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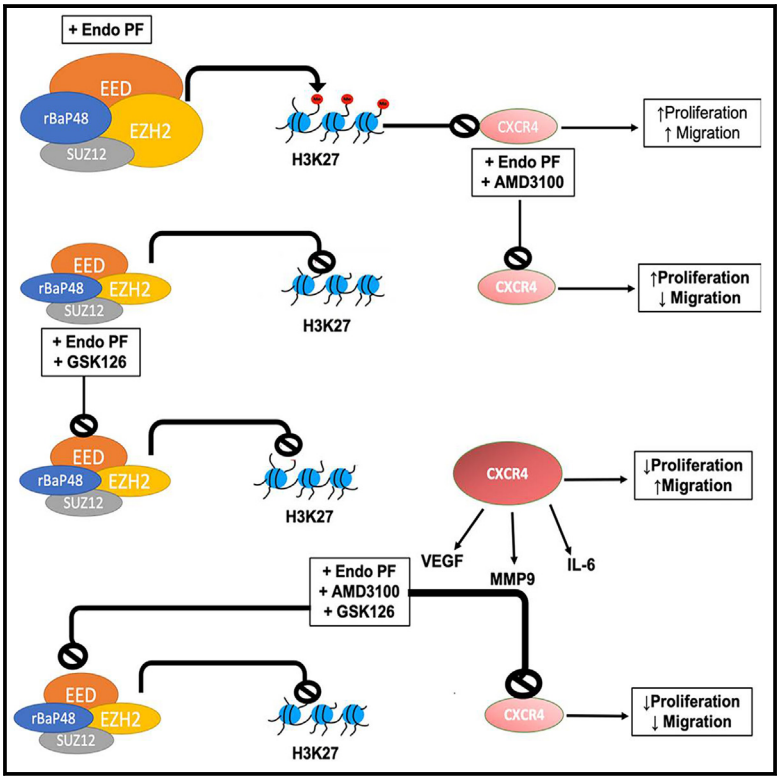
Dual targeting of CXCR4 and EZH2 in endometriosis

Graphical abstract



Highlights

- Endometriosis is an inflammatory disease that can be epigenetically regulated
- The CXCR4-CXCL12 axis plays a role in endometrial cell growth and migration
- This axis is epigenetically regulated by the EZH2 pathway
- Dual targeting CXCR4 axis and EZH2 prevents endometrial growth

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In brief

Body substance sample; Disease; Epigenetics; Small molecule; Treatment

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Dual targeting of CXCR4 and EZH2 in endometriosis

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SUMMARY

We recently showed that endometriotic peritoneal fluid (PF) altered the regulation of enhancer of zeste homolog 2 (EZH2) and H3K27me3. This study aimed to determine if PF by regulating EZH2/H3K27me3 modulated C-X-C chemokine receptor type 4 (CXCR4), a major chemokine involved in the proliferation and migration processes in endometriosis. Endometriotic PF induced the mRNA expression of CXCR4 and EZH2 and protein expression of H3K27me3 in human endometrial stromal cells (hESCs) and eutopic endometrium (Eu). CXCR4 inhibitor, AMD3100, decreased the PF-induced expression of these factors and reduced migration, but increased the proliferation of hESCs. In contrast, the EZH2 inhibitor, GSK126, decreased the expression of EZH2 and H3K27me3 and reduced proliferation, but increased the expression of CXCR4 and migration of hESCs. A combination of both inhibitors decreased the expression of CXCR4, EZH2, and H3K27me3, as well as reduced cell proliferation and migration. Our study suggests that targeting both CXCR4 (inflammation) and EZH2 (epigenetics) may be a better alternative for women with endometriosis.

INTRODUCTION

Endometriosis affects 1 in 10 women, though recent studies indicate that this ratio may have increased to 1 in 9 women,[1,2] suggesting that more than 176 million women worldwide are afflicted with this disease. Some of the major symptoms associated with endometriosis include severe dysmenorrhea, dyspareunia, and infertility.[3,4] Though the pathophysiology of this disease is not completely understood, several theories have been proposed,[5–7] which include Sampson's theory, also known as the retrograde menstruation theory, the coelomic metaplasia theory, and the embryonic origin theory. Other recent theories include stem cells, genetics, and autoimmune disease.[8,9]

All the proposed theories suggest a pathological role for an inflammatory peritoneal microenvironment that differs between women with and without endometriosis. In the former group, peritoneal fluid (PF) is present in larger quantities and contains excess levels of inflammatory cells, pain-inducing molecules, growth promoters, and cytokines/chemokines.[10–22] We and others have also shown that oxidants present in abundance in the PF can increase the proliferation and growth of endometrial cells.[10,23]

Lymphokines such as interleukins 4, 6, and 10 and tumor necrosis factor α are present in excess in the PF of women with endometriosis. C-X-C motif chemokine receptor 4 (CXCR4) is one of the most widely expressed chemokine receptors,[24] which, upon binding to its only ligand CXCL12, or SDF-1, activates mechanisms that are important in reproductive biology[25] as well as triggers signaling pathways important in cell proliferation and migration.[24] Its ligand, CXCL12, can also interact with CXCR7, another heptahelical G protein-coupled receptor.[26] Patients with deep infiltrating endometriosis had increased concentrations of CXCL12 in the PF and thus showed a higher expression of CXCR4 in eutopic endometrial stromal cells compared to control cells.[27]

Targeting CXCR4 has been explored as therapy in other diseases.[28,29] One such compound, AMD3100 (plerixafor), is a highly selective CXCR4 chemokine receptor antagonist with an IC_{50} value of 44 nM in cell-free assay that was originally developed as an inhibitor for HIV-1 and HIV-2 replication. This compound inhibits the intracellular calcium signaling and chemotactic response elicited by CXCL12.[30] In endometriosis, Ruiz et al. showed that AMD3100 alone decreased migration but increased invasion of an endometriotic cell line, 12Z cells; however, when AMD3100 was added in the presence of the ligand CXCL12, both migration and invasion were decreased.[31] These studies proposed targeting the CXCR4-CXCL12 axis as a potential non-hormonal therapy for endometriosis.

