EZH2 Is Required for Breast and Pancreatic Cancer Stem Cell Maintenance and Can Be Used as a Functional Cancer Stem Cell Reporter

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INTRODUCTION

Histone modification through polycomb group (PcG) proteins is a major driver in stem cell biology through the regulation of genes involved in stemness and differentiation. PcG proteins function within two main polycomb repressive complexes (PRC), PRC1 and PRC2 [1]. Together, these PRCs mediate gene repression through chromatin remodeling, specifically by catalyzing post-translational modifications on histone proteins [1, 2]. PRC2 is a multiprotein complex, consisting of EZH2, SUZ12, EED, and RBAP48. The PRC2 component enhancer of zeste homolog (EZH2) catalyzes the methylation of histone H3 at lysine 27, leading to a trimethylation at this site (H3K27me3) [2, 3]. H3K27me3 then acts as a binding site for PRC1, which catalyzes the ubiquitination of lysine 119 on histone H2A [4]. Ultimately, this cooperation is believed to lead to initiation (by PRC2) and maintenance (by PRC1) of gene repression [1], further believed to specifically repress differentiation genes, thereby maintaining cells in a “stemness” state [1].

Complementary to polycomb repression is the trithorax group (TrxG), a complex of proteins that catalyzes methylation of lysine 4 on histone H3 (H3K4me3) [5]. Chromatin modulation by H3K4me3 demarkation regulates gene activation rather than repression and thus accomplishes an opposite drive from that of PcG proteins [4, 5]. It is now known that key developmental regulators can be bivalently marked by both H3K27me3 and H3K4me3 [6], leading to a fine balance between repression and activation of the gene rather than regulation through more permanent silencing mechanisms. This flexibility is further extended with the discovery of H3K27me3 demethylases JMJD3 and UTX, which could further support the mechanism whereby a repressed domain becomes active [7].

Overexpression of several PcG proteins, EZH2 included, has not only been identified in many clinical tumor specimens but has also been implicated as a negative prognostic factor in these tumors [8, 9]. In prostate cancer, levels of EZH2 are gradually upregulated with progression of the disease, and high levels of EZH2, detected by immunohistochemistry, significantly correlate with a greater risk of recurrence [10]. In accordance with this finding, Crea et al. [11] found
that therapeutic inhibition of PRC2 with the small molecule inhibitor 3-dezaneplanocin-A (DZneP) decreased invasion and tumor growth of two prostate cancer models, leading to a significant increase in tumor-free survival in animals. Similar results have been found in pancreatic ductal adenocarcinoma, whereby patients with increased expression of EZH2 not only had a greater occurrence of node positivity but also presented with significantly larger tumor size [12]. Similar to prostate cancer, high EZH2 expression in pancreatic cancer led to a decrease in overall patient survival [12]. Chen et al. [13] used RNA interference-mediated knockdown of EZH2 to show that EZH2 loss decreased tumor growth and the occurrence of liver metastasis of a pancreatic cancer model. In breast cancer, EZH2 overexpression similarly correlates with poor prognosis. Pietersen et al. [14] conducted a meta-analysis of 295 stage 1 and 2 breast cancer patients and concluded that high EZH2 levels not only correlated with shorter overall survival but also positively correlated with tumor cell proliferation markers. Interestingly, however, they found an inverse relationship for the PRC1 protein BMI-1, in that high BMI-1 levels were correlated with increased survival and good prognosis, a finding that they attribute to the differential expression of EZH2 and BMI-1 in breast cancer subtypes. EZH2 overexpression was seen in the more aggressive basal type, whereas BMI-1 overexpression was noted in the luminal type [14].

The field of cancer biology is gaining increasing support for the findings that initial tumorigenicity and tumor progression is driven by a tumor cell subtype that has “stem-like” characteristics, termed the cancer stem cell (CSC), or tumor initiating cell. CSCs have been implicated in a plethora of studies as the cells responsible for drug resistance and disease relapse following therapy [15], disease progression [16], and metastasis [16, 17]. In many instances, CSCs are driven by similar stemness and differentiation pathways (such as Wnt, Notch, and Hedgehog) that regulate embryonic and adult tissue stem cells [18]. Thus, the idea that epigenetic modification by PcG proteins is implicated in differentiation pathways (such as Wnt, Notch, and Hedgehog) that regulate embryonic and adult tissue stem cells [18].

