Survival of skin cancer stem cells requires the Ezh2 polycomb group protein

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Abstract

Polycomb group proteins, including Ezh2, are important candidate stem cell maintenance proteins in epidermal squamous cell carcinoma. We previously showed that epidermal cancer stem cells (ECS cells) represent a minority of cells in tumors, are highly enriched in Ezh2 and drive aggressive tumor formation. We now show that Ezh2 is required for ECS cell survival, migration, invasion and tumor formation and that this is associated with increased histone H3 trimethylation on lysine 27, a mark of Ezh2 action. We also show that Ezh2 knockdown or treatment with Ezh2 inhibitors, GSK126 or EPZ-6438, reduces Ezh2 level and activity, leading to reduced ECS cell spheroid formation, migration, invasion and tumor growth. These studies indicate that epidermal squamous cell carcinoma cells contain a subpopulation of cancer stem (tumor-initiating) cells that are enriched in Ezh2, that Ezh2 is required for optimal ECS cell survival and tumor formation and that treatment with Ezh2 inhibitors may be a strategy for reducing ECS cell survival and suppressing tumor formation.

Introduction

Epidermal squamous cell carcinoma ranks among the most common forms of cancer. Moreover, due to exposure to environmental irritants and ultraviolet radiation, the incidence continues to increase (1). Early lesions can be removed by surgical excision, but the 5 year recurrence rate is still 8% (2). Advanced disease is life threatening and there are no effective treatments (3). Moreover, the high rate of skin cancer occurrence in the population means a high cost to society.

Recent findings suggest that epidermal squamous cell carcinoma includes a subpopulation of tumor-initiating cells we call epidermal cancer stem cells (ECS cells), which exhibit self-renewal capacity, proliferate infrequently and are required for tumor maintenance and metastasis (4–6). Since the cancer stem cells are thought to give rise to non-stem cancer cells, eliminating the stem cell population may be necessary to halt tumor formation (7). However, these cells are resistant to the action of traditional anticancer agents that kill rapidly growing tumor cells (7).

On a practical level, stem cells can be identified by the presence of protein epitopes that are associated with stem cells from the corresponding normal tissue. In breast cancer, the stem cell population displays a CD44+CD24- phenotype (8), and CD133 marks cancer stem cells in brain tumors, colorectal carcinoma and pancreatic carcinoma (9–12). In head and neck cancer, CD44+ cells display cancer stem cell properties (13), and aldehyde dehydrogenase 1 activity identifies cancer stem cells in a host of cancer types (14–17). The human epidermis contains multiple stem cell populations (4), including the CD200+/K15+/K19+ hair bulge stem cells (18) and the α6β1/CD71+ interfollicular stem cells (19,20). CD133 has
also been reported to identify human skin cancer stem cells (5,21,22).

Epidermal squamous cell carcinoma cells and tumors are enriched for expression of the polycomb group (PcG) proteins, which are a conserved family of proteins that act epigenetically to silence tumor suppressor gene expression (6,23,24). These regulators repress gene expression by covalently modifying histones to produce closed chromatin (24-29). PcG proteins operate as two multiprotein chromatin-binding complexes—polycomb repressive complex 1 (PRC1) and PRC2 (27). The PRC1 complex includes Bmi-1, Ph1, CBX and Ring 1A/B, whereas the PRC2 multiprotein complex contains Ezh2, EED, Suz12 and RbpAp46 (30). As an initial step in regulation, trimethylation of lysine 27 of histone H3 (H3K27me3) occurs via the action of the Ezh2 protein (28,31). In the second step, H3K27me3 serves as a binding site for the chromodomain of the CBX protein of the PRC1 complex (31). Once bound, the PRC1 complex Ring1B protein ubiquitinates histone H2A at lysine 119 (25,31). The sequential trimethylation and ubiquitination events result in chromatin condensation leading to gene silencing (27,28).