We recently showed that the PF from women with endometriosis alters epigenetic mechanisms such as the expression and function of polycomb repressive complex 2 (PRC2) and its major subunit enhancer of zeste homolog 2 (EZH2).[32] EZH2 is a histone methyltransferase that plays a key role in transcriptional repression through chromatin remodeling, thus regulating gene expression, maintenance of cell identity, and oncogenesis.

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
1% Control PF	1.23	0.51	1.63	1.29
1% Endo PF	1.06	0.42	1.23	2.77
1 μM AMD3100	0.42	0.20	0.68	0.77

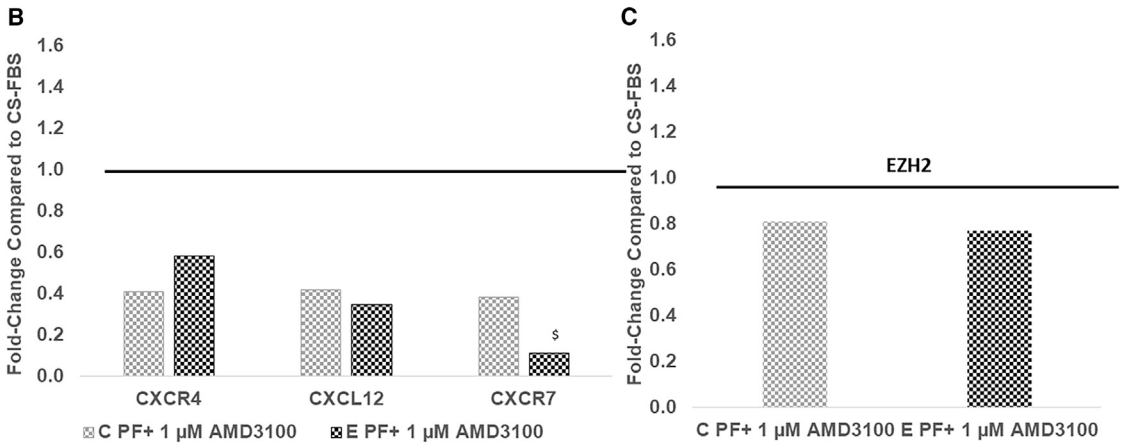


Figure 1. mRNA expression of *CXCR4* axis and *EZH2* in hESCs treated with PF and/or AMD3100

(A) Relative mRNA expression of genes detected in 1% PF alone-treated cells showed endo PF increasing *EZH2* expression approximately by 2.77-fold, but not significant. 1 μM AMD3100 treatment lowered the expression levels of all genes determined.

(B) 1% PF + 1 μM AMD3100-treated cells showed a decrease in mRNA expression of all three genes in the *CXCR4* axis, with *CXCR7* ($p = 0.0196$) decreasing significantly.

(C) A relative lowering in mRNA expression of *EZH2* was also observed in 1% PF + 1 μM AMD3100-treated cells. $ = significant p value compared to endo PF alone.

EZH2 activates H3K27me3, which in turn silences a broad range of genes.[33–36] EZH2 expression is elevated in various cancers,[37–39] making it a major drug target in the treatment of cancer.[40] We and others have observed an increased expression of this complex in both the eutopic and ectopic endometriotic tissues of women with endometriosis,[41,42] as well as endometrial stromal cells treated with endometriotic PF.[32] This response was inhibited by GSK126, a selective, S-adenosyl-methionine-competitive small-molecule inhibitor of EZH2 methyltransferase activity, which is more than 1,000-fold selective for EZH2 over other histone methyltransferases and an IC_{50} value of 9.9 nM.[43] Inhibition of EZH2 also decreased lesions in a rat model of endometriosis.[44]

A positive correlation between the expression of EZH2 and CXCR4 exists,[45–48] and H3K27me3 histone modifications are enriched at the CXCR4 promoter.[49] We hence speculated that the epigenetic regulation of the CXCR4 axis by the PRC2 complex might be involved in the growth-promoting effects of endometriotic PF. Blocking this axis should inhibit the PF-mediated growth and migration of endometrial cells. Using endometriotic PF-treated human endometrial stromal cells (hESCs) and eutopic (endometrium) tissues, we observed an association between EZH2 activation and the ability of the CXCR4 axis to alter the proliferation and migration of cells. Our findings showed that a combination of both CXCR4 and EZH2 inhibitors, and not the individual drugs, was significantly more effective in lowering the growth and migration of hESCs.

RESULTS

mRNA and protein expression of CXCR4 axis and EZH2 in hESCs

hESCs treated with either 1% control ($n = 8$) or endo PF ($n = 6$) alone had minimal effects on the mRNA expression levels of the CXCR4-CXCL12-CXCR7 axis. Only endo PF alone treatment increased the expression of *EZH2* nearly 3-fold but did not reach statistical significance. When 1 μM AMD3100 alone was added to the cells ($n = 3$), the expression of all genes showed a trend of lowered expression (Figure 1A). The expression of *CXCR4*, *CXCL12*, and *CXCR7* mRNA also decreased when cells were treated with AMD3100 in the presence of PF. The greatest decrease in expression was observed in *CXCR7* levels in cells that were treated with 1% endo PF + 1 μM AMD3100 (0.11-fold, $p = 0.0196$ vs. endo PF alone) (Figure 1B). Cells treated with 1 μM AMD3100 in the presence of PF had a trend of lowering *EZH2* mRNA expression but it did not reach significance (Figure 1C).

In contrast to the mRNA expression, protein levels were significantly altered in hESCs treated with 1% PF or AMD3100 alone. Endo PF increased EZH2 protein expression by 1.55-fold ($p = 0.0221$) and H3K27me3 by 2.03-fold ($p = 0.0011$). When hESCs were treated with 1 μM AMD3100 alone, expression of both EZH2 and H3K27me3 showed a significant downregulation when compared to both the control and endo PF alone-treated cells. EZH2 levels were decreased to 0.22-fold ($p < 0.0001$ vs.