The findings that initial tumorigenicity and tumor progression is driven by a tumor cell subtype that has “stem-like” characteristics, termed the cancer stem cell (CSC), or tumor initiating cell. CSCs have been implicated in a plethora of studies as the cells responsible for drug resistance and disease relapse following therapy [15], disease progression [16], and metastasis [16, 17]. In many instances, CSCs are driven by similar stemness and differentiation pathways (such as Wnt, Notch, and Hedgehog) that regulate embryonic and adult tissue stem cells [18]. Thus, the idea that epigenetic modification by PcG proteins is implicated in the CSC phenotype is not a far stretch. In fact, transduction of the idea that epigenetic modification by PcG proteins is implicated in the CSC phenotype is not a far stretch. In fact, transduction of

**MATERIALS AND METHODS**

**Cell Culture and Treatment**

Human pancreatic adenocarcinoma cell lines HPAC and Panc-1 and breast adenocarcinoma cell lines HCC1937 and T47D were maintained in two-dimensional culture under humidified incubation in 5% CO₂, controlled atmosphere, using cell culture medium for each line recommended by the American Type Culture Collection (Manassas, VA, http://www.atcc.org). For small interfering RNA (siRNA) knockdown experiments, cells were reverse transfected with 10 nM siRNA for T47D, HCC1937, and Panc-1 cells, and 20 nM for HPAC cells, using nontarget (control C) or EZH2-targeted pooled siRNA constructs (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) and RNAiMax (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) as the carrier, under reduced (2%) serum conditions for 4 days prior to gene, protein, or flow cytometric analysis. For proof-of-concept studies, HPAC cells were treated with salinomycin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), diluted from a 5 mM stock prepared in dimethyl sulfoxide (DMSO), N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyloxycarbonyl (DAPT; EMD Millipore, Billerica, MA, http://www.emdmillipore.com) diluted from a 25 mM stock prepared in DMSO, both alongside DMSO as a vehicle control, or gemicitabine (Gemzar; Hanhs, New York, NY, http://www.hanschein.com), diluted from a 1 mg/ml stock prepared in distilled water for 72 hours. Cells cultured under spheroid growth conditions were plated at 3,000 cells per ml to ultra-low attachment tissue culture plates (Corning Costar, Acton, MA, http://www.corning.com/lifesciences) in serum-depleted defined medium. The cells were incubated for 4 days at 37°C with 5% CO₂ controlled atmosphere to form spheroid colonies.

**Cell Sorting and Flow Cytometric Analysis**

The samples were prepared for any flow cytometry experiments by harvesting cells with 0.25% trypsin-EDTA, followed by a wash and resuspension at 5 × 10⁶ cells per ml into Hank’s balanced saline solution (HBSS). The cells were stained with anti-human CD24-fluorescein isothiocyanate, and anti-human CD44-allophycocyanin antibodies at a concentration of 5 μl of antibody per 1 × 10⁶ cells or anti-human ESA-PerCP-cy5.5 antibody at a concentration of 10 μl antibody per 1 × 10⁶ cells (all antibodies obtained from BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). The cells were incubated with antibodies for 15 minutes at 4°C, followed by two washes in HBSS. Prior to fluorescence-activated cell sorting (FACS) experiments, 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) was added to each sample at 30 μM to identify live cells by DAPI exclusion. All cell sorting experiments were run on an Ariall (BD Biosciences), and all flow cytometry analysis experiments were run on an LSRII (BD Biosciences).

**Molecular Characterization of Gene and Protein Activity**

RNA was extracted from all cell types using the Ambion RNAqueous kit (Life Technologies, Carlsbad, CA, http://www.
lifetecnologies.com) and analyzed for quantity and quality by spectrophotometry. For quantitative reverse transcription-polymerase chain reaction (qPCR) experiments, 10–100 ng of RNA was reverse transcribed using Superscript III enzyme (Invitrogen) and preamplified for 14 cycles using pooled primers for each gene of interest (primers at 0.2× concentration each), followed by qPCR of 1:20 diluted preamplified cDNA on an 7900HT fast thermocycler (all qPCR primers, reagents, and instrumentation were from Life Technologies). The values were standardized to an endogenous control gene and expressed as relative quantitation relative to non-CSCs or control siRNA.

