stem cells. Their role in physiology and pathophysiology is depicted in the figure.

chromatin states through multiple cell divisions and for extended periods of time—sometimes even in the absence of the initial transcription factors (Schuettengruber et al., 2017).

Methylation dynamics in stem cells

The stem cells have been excellent cellular models to understand the molecular mechanisms of epigenetics. Stem cells are capable of self-renewal and differentiation to all three lineages, and can be classified as follows: a) Naïve stem cells (derived from the zygote of the mammalian embryo, capable of self-renewal and unrestricted differentiation potential), b) Primed stem cells/Epiblast stem cells (EpiSCs) (that originate from the zygotic stage immediately after maternal redetermination post implantation, capable of self-renewal but have a more lineage restricted differentiation potential, c) Embryonic stem cells (ESCs) (derived from the inner cell mass of the blastocyst, capable of self-renewal and multi-lineage differentiation potential, d) Adult stem cells (ASCs), found in adult tissues and organs within their respective niche responsible for maintaining tissue homeostasis, repair and regeneration. These stem cells remain in a quiescent state till activation by a signal like cell damage, and capable of self-renewal and multi-lineage differentiation potential, e) Cancer stem cells (CSCs) that are derived from the dedifferentiation of cancer cells or from the malignant transformation of normal stem cells. These cells like any other stem cells have self-renewal abilities and multi-lineage differentiation potential and play a major role in the prognosis of the disease (Zhou and Zhang, 2008; Harikumar and Meshorer et al., 2015; Morena et al., 2018).

These unique characteristics of a stem cell are regulated by molecular mechanisms that involve transcription factors, signalling pathways, epigenetics and epitranscriptomics. Transcription factors such as Oct3/4, Sox2, c-Myc and Nanog bind to their target genes and regulate their expression (Harikumar and Meshorer et al., 2015). Many signalling pathways such as the JAK/STAT, PI(3)K, MAPK, Wnt, Notch, Smad and FGF pathways play major roles in regulating stemness. The epigenome dynamics contributes to the regulation of stemness which includes biochemical modification of DNA, RNA, histone proteins, and chromatin. These modifications

are carried out by specific enzymes where the "writer" and "eraser" proteins catalyse the addition and removal of the modifications respectively, while other proteins called "reader" proteins specifically recognize these modifications (Figure 1).

DNA Methylation: A family of DNA methyltransferases (DNMTs), catalyzes the Methylation of cytosine's fifth carbon position, leading to 5-methylcytosine (5 mC) formation. DNMT1 copies existing methylation patterns for inheritance during DNA replication, while DNMT3A and DNMT3B act as *de novo* methyltransferases to create new methylation patterns (Bestor, 2000). A group of methyl-CpG-binding proteins acts as readers, interpreting the 5 mC signal and mediating its role. While DNA methylation can be 'passively diluted' by cell division, mechanisms for enzymatic DNA methylation removal have been recently discovered. The ten eleven translocation 1 (TET1) enzymes, catalyzes the conversion of 5mC to 5hydroxymethylcytosine (5hmC) (Tahiliani et al., 2009). Following that, three TET family proteins were discovered to be able to oxidize 5hmC to 5formylcytosine (5 fC) and then to 5carboxylcytosine (5caC) (Ito et al., 2010; He et al., 2011; Ito et al., 2011). In addition, the deaminases activation induced cytidine deaminase (AID; also known as AICDA) and apolipoprotein B mRNA editing enzyme catalytic polypeptides (APOBECs) can convert 5hmC to 5-hydroxymethyluracil (5hmU). To complete the active DNA demethylation process, thymine DNA glycosylase excises all of these derivatives and replaces them with an unmodified cytosine through the base-excision repair (BER) pathway (Wu and Zhang, 2014).