A

Treatment	EZH2	H3K27me3
1% Control PF	$1.30_{+/-0.03}$	$1.84_{+/-0.06}$
1% Endo PF	$1.55^{*}_{+/-0.007}$	$2.03^{**}_{+/-0.03}$
1 μM AMD3100	$0.53^{**}_{\$\$\ +/-0.015}$	$0.23^{*}_{\$\$\$\$\ +/-0.001}$

B

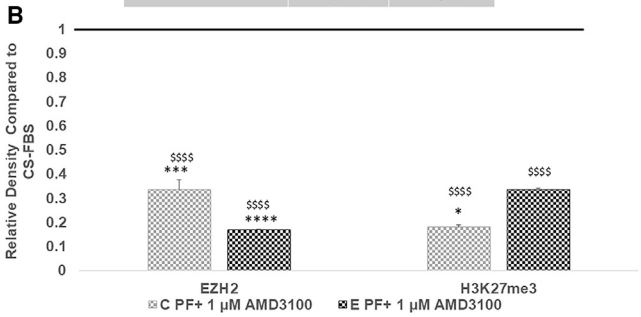


Figure 2. Protein expression of EZH2 and H3K27me3 in hESCs treated with 1% PF and/or 1 μM AMD3100

(A) Densitometric analysis of EZH2 protein, as determined by whole-exome sequencing (WES), showed a significant increase in 1% PF alone-treated cells (1.55-fold, $p = 0.0221$, endo PF compared to 1% CS-FBS media), but a significant decrease in 1 μM AMD3100 alone-treated cells (0.53-fold, $p = 0.0221$ compared to endo PF). H3K27me3 protein expression also significantly increased by 2.03-fold ($p = 0.0011$) in endo PF as well as in 1 μM AMD3100 alone-treated cells (0.23-fold, $p = 0.0207$ vs. CS-FBS media and $p < 0.0001$ vs. endo PF alone).

(B) EZH2 protein significantly decreased in hESCs treated with both control PF + 1 μM AMD3100 (0.33-fold, $p = 0.0001$ vs. CS-FBS media and $p < 0.0001$ vs. endo PF alone) or endo PF + 1 μM AMD3100 (0.17-fold, $p < 0.0001$ vs. CS-FBS media and endo PF alone)-treated hESCs. The expression of H3K27me protein was also statistically decreased when compared to endo PF alone for both control (0.11-fold) and endo PF (0.24-fold) + 1 μM AMD3100 ($p < 0.0001$) and when compared to CS-FBS media alone for control PF + 1 μM AMD3100 ($p = 0.0297$). * = significant p value compared to CS-FBS media alone. $ = significant p value compared to endo PF alone.

1% CS-FBS media and 0.0009 vs. endo PF) and H3K27me3 to 0.23-fold ($p = 0.0207$ vs. 1% CS-FBS media and <0.0001 vs. endo PF) (Figure 2A). PF + 1 μM AMD3100 showed a significant downregulation of EZH2 when added in the presence of both 1% control PF and endo PF (0.33-fold, $p = 0.0001$ for control PF, and 0.17-fold, $p < 0.0001$ for endo PF vs. 1% CS-FBS media). H3K27me3 also showed significant downregulation of expression in both 1% control PF + 1 μM AMD3100 (0.11-fold, $p = 0.0297$ vs. 1% CS-FBS media alone and <0.0001 vs. endo PF alone) or endo PF + 1 μM AMD3100 (0.24-fold, $p < 0.0001$ vs. endo PF alone)-treated cells (Figure 2B).

CXCR4 inhibitor, AMD3100, increased cell proliferation but decreased migration

The rates of proliferation of the hESCs treated with PF and/or AMD3100 alone or in combination were measured using the xCELLigence technology. At 48 h, hESCs treated with control PF had a 141.5% increase in proliferation, and endo PF had a 142.4% increase when compared to hESCs grown in 1% CS-FBS media alone ($p < 0.0001$ for both). At 96 h, proliferation in hESCs treated with control PF was 126.5% and 128.1% for endo PF-treated cells ($p < 0.0001$ for both) compared to untreated cells. Surprisingly, the addition of AMD3100 increased proliferation in the presence of PF. At 48 h, cell proliferation was increased to 190.3% for control PF + 1 μM AMD3100 ($p < 0.0001$) and 155.6% ($p < 0.0001$) for control PF + 2.5 μM AMD3100. Similarly, the increase was 180.7% for endo PF + 1 μM AMD3100 ($p < 0.001$) and 188.6% ($p < 0.0001$) for endo PF + 2.5 μM AMD3100 when compared to 1% CS-FBS media alone. At 96 h, proliferation lowered but it remained higher than 150% for both treatments ($p < 0.0001$) at 48 h and 163.3%/158.77% ($p < 0.0001$) for control PF and 136.2/154.97% ($p < 0.0001$) for endo PF-treated cells at 96 h. AMD3100 alone did not lower proliferation at 1 μM, but at 2.5 μM, AMD3100 decreased proliferation to 71.2% compared to media alone at 48 h and to 59.5% proliferation ($p = 0.0107$) at 96 h (Table S2A).

The rate of migration of hESCs also determined using xCELLigence technology showed that the rate of migration increased to 147% and 180% by hour 6 when treated with control or endo PF alone, respectively. These rates began to slowly decrease by hour 12 but were still higher than the 1% CS-FBS media alone. Contrary to its effects on proliferation, AMD3100 in the presence of PFs decreased migration. While an initial increase was noticed for treatments with AMD3100, by hour 6, all treatments were below or around the same rate of the 1% CS-FBS media alone. Both endo PF + 1 μM AMD3100 and endo PF + 2.5 μM AMD3100 decreased to 71.66% and 77.59% at hour 9, respectively, compared to endo PF alone ($p = 0.0080$, 0.0202)-treated hESC migration. By hour 12, both concentrations (1 and 2.5 μM AMD3100) lowered migration to 65.56% and 67.63% ($p = 0.0232$, 0.0315 compared to endo PF alone). The biggest decrease in the rate of migration by hour 12 was observed in control PF + 1 μM AMD3100 at 52% ($p = 0.0016$ compared to endo PF alone) (Table S2B).

