Dysregulation of the Repressive H3K27 Trimethylation Mark in Head and Neck Squamous Cell Carcinoma Contributes to Dysregulated Squamous Differentiation

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Abstract

Purpose: Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent cancers diagnosed worldwide and is associated with a 5-year survival rate of 55%. EZH2, a component of the polycomb repressor complex 2, trimethylates H3K27 (H3K27me3), which has been shown to drive squamous differentiation in normal keratinocytes. This study determined whether inhibition of EZH2-mediated epigenetic silencing could induce differentiation or provide therapeutic benefit in HNSCC.

Experimental Design: We determined the effects of inhibiting EZH2, by either RNA interference or pharmacologically, on HNSCC growth, viability, and differentiation in vitro. Xenografts of HNSCC cell lines were used to assess efficacy of 3-deazaneplanocin A (DZNep), an inhibitor of H3K27 trimethylation, in vivo.

Results: EZH2 was highly expressed in HNSCC cell lines in vitro and tissue microarray analysis revealed high expression in (n = 59) in situ relative to normal oral epithelium (n = 12). Inhibition of EZH2 with siRNA could induce expression of differentiation genes in differentiation-refractory squamous cell carcinoma cell lines. Differentiation-refractory HNSCC cell lines displayed persistent H3K27me3 on the promoters of differentiation genes. DZNep caused cancer-cell–specific apoptosis in addition to a profound reduction in colony-forming efficiency and induction of some squamous differentiation genes. Furthermore, in vivo, DZNep attenuated tumor growth in two different xenograft models, caused intratumor inhibition of EZH2, and induction of differentiation genes in situ.

Conclusions: Collectively, these data suggest that aberrant differentiation in HNSCC may be attributed to epigenetic dysregulation and suggest that inhibition of PRC2-mediated gene repression may represent a potential therapeutic target.

Introduction

Squamous differentiation is a complex, tightly regulated process involving the coordinated induction and repression of genes involved in keratinocyte, proliferation, differentiation, and stem cell maintenance. Maintaining a balance between the relative abundance and spatial positioning of stem cells, proliferating transit amplifying cells and the terminally growth-arrested differentiated keratinocytes in stratified squamous epithelium is primarily achieved via transcriptional activation and repression (reviewed in ref. 1). Earlier studies have shown that many squamous differentiation-specific genes are induced following the binding and activation of specific transcription factors such as AP1, AP2, E2F, C/EBP, and SP1 to the promoters of differentiation-specific genes (reviewed in ref. 2). More recently, it has become apparent that gene expression can be regulated by epigenetic modifiers such as the histone methyltransferases and histone demethylases (reviewed in refs. 3, 4). In particular, an epigenetic modification, which has attracted much interest is tri-methylation of lysine 27 on histone H3 (H3K27me3) by the polycomb repressor complex 2 (PRC2).

PRC2 is composed of EZH2, SUZ12, EED, and Rb AP46/48 (5), and catalyses the methylation of lysine 27 on histone H3 (H3K27me3) by the polycomb repressor complex 2 (PRC2). PRC2 is composed of EZH2, SUZ12, EED, and Rb AP46/48 (5), and catalyses the methylation of lysine 27 on histone H3, mediated by the N-terminal set domain of the catalytic subunit, EZH2 (6). PRC2 plays a critical role in embryonic development, lineage commitment, and the terminal differentiation of somatic tissues. For instance, squamous differentiation (7, 8), adipogenesis (9), and myogenesis (10, 11) are characterized by dynamic changes in H3K27me3 occupation of key differentiation-associated genes. In particular, H3K27me3 is found to be associated with the promoters of a subset of differentiation genes in proliferating keratinocytes (12). Demethylation of H3K27me3 is catalyzed by the lysine 27-specific demethylases, JMJD3 and UTX (13), with JMJD3...
Translational Relevance

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent cancers diagnosed worldwide and is associated with a 5-year survival rate of 55%. The overall survival of patients with HNSCC has not improved greatly in the past 30 years. Underlying this lack of progress is a poor understanding of the drivers of HNSCC and a lack of targeted therapies (with the exception of anti-EGFR therapies). A fundamental defect in HNSCC is their inability to undergo differentiation in response to normal differentiation-inducing stimuli. In this article, we show that differentiation insensitivity is attributable to defects in the epigenetic control of differentiation gene transcription. We show that genetic and pharmacologic inhibition of the PRC2 epigenetic repressor can reinstate markers of differentiation in differentiation-refractory SCC cells and reduce tumor growth in a preclinical xenograft tumor model. This is the first report where the epigenetic modifier 3-deazaneplanocin A has been used in vivo to treat SCC and suggests that polycomb-mediated gene repression may be a potential therapeutic target in HNSCC.

induced demethylation sufficient to induce the expression of terminal differentiation genes in the keratinocyte (12). Thus, the activation of a squamous differentiation program is, in part, driven by the selective loss of the repressive H3K27me3 mark associated with a selected group of differentiation genes.