Histone methylation and demethylation: Histone methylation is a dynamic process that plays important functions in differentiation and development (Eissenberg and Shilatifard, 2010). Basic residues like lysine and arginine undergo methylation and can have several methylations on their side chains (Greer and Shi, 2012). H3K4me3 and H3K27me3 are two histone modifications that have been linked to active and repressive transcription, respectively. A variety of lysine methyltransferases (KMTs) as writers and lysine demethylases as erasers can mediate dynamic methylation of lysine residues. Many proteins, including the well-known PcG repressive complex (PRC) and Trithorax active complex (TRXG), have KMT properties (Schwartz and Pirrotta, 2007; Greer and Shi, 2012). Methylation of H3K4, H3K36, and H3K79 is associated with transcriptional activation, and methylation of H3K9, H4K20, and H3K27 is related with transcriptional repression. Notably, "bivalent domains" which are thought to be crucial for maintaining pluripotency by silencing developmental genes in embryonic stem cells (ESCs) and keeping them ready for activation during developmental stage—are formed when large regions of H3K27 methylation co-occur with smaller regions of H3K4 methylation marks (Bernstein et al., 2006; Hu et al., 2013).

RNA Methylation: More than 100 post-transcriptionally modified ribonucleosides have been found in various forms of RNA (Jia et al., 2013). N6-methyladenosine (m6A) is a conserved modification found in most eukaryotic nuclear RNAs, as well as some viral RNAs replicating in the host nuclei (Carroll et al., 1990). m6A was discovered as an abundant nucleotide modification in eukaryotic messenger RNA in 1970 (Desrosiers et al., 1974). In global cellular RNAs, m6A is found in 0.1–0.4% of all adenosines and accounts for almost half of all methylated ribonucleotides. m6A modification is enriched in long internal exons, upstream of stop codons, and the 3′-UTR of mRNA, suggesting roles in translational regulation, affecting RNA binding protein affinities, or distinctive m6A derived transcriptome topology (Dominissini et al., 2012; Meyer et al., 2012; Batista et al., 2014). The discovery of proteins involved in m6A regulation, as well as their roles as "writers" (m6A methyltransferases), "erasers" (m6A demethylases), and "readers" (effectors recognizing m6A), has been one of the most significant achievements in this field of study (Lee et al., 2014), together facilitate various functional outcomes, including nuclear RNA export, splicing, mRNA stability, circRNA translation, miRNA biogenesis, and lncRNA metabolism (Roignant and Soller, 2017; Yang et al., 2017) thus regulating physiological and pathological events such as Yeast meiosis, plant development, immunoregulation obesity, and carcinogenesis (Wang et al., 2017; Wei et al., 2017).

The epigenome in embryonic stem cells

Nucleosomes of stem cells show a higher level of modifications marks that are involved in active gene expression such as histone H3 lysine four trimethylation (H3K4me3), histone H4 lysine 9 and 14 acetylation (H3K9ac, H3K14ac). The two methyl modifications on H3K4 and H3K27 form a bivalent chromatin mark which is seen in the chromatin of stem cells. In stem cells, the highly conserved non-coding elements (HNCE) were found to be enriched with bivalent histone modifications, an active chromatin mark, H3K4me3 and a repressive chromatin mark, H3K27me3 (Bernstein, et al., 2006; Harikumar and Meshorer, 2015). These modifications are also abundant at promoter regions of genes that code for other factors required during development (Lessard and Crabtree, 2010). It is proposed that this bivalent chromatin mark resolves and there is activation of a few genes to regulate stemness while keeping other genes required for development poised for activation during development and cell differentiation (Bernstein et al., 2006; Lessard and Crabtree, 2010; Harikumar and Meshorer, 2015; Paranjpe and Veenstra, 2015). Recent studies have shown that many lineage-commitment genes have the bivalent mark and RNA polymerase II may be stalled at the promoters of these genes. During differentiation, the chromatin modifications are resolved into either an active or repressed state depending on the lineage commitment and these modifications can be newly established or