The PcG proteins, by suppressing tumor suppressor expression, have been implicated as important in maintenance of stem cell survival (24,32-36). Indeed, we have shown that several PcG proteins are overexpressed in epidermal squamous cell carcinoma (30,37,38) and that this overexpression enhances epidermal cancer cell survival (6,39-41). Ezh2 is a particularly important PcG protein as it is the key catalytic protein in the PRC2 complex and is highly elevated in skin cancer (30). We have shown that Ezh2 is overexpressed in ECS cells (6). Moreover, ECS cells form large, aggressive and highly invasive and vascularized tumors following injection of as few as 100 cells in immune compromised mice (6). A key question is whether the Ezh2 protein is required for ECS cell survival and tumor formation. In the present study, we show that Ezh2 is required for ECS cell survival, migration and invasion and spheroid and tumor formation. We also show that Ezh2 inhibitors reduced these processes including tumor formation.

Materials and methods

Abbreviations

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<tr>
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<th>Description</th>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>ECS cell</td>
<td>epidermal cancer stem cell</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PcG</td>
<td>polycomb group</td>
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<td>PRC</td>
<td>polycomb repressive complex</td>
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Antibodies and reagents

Duulbecco’s modified Eagle’s medium (11960-077), sodium pyruvate (11360-070), l-glutamine (25030-164), penicillin-streptomycin solution (15140-122) and 0.25% trypsin–ethylenediaminetetraacetic acid (12500-056) were purchased from Gibco (Grand Island, NY). Heat-inactivated fetal calf serum (FCS, F4135) was obtained from Sigma. Antibodies for Ezh2 (612667) and Oct4 (611203) were obtained from BD Transduction laboratories (San Jose, CA). Anti-H3K27me3 (67-499) was from EMD Millipore (Bedford, MA). Antibodies for Sox2 (ab15830-100) and Bmi-1 (ab14389) were obtained from Abcam (Cambridge, UK). Anti-Ki5 (10137-1-AP) was obtained from Proteintech (Chicago, IL). β-Actin (A5441) antibody was purchased from Sigma (St Louis, MO). Alexa Fluor 594 goat anti-rat Immunoglobulin G (IGG) (A11007), Alexa Fluor 488 goat anti-mouse IgG (A21121) and Alexa Fluor 594 goat anti-rabbit IgG (A11012) secondary antibodies were obtained from Invitrogen and used at 1:500 dilution. Peroxidase-conjugated anti-mouse IgG (NA931) and anti-rabbit IgG (NA934V) were obtained from GE Healthcare (Buckinghamshire, UK) and used at a 1:5000 dilution. Anti-PARP (556494) was purchased from BD Pharmingen (Dan Diego, CA), anti-active caspase-3 (9665) was obtained from Cell Signaling (Danvers, MA), anti-Ring 1B (ab3832) was from Abcam and anti-H2AK119ub (AB10029) was obtained from Millipore.

Ezh2 inhibitors

EPZ-6438 (A-1623) was obtained from Active Biochemicals (Wan Chai, Hong Kong) and GSK126 (CT-GSK126) was purchased from Chemie Tek (Indianapolis, IN). JQ-EZ-005 was a gift from Dr J.Bradner (Harvard). For cell culture studies, all compounds were prepared as stocks in dimethylsulfoxide. For animal experiments, GSK126 was dissolved in captisol, a non-toxic delivery vehicle (42). In brief, 40% captisol (RC-0C7-020, CyDex Pharmaceuticals, Lawrence, KS) was prepared in sterile water by stirring overnight at 25°C followed by filter sterilization. The 40% captisol solution (50 ml) was diluted 1:1 in sterile water and supplemented with 0.5 ml of 1 N acetic acid. GSK126 was dissolved in 20% captisol in an amber vial to a final concentration of 5 mg/ml by sonication in water bath sonicator at 37°C with adjusting to pH 4.5 by the addition of 1 N acetic acid. Control mice were treated with 20% captisol solution. GSK126 is stable for at least 5 days in 20% captisol and so fresh solutions were made weekly. GSK126 was delivered by intraperitoneal injection, on alternate days, of 200 μl of the 5 mg/ml stock (50 mg/kg body weight).
4,6-diamidino-2-phenylindole (DAPI) using microscopic fluorescence detection.