Given the key role of polycomb-mediated gene silencing in terminal differentiation of adult tissues, it is not surprising that this mechanism is dysregulated in malignancy. For example, overexpression or amplification of EZH2 has been detected in breast cancer (14, 15), prostate cancer (6), and squamous cell carcinoma (SCC; refs. 16–18). Recurrent mutations of EZH2 have also been detected in B-cell lymphoma, leukemia, and myelodysplastic syndrome (19). Furthermore, the demethylase UTX has been shown to be recurrently mutated in esophageal carcinoma and renal cell carcinoma (20).

The emerging importance of the regulation of the H3K27me3 mark as a driver of squamous differentiation suggests that SCCs may harbor defects in the epigenetic regulation of squamous differentiation. Dysregulation of squamous differentiation is fundamental to the development of SCC and has been reported to occur early in premalignant lesions (21). In particular, there have been several recent studies that report an association between PRC2-mediated regulation of gene expression and head and neck squamous cell carcinoma (HNSCC). For instance, EZH2 has been shown to be highly expressed in a cohort of oral SCC lesions and correlates with poor patient outcome (16). Moreover, a recent sequencing project found that EZH2 was mutated in 6% of HNSCC lesions relative to paired normal tissue (22). Given the evidence for the involvement of epigenetic mechanisms, particularly PRC2 in malignant transformation, it has been suggested that drugs that inhibit epigenetic modifiers may be of clinical value. At present, the histone deacetylase inhibitor vorinostat (23) and DNA methylation inhibitor 5-azacytidine (24) have been approved for use in several myeloid cancers. While no specific PRC2 inhibitor has been described, the homocysteine hydrolase inhibitor 3-deazaneplanocin A (DZNep) has been shown to deplete PRC2 components with a resultant reduction in H3K27me3 (25). In this study, we examine whether the differentiation-refractory behavior of HNSCC can be causally attributed to the dysregulation of the repressive H3K27me3 mark associated with differentiation-specific genes. We also examine whether pharmacologic or genetic modification of the H3K27me3 mark can reinstate differentiation in vitro and whether it has therapeutic potential in a xenotransplant model of HNSCC.

Materials and Methods

Tissue culture

Epidermal keratinocytes were isolated from human neonatal foreskins and cultured in keratinocyte serum-free medium (Invitrogen) as described in ref. (26). Detroit 562 and SCC25 cells were obtained from the American Type Culture Collection (ATCC). Cal27, FaDuI, SCC9, SCC15, and COLO16 were a kind gift from Dr. Elizabeth Musgrove (Garvan Institute, New South Wales, Australia) and were verified by short tandem repeat (STR) genotyping. SCC cells were maintained in mycoplasma-free conditions and were cultured as per ATCC recommendations. KJD cells were cultured as per ref. (27). Cells were treated with DZNep, a kind gift from Yu Qiang (Genome Institute of Singapore, Singapore). To induce differentiation, cells were maintained at confluence for 7 days.

siRNA delivery

Control or EZH2 siRNA (Cell Signaling) was diluted in Opti-Mem serum-free media (Invitrogen; for SCC cell lines) or minimal keratinocyte serum-free medium (for primary keratinocytes) and Lipofectamine 2000 (Invitrogen; for SCC cell lines) or Opti-Mem serum-free media (for primary keratinocytes) and Lipofectamine 2000 (Invitrogen). siRNA complexes were delivered to cells at a final concentration of 40 nmol/L in accordance with the manufacturer’s instructions.

Colony forming assay

Known numbers of cells were placed into 6-well plates and allowed to grow for 14 days, following which plates were fixed and stained with Coomassie Blue (21) and counted. Colony forming efficiency (CFE) was expressed as the total number of colonies/total number of cells plated × 100.

DNA synthesis

DNA synthesis was measured by determining 5-bromo-2-deoxyuridine (BrdUrd) incorporation using a colorimetric ELISA assay (Roche Diagnostics) in accordance with the manufacturer’s instructions.
Viability assay
Cell viability was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) in accordance with the manufacturer’s instructions.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was conducted as per the Farnham lab protocol. Normal or tumor epithelial tissue was isolated using dispase digestion as described (28). One microgram of antibody was used for immunoprecipitation. Antibodies used were H3K27me3 (Upstate) and normal rabbit IgG (Cell Signaling). Quantitative PCR was conducted as described below and the formula $2^{(\text{Input}_{\text{Ct}} - \text{ChIP}_{\text{Ct}})} \times 10$ was used to calculate ChIP enrichment. Standard PCR was conducted using ThermoPol Taq Polymerase (New England Biolabs) in accordance with the manufacturer’s instructions. Primer sequences are provided in the Supplementary Data.

Flow cytometry
Annexin V staining was assessed with fluorescence-activated cell sorting (FACS) using a FACS Canto acquisition instrument (Beckman Coulter). One hundred thousand trypsinized cells were re-suspended in 100 µL of 1× FACS binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2 in distilled water) followed by the addition of Annexin V-Cy5 (BD Pharminagen) and 7-aminoactinomycin D (7-AAD; BD Pharminagen). Compensation was determined using unstained and single color-stained cells for each experiment. Data were analyzed with Flow Jo Software (TreeStar). Cell-cycle analysis with propidium iodide was conducted as per ref. (28).