maintained in differentiating cells (De Gobbi et al., 2011). Many early genes involved in the determination of the mesodermal lineage including various members of the GATA and Tbx families, Mixl1, and Brachyury, have bivalent domains in ES cells, supporting the notion that they are important early contributors (Pan et al., 2007). Histone arginine methylation has been shown to be important for pluripotency maintenance as well as lineage specification (Torres-Padilla et al., 2007; Selvi et al., 2015; Cui et al., 2017). Recent studies have shown that the RNA modifications have an important role in stem cell maintenance. The writer proteins are involved in controlling the expression of critical transcripts that are essential for stem cell self-renewal. m^6A is shown to regulate molecular switches for differentiation and generation of EpiSCs, as well as in adult stem cells, like myeloid differentiation of hematopoietic stem cells (HSCs) (Morena et al., 2018).

The epigenome during differentiation

The embryonic stem cells undergo multiple rounds of differentiation, resulting in multipotent or unipotent adult stem cell progenitors. Extrinsic differentiation signals and intrinsic pathways interact and tightly regulate how stem cells differentiate. The formation of neurons and other ectodermal lineage cell types, has been one of the most well studied differentiation pathways. The perturbation of DNA methylation, histone methylation or RNA methylation leads to defects in neurogenesis. In mice, a mutation in any of the three main *Dnmt* genes causes significant developmental defects and embryonic or early postnatal death (Li et al., 1992; Okano et al., 1999). Methyl-CpG binding domain protein 1 (MBD1) binds to hypermethylated CpG islands in gene promoter regions preferentially, and its absence impairs adult hippocampal neurogenesis and genomic stability *in vitro* (Zhao et al., 2003). PcG proteins and TRXG have also been linked to neurogenesis regulation. In embryonic cortical NPCs, deletion of Enhancer of zeste homologue two in PRC2 (Ezh2) causes a global loss of H3K27me3, derepression of a large number of neuronal genes, and disrupted neuronal differentiation (Pereira et al., 2010). The RNA demethylase FTO has been shown to be expressed in adult neural stem cells and neurons and exhibits dynamic expression during postnatal neurodevelopment.

The role of the epigenome on differentiation has also been well studied in the hematopoietic stem cells (HSC). *Hox* genes, critical for maintaining the balance between self-renewal and differentiation of HSC and progenitor cells are associated with bivalent domains in undifferentiated ESCs and its sequential expression during differentiation is regulated by PcG and TRXG proteins (Deng et al., 2013). Hematopoietic specific genes such as CD45, CD34 among others exhibited repressive DNA methylation marks prior to differentiation of the ESC which are subsequently lost upon differentiation correlating with gene expression (Suelves et al., 2016). DNMT3a and DNMT3b act to repress self-renewal genes in HSCs and their combined loss enhances self-renewal by activating β-catenin signalling (Sharma and Gurudutta, 2016). DNMT1 aids in efficient hematopoietic differentiation and is crucial for the progression of cells to multipotent progenitors to lineage-restricted myeloid and lymphoid progenitor cells. DNMT3b is responsible for the *de novo* methylation of hematopoietic genes during early embryogenesis (Suelves et al., 2016). Deletion of the histone demethylase JARID1b compromises the self-renewal capability of the HSCs (Sharma & Gurudutta, 2016). The RNA m6A modification writer METTL, has also been shown to be essential for the symmetric division of HSCs (Cheng et al., 2019).