EZH2 inhibitor, GSK126, upregulated the PF-mediated expression changes of the CXCR4 axis

To determine if the increased proliferating effects seen with endo PF treatment were due to its activation of EZH2, the aforementioned experiments were repeated in the presence of an EZH2 inhibitor, GSK126. hESCs were treated with 1 or 2.5 μM GSK126 either alone ($n = 3$) or in combination with 1% PF (control [$n = 6$] or endo [$n = 7$]). mRNA analysis revealed that when GSK126 was added to the cells alone, 2.5 μM GSK126 increased the expression of *CXCR4* and *CXCR7*, while slightly decreasing the expression of *EZH2* (Figure 3A). When GSK126 was added in the presence of endo PF, the expression of both *CXCR4* and *CXCR7* increased 4- and 5.15-fold, respectively, but these results were not statistically significant (Figure 3B). When the cells were treated with PF and GSK126, *EZH2* expression was decreased for all treatments when compared to 1% CS-FBS media alone but none reached significance (Figure 3C).

When GSK126 was added to the hESCs alone, significant downregulation of EZH2 protein expression was seen at both concentrations compared to endo PF alone-treated cells ($p = 0.0009$, 0.0053, respectively). Protein levels of H3K27me3 also showed a lower trend in expression (Figure 4A). However, when hESCs were treated with either control or endo PF in the presence of 1 or 2.5 μM GSK126, there was a decrease in the protein levels of EZH2 in all treated cells when compared to

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
1 μM GSK126	0.33	0.45	0.59	1.11
2.5 μM GSK126	2.13	0.82	1.16	0.92

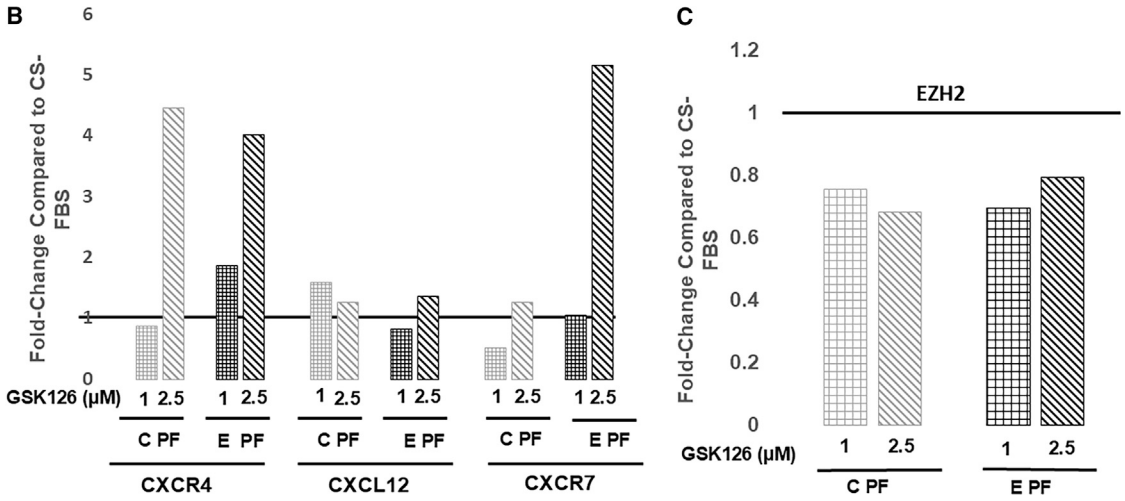


Figure 3. mRNA expression of *CXCR4* axis and *EZH2* in hESCs treated with GSK126

(A) No significant changes in relative mRNA expression of the CXCR4 axis genes or *EZH2* in hESCs treated with GSK126 alone were observed although a 2-fold increase in *CXCR4* was seen in the 2.5 μM GSK126 alone treatment.

(B) Over a 4-fold increase in both *CXCR4* and *CXCR7* expression was observed in hESCs treated with 1% endo PF + 2.5 μM GSK126 ($p > 0.05$).

(C) 1% PF + GSK126 lowered *EZH2* expression, but this did not reach significance.

1% CS-FBS media alone (Figure 4B). The greatest lowering in EZH2 expression was seen in hESCs treated with endo PF + 2.5 μM GSK126 (0.33-fold, $p = 0.0334$) and control PF + 2.5 μM GSK126 (0.44-fold, $p = 0.0307$) when compared to 1% CS-FBS media alone cells or compared to endo PF alone ($p < 0.0001$). When GSK126 was added to the hESCs in the presence of PF, expression of H3K27me3 was shown to be significantly downregulated when compared to hESCs treated with 1 μM GSK126 alone. The biggest decrease was seen in cells treated with control PF + 1 μM GSK126 (0.37-fold, $p = 0.0297$).

EZH2 inhibitor decreased proliferation but increased migration of hESCs

GSK126 alone at 1 μM decreased hESC proliferation to 55.7% ($p = 0.0013$) and at 2.5 μM to 38.9% ($p < 0.001$) compared to 1% CS-FBS media alone at 48 h. In the presence of PF, GSK126 at 1 μM only slightly decreased proliferation (99.34% for control PF + 1 μM GSK126 and 92.95% for endo PF + 1 μM GSK126), but at 2.5 μM, GSK126 decreased proliferation to 75.7% for control PF + 2.5 μM GSK126 and to 88.01% for endo PF + 2.5 μM GSK126, though neither reached significance. However, at 96 h, GSK126 alone at 1 μM (46.45%, $p < 0.0001$) or 2.5 μM (38.89%, $p < 0.0001$) decreased proliferation even further when compared to 1% CS-FBS media. In the presence of PF, GSK126 significantly decreased proliferation at both concentrations, to 76.3%, $p = 0.0067$ for control PF + 1 μM GSK126 and to 67.98%, $p = 0.0002$ for endo PF + 1 μM GSK126. In the presence of control and endo PF, 2.5 μM GSK126 decreased proliferation to 61.22% ($p < 0.0001$) and 71.62% ($p = 0.0025$), respectively (Table S3A).