Protein was extracted from all cell types using radioimmunoprecipitation buffer supplemented with protease and phosphatase inhibitors and analyzed for quantity using the BCA assay (ThermoFisher Scientific, Waltham, MA, http://www.thermofisher.com). Ten micrograms of protein was loaded onto a 4%–12% 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol gel, transferred to nitrocellulose, and blocked with 3% non-fat dry milk-Tris buffered saline + Tween 20 (TBST) for actin, and 3% bovine serum albumin-TBST for EZH2 and H3K27me3. Both primary and horseradish peroxidase-conjugated secondary antibodies were incubated at 1:1,000 dilution (all antibodies from Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com).

Whole Genome Array and Histone-H3 Trimethylation ChIP Assay
Whole genome array was run using an Affymetrix array (Affymetrix, Santa Clara, CA, http://www.affymetrix.com). Generation of biotin-labeled amplified cRNA from 250 ng of total RNA was accomplished with the MessageAmp II-Biotin Enhanced RNA Amplification Kit (Life Technologies). The concentration and purity of the cRNA product were determined spectrophotometrically. Fifteen micrograms of each biotin-labeled cRNA was fragmented for hybridization on Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays. All GeneChip washing, staining, and scanning procedures were performed with Affymetrix standard equipment. Data capture and initial array quality assessments were performed with the GeneChip Operating Software tool.

Histone H3 trimethylation chromatin immunoprecipitation (ChIP) was performed essentially as described [21]. Briefly, chromatin was prepared from HCC1937 cells after knockdown with nontarget or EZH2-targeted siRNAs as described in Cell Culture and Treatment. The cells were cross-linked in 1% formaldehyde, fixed in 4% formaldehyde for 15 minute, followed by a 15-minute permeabilization step in 0.1% Triton X-100 (Sigma-Aldrich). The plates were blocked in HCS blocking buffer (ThermoFisher Scientific) supplemented with 2% FBS, followed by a 1-hour incubation in mouse anti-human EZH2 or rabbit anti-human H3K27me3 both at 1:200 dilution (Cell Signaling Technology). The plates were first washed twice in Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 0.05% Tween 20 (Sigma-Aldrich) and then washed an additional two times in DPBS. Dylight-549-conjugated anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) and Dylight-488-conjugated anti-mouse (Jackson Immunoresearch) were then added to the plates at 1:200 dilution in addition to Hoechst 33342 (Life Technologies) at 1:10,000 dilution. The plates were incubated for 45 minutes, followed by the same washing procedure as before. Subsequently, the plates were scanned and analyzed on the Cellemics Arrayscan VTi reader (ThermoFisher Scientific). Additional details and materials and methods can be found in the supplemental online text.

RESULTS
Characterization of Breast and Pancreatic CSCs
Various independent reports have identified the breast CSC population as CD44+CD24low− [23] and the pancreatic CSC population as EpCam+CD44+CD24− [24]. We have confirmed that these markers identify phenotype-positive CSCs in our models as well by validating tumorigenic potential of cells isolated by these markers compared with non-CSC control populations (supplemental online Fig. 1, with gating strategy and controls in supplemental online Fig. 2; supplemental online Table 1).

Following validation of the CSC population in both tumor types, isolated CSCs were profiled for their relative EZH2 and H3K27me3 levels. Total protein was collected from HPAC and HCC1937 isolated CSCs and non-CSCs and subjected to Western blot analysis. Both the pancreatic and the breast CSCs have clearly elevated levels of H3K27me3 compared with the non-CSC control population (using actin staining as an internal loading control) (supplemental online Fig. 1C). Similarly, EZH2 levels are elevated in the CSCs, albeit not as drastically as H3K27me3 levels. We further confirmed elevation of EZH2 levels in CSCs by qPCR analysis in supplemental online Figure 1D. Extension of this profiling to an additional pancreatic cancer line (Panc-1) and an additional breast cancer line (T47D) supports the finding that CSCs have elevated EZH2 levels (supplemental online Fig. 1D). This elevation of EZH2 expression in CSCs carries over to clinical pancreatic and breast tumors, whereby sorted CSCs from breast and pancreatic patient-derived xenografts (PDX) were found to have elevated EZH2 levels (supplemental online Fig. 3A).

Characterization of cell type and environment of elevated EZH2 in the CSC population of breast and pancreatic cancer.