**Immunoblot**

For immunoblot, equivalent amounts of protein were electrophoresed on denaturing and reducing 8% polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked for 1 h with 5% non-fat dry milk and then incubated with the appropriate primary (1:1000) and secondary antibody (1:5000). Secondary antibody binding was visualized using chemiluminescence detection technology.

**Production of Ezh2 knockdown cell lines**

SCC-13 cells (1 × 10⁶) were plated in 24-well plates and allowed to attach overnight. The cells were then infected with SMARTVector 2.0 Lentivirus encoding control-shRNA (Thermo Scientific, S-000500-01) or SMARTVector 2.0 Lentivirus constructs encoding each of three distinct Ezh2-shRNA (Thermo Scientific, SH-0024218-01-10, SH-0024218-02-10, SH-0024218-03-10). SCC13-Ezh2-shRNA3 Ezh2 knockdown cells were produced using SMARTVector 2.0 SH-0023218-03-10. A similar procedure was used to generate the HaCaT-Control-shRNA and HaCaT-Ezh2-shRNA3 cells. Viral infection was in serum-free growth medium containing 8 μg/ml polybrene at 37°C for 4 h. The medium was changed to fresh serum-containing growth medium and 80% confluent cultures were split into 35 mm dishes and maintained in the presence of 0.25 μg/ml puromycin for 2 weeks to develop the lines. Additional lines were produced from independent viral infections. Parallel Ezh2 knockdown lines were developed using SMARTVector 2.0 SH-0024218-01-10. These cell lines also displayed reduced Ezh2 level. Data from the SCC13-Ezh2-shRNA3 and HaCaT-Ezh2-shRNA3 lines are reported here, but identical results were observed using these alternate lines.

Cell lines were purchased from vendors (ATCC, etc.), which confirm identity by short tandem repeat analysis. Fresh batches of cells are thawed for use at 6 month intervals to reduce chance of contamination and genetic drift.

**Tumor xenograft growth assays**

Spheroid-selected (ECS) cells were trypsinized to prepare single cell suspensions, resuspended in PBS containing 30% Matrigel and 100 000 cells, in 100 μl, were injected subcutaneously into the two front flanks of NOD scid IL2 receptor gamma chain knockout mice (NSG mice) using a 26.5 gauge needle. Five mice were used per data point (6). Tumor growth was monitored by measuring tumor diameter and calculating tumor volume = (4/3π × (diameter/2)²) (45). Tumor samples were harvested to prepare extracts for immunoblot and sections for immunostaining. These studies were approved by the institutional board and followed accepted national and international practices for the treatment and welfare of animals.

**Results**

**Biological impact of Ezh2 knockdown on spheroid-forming cells**

Ezh2 and other PcG genes have been proposed to have a role in cancer stem cell survival (25,29,37,38,46–48). Our previous studies show that PcG proteins are highly expressed in epidermal squamous cell carcinoma, compared with normal tissue (6,37,38,47,48), and that tumor-initiating ECS cells express the highest levels of Ezh2 (6). Moreover, Ezh2-positive ECS cells form highly vascularized and aggressive tumors (6).

In the present study, we investigate whether Ezh2 expression and activity is required for ECS cell survival. We first compare wild-type and stable Ezh2 knockdown SCC-13 cell lines. The Ezh2 knockdown cells were produced by lentivirus-mediated delivery of Ezh2-specific shRNA followed by puromycin selection. We assessed the ability of wild-type and Ezh2 knockdown cells to form spheroids. Figure 1A shows the spheroid morphology and suggests a reduction in spheroid number for Ezh2 knockdown cultures. Figure 1B plots the reduction in spheroid formation following Ezh2 knockdown. A substantial reduction in spheroid number is observed at days 6, 8 and 10, suggesting that Ezh2 expression is required for spheroid growth and survival.