In contrast to its inhibitory effects on hESC proliferation, GSK126 increased migration at both concentrations and was higher when compared to 1% CS-FBS media alone treatment. Control PF + 1 μM GSK126 and endo PF + 1 μM GSK126 were significantly higher when compared to 1% CS-FBS media alone with migration rate of 205.98% and 200.63% at 6 h, respectively ($p = 0.0075, 0.0161$). Even at 12 h, the rate was 180% higher than that of 1% CS-FBS media alone. At hour 3, endo PF + 2.5 μM GSK126 was 242.93% higher compared to 1% CS-FBS media alone ($p < 0.0001$) and remained significantly higher until hour 12 (Table S3B).

Combined inhibition of CXCR4 and EZH2 provided optimal overall effects

To examine if the combination treatment with AMD3100 and GSK126 would have a synergistic effect than each drug individually, hESCs were treated with a combination of 1 μM AMD3100 and 1 μM GSK126 alone ($n = 3$) or along with 1% control or endo PF ($n = 4$/each). The expression of *CXCR4* and *CXCR7* was slightly increased by the combination drug treatments when compared to the 1 μM AMD3100 alone treatments when the drugs were added without PF. This may be due to the addition of GSK126 in the treatments. The expression of *EZH2* decreased further in the combination treatment than with AMD3100 alone (Figure 5A). In the presence of PF, *CXCR4* expression increased by 1.8-fold when compared to media control in hESCs treated

A

Treatment	EZH2	H3K27me3
1 μM GSK126	0.87 $^{\$\$\$}_{+/-\ 0.05}$	0.69 $_{+/-\ 0.23}$
2.5 μM GSK126	0.91 $^{\$\$}_{+/-0.016}$	0.45 $_{+/-\ 0.002}$

B

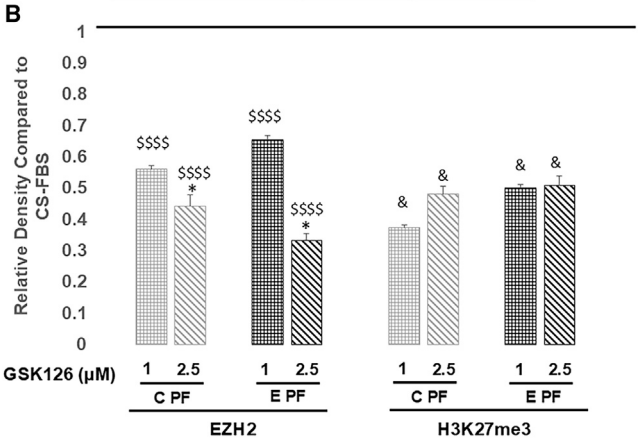


Figure 4. Protein expression of EZH2 and H3K27me3 in hESCs treated with GSK126

(A) Densitometric analysis showed a significant decrease of EZH2 protein as determined using WES, in hESCs treated with 1 or 2.5 μM GSK126 alone compared to endo PF alone ($p = 0.0009$ and 0.0053, respectively). The protein levels of H3K27me3 trended toward lower expression but did not reach significance.

(B) Densitometric analysis of EZH2 protein in EOOF5 cells treated with 1% PF + GSK126 showed a significant decrease in all treatments when compared to endo PF alone ($p < 0.0001$). EZH2 protein was significantly decreased in 1% control PF or endo PF+ 2.5 μM GSK126-treated hESCs (0.44-fold, $p = 0.0307$ and 0.33-fold, $p = 0.0334$, respectively). The protein expression of H3K27me3 was also significantly downregulated in all treatments of 1% PF + GSK126 compared to 1 μM GSK126 alone-treated hESCs. Both concentrations of GSK126 added in the presence of endo PF showed a decrease of approximately half that of CS-FBS media alone hESCs (0.48-fold, $p = 0.0271$ and 0.51-fold, $p = 0.0430$). * = significant p value compared to CS-FBS media alone. $ = significant p value compared to endo PF alone. & = significant p value when compared to 1 μM GSK126 alone.

with control PF + 1 μM AMD3100 + 1 μM GSK126, but *CXCL12* expression decreased even more than with 1 μM AMD3100 treatment alone. In the presence of endo PF + 1 μM AMD3100 + 1 μM GSK126, *CXCL12* expression significantly decreased (0.12-fold, $p = 0.0385$), compared to media alone (Figure 5B). *EZH2* expression decreased from 0.77-fold for endo PF + 1 μM AMD3100 to 0.32-fold in the endo PF + 1 μM AMD3100 + 1 μM GSK126 ($p = 0.0076$ vs. endo PF alone) (Figure 5C).

Protein levels for EZH2 showed a significant downregulation when 1 μM AMD3100 + 1 μM GSK126 alone was added to hESCs when compared to both 1% CS-FBS media and endo PF alone-treated cells (0.31-fold, $p < 0.0001$ for both). The expression of EZH2 was shown to decrease even further when the combination was added in the presence of control and endo PF to 0.11- and 0.10-fold, respectively ($p < 0.0001$ for both when compared to both CS-FBS media alone and endo PF alone cells). The expression of H3K27me3 was also significantly downregulated, control PF + 1 μM AMD3100 + 1 μM GSK126 showed an expression of only 0.11-fold ($p = 0.172$ vs. CS-FBS media and <0.0001 vs. endo PF), and endo PF + 1 μM AMD3100 + 1 μM GSK126 was 0.24-fold ($p = 0.0235$ vs. CS-FBS media and <0.0001 vs. endo PF) (Figures 5D and 5E).