EZH2/H3K27me3 Loss Has a Direct Effect on CSC Regulation
To examine the phenotypic role of EZH2 and H3K27me3 on the CSC compartment, EZH2 was knocked down by siRNA, and the

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CSC percentage was subsequently measured by flow cytometric analysis, which can effectively monitor subdivision of tumor cells into CSCs and respective progenitor and differentiated populations. In T47D breast cancer cells, EZH2 loss decreased the CD44<sup>-</sup>CD24<sup>low</sup> frequency 4.5-fold from 23.1% in control cells to 5.1% with EZH2 siRNA (Fig. 1A, \( p < .001 \)). In HCC1937 breast cancer cells, a similar significant loss of CSCs was seen, where a 4.5% drop in the CSC population was seen after EZH2 knockdown (Fig. 1B, \( p < .01 \)). Both pancreatic cancer modes, HPAC and Panc-1, showed similar results. In Panc-1 cells, the EpCam<sup>-</sup>CD44<sup>-</sup>CD24<sup>-</sup> frequency dropped 1.6-fold from 12.7% frequency in the control to 7.8% frequency after EZH2 loss (Fig. 1C, \( p < .001 \)). Lastly, in HPAC cells, the CSC frequency was similarly reduced twofold from 13.8% under control conditions to 7% after EZH2 knockdown (Fig. 1D, \( p < .001 \)). Although CSC frequency was significantly diminished with EZH2 loss in all models tested, a complete elimination of the CSC population was not seen. This could, however, be attributed to the presence of some residual detectable EZH2 and H3K27me<sub>3</sub> levels following siRNA transfection as seen in Figure 2A. It remains to be determined whether complete EZH2/H3K27me<sub>3</sub> loss will eradicate the CSC population or whether other molecular pathways can allow for some resistance. Nonetheless, the data clearly show a critical role for EZH2 and H3K27me<sub>3</sub> in CSC maintenance.

It is unclear whether EZH2 loss leads to a specific killing of the CSCs or whether loss of this polycomb repression simply drives the cells into differentiation. However, analysis of the corresponding populations can shed some light on the cellular fate following EZH2 loss. We hypothesize that EZH2 knockdown does not affect all populations uniformly but rather appears to compensate for the loss in CSCs. The breast cancer cells show a significant gain in the more differentiated CD24<sup>+</sup> populations in response to a loss of CSCs, whereby T47D cells compensate for the 4.5-fold loss in CSCs with a 4.4-fold gain in CD44<sup>-</sup>CD24<sup>+</sup> cells (Fig. 1A). HCC1937 cells similarly adjust their 4.5% CSC loss with a 2.5% gain in CD44<sup>low</sup>CD24<sup>+</sup> cells, although a 2% gain in the fully differentiated CD44<sup>+</sup>CD24<sup>+</sup> population was also seen (Fig. 1B).

In the HPAC pancreatic cancer model, the 9% loss of cells from the EpCam<sup>+</sup>CD44<sup>-</sup> populations was opposed by a gain in the EpCam<sup>+</sup>CD44<sup>-</sup>CD24<sup>-</sup> fractions (Fig. 1D). In Panc-1 cells, however, the nearly 10% combined loss of CD44<sup>-</sup>CD24<sup>+</sup> cells was mirrored by a 10% gain in a different subpopulation, mainly the EpCam<sup>+</sup>CD44<sup>+</sup>CD24<sup>-</sup> cells (Fig. 1C). Although observational, these data do suggest that EZH2 loss drives the cells into more differentiated populations, as expected from the epigenetic regulation by EZH2 and H3K27me<sub>3</sub>.

Several factors can compensate for or override the catalytic activity of EZH2 in methylating histone 3 on K27. EZH1 has been shown to compensate for EZH2 loss and facilitate histone methylation at this site [25]. Inversely, JMJD3 and UTX are histone demethylases at this site [7]. Although knockdown of EZH2 to the total tumor population did result in a loss of H3K27me<sub>3</sub> (Fig. 2A), it is possible that expression of EZH1, JMID3, and UTX could be modified to compensate for the loss. Therefore we monitored the response of these three additional proteins following EZH2 knockdown in the total tumor population. Figure 2B shows by qPCR analysis that EZH2 knockdown does not alter gene expression patterns that would favor remethylation but surprisingly leads to an alteration of proteins that would favor enhancement of methylation loss. In HPAC cells, EZH2 knockdown also leads to a twofold decrease in EZH1 compared with control siRNA, with