RNA isolation and quantitative reverse transcriptase PCR
Total RNA was isolated and cDNA prepared as described in ref. (29). Quantitative reverse transcriptase PCR (qRT-PCR) was conducted as described (28). Primer sequences are provided in the Supplementary Data.

Immunoblot
Protein extractions and Western blot assays were conducted as previously described (30). Membranes were incubated with the following primary antibodies: EZH2 1:1,000, SUZ12 1:1,000 (Cell Signaling), H3K27me3 1:1,000, H3 1:1,000 (Upstate), β-actin 1:10,000 (Sigma Aldrich), CDC2 1:500, and involucrin 1:1,000 (Santa Cruz Biotechnology).

Immunohistochemistry
Immunohistochemistry was conducted as described (31). Sections were incubated with the following primary antibodies: H3K27me3 1:100 (Upstate), BrdUrd 1:100 (Roche), Ael/Ae3 1:600 (ICN Biomedical), involucrin 1:100 (Santa Cruz Biotechnology), Keratin 1 (Novus Biologicals), cleaved caspase III 1:500, and EZH2 1:50 (Cell Signaling). Secondary antibodies were Starr Trek Universal HRP Detection System (Biocare Medical) or Alexa Fluor 488 or 568 conjugated anti-mouse or rabbit 1:1,000 (Invitrogen). DAPI (4′,6-diamidino-2-phenylindole; Cell Signaling) was used as a nuclear stain for immunofluorescent images which were imaged on a confocal microscope (Zeiss Inverted Confocal LSM510 META) and acquired with Zen 2008 software. For tumors that were subjected to colorimetric immunohistochemical staining, the ImageJ (Wayne Rasband; National Institutes of Health, Bethesda, MD) macro Immunoratio (32) was used to count the number of hematoxylin and 3,3′-diaminobenzidine (DAB)-stained nuclei on multiple fields of each tumor. For quantification of total expression of an antigen in immunofluorescent images, the threshold function of Image J and “area fraction” measurement was applied to a tile scan image (total area 3.61 um²) that had been split into red, green, and blue channels. The area fraction for the fluorescent antibody stain was then expressed as a proportion relative to the total DAPI stain for the image for AE1/AE3, or as a raw percentage for KRT1 and IVL.

Tissue microarray
Immunohistochemistry was conducted on tissue microarray slides spotted with 1.0 µm cores of paraffin-embedded, pathologist-diagnosed SCC or normal oral epithelium (Biomax). Samples were scored using a previously published and validated tissue microarray scoring system where tissue was stratified as EZH2 or H3K27me3 high or EZH2 or H3K27me3 low, where a low-score system where tissue was stratified as EZH2 or H3K27me3 high or EZH2 or H3K27me3 low, where a low-score incorporated tissue, which received a score of 1 (negative) or 2 (<25% cells staining positive with any intensity). Tissue scored as EZH2 or H3K27me3 high received a score of 3 (>25% to <75% cells staining positive with any intensity) or 4 (>75% of cells staining positive with any intensity).

In vivo DZNep administration
All animal experiments were approved by the Institutional Animal Ethics Committee. Six-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were injected subcutaneously on the flank with 1×10⁶ Cal27 or Detroit 562 cells. Groups of 4 mice received the following treatments when tumors were between 2 and 4 mm diameter: (i) vehicle only, (ii) DZNep (Cayman Chemicals) 2 mg/kg twice per week by intraperitoneal (i.p) injection, (iii) DZNep 5 mg/kg daily intraperitoneally, or (iv) 6 mg/kg cisplatin (InterPharma) weekly by an i.p. injection. Stocks of DZNep were prepared in dimethyl sulfoxide (DMSO; 10 mg/mL). Immediately before use, the stocks were diluted in PBS. Tumor growth and animal weights were monitored for a period of up to 5 weeks and experiments terminated when tumors reached 10 mm. Animals were injected with 20 µL/g of 10 µmol/L BrdUrd before euthanasia.

Statistical analysis
Statistical significance was calculated by a Student t test or Fisher exact t test using GraphPad Prism v5 (GraphPad Software).
Results