Both proliferation and migration of hESCs decreased with use of combination drug treatment

Using xCELLigence, we examined the effects that the combination of 1 μM AMD3100 + 1 μM GSK126 had on hESC proliferation and migration. The combination drug treatment, when added alone to the hESCs, decreased proliferation by 47.8% at 48 h ($p = 0.0046$) and 39.8% at 96 h ($p < 0.0001$). However, when the combination drugs were added in the presence of PF, the cell proliferation initially increased at 48 h to 143.1% when added with control PF and 133.6% when added with endo PF, but neither was statistically significant. By 96 h, proliferation significantly decreased in both control and endo PF + 1 μM AMD3100 + 1 μM GSK126 to 69.6% ($p = 0.0420$) and 61.6% ($p = 0.0010$), respectively (Table S4A).

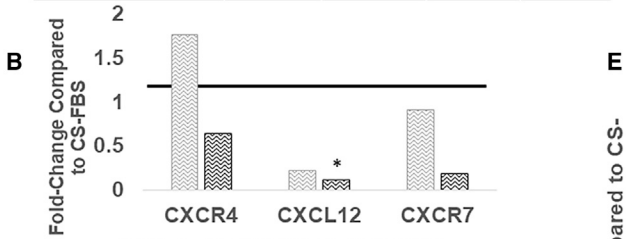
The rate of migration also decreased with the combination of the drugs, but not as low as 1 μM AMD3100 alone. 1 μM AMD3100 + 1 μM GSK126 alone had the lowest rate of migration and was statistically significant when compared to endo PF alone beginning at hour 6 at 72.43% ($p = 0.0088$) and continually decreased. By hour 12, the rate of migration was shown to be 57.71% ($p = 0.0297$ compared to endo PF alone). In the presence of PF, the migration was lower than 1% CS-FBS media alone at 93.22% and 92.2%, respectively (Table S4B).

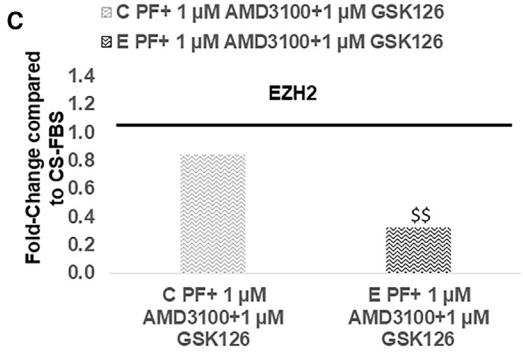
CXCR4 and EZH2 inhibitors had similar effects on Eu tissue as that seen in hESCs

To more fully explain if the changes that were observed due to the PF treatment on the CXCR4 axis and EZH2/H3K27me3 in primary hESCs would also occur in whole tissues, we performed similar studies in eutopic endometrium (Eu tissue). Eutopic endometrium consists of heterogeneous cell populations (stromal, epithelial, and other vascular cells), similar to endometriotic lesions, rather than just stromal cells. Control and endo PF alone, as well as varying concentrations of AMD3100 and GSK126, and the combination of AMD3100 + GSK126 that was used in the hESCs treatments were added to Eu tissues ($n = 3$ for all compound alone treatments, $n = 6$ for PF + compound treatments). When endo PF alone was added to the tissues, we noted an increase in both *EZH2* and *CXCR4* expression by over 2-fold, which correlated with what was observed in hESCs for *EZH2* but was higher in expression for *CXCR4.* At almost all concentrations of the drug treatments, it either slightly lowered or did not change the mRNA expression compared to media alone-treated Eu tissues. *EZH2* was significantly decreased in the 5 μM GSK126 alone treatment when compared to endo PF alone (0.27-fold, $p = 0.0467$ vs. endo PF alone) (Figure 6A). 1 or 2.5 μM AMD3100 in the presence of PF lowered the mRNA expression of the CXCR4-CXCL12-CXCR7. The largest decreases in expression were seen in *CXCR7* with endo PF + 1 μM AMD3100 being lowered to 0.39-fold ($p = 0.0208$). The only exception was *CXCL12* in the endo PF + 2.5 μM AMD3100 treatment, which showed a 1.16-fold increase in expression, but this was not significant (Figure 6B). Expression

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
1 μM AMD3100 + 1 μM GSK126	0.93	0.26	0.81	0.72





D

Treatment	EZH2	H3K27me3
1 μM AMD3100+ 1 μM GSK126	0.31**** +/- 0.003	0.54 +/-0.26

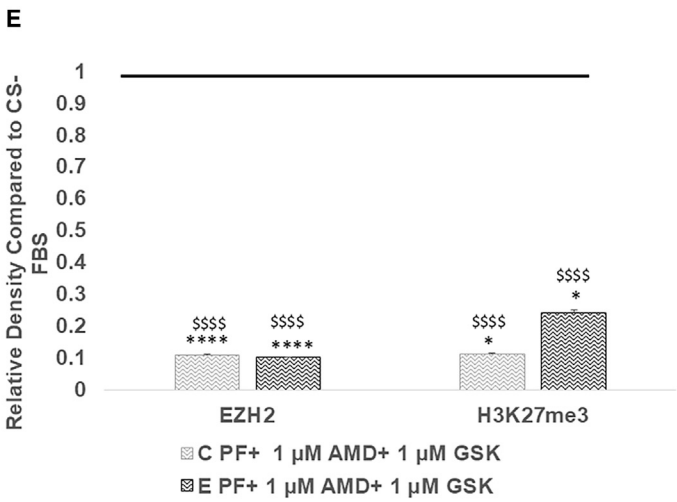


Figure 5. mRNA and protein expression of CXCR4 axis and EZH2/H3K27me3 in hESCs treated with a combination of AMD3100 and GSK126 drugs

(A) Relative lowering of *CXCR4* axis genes and *EZH2* mRNA expression was seen in 1 μM AMD3100 + 1 μM GSK126 alone-treated hESCs ($p > 0.05$).