**Figure 1.** Phenotypic effect of EZH2 knockdown establishes a critical role for EZH2/H3K27me<sub>3</sub> in CSC maintenance. Shown are flow cytometry analysis of breast cancer population distribution in T47D (A) and HCC1937 (B) cells based on CD44 and CD24 surface marker expression and pancreatic cancer population distribution in Panc-1 (C) and HPAC (D) cells based on EpCam, CD44, and CD24 surface marker expression. All of the data were collected at day 4 after control or EZH2 siRNA transfection. *(), **(), and ***() denote statistical significance between control and EZH2 siRNA at \( p < .05 \), \( p < .01 \), and \( p < .001 \), respectively (\( n = 3 \) biological replicates per treatment per cell type). Abbreviation: siRNA, small interfering RNA.
no significant changes in UTX or JMJD3, thereby complementing the loss of EZH2 rather than compensating for it. In HCC1937 cells, a similar change occurred whereby EZH2 knockdown also led to a 1.5-fold increase in the demethylase protein JMJD3 compared with control siRNA, also complementing EZH2 loss rather than compensating for it.

Transcriptional Regulation Following EZH2 Loss Correlates to a CSC Signature

To better understand the molecular consequences of the loss of EZH2 expression, whole genome Affymetrix arrays were performed. Several groups have demonstrated that breast cancer cell lines cultured under anchorage-independent, serum-free conditions develop into tumor spheroids composed of multilineage progeny and an enrichment of CSCs [26]. We have also confirmed this CSC-enriched phenotype for breast cancer spheroids by showing increased tumorigenicity of HCC1937 cells when cultivated under mammosphere-forming conditions (supplemental online Fig. 4). To relate gene expression changes observed upon EZH2 loss to CSCs, we compared these arrays with arrays performed using RNA isolated from mammospheres. Upon examination of the gene regulation, a striking inverse correlation was observed. Figure 2C illustrates this clear relationship between EZH2 and CSCs in HCC1937 cells. Genes downregulated in response to EZH2 knockdown, and thus CSC inhibition, are conversely increased in CSC-enriched spheroids. Likewise, genes upregulated with EZH2-dependent CSC inhibition are then decreased in spheroids.

Epigenetic Regulation of CSC-Related Genes by EZH2/H3K27me3

To further validate the mechanisms whereby EZH2 loss leads to CSC inhibition by regulation of H3K27me3 specifically, we subjected HCC1937 cells treated with control or EZH2 siRNA to ChIP analysis against H3K27me3 and its bivalent marker H3K4me3. Figure 3 illustrates this relationship between histone methylation and gene expression. The data indicate that EZH2 knockdown, leading to gene activation of differentiation regulators such as WNT5a and HS6ST2, is mirrored by a decrease in the transcriptional repressor H3K27me3 on the locus (Fig. 3). Conversely, we observed that genes that showed transcriptional inhibition following EZH2 loss had increased levels of H3K27me3 demarkation, further supporting the role for histone methylation at this site to regulate this transcriptional activity. A loss of the repressive H3K27me3 is usually accompanied by a gain in the activating mark H3K4me3 and vice versa, an observation that held true in our analysis as well (Fig. 3). Again, we observed an inverse correlation between EZH2-regulated transcription and known CSC mediators. Developmental regulators such as KLF4 and GATA6 were decreased with EZH2 knockdown. Both of these genes have a demonstrated role in maintenance of CSCs either directly [27] or through a direct linkage to Wnt signaling [28], thereby tying their expression patterns to CSCs. Similarly WNT5a
and H56ST2, two genes thought to influence cell motility and differentiation in cancer [29, 30], were upregulated when EZH2 was knocked down. These results not only tie together the direct effect of EZH2-mediated histone methylation on transcriptional activation, but they also suggest the epigenetic regulation whereby EZH2 and H3K27me3 influence CSC maintenance.