The epigenome in CSCs, during dedifferentiation

Cancer Stem Cells (CSCs) are a small subpopulation of cells within tumors, which are capable of self-renewal, differentiation, and tumorigenicity when transplanted into an animal host. CSCs can be distinguished from other cells within the tumor by differences in their cell division and gene expression (Rosen and Jordan, 2009). The first evidence for the presence of CSCs was shown in a study where leukemia initiating cell population from AML patients was identified based on the expression of (CD34+/CD38−) cell surface markers, by transplantation into severe combined immune-deficient (SCID) mice (Lapidot et al., 1994). The existence of Glioma stem cells (GSC) was first hypothesized in 2002, when it was considered to have migrated from the sub-ventricular niche. (Ignatova et al., 2002). It has now been shown that the genome-wide distribution of epigenetic signatures is associated with the differential programming of GSC and Neuronal Stem Cells (NSC) (Valor Luis and Hervás-Corpión 2020). CSCs are resistant to conventional chemotherapy or radiation treatment and can contribute to metastasis through the dedifferentiation process (Meirelles et al., 2012). High methylation can contribute to the self-renewing ability of CSCs during tumor progression (Muñoz et al., 2012). The epigenome modifications of CSCs play a major role in recurrence, metastasis, and therapeutic failure.

Resetting the epigenome through methylation dynamics

The dynamicity of the methylation mark on DNA, histones or RNA serves as an important biochemical rheostat for regulating stem cell pluripotency and lineage commitment along with other regulatory factors (Berdasco and Esteller, 2011; Völker-Albert et al., 2020; Sun et al., 2021). The reversible nature of these modifications provide an easy and efficient modulatory node that is used by cancer stem cells