HNSCC display dysregulation of H3K27me3-mediated differentiation gene repression

Loss of H3K27me3 in proliferating keratinocytes is catalyzed by the induction of JMJD3 and is sufficient to induce markers of squamous differentiation in human keratinocytes (12). We therefore examined whether the differentiation-refractory behavior of HNSCC could be attributed to the dysregulation of H3K27 methylation status on the promoters of differentiation genes. First, we examined the expression of EZH2 in established human HNSCC cell lines (Fig. 1A) by immunoblot. Relative to normal human epidermal keratinocytes (HEK), EZH2 is elevated at the protein level in 6 of the 8 SCC cell lines examined. The expression of EZH2 and H3K27me3 protein. Tissue was scored as low (score of 1 or 2) or high (score of 3 or 4). Representative images are shown where normal is nonmalignant epithelial tissue from the oral cavity and SCC is a head and neck SCC. Two tissue microarrays were used for EZH2 for a total of 59 SCC samples and 12 normal mucosal samples. C, the stained tissue sections were scored and EZH2-staining intensity was stratified by tumor grade, being well differentiated, moderately differentiated, or poorly differentiated (1, 2, and 3, respectively). All normal tissue was EZH2 low. D, one tissue microarray was stained for H3K27me3 for a total of 38 SCC samples and 8 normal mucosal samples. The stained tissue sections were scored and H3K27me3 staining intensity was stratified by tumor grade, being well differentiated, moderately differentiated, or poorly differentiated (1, 2, and 3, respectively). E, whole-cell lysate was isolated from proliferative keratinocytes or keratinocytes treated with 1.2 mmol/L calcium or growth to confluence to induce differentiation. Lysates were then immunoblotted for involucrin (IVL), CDC2, H3K27me3, and H3 expression. H3 is a loading control. F, ChIP was conducted on normal tissue and SCC lesions to determine the relative occupancy of the IVL promoter with H3K27me3. Data represent the mean ± range; n = 5, * P = 0.05.
EZH2 protein in Detroit 562, SCC25, and Cal27 cells was verified in multiple experiments (Supplementary Fig. S1). Using tissue microarrays, we determined the in situ expression of EZH2 and H3K27me3 in a cohort of head and neck SCC tissue (n = 59) and normal oral epithelial tissue (n = 12) by quantitative immunohistochemistry. Tissue sections were scored as EZH2 high or low in accordance with a previously published and validated system (6). Representative images for immunohistochemistry are shown in Fig. 1B. HNSCC lesions expressed high levels of EZH2 in 50% of the sections evaluated, whereas all of the normal mucosal sections expressed low levels of EZH2 (P = 0.0009, Fisher exact t test; Fig. 1C). The mean EZH2-staining scores for normal tissue and grades (tumor grade 1 = well, 2 = moderately, or 3 = poorly differentiated) 1, 2, or 3 of SCC were 1 (SE 0.0), 2.47 (SE 0.23), 2.24 (SE 0.23), and 3.42 (SE 0.37), respectively. Thus, EZH2 is overexpressed in 50% of HNSCC and is independent of disease grade. Similarly, EZH2 is overexpressed in approximately 70% of SCC cell lines studied compared with normal keratinocytes.

As the major function of EZH2 and PRCA2 in general is to methylate H3K27, we examined whether the overexpression of EZH2 in HNSCC tumors was associated with corresponding alterations in H3K27 methylation status. We examined H3K27me3 expression in HNSCC lesions (n = 38) and normal mucosa (n = 8) using tissue microarray and the same scoring system as described for EZH2. All of the HNSCC and normal tissue examined was positive for H3K27me3, with 12.5% of the normal tissue and 10% of the SCC tissue H3K27me3 low (not significant, Fisher exact t test; Fig. 1D). The mean H3K27me3-staining scores for normal tissue and grade 1, 2, or 3 of SCC were 3.23 (SE 0.25), 3.4 (SE 0.13), 3.19 (SE 0.19), and 3.29 (SE 0.29), respectively. These data indicate that EZH2 overexpression in HNSCC is not associated with global alterations in H3K27 methylation status.

The lack of an association between EZH2 expression and H3K27me3 status could be explained by enhanced activity of demethylases such as JMJD3 or UTX or by compensatory loss of methylation pathways such as EZH1. Regardless of the mechanism, it is established that global changes in H3K27me3 status are not indicative of the selective regulation of specific promoters (12). This is clearly shown in Fig. 1E, where global H3K27me3 status is unchanged in proliferating and differentiated keratinocytes despite previous reports showing an unequivocal and selective loss of H3K27me3 status on the promoters of differentiation genes in keratinocytes undergoing differentiation (12). Thus, global H3K27me3 levels are not indicative of H3K27me3 levels on specific promoters. Therefore, we examined whether there was evidence for selective dysregulation of the association of H3K27me3 with the promoters of differentiation genes in SCC samples in vivo. ChIP on normal and HNSCC tissue showed that there was more H3K27me3 bound to the promoter of the differentiation gene involucrin in SCC lesions relative to nonmalignant tissue (Fig. 1F; P = 0.05, Student t test). This is consistent with persistent repression of differentiation and is consistent with the differentiation-refractory behavior of SCC.