**EZH2 HCS Assay Setup and Validation**

Our results not only demonstrate that EZH2 is a critical component of CSC function in breast and pancreatic cancer but have also found that CSCs are set apart from non-CSCs through their elevated levels of EZH2 and H3K27me3. This finding provided the basis for development of a novel assay aimed to quantitatively measure perturbations in the CSC population of the total tumor or cell line population by monitoring EZH2 levels under conditions that support high-throughput screening. This assay would be advantageous by eliminating the need to isolate CSCs directly and could measure alterations in CSCs in the context of other cell types present more closely mimicking the physiological condition. For this assay, the cells were simultaneously stained in multwell tissue-culture plates for EZH2 and H3K27me3 using an immunofluorescence technique. Using the Arrayscan VTI HCS platform, each well was imaged by epifluorescence microscopy and quantitatively analyzed for fluorescence intensity of each cell scanned per well. As illustrated in Figure 4A, the fluorescence intensity of each cell measured in any given well can be plotted to look at the distribution of EZH2 and H3K27me3 staining patterns across the population. Analysis of the EZH2 (middle panel) and H3K27me3 (bottom panel) staining distribution clearly revealed a subset of cells that have elevated levels of EZH2 and H3K27me3, whereas Hoechst staining (top panel), which serves as an internal staining control, is uniformly distributed across the population. Using the analysis software, gates can be set to specifically collect data only in the EZH2high and/or H3K27me3-high cells, as a reporter for the CSC subpopulation; such gating is illustrated in Figure 4A by the overlay of red boxes. In this assay, a negative control treatment is used to determine the gating for the EZH2high and H3K27me3-high subpopulations, and this gating is then universally applied to the whole plate to measure changes in these populations following treatment. Figure 4B shows how RNA interference-mediated EZH2 knockdown serves as a validation of this gating concept across all four cell line models examined. Gates for EZH2high and H3K27me3-high populations are set on the control siRNA-treated cells and then applied to the EZH2 siRNA-treated cells. EZH2 siRNA universally reduced the percentage of EZH2high cells as shown in the left series of panels, with each dot in the data plot representing the percentage of EZH2high cells in an individual well (n = 30 wells per treatment for HPAC and HCC1937 cells, and n = 15 wells per treatment for Panc-1 and T47D cells). For example, on average, EZH2 knockdown reduced the percentage of EZH2high cells from 25.5 ± 0.8% to 2.4 ± 0.1% (p < .0001) in the HCC1937 cells, a measure that is well quantifiable despite the variability of the percentage of EZH2high distribution across the individual wells measured. Simultaneously, changes in the H3K27me3-high population can also be measured as reporter for CSCs, as shown in the right series of panels. However, because we found greater variability in the distribution of H3K27me3-high staining, there was a lower sensitivity in using H3K27me3-high as the CSC reporter over EZH2. This is illustrated in Figure 4B where a significant reduction in the percentage of H3K27me3-high cells can be measured following EZH2 siRNA treatment in HCC1937, T47D, and Panc-1 cells (p < .0001, p = .0003, and p = .0028, respectively), but the high variability of H3K27me3-high distribution in the HPAC cells prohibits any measure of an effect on this population with EZH2 knockdown. Thus, EZH2 was chosen as the reporter for CSC activity.

**Proof-of-Concept Use of an EZH2 Reporter Assay to Detect CSC Activity**

As a proof-of-concept, we sought to determine whether the EZH2 assay can measure both therapeutic inhibition of CSCs, as well as an enrichment of CSCs following chemotherapy treatment. Several recent reports have identified the antibacterial drug salinomycin as a selective CSC inhibitor [31–33]; thus we opted to use salinomycin as a CSC-inhibiting agent. Similarly, antagonism of the Notch pathway with the γ-secretase inhibitor DAPT is also expected to inhibit cancer stem cell activity [34]. Conversely, it is well known that treatment of pancreatic cancer with the chemotherapeutic gemcitabine enriches the CSC population because of the inherent resistance of pancreatic CSCs to chemotherapy [35, 36]. To validate the effect of salinomycin in reducing the CSC population, HPAC pancreatic cells were treated with 2 μM salinomycin for 72 hours, prior to population analysis by flow cytometry. Figure 5A shows how salinomycin treatment causes a visible loss of total EZH2 protein levels, mirrored by a reduction in the frequency of EpCamCD44CD24 CSCs by 13.5% compared with vehicle (DMSO) control (p < .001) (Fig. 5B). The same 2 μM salinomycin treatment on HPAC cells caused a similar 11.6% reduction in the EZH2high population (p < .05), as demonstrated in Figure 5C. Furthermore the decrease in CSCs by salinomycin treatment was dose-dependent. In concordance, a 72-hour gemcitabine treatment at 1 ng/ml on HPAC cells significantly increased the percentage of phenotype-positive CSCs from 8.2% to 27.8% (p < .001) (Fig. 5E), whereas the same 72-hour 1 ng/ml gemcitabine treatment also significantly increased the percentage of EZH2high cells from 6.7% to 54.4%.
EZH2high staining correlates well with the CSC population percentage of H3K27me3-high (right panels) from one well to another in each of the four cell types (HCC1937, T47D, Panc-1, and HPAC) measured.