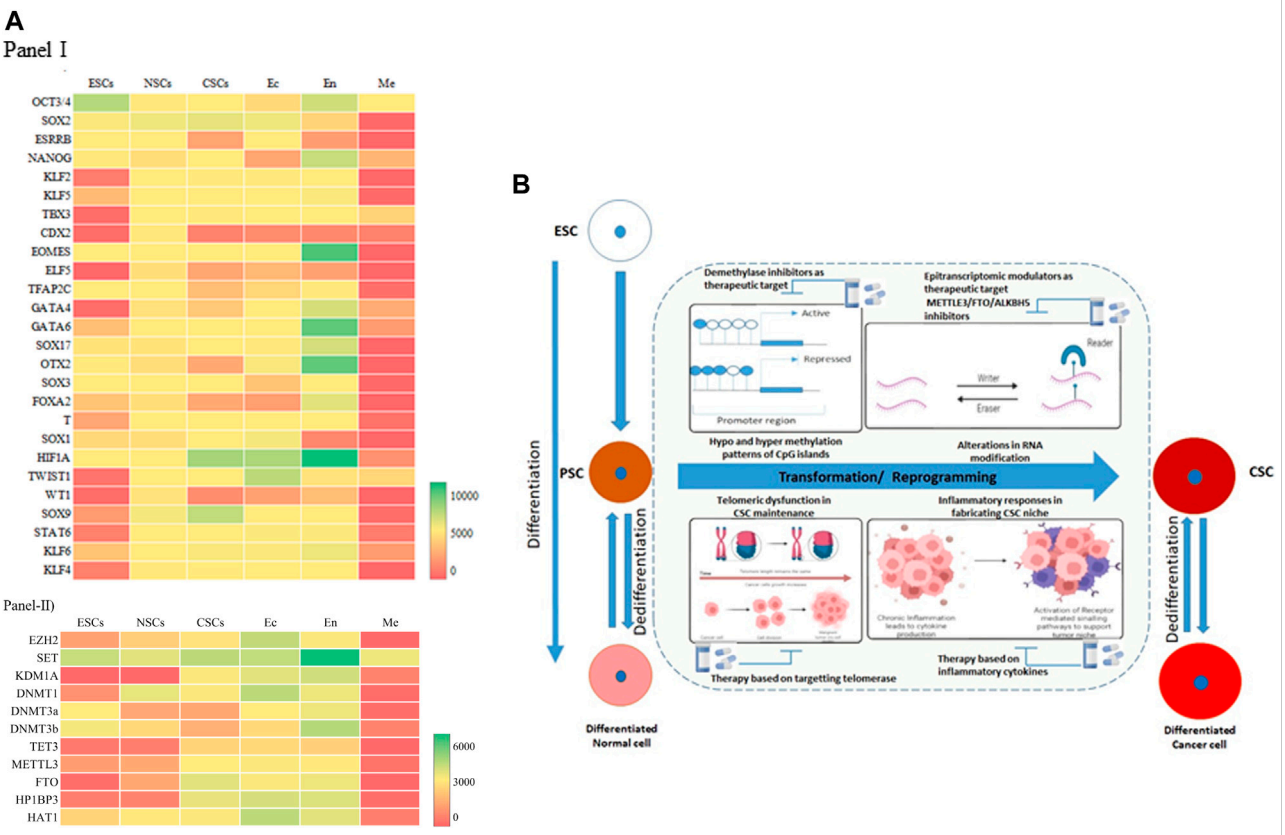

FIGURE 2
Resetting the Epigenome Dynamics in Differentiation and Dedifferentiation. **(A)** Heat map of Panel I, Transcription Factors (26) expression and Panel II modifiers (11) such as Histone methyltransferase/demethylase, DNMT/demethylase, RNA methyltransferase/demethylase in different cell types- embryonic stem cells (ESCs), neural stem cells (NSCs), cancer stem cells (CSCs), ectoderm (Ec), endoderm (En), mesoderm (Me). The red color indicates a lower expression level, and the green indicates a high expression level. Yellow represents intermediary expression levels. The intensity of the color indicates the expression level. Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was used to gather the data for the six types of cells/tissues EMBRYONIC STEM CELLS (Datasets- GSE220881 and GSE775182), NEURAL STEM CELLS (Datasets- GSE380453 and GSE353904), CANCER STEM CELLS (Datasets- GSE433785 and GSE42906), ECTODERM (Datasets- GSE339037 and GSE1442418), ENDODERM (Datasets- GSE1080479, GSE5528310, and GSE2413511), MESODERM (Datasets- GSE18216112 and GSE11477613). Excel software was used to plot the heatmap. The selected data is converted into the heat map using conditional formatting (color scales). **(B)** A combination of various oncogenic events triggers transition of pluripotent stem cells and differentiated cells to cancer stem cells. Differential methylation at the CpG islands triggers oncogenes and transcription factors leading to emergence of tumor heterogeneity and CSCs. Alterations in global epitranscriptomic profile also regulate the reprogramming or dedifferentiation events. Cytokine and Interleukins acts in paracrine manner leading to cancer inflammation and crafts a niche for emergence of stem like cells by activating downstream signalling pathways. Telomere associated quiescence supports the stem like cellsin the tumor micro environment elevating their self-renewal capacity.

(Vincent et al., 2019; Carvalho, 2020). The expression of transcription factors, signalling pathways and other regulatory proteins in stem cell biology are under the control of this reversible modification.

A meta analysis of the available datasets was done to assess the changes that occur during these stages, as shown in Figure 2A. The transcription factors Oct3/4, Sox2, and Nanog expression are upregulated in ESCs because they are the core transcription factors in maintaining the pluripotency of the embryonic stem cells (Boyer et al., 2005). At the same time, Elf5, Gata4, Wt1, Stat6, Klf2, Tbx3, Cdx2, etc., are downregulated in ESCs. In Neural Stem Cells (NSCs) (NSCs), almost all of the TFs have average expression levels (Figure 2A, Panel I), with Sox2 at the highest level of expression. The cancer stem cells (CSCs) in gliomas, that would have undergone a dedifferentiation, show a very different expression level compared to the NSCs. Sox2, Sox9, and HIF1A show increased expression, whereas Cdx2, Esrrb, Wt1, etc., show decreased expression in CSCs. These expression levels could be the markers of cancer stem cells (Zhao et al., 2017). On comparing the three germ layers (Ectoderm, Endoderm, and Mesoderm), the expression level of TFs changes significantly,

especially in the mesodermal lineage. The cells or tissues showing the elevated expression of the Eomes, Hif1a, Gata6, Gata4, Sox17, Otx2, etc., can be identified as an endodermal lineage. In addition to this, there is an expression of pluripotency factors such as Oct3/4 and Nanog. In ectodermal cells, we see the upregulated expression of Hif1a, Twist1, Sox2. Interestingly, the expression profile of ectodermal cells is somewhat similar to the CSCs.