**Inhibition of H3K27me3 status in HNSCC can induce markers of squamous differentiation**

Given that HNSCC cells and tumors are characterized by persistent H3K27me3 binding to the promoters of the differentiation gene, involucrin, we investigated whether knockdown of EZH2 by siRNA could restore differentiation capacity in HNSCC cell lines. A subset of terminal differentiation genes, such as S100A8, involucrin and keratin 1 have been shown to be bound and repressed by EZH2 and H3K27me3 in proliferating keratinocytes (12); a mark which is lost during differentiation. In SCC cells, EZH2 was inhibited with siRNA and caused depletion of global H3K27me3 (Fig. 2A), an increase in the fraction of cells arresting in the G1 and G2 phases of the cell cycle and reduction in the fraction of cells in S-phase: consistent with reduced proliferation (Fig. 2B). We next examined the differentiation status of 3 HNSCC cell lines in response to differentiation stimuli. Primary HEKs induced the expression of involucrin in response to growth to confluence or treatment with 1.2 mmol/L CaCl2 (Fig. 1E). In contrast, SCC25, Detroit 562, and Cal27 cell lines did not induce involucrin in response to 1.2 mmol/L CaCl2 treatment, whereas SCC25 and Cal27 cells could induce involucrin at confluence (Fig. 2C). The differentiation-refractory status of the Detroit 562 cell line was coupled with persistent H3K27me3 bound to the involucrin promoter upon confluence, in contrast to the loss of trimethylated H3K27, which is observed in normal keratinocytes (Fig. 2D). However, when EZH2 was inhibited by siRNA, involucrin protein, and involucrin, S100A8 and keratin 1 transcripts were upregulated in the differentiation refractory SCC cell lines (Fig. 2E and F). These data show that selective inhibition of EZH2 is able to deplete H3K27me3 and induce the expression of differentiation-specific genes in differentiation-refractory HNSCC cell lines. This confirms that HNSCC cell lines retain a capacity for squamous differentiation.

Given that inhibition of EZH2 by siRNA decreased proliferation markers and increased expression of differentiation genes in HNSCC cell lines, we examined whether pharmacologic inhibition of PRCA2 activity could also induce differentiation in HNSCC. We used DZNep to inhibit PRCA2 in HEKs and HNSCC cells. First, we characterized the effects of DZNep on the expression of the PRCA2 components EZH2 and SUZ12 in addition to methylation of H3K27 in HEKs and 3 HNSCC cell lines: Detroit 562, SCC25, and Cal27. Cells were incubated with different concentrations of DZNep (0.5–5 mmol/L) for varying times and subjected to Western blot analysis. There was a dose-dependent decrease in global H3K27me3 after 48 hours incubation with DZNep in HEKs and HNSCC cell lines (Fig. 3A). DZNep also induced a time-dependent decrease in EZH2 expression, with the HEKs showing the most robust changes (Fig. 3B), although the SCC cells were more sensitive to loss of H3K27me3 than the keratinocytes (Fig. 3A).
We next determined whether DZNep exerted effects on cell viability and proliferation. The normal keratinocytes and SCC cell lines exhibited a dose-dependent decrease in proliferation after 48 hours treatment (Fig. 3C and D). The proportion of Annexin V$^+$/7-AAD$^-$ (apoptotic) cells was determined for Detroit 562, SCC25, and HEK cells following treatment with 5 μmol/L DZNep. After 96 hours of treatment with 5 μmol/L DZNep, there was an approximate
EZH2

**A**

HEK

DZNep

DZNep

DZNep

DZNep 24 h

DZNep 48 h

H3K27me3

β-Actin

**B**

HEK

5 μmol/L DZNep

Detroit 562

3 μmol/L DZNep

SCC25

5 μmol/L DZNep

Cal27

3 μmol/L DZNep

β-Actin

**C**

Proliferation

BrdUrd incorporation (%)

DZNep Concentration

HEK

SCC25

Detroit 562

**D**

SCC25

Cell cycle

G0–G1

S

G2–M

Detroit 562

Cell cycle

**E**

Annexin V

Apoptotic cells (%)

HEK

Detroit 562

SCC25

Cal27

**F**

CFE (%)

CFE (%)