The PRC2 multiprotein complex contains Ezh2, EED, Suz12 and RbAp46 (30). The major role of this complex is to facilitate Ezh2-dependent trimethylation of histone H3 at lysine 27 (H3K27me3) (28,31). Figure 1C characterizes the impact of Ezh2 knockdown and shows that this is associated with reduced H3K27me3 formation. Since the PRC2 complex acts as a unit, we wondered whether loss of Ezh2 may also alter the level of other PRC2 complex proteins. However, we observe no change in expression of Suz12, suggesting that loss of Ezh2 does not necessarily alter the level of other components.

We also examined the impact on the PRC1 complex. This complex includes Bmi-1, Ph1, CBX and Ring 1A/B (30). H3K27me3 serves as a binding site for the chromodomain of the CBX protein (31). The CBX protein anchors the PRC1 complex to chromatin and then the Ring1B protein ubiquitinates histone H2A at lysine 119 (25,31). Therefore we monitored the impact on the level of Ring1B, the catalytic subunit of the PRC1 complex (25,40). No impact on Ring1B level is observed; however, the product of Ring1B catalytic activity, ubiquitination of lysine 119 of histone H2A, is reduced (Figure 1C). This is perhaps not unexpected, considering that H3K27me3 constitutes the binding site for PRC1 complex interaction with chromatin (25,40). Thus, a reduction in H3K27me3 level would preclude Ring1B interaction with chromatin and reduce H2AK119ub formation. It is interesting that Bmi-1, a PRC1 complex protein, is increased in expression. This increase in Bmi-1 level may be a compensatory change in response to the reduction in H2AK119ub formation, as Bmi-1 interaction with Ring1B enhances Ring1B catalytic activity (24–29). We also checked the impact of Ezh2 loss on the level of other stem cell marker proteins. As shown in Figure 1C, Ezh2 knockdown cells display a marked reduction in Sox2 level, but Oct4 and K15 levels remain unchanged.

**Pharmacologic inhibition of Ezh2 activity**

As a second approach to assess the role of Ezh2, we examined the impact of Ezh2 inhibitors, JQ-EZ-005, GSK126 (42) and EPZ-6438 (49–51), on spheroid formation. GSK126 and EPZ-6438 are commercially available Ezh2 methyltransferase activity inhibitors (42,49). JQ-EZ-005 is an Ezh2 activity inhibitor that was developed by Dr J Bradner (Harvard). As shown in Figure 1D, treatment of preformed SCC-13-derived spheroids with JQ-EZ-005 causes a reduction in Ezh2 level and an associated reduction in H3K27me3 formation. Moreover, JQ-EZ-005 treatment reduces spheroid number (Figure 1B) and causes fragmentation of the normally well-circumscribed spheroids (Figure 1F). JQ-EZ-005 treatment also reduces spheroid formation (Figure 1G). We next tested the impact of GSK126 and EPZ-6438 (42,49–51). Spheroids were permitted to form for 8 days before treatment with these agents. Treatment reduced spheroid number (Figure 2A/D). Like JQ-EZ-005, treatment with EPZ-6438 causes spheroid fragmentation (Figure 2E). This is in contrast to GSK126, which reduces spheroid number, but does not cause fragmentation (Figure 2B). We also examined the impact of these agents on PcG protein and stem cell marker expression. EPZ-6438 and GSK126 reduce Ezh2 protein level and H3K27me3 formation (Figure 2C and F) and this is associated
with loss of Suz12, another PRC2 protein. Moreover, each compound also reduces expression of Bmi-1 and Ring1B (PRC1 complex proteins) and H2AK119ub formation (Figure 2C and F). These compounds reduced Oct4 level but did not influence Sox2, GSK126 and EPZ-6438 did not alter K15 level (Figure 2F).