Shifts in the population frequency can be effectively measured within the representative well, in each of the three fluorescence channels (Hoechst, EZH2, and H3K27me3). Red boxes illustrate the gating setup to identify cells in the population as EZH2high or H3K27me3-high. (B): Variance of percentage of EZH2high (left panels) and percentage of H3K27me3-high (right panels) from one well to another in each of the four cell types (HCC1937, T47D, Panc-1, and HPAC) examined. Each dot indicates the percentage of the population in a well, treated with control or EZH2 siRNA for 4 days. The data display the intrawell variance across n = 15 wells per treatment for T47D and Panc-1 and n = 30 wells per treatment for HPAC and HCC1937.

**DISCUSSION**

With the emerging evidence that tumor initiation and progression are driven by CSCs, we hypothesized that epigenetic modification by PcG proteins helps maintain the CSC phenotype and that these epigenetic modifications can in turn be used as a reporter for CSC activity in an in vitro high-throughput screen (HTS) assay. Specifically, we investigated activity of EZH2 and its catalytic outcome H3K27me3 for their involvement in CSC maintenance, given the correlative role of EZH2 in tumor growth and metastasis of several tumor types. EZH2 was consistently upregulated in CSCs specifically across the panel of breast and pancreatic cancer models that we examined. Furthermore, EZH2 knockdown clearly reduced H3K27me3 levels as determined by Western blot in cell line models and led to a significant reduction in the CSC population as measured by flow cytometry.

Genomic analysis further strengthened the relationship between EZH2 and CSCs. We found that a series of developmental genes were decreased when EZH2 was knocked down and similarly that this knockdown in turn led to an increase of genes involved in differentiation and cell motility, all clues that EZH2 loss was affecting CSCs directly. Several genes known to have roles in cell development were observed, such as the upregulation of tissue development mediators, FOXC2, TNC, Rac1, and NT5E (CD73) in spheroids. In addition to their role in tissue development, all of these mediators have a strong linkage to cancer. FOXC2 is involved in epithelial-to-mesenchymal (EMT) transition of tumor cells and is associated with metastatic progression [37]. A similar role for TNC in tumor cell proliferation and migration has also been described [38]. NT5E, a marker of mesenchymal stem cells [39], has been shown to protect tumors from immune-mediated killing and promote metastasis [40]. Rac1 similarly has roles in invasion and metastasis of tumor cells, attributed to a role within CSCs specifically [41]. If genes upregulated in spheroids and downregulated upon EZH2 loss are associated with CSCs...
and tumorigenesis, then it follows suit that genes stemming from the converse situation should be tumor-suppressing. Several genes, including XAF1, TF, and TIMP3, exhibited this relationship in our analysis. XAF1, involved in promoting apoptosis [42], has been shown to prohibit tumorigenesis in prostate cancer [43]. Receptors for TF have been shown to be associated with non-CSC differentiated cells [44, 45], and TIMP3, frequently silenced in many cancers, has been shown to directly inhibit tumor growth and metastasis [46]. The validation of genes identified in our analysis with their hypothesized role strengthens the conclusion that EZH2 plays a role in CSC regulation. Overall, we observed regulation of several genes known to play roles in stem cell maintenance (KLF4 [47], Rac1 [48], NTSE [39], GATA6 [49]), organogenesis (FOXC2 [50] and FRAS1 [51]), and EMT and/or metastasis (NTSE [40], TNC [38], Rac1 [41], FOXC2 [37], CCL20 [52], and CEACAM5 [53]). Recent reports have suggested an intimate link between EMT and CSCs [54–56]; thus the strong linkage of EMT and metastasis-associated genes in our analysis correlates well to that hypothesis. However, the more striking evidence linking EZH2 and CSCs came when we examined regulation of these same genes from cells grown in CSC-enriching spheroid conditions, whereby we observed an inverse correlation in transcriptional regulation.