DZNep

5 μmol/L DZNep

DZNep

3 μmol/L DZNep

3 μmol/L DZNep

β-Actin

Figure 3. DZNep can deplete H3K27me3 and PRC2 components and reduce the viability and proliferation of SCC cells. A, whole-cell lysate was isolated from keratinocytes, SCC25, and Detroit 562 cells after treatment with 0.5 to 5 μmol/L DZNep. Immunoblot was used to detect total H3K27me3. β-Actin is a loading control. B, whole-cell lysate was isolated from keratinocytes, Detroit 562, SCC25, and Cal27 cells after treatment with DZNep for 48, 96 hours, or 7 days. Cells treated with an equivalent volume of DMSO were the control. Immunoblot was used to detect EZH2 and SUZ12 expression. β-Actin is a loading control. C, proliferation of primary keratinocytes (HEK), SCC25, and Detroit 562 cells was determined 48 hours after treatment with 1 to 5 μmol/L DZNep. Proliferation was determined by calculating BrdUrd incorporation. n = 3. D, SCC25 and Detroit 562 cells were stained with propidium iodide before analysis by flow cytometry to determine the proportion of cells in different phases of the cell cycle. n = 3. E, annexin V and 7-AAD staining was used to determine the proportion of apoptotic cells after 5 μmol/L DZNep treatment. n = 3. F, CFE of HEKs was determined 48 hours after treatment with 5 μmol/L DZNep. n = 4. Data have been normalized such that the control is 100%. CFE was determined 96 hours after treatment with 3 or 5 μmol/L DZNep. Data have been normalized such that the control is 100%. n = 3 and n = 5 for Cal27 at 3 or 5 μmol/L, respectively. All data are the mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
3-fold increase in the proportion of apoptotic SCC cells, which was not evident in the HEKs (Fig. 3E). This is consistent with previous reports that primary cells are not killed by DZNep (25), and indicates that DZNep possesses cancer cell selectivity. Interestingly, siRNA targeting of EZH2 expression in SCC cells did not induce apoptosis 48 hours or 4 days after gene knockdown (Supplementary Fig. S2). Furthermore, when we investigated effects of 96-hour exposure to DZNep by in vitro colony-forming assay, we observed a profound loss in CFE of SCC cells, to 10% and 40% of control (Fig. 3F). This is unlikely to be attributable solely to the cytotoxic effects as HEKs also exhibited a 50% reduction in CFE after 48 hours administration of the drug in the absence of cytotoxicity (Fig. 3E). The reduction in CFE indicates that the effects of DZNep last beyond the administration of the drug.

As we had shown that knockdown of EZH2 could restore differentiation capacity to SCC cells, we wanted to determine whether DZNep had a similar effect. HEKs, SCC25, and Detroit 562 cells were treated with DZNep for 48 hours or 7 days and assayed for H3K27me3-marked differentiation gene expression by quantitative real-time PCR and immunoblot. DZNep could induce expression of the differentiation gene involucrin, S100A8, and cytokeratin 1 in normal keratinocytes (Fig. 4A and B) after 48 hours of treatment. In contrast, SCC25 and Detroit562 cells lines did not induce either involucrin protein (Fig. 4B) or mRNA (Fig. 4C) in response to DZNep but did induce S100A8 mRNA in response to DZNep (Fig. 4C). The Cal27 cell lines induced involucrin protein and transcript and S100A8 transcript (Fig. 4B and C). Combined, these data indicate that targeted inhibition of H3K27 methylation, with DZNep, can induce long-term growth arrest, cytotoxicity, and the selective induction of differentiation markers.

Characterization of DZNep activity in a xenograft model of SCC

Given that DZNep displayed cancer cell-selective cytotoxicity in HNSCC cell lines in addition to differentiation inducing capacity, we examined whether DZNep was efficacious against a xenotransplant model of HNSCC in immunocompromised mice. One million Cal27 or Detroit 562 cells were used to inoculate NOD/SCID mice, and tumors were allowed to establish subcutaneously. These cell lines were selected for in vivo analysis as they represent models that express high levels of EZH2 that are differentiation competent (Cal27) and differentiation refractory (Detroit562) in vitro. When tumors were 4 mm (Cal 27) or 2 mm (Detroit 562) in diameter, the mice were administered DZNep by i.p. injection. The mice bearing Cal 27 tumors were administered 2 mg/kg DZNep twice per week, whereas the Detroit 562 tumor-bearing mice were administered 5 mg/kg DZNep daily. The different doses were chosen on the basis of relative growth rates of the tumors, with the Detroit 562 tumors reaching 10 mm diameter after 20 to 25 days compared with 40 days for the Cal 27 cells (unpublished data). When the tumors reached a diameter of 10 mm, animals were administered BrdUrd, euthanized, and the tumors resected and analyzed.

DZNep was well tolerated by the NOD/SCID mice, with no behavioral changes, signs of systemic toxicity, or weight loss observed (Fig. 5A). When tumor volume was calculated at 24 days after tumor cell inoculation, DZNep inhibited tumor growth (Fig. 5B) for both Cal27 and Detroit 562 cells (Fig. 5B). However, if the DZNep-treated Cal27 or Detroit tumor-bearing mice were followed beyond 24 days, the tumors continued to grow to a size, which required that the
animals be sacrificed. As a positive control, a different cohort of mice that received cisplatin 6 mg/kg weekly is shown.