Figure 1. Ezh2 expression is required for spheroid formation. (A) Ezh2 knockdown and control cell lines were plated at 40,000 cells per 9.5 cm² dish in non-attachment plates and grown in spheroid medium. After 10 days, the spheroids were photographed. (B) Ezh2 knockdown cells fail to efficiently form spheroids. Cells were plated as above and spheroid number was monitored at 0–10 days. The values are mean ± SEM, n = 3. The asterisks indicate a significant difference between Ezh2-shRNA and control-shRNA cell spheroid formation (P < 0.01). (C) Level of Ezh2 in control and Ezh2 knockdown cells. Control and Ezh2 knockdown cells were grown for 10 days in spheroid-selection medium and total cell extracts were prepared for immunoblot detection of the indicated proteins. Similar results were observed in each of three independent experiments. (D) Treatment with Ezh2 inhibitor reduces spheroid survival. SCC-13 cells were seeded at 40,000 cells in 9.5 cm² non-attachment plates and grown as spheroids for 8 days before addition of the indicated concentration of JQ-EZ-005. After treatment with JQ-EZ-005 for 48 h, the extracts were prepared for immunoblot detection of the indicated proteins. (E) The spheroids were photographed after 48 h of treatment with JQ-EZ-005. Bars = 125 μm. The arrow indicates spheroids that are fragmenting and panel (F) presents an enlarged image of this spheroid. (G) Spheroid number was determined after treatment with JQ-EZ-005 for 48 h as outlined above. The values are mean ± SEM, n = 3 and the asterisks indicate a significant difference, P < 0.005.

Ezh2 is required for ECS cell migration

Enhanced ability to migrate is a property of tumor-initiating cells that is associated with cancer metastasis. We previously showed that ECS cells efficiently invade and rapidly migrate compared with non-stem cancer cells and that these properties are associated with enhanced tumor formation (6). To assess whether elevated Ezh2 is important for enhanced migration, we monitored the ability of Ezh2 knockdown cells to migrate through matrigel and to close a scratch wound. Figure 3A and B shows that the Ezh2 knockdown cells display a 50% reduction in ability to migrate through matrigel compared with the control cells. Figure 3C examines the ability of spheroid-derived control and Ezh2 knockdown cells to close a wound in a cell-attached scratch wound-healing assay. This experiment shows
the reduced migratory capacity of Ezh2-negative ECS cells. We next examined the impact of Ezh2 inhibitor treatment on ECS cell invasion and migration. As shown in Figure 3, treatment with these agents (GSK126, EPZ-6438) reduces cell migration in both the matrigal invasion (Figure 3D and E) and scratch wound closure (Figure 3F) assays.

**Loss of Ezh2 leads to reduced tumor formation**

We next examined the impact on tumor growth. Control and Ezh2 knockdown cells were assessed for ability to form tumors in immune-compromised NSG mice. Subcutaneous injection of cells at 0.1–1.5 million per site revealed a dose-dependent increase in tumor formation when monitored at 4 weeks post-injection (Figure 4A) and increased tumor size with time (Figure 4B). However, in each case, tumor size is reduced by Ezh2 knockdown. Figure 4C shows that the reduction in Ezh2 level is associated with reduced H3K27me3 formation. This analysis also shows minimal changes in Bmi-1 and Suz12 level. In some experiments, SCC13-Ezh2-shRNA3 cells formed tumors as efficiently as control cells, but this was always associated with re-expression of Ezh2. We also examined the impact of treatment with GSK126 on ECS cell ability to form tumors. Figure 4D shows a marked reduction in tumor growth rate in response to GSK126 treatment. Figure 4E shows representative images of the tumors from the GSK126 treated and non-treated groups. As shown in Figure 4F, inhibition of Ezh2 is associated with reduced Ezh2 level and activity (H3K27me3 formation). This is associated with reduced expression of Suz12, Bmi-1 and Oct4. PARP is slightly reduced and caspase-3 level is increased, suggesting that GSK126 treatment activates apoptosis.
Ezh2 is required for HaCaT cell spheroid formation, migration and invasion