Our findings support the conclusion that EZH2 and its catalytic activity H3K27me3 directly affect the level of CSCs present. These results translated to the development of a cell-based assay using high-content screening. Using high-content imaging, elevated EZH2 and/or elevated H3K27me3 levels could be gated out to quantitatively measure changes to the CSC population. Therapeutic treatment of these cells with a known CSC-targeting compound led to a significant reduction of the EZH2high population in this assay. In correlation, a significant reduction in CSCs was also measured by flow cytometry, a more conventional method to enumerate CSCs. In converse, chemotherapeutic enrichment of CSCs, as confirmed by flow cytometric analysis, could also effectively be measured by this assay. These results not only demonstrate that EZH2 and its catalytic activity, histone trimethylation at K27, are involved in CSC maintenance but also show that this event can also be used as a reporter for CSC efficacy in a HTS assay for rapid phenotypic screening of compounds that effect the CSC compartment. We anticipate that this assay can become useful as a first-pass screening tool to determine whether test compounds affect the CSC population in either an inhibiting or an enriching manner. More conventional follow-up studies could then serve to further validate any compounds identified and characterize the mechanism whereby CSCs are affected.

Figure 5. Proof-of-concept use of the EZH2 assay to quantitatively measure inhibition or enrichment of CSCs following therapy with salinomycin, gemcitabine, or DAPT. (A, B): Western blot analysis (A) of total EZH2 protein levels and corresponding flow cytometric analysis of CSC frequency (B) in HPAC cells treated with 2 μM salinomycin for 72 hours, using DMSO treatment as a vehicle control. (C): Effect of salinomycin at a dose-response curve on the percentage of EZH2high population in the HPAC cells treated for 72 hours, using DMSO as a vehicle control. (D, E): Western blot analysis (D) of total EZH2 protein levels and corresponding flow cytometric analysis of CSC frequency (E) in HPAC cells treated with 1 ng/ml gemcitabine for 72 hours. (F): Effect of gemcitabine treatment at a dose-response curve on the percentage of EZH2high population in HPAC cells treated for 72 hours. (G–I): Western blot analysis (G) of total EZH2 protein levels, corresponding flow cytometric analysis of CSC frequency (H), and percentage of EZH2high population analysis (I) in HPAC cells treated with 1 μM DAPT for 72 hours, using DMSO treatment as a vehicle control. β-Actin served as a loading control for all Western blot data. For all experiments, n = 3 biological replicates per treatment. * and ** indicate statistical significance as determined by t test between compound and control treatment at p ≤ .05, p ≤ .01 and p ≤ .001, respectively. Abbreviations: DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide.
Current technology to investigate CSC activity can be labor intensive and low throughput. The most direct measure of effects on the CSC population occurs through flow cytometry analysis, whereby antibody-directed cell surface marker staining can quantitatively gate in on a subpopulation of cells that bear CSC surface marker characteristics. This procedure can be lengthy in both experimental run time and data analysis and lacks universality across different tumor types. The EZH2 assay described here facilitates CSC research by both providing a significantly reduced runtime compared with conventional technology and providing uniformity for CSC identification across different tumor types. Taken together, these advantages allow for high-throughput screening with this assay, a feat that is difficult to achieve with conventional technology. More importantly, however, patterns of cell surface markers defining the CSC population can differ from one tumor type to another; thus individual flow cytometry assays needed to be established for each tumor type. CSC identification by the EZH2 assay described here solves this problem. We believe that this single assay format can be used to monitor activity of CSCs for a multitude of tumor types because of the wide reports of the role of EZH2 in the maintenance of CSCs, allowing for uniformity of experimental output. Our results not only demonstrate the importance of EZH2 and H3K27me3 in CSC regulation, but use of this target as a novel screening tool for CSC activity could greatly facilitate therapeutic development against this critical tumor subpopulation.

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AUTHOR CONTRIBUTIONS

L.E.v.V.: primary author, study design, collection and analysis of data, manuscript writing; C.M.K.: collection and analysis of ChiP data; C.M.: collection and analysis of genomics data; Y.L.: collection and analysis of FACS data; C.G., S.D.W., and Y.Y.: data analysis and interpretation; R.E.H.: final approval of manuscript; E.M.H.: conception and design, data analysis and interpretation, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

L.E.v.V., C.M.K., C.G., S.D.W., Y.Y., R.E.H., and E.M.H. have employment and stock compensation with MedImmune.
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