We next examined levels of H3K27me3 (Fig. 5C). EZH2 (Fig. 5D), BrdUrd positivity (Fig. 5E), and differentiation gene expression (Fig. 5F and G) within the tumors resected...
from mice treated with 2 mg/kg DZNep or 5 mg/kg DZNep. Quantitative immunohistochemistry showed that global H3K27me3 was unchanged in Cal27 tumors from mice that received 2 mg/kg DZNep (Fig. 5C), whereas 5 mg/kg DZNep produced a modest reduction in global H3K27me3 in Detroit 562 tumors. EZH2 was significantly depleted in Cal27-derived tumors from mice treated with 2 mg/kg DZNep, as measured by quantitative immunohistochemistry. Similarly, immunoblot of a total cell lysate from Detroit 562 tumors showed that DZNep also depleted EZH2 (Fig. 5D). Quantitative immunohistochemistry revealed that DZNep did not inhibit intratumoral proliferation at either dose, in either cell line as measured by BrdUrd positivity (Fig. 5E). We next examined differentiation marker levels in Cal27 and Detroit 562 tumors that had been administered DZNep. Immunohistochemical staining with antibodies against involucrin and KRT1 revealed that there was an increase in involucrin positivity in DZNep-treated tumors, whereas KRT1 was unaltered (Fig. 5F). Furthermore, the H3K27me3-marked differentiation gene involucrin, S100A8, and transglutaminase 1 transcripts were 1.5- to 2-fold upregulated in Detroit 562 tumors, which received 5 mg/kg DZNep daily (Fig. 5G). Finally, we investigated whether there was a change in the tumor/stromal composition or apoptotic indices of the tumors in mice that had received 5 mg/kg DZNep. This was determined by immunofluorescent staining of tumor sections by colocalization of the pan epithelial marker AE1/AE3 (33), and cleaved caspase III (Fig. 6A and B). While there was a trend toward a decrease in the epithelial portion of the tumors from mice that had received 2 mg/kg DZNep and 5 mg/kg DZNep, this failed to reach statistical significance (Fig. 6C). Furthermore, there was no change in the proportion of cleaved caspase III-positive cells in either treatment groups (Fig. 6C). Combined, these data indicate that DZNep has anticancer activity in vivo and is a pharmacologic inhibitor of EZH2 in vivo. These data also indicate that DZNep treatment can induce markers of squamous differentiation in a xenotransplant model of differentiation-refractory HNSCC.

Discussion

The present study makes several significant and novel findings. Specifically, we show that, (i) loss of the H3K27 trimethylation mark from the promoters of differentiation genes induced by genetic or pharmacologic means is sufficient to induce growth inhibition and the expression of differentiation marker genes in normal keratinocytes, (ii) that the H3K27me3 mark persists on the promoters of differentiation genes in HNSCC in vitro and in vivo, (iii) that the dysregulation of H3K27 methylation status contributes to the differentiation-refractory behavior of HNSCC, (iv) that characteristics of differentiation (e.g., growth inhibition and differentiation gene expression) can be reinstated in HNSCC in vitro and in vivo by manipulating H3K27me3 status, and (v) that the PRC2 inhibitor, DZNep, has anticancer activity in xenotransplant models of HNSCC.

Previous studies have shown that the loss of H3K27me3 bound to the promoters of differentiation genes is sufficient to initiate squamous differentiation (12). Our study confirms these findings in normal human keratinocytes and extends them to show that pharmacologic inhibition of PRC2 activity by DZNep is also able to induce markers of differentiation in human keratinocytes. Furthermore, we show that EZH2 is highly expressed in HNSCC both in vitro and in vivo. These observations have significant implications for our understanding of HNSCC formation and potential therapeutic targets. For example, our data clearly show that H3K27me3-mediated binding and repression of squamous differentiation genes persists in HNSCC cells that have been exposed to differentiation stimuli. Resistance to differentiation stimuli and growth inhibitors [e.g., protein kinase C activators (34), TGFβ-1 (35), and IFN-γ (36)] has been reported previously for SCC cells and remains a fundamental characteristic of SCC (37). For example, recent profiling studies have shown that SCCs harbor mutations in key regulators of squamous differentiation, most notably NOTCH1 (38, 39) and p63 (40) in addition to other differentiation genes, including EZH2 (22). These reports, in combination with the data presented in this article, highlight the consistent dysregulation of normal differentiation in SCC. The present study shows that the persistence of the H3K27me3 mark on the promoters of differentiation genes contributes causally to the differentiation-refractory behavior of SCC. Evidence for a role of persistent H3K27me3 binding in the differentiation-refractory behavior of SCC is supported by the abundant binding of H3K27me3 to the involucrin promoter in human SCC samples and the persistence of H3K27me3 binding in SCC cells treated with a differentiation stimulus. In addition, reduction of H3K27 trimethylation via DZNep treatment results in long-term growth inhibition and induction of the differentiation marker, S100A8, in HNSCC cells. The induction of differentiation genes is also observed in xenotransplants of HNSCC in mice treated with the epigenetic inhibitor DZNep. These data are significant in so far as they show (i) a causal relationship between the dysregulation of squamous differentiation in SCC and the persistent binding of the repressive H3K27me3 mark to the promoters of differentiation genes and (ii) HNSCC cells retain a capacity to undergo squamous differentiation. This latter point is consistent with earlier studies which showed that E2F transcription factor inhibition in conjunction with SP1 transcription factor overexpression can induce differentiation gene expression in SCC cells (41, 42). Nonetheless, while loss of H3K27me3 from promoter binding sites is an important regulatory event in the induction of squamous differentiation, it is clear that this process is only one part of a complex program of events that controls squamous differentiation. For instance, loss of E2F expression (via inhibition of E2F activity) during squamous differentiation results in the derepression of a major differentiation regulator, Sp1, that in turn induces expression of a select suite of differentiation genes (28). Furthermore, differentiation is accompanied by changes in DNA methylation and DNMT1.
activity (43). Thus, H3K27me3 and EZH2-mediated regulation of squamous differentiation represents one part of a complex regulatory network that coordinates the initiation and maintenance of squamous differentiation.