To assess whether these changes are observed in other epidermis-derived lines, we assessed the impact of Ezh2 treatment on HaCaT cells. HaCaT cells are epidermis-derived cells that are immortalized, but do not form tumors (44). We compared HaCaT-control-shRNA and HaCaT-Ezh2-shRNA3 cells. Approximately, 0.05% of the HaCaT cells (20–25 spheroids/40 000 cells plated) are able to form spheroids in non-attached growth conditions and Ezh2 knockdown reduces spheroid number (Figure 5A and B) and H3K27me3 formation (Figure 5C). Ring 1B level and H2AK119ub formation are also reduced (Figure 5C). Loss of Ezh2 also leads to reduced invasion in a matrigel invasion assay (Figure 5D) and reduced migration in a wound closure assay.
We further show that GSK126 and EPZ-6438 reduce HaCaT cell spheroid formation (Figure 6A, B, D and E) and Ezh2 level and activity (as measured by reduced H3K27me3 formation) (Figure 6C and F). Analysis of polycomb expression reveals reduced Suz12 and Bmi-1 expression following treatment. Sox2 levels increase and Oct-4 level declines with treatment, and keratin 15 expression is unchanged (Figure 6C and F). GSK126 and EPZ-6438 also activate apoptosis, as evidenced by increased level of active caspase-3 and/or reduced PARP level (Figure 6C and F).

Discussion

Epidermis-derived cancer stem cells

Our strategy for studying tumor-initiating cells is based on that of Wicha et al. (52) where mammary tumor-initiating cells were selected by culture as spheroids. In the mammary cancer system, these cells are multipotent and express a specific set of stem cell marker proteins (52). Skin tumors also include a subpopulation of cells that can be grown as spheroids under non-attachment conditions. We observed that 0.15% of SCC-13 cells survive under spheroid-selection conditions (54). This is consistent with the idea that tumor-initiating cells represent a small proportion of the total mass of the tumor (52).

To characterize these cells, we measured expression of stem cell markers, as cancer stem cells can be identified based on whether they express markers typical of normal tissue stem cells and embryonic stem cells (52–55). We also showed that these cells are enriched for expression of aldehyde dehydrogenase 1 (6), a marker that is expressed in many types of stem cells (56–58), as well as embryonic stem cell markers, Oct4 and Sox2 (6). Using this model system, we have studied the role of PcG proteins in stem cell maintenance.
Ezh2 is required for ECS cell survival

PcG protein levels are elevated in skin cancer cells and tumors (30,37,38,47,59,60) and our recent study shows that these proteins are highly enriched in ECS cells (6). PRC1 and PRC2 (27) suppress gene expression via covalent modification of selected histones (24–29). Ezh2 is a PRC2 complex component that trimethylates lysine 27 of histone H3 (H3K27me3) (28,31). This is the first step in gene silencing. H3K27me3 then serves as a binding site for the chromodomain of the CBX protein of the PRC1 complex, which anchors the PRC1 complex to the chromatin (31). Ring1B is the key activity of the PRC1 complex and it ubiquitinates histone H2A at lysine 119 (25,31). The sequential
trimethylation and ubiquitination events result in chromatin condensation leading to silencing of tumor suppressor gene expression (27, 28).

Our present study shows that Ezh2 knockdown reduces ECS cell spheroid formation, suggesting that Ezh2 is required for skin cancer stem cell survival. Moreover, the reduction in Ezh2 is associated with reduced Ezh2 activity as measured by H3K27me3 formation. Since the PRC2 complex includes Ezh2, EED, Suz12 and RbAp46 (30), we might anticipate that loss of Ezh2 may destabilize the complex and cause loss of the other PRC2 proteins. However, we found that loss of Ezh2 does not predict loss of other polycomb proteins. In Ezh2 knockdown SCC-13 cells, H3K27me3 formation is reduced, Ring 1B levels remain the same, H2AK119ub formation is reduced and Bmi-1 levels are increased. In contrast, when SCC-13 cells are treated with GSK126 or EPZ-6438, different changes are observed. Moreover, a different pattern of response is observed for Ezh2 knockdown and inhibitor-treated HaCaT cells. Thus, the impact of Ezh2 loss or inactivation on the level and function of other polycomb proteins is cell-type and treatment dependent. Moreover, the inhibitors produce slightly different responses in terms of biochemical markers. We are not sure why this is the case, but it is likely that difference in mechanism of action account for some of these differences.