The epigenome modifiers such as Histone methyltransferases/demethylases, DNMTs/demethylases, RNA methyltransferases/demethylases also have dynamic expressions in the different cell types (Rwigemera et al., 2021). In ESCs, most of the transcription factors have moderate expression. SET has a higher expression level as opposed to the KDM1A, TET3, and FTO (Chung and Sidhu, 2008). NSCs also follow the same trends as ESCs (Figure 2A, Panel II). Ectodermal cells have higher expression levels of epigenome modifiers. Most epigenome modifiers have lower expression in the mesodermal cells except SET, KDM1A, DNMT3b, and HAT1. In CSCs, all the modifiers express moderately, except SET, FTO, and HP1BP3. This suggests an intermediary state of gene expression in the CSCs, where additional environmental factors can then come into play and facilitate tumour manifestation. It has been shown that Glioma stem cells (GSC), once formed, are also regulated by various signalling pathways, coordinated by epigenetic reprogramming. GSCs are reported to overexpress histone demethylase KDM4C, which removes H3K9me3 from Wnt target genes, promoting Wnt/Signalling Pathway and thereby stem cell maintenance (Chen et al., 2020; Kumar et al., 2022). Epigenetic regulators maintain tumoral hierarchy through two mechanisms, either through inhibition of self-renewal property of cancer cells thereby maintaining heterogeneity, or by facilitating CSCs in evading differentiation and maintenance of stem cell phenotype (Wainwright and Scaffidi, 2017; Valor Luis and Hervás-Corpión, 2020; Tao et al., 2022). RNA Methyltransferase, METTL3-mediated RNA stabilization positively regulates major signalling pathways such as Notch, NFκB, Wnt, c-Myc, TGFβ, involved in cancer stem cell maintenance and proliferation in several cancers including Glioma and Leukemia maintenance and tumorigenesis implying its oncogenic role (Visvanathan et al., 2018).

In this context, the inflammatory pathway has been shown to be recognized as a major component of tumorigenesis in various cancers. Solid tumors are also associated with Tumor Associated Macrophages (TAM) which constitute various immune infiltrating cells. These TAMs and stromal cells secrete cytokines such as Interleukin 1(IL1), IL6 and TNFα acts in paracrine fashion for sustenance and reprogramming of CSCs, by altering epigenetic mechanisms and thereby regulating transduction pathways such as NFκB, STAT3 and SMADs. (Biswas et al., 2013). These inflammatory pathways interconnect to form molecular regulatory circuits in resetting the networks for maintaining CSCs (Liu et al., 2021). Chronic inflammation can initiate DNA damage response in preneoplastic lesions, leading to telomere loss (Shay and Wright 2010). This triggers segregational defects, activation of telomerase and setting in of genomic instability, one of the major hallmarks of cancer. Patient derived CSCs in glioma have demonstrated shortened telomeres along with telomerase expression indicating the fact that GSCs are not quiescent and have the capacity for aberrant self-renewal properties (Koeneman et al., 1998). A summary of the alterations in reprogramming/transformation and de-differentiation is represented in Figure 2B. Inflammation regulates the acquisition and maintenance of the cancer stem cell phenotype by stimulating epithelial mesenchymal transitions. Many inflammatory factors like IL-1β, TGF-β, IL-6 can regulate the DNA methylation patterns that induce cancer initiation and progression in cancers such as gastric cancer, ovarian cancer, and liver cancer (Liu et al., 2021). The exact mechanisms of how the epigenome dynamics facilitates this process warrants further investigation which will provide useful therapeutic intervention prospects.

Author contributions

AR, SSP, IK, SN, HS, and RSB wrote the manuscript. IK and HS created the Figure 1, SN created Figure 2A, SP created Figure 2B.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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