(B) While a slight increase in mRNA expression of the *CXCR4* axis was seen when the combination drugs (1 μM AMD3100 + 1 μM GSK126) were added in the presence of 1% control PF, it was lowered in the presence of 1% endo PF + 1 μM AMD3100 + 1 μM GSK126 with CXCL12 expression reaching significance.

(C) Relative mRNA expression of EZH2 was significantly decreased in the presence of 1% endo PF + 1 μM AMD3100 + 1 μM GSK126 ($p = 0.0076$ compared to endo PF alone).

(D) Densitometric analysis of EZH2 in hESCs treated with 1 μM AMD3100 + 1 μM GSK126, as determined by WES, showed a significant decrease in expression (0.31-fold) when compared to both the CS-FBS media and the 1% endo PF alone-treated cells ($p < 0.0001$ for both). H3K27me3 protein levels also decreased but did not reach significance.

(E) A significant decrease in expression of EZH2 was also observed when the combination drugs were added in the presence of 1% control or endo PF (drugs + control PF, 0.11-fold or drugs + endo PF, 0.10-fold, $p < 0.0001$ compared to both CS-FBS media and endo PF alone). Expression of H3K27me3 was also significantly downregulated in both the control (0.11-fold) and endo (0.24-fold) PF treatments ($p < 0.0001$ when compared to endo PF alone for both and $p = 0.0172$ and 0.235, respectively vs. CS-FBS media alone). * = significant p value compared to CS-FBS media alone. $ = significant p value compared to endo PF alone.

of *EZH2* was shown to increase when 2.5 μM AMD3100 was added in the presence of endo PF to 1.31-fold, but this was not significant (Figure 6C).

Tissues treated with 1% endo PF and GSK126 showed a significant increase in *CXCR4* expression by over 5-fold at all three concentrations ($p = 0.0122$, 0.0301, and 0.0143, at 3, 5, and 9 μM GSK126, respectively). 1 μM GSK126 in the presence of 1% endo PF also showed a 3.5-fold increase in expression of *CXCR4*, but this was not statistically significant. For *CXCL12* and *CXCR7*, a significant upregulation was seen in the endo PF + 9 μM GSK126 treatments with a 3.84-fold increase for *CXCL12* ($p = 0.0261$ compared to tissue alone) and 3.67-fold for *CXCR7*, which was shown to be statistically significant when compared to both 1% CS-FBS alone and endo PF alone ($p = 0.0079$ and 0.0089, respectively). Expression was also increased for the 3 μM GSK126 + 1% endo PF, but these results were not statistically significant. While upregulation was also seen in Eu tissues treated with 1% control PF + GSK126, none were shown to be statistically significant (Figure 6D). *EZH2* expression was lowered at all concentrations of GSK126 added in the presence of PF when compared to endo PF alone tissues and was significantly downregulated for 1 and 5 μM GSK126 treatments in the presence of endo PF ($p = 0.0070$ and 0.0044, respectively) (Figure 6E).

The combination of 1 μM AMD3100 + 1 μM GSK126 showed a downregulation of the CXCR4-CXCL12-CRCR7 axis, with a greater decrease in expression seen when drugs were added in the presence of PF. Though it did not reach significance, all treatments showed an expression less than the media alone-treated tissue (Figure 6F). *EZH2* expression showed little change in expression in the PF + AMD3100 + GSK126 treatments (data not shown).

Protein expression of EZH2 was significantly increased in endo PF-treated Eu tissues compared to untreated Eu tissue (2.19-fold, $p = 0.0348$) (Figure 7A). An increase in expression of H3K27me3 was also noted in the endo PF-treated Eu tissues

A

Treatment	CXCR4	CXCL12	CXCR7	EZH2
Control PF	1.23	0.69	0.60	1.17
Endo PF	2.00	1.04	0.88	2.11
1 μM AMD3100	0.98	0.46	0.24	0.67
2.5 μM AMD3100	1.84	0.68	1.14	1.12
1 μM GSK126	2.70	1.37	1.11	0.49
3 μM GSK126	1.84	0.68	1.14	1.12
5 μM GSK126	1.88	0.97	0.76	0.27 $
9 μM GSK126	0.77	0.64	0.59	0.52
1 μM AMD3100 + 1 μM GSK126	0.87	1.07	0.76	0.68