Treatment with the agents that inhibit Ezh2 catalytic activity (GSK126, JQ-EZ-005 and EPZ-6438) reduced ECS cell spheroid formation. This is associated with a reduction in spheroid number and also in spheroid growth. These compounds enhance apoptosis-associated spheroid fragmentation and can destroy pre-existing spheroids. De novo spheroid survival is also inhibited by these agents including GSK126 (Supplementary Figure 2S is available at Carcinogenesis, Online). It is interesting that although these agents are designed to inhibit Ezh2 catalytic activity, they also consistently reduce Ezh2 protein level. We have not explored why this happens, but it is possible that inhibition of activity alters the conformation of the methyltransferase and makes it susceptible to proteasome degradation. This would be consistent with our previous report using DZNep, a 3-deazaadenosine analog and potent inhibitor of S-adenosylhomocysteine hydrolase (61–64). Inhibiting S-adenosylhomocysteine hydrolase

Figure 6. GSK126 and EPZ-6438 reduce HaCaT cell spheroid formation. HaCaT cells were seeded at 40 000 cells in 9.5 cm² non-attachment plates and grown as spheroids for 8 days before 3 day treatment with the indicated concentration of GSK126 or EPZ-6438. (A and D) Spheroid number was determined at day 3 after initiation of inhibitor treatment. The values are mean ± SEM, n = 3 and the asterisks indicate a significant difference, P < 0.01. (B and E) Spheroid images were collected at 3 days after initiation of inhibitor treatment. (C and F) Extracts were prepared for immunoblot detection of the indicated proteins at 3 days after initiation of inhibitor treatment.
results in accumulation of S-adenosylhomocysteine, which leads to product inhibition of S-adenosyl-L-methionine-dependent methyltransferases (63), which indirectly inhibits methyltransferase activity by limiting methyl donor availability (65). Treatment with DZNep enhances proteasome-dependent Ezh2 degradation (60). DZNep also promotes apoptosis as measured by increased PARP cleavage and caspase-3 activation (60). This is similar to the present study, where we observe increased cell death in inhibitor-treated cells evidenced by an increase in the number of trypan blue-positive cells and enhanced PARP cleavage and caspase-3 activation.

**Ezh2 is required for ECS cell invasion, migration and tumor formation**

Increased ability to invade tissue and migrate are known properties of cancer stem cells. Indeed, our previous study shows that ECS cells more efficiently invade matrigel and migrate on plastic compared with non-stem cancer cells (6). The present studies show that knockdown of Ezh2 reduces both invasion and migration and that the Ezh2 selective inhibitors also reduce these events. We also tested the impact of Ezh2 knockdown and inhibitor treatment on tumor formation. It is known that ECS cells form rapidly growing, aggressive and vascularized tumors compared with non-stem cancer cells (6). Aggressive tumor formation is observed following injection of as few as 100 cells into immune-compromised mice (6). Our present studies show that Ezh2 knockdown or treatment with GSK126 reduces tumor formation by 60%. Moreover, these tumors appear to be undergoing substantial destruction and are in some cases highly necrotic. We further show that GSK126 treatment is also consistently associated with reduced Ezh2 activity as evidenced by reduced H3K27me3 formation.

**Targeting Ezh2 as a cancer stem cell survival protein**

The present studies show that Ezh2 expression is essential for ECS cell survival and that reducing Ezh2 level by knockdown or treatment with agents that inhibit catalytic activity inhibits ECS cell invasion, migration and tumor formation. The observation that Ezh2 is highly enriched in ECS cells suggests that it may serve as a target to reduce stem cell survival in squamous cell carcinoma. This is potentially important, as although ECS cells comprise only a small subpopulation of the tumor, they are the most highly tumorigenic cell type in the tumor (6). Thus, targeted inhibition of Ezh2 may make it possible to reduce ECS cell survival and thereby reduce tumor formation.

**Supplementary material**

**Supplementary Figures 1S–3S** can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

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