Dysregulation of H3K27 methylation status is selectively disrupted in SCC and contributes significantly to the differentiation-refractory behavior of SCC. The basis for the persistence of H3K27me3 on differentiation promoters in either normal homeostasis or in SCC is unclear. Our data would suggest that overexpression of EZH2 could catalyze the methylation of H3K27; however, it may be that the H3K27me3 demethylases, JMJD3 and UTX, undergo differential expression during differentiation or disease. However, such explanations may be simplistic. For example, we

Figure 6. DZNep does not affect cellularity or apoptotic fractions in tumors. Immunohistochemistry was conducted with an antibody against AE1/AE3 and cleaved caspase III in Detroit 562 and Cal27 tumors that had received control (PBS) or DZNep. Confocal microscopy was used to generate a tile scan image. AE1/AE3-positive cells are AlexaFluor 488-positive (green) and cleaved caspase III-positive cells are AlexaFluor 568 positive (red). DAPI was used to stain nuclei. A, a representative image of a control tumor and (B) a representative image of a tumor that had received 5 mg/kg DZNep daily. C, ImageJ was used to calculate the proportion of epithelial and apoptotic cells relative to the total number of cells (DAPI stained) for Cal27 and Detroit 562 tumors that received 2 mg/kg DZNep or 5 mg/kg DZNep, respectively. An area of 3.61 μm² was quantified for 4 tumors from each group. Data represent the mean ± SEM.
know that global levels of H3K27me3 do not change during differentiation (Fig. 1E) and are similar in normal mucosa and SCC samples (Fig. 1C), yet select suites of differentiation genes display altered patterns of association with H3K27me3 during squamous differentiation (7, 12). Thus, it is clear that H3K27me3-mediated regulation is gene-specific and context-specific. This would suggest that factors, in addition to EZH2 overexpression, also contribute to the selective persistence of repressive H3K27me3 marks on the promoters of a subset of squamous differentiation genes. While no studies have identified a universal mechanism for recruitment of PRC2 in humans, it is possible, yet unproven, that such a mechanism is dysregulated in SCC.

Our in vivo studies with DZNep suggest that therapies targeting PRC2 may have some value as a differentiation therapy in HNSCC. Our results, with 2 different HNSCC cell lines, indicated that DZNep could reduce tumor growth in a xenotransplant model. However, it should be noted that DZNep did not induce cures in the mice and that tumors continue to grow, albeit at a slower rate. This may be due to, in part, the unfavorable pharmacokinetics of DZNep: the half-life of DZNep in rats is reported to be 1.1 hours (44). Although our data clearly show that DZNep was pharmacologically active within the tumor (Fig. 5D), it is not clear whether the growth inhibitory or cytotoxic effects of DZNep require sustained concentrations, or whether the tumor cycles between proliferation and growth inhibition as the DZNep levels flux during administration and elimination. Significantly, although the tumor growth rates slowed in the DZNep-treated mice, we found no statistically significant difference in proliferation or apoptotic indices. However, we did find evidence for the induction of differentiation genes, S100A8 and involucrin, in tumors at completion of the treatment cycle. The reason for the reduced tumor growth rate remains unclear; however, it may potentially result from apoptotic or antigrowth effects early in the treatment cycle.

While this is the first study to report on the anticancer activity of DZNep in HNSCC, earlier studies have reported anticancer effects of DZNep in acute myeloid leukemia (45, 46, 47) and hepatocellular carcinoma (47, and mesothelioma (48). However, our study would suggest that the anticancer activity of DZNep in SCC cell lines and tumors may not be solely attributable to PRC2 inhibition. For example, post-translational modifications on other histone residues can also be affected by DZNep (25, 46, 49). In our hands, DZNep can be considered to be a selective but nonspecific inhibitor of H3K27me3. Furthermore, depletion of EZH2 with siRNA does not induce apoptosis in SCC cells; which suggests that DZNep may exert "off-target" effects, that is, beyond inhibition of PRC2-mediated gene repression. Notwithstanding this, the PRC2-inhibitory effects of DZNep are still important for the differentiation-inducing and anticancer activity observed in this study. Supporting this, other novel chemotherapeutic compounds such as the green tea polyphenol (−)-Epigallocatechin-3-gallate (50) also decrease expression of PRC2 components, suggesting that "off-target" inhibition of EZH2- and H3K27me3-mediated gene silencing may be involved in the therapeutic activity of other novel compounds. Collectively, these data suggest that inhibition of PRC2-mediated gene silencing may represent a new and novel therapeutic target for head and neck SCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: O. Gannon

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Gannon, N.A. Saunders

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Gannon, N.A. Saunders

Writing, review, and/or revision of the manuscript: O. Gannon, L.B. endo-munoz, N.A. Saunders

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Gannon, L.M. Long, M. Hazarnethinam, N.A. Saunders

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References


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