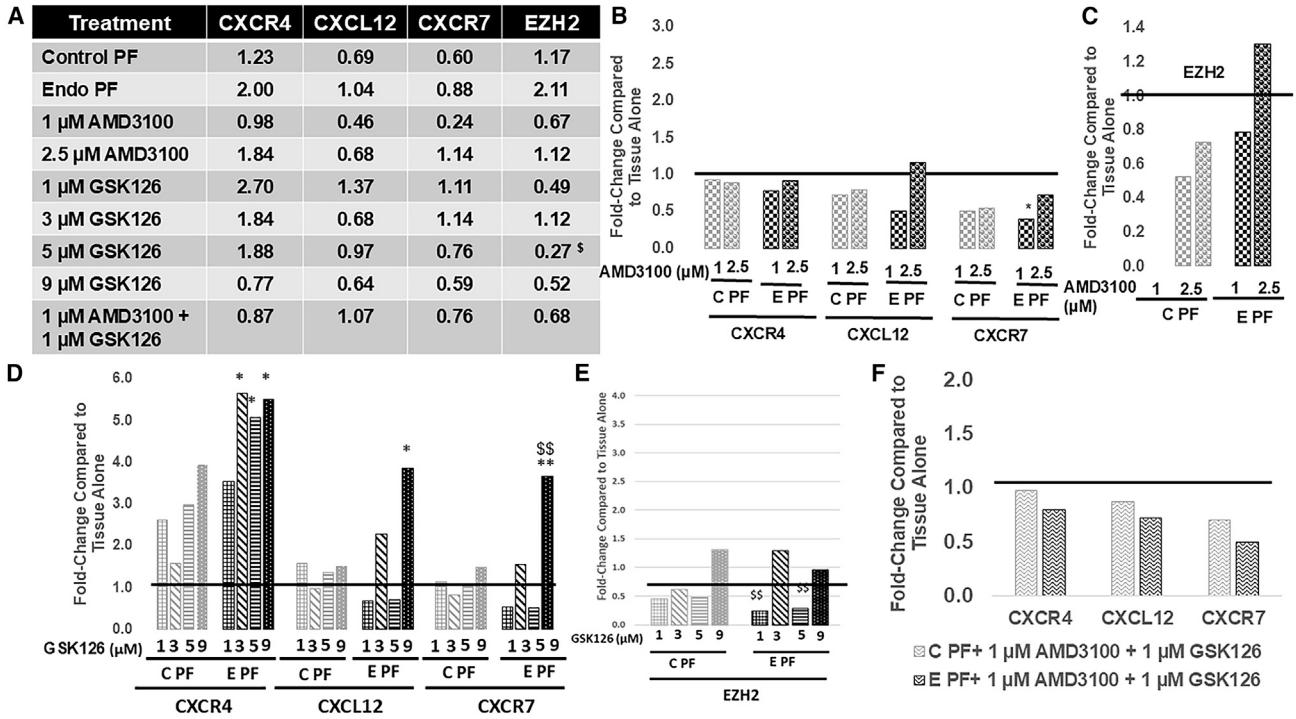


Figure 6. mRNA expression of CXCR4 axis genes and EZH2 in eutopic endometrium treated with PF and/or inhibitors

(A) mRNA expression results paralleled that was observed with similar treatments in hESCs. A statistically significant decrease in the expression of *EZH2* in the 5 μM GSK126 alone-treated Eu tissues (0.27-fold, $p = 0.0467$ vs. endo PF alone tissues) was observed.

(B) mRNA expression of the *CXCR4* axis showed a decrease in expression, with *CXCR7* showing a significant decrease when 1 μM AMD3100 was added in the presence of endo PF (0.39-fold, $p = 0.0208$).

(C) *EZH2* mRNA expression increased in the endo PF + 2.5 μM AMD3100-treated Eu tissues, while all other treatments decreased its expression.

(D) mRNA expression of the *CXCR4* axis showed a significant increase in expression when GSK126 was added in the presence of endo PF. *CXCR4* increased by over 5-fold at the 3, 5, and 9 μM GSK126 treatments ($p = 0.0122$, 0.0301, and 0.0143, respectively), while *CXCL12* and *CXCR7* significantly increased at the 9 μM GSK126 + PF-treated Eu tissues (3.84-fold, $p = 0.0261$ and 3.67-fold, $p = 0.0079$ vs. tissue alone and $p = 0.0089$ vs. endo PF alone-treated Eu tissue).

(E) mRNA expression of *EZH2* showed a significant decrease when 1 or 5 μM GSK126 was added to the tissues in the presence of endo PF ($p = 0.0070$, $p = 0.0044$).

(F) While no significant p values were noted, 1 μM AMD3100 + 1 μM GSK126 added in the presence of PF decreased the expression of all 3 genes of the CXCR4 axis compared to their expression seen in media alone-treated Eu tissues. * = significant p value compared to CS-FBS media alone. $ = significant p value compared to endo PF alone.

correlating with what was previously seen in the endo PF-treated hESCs. When 1 μM AMD3100 alone was added to the Eu tissues, H3K27me3 showed a significant downregulation when compared to endo PF alone-treated Eu tissues (0.61-fold, $p = 0.0231$). Significant downregulation was also seen for H3K27me3 for the 3 and 5 μM GSK126 alone-treated tissues (0.46-fold, $p = 0.0495$ and 0.20-fold, $p = 0.0109$, respectively) when compared to endo PF alone tissues. EZH2 also showed a significant decrease in expression in the 1 and 5 μM GSK126 alone-treated tissues (0.71-fold, $p = 0.0082$ and 0.81-fold, $p = 0.0178$, respectively) when compared to endo PF alone.

Protein expression of EZH2/H3K27me3 in Eu tissues treated with PF + AMD3100 confirmed the results seen for the mRNA. Both concentrations of AMD3100 (1 and 2.5 μM) added in the presence of endo PF showed significant downregulation of EZH2 to 0.18- and 0.20-fold when compared to 1% endo PF alone-treated Eu tissues ($p = 0.0016$ and 0.0004). Expression of H3K27me3 was also significantly downregulated in the tissues treated with endo PF + 1 μM AMD3100 compared to the 1% endo PF alone-treated tissues (0.89-fold, $p = 0.0037$) as well as when 2.5 μM AMD3100 was added in the presence of 1% control or endo PF compared to 1% endo PF alone-treated tissues (0.79-fold, $p = 0.0238$ and 0.67-fold, $p = 0.0171$, respectively) (Figure 7B).

Eu tissues treated with all concentrations of GSK126 (1, 3, 5, and 9 μM) in the presence of control or endo PF showed significant downregulation of EZH2 expression when compared to tissue alone. Endo PF + 5 μM GSK126 showed a decrease in expression at 0.54-fold ($p < 0.0001$), and 9 μM GSK126 showed the highest decrease in expression at 0.47-fold ($p = 0.0002$). Tissues treated with control PF + 1 or 5 μM GSK126 showed significant downregulation of H3K27me3 when compared to endo PF alone (0.63-fold, $p = 0.0273$ and 0.68-fold, $p = 0.0365$) (Figure 7C).

EZH2 expression was shown to be significantly decreased when the combination drugs were added in the presence of

A

Treatment	EZH2	H3K27me3
Control PF	$1.53_{+/-0.002}$	$0.80_{+/-0.04}$
Endo PF	$2.19^{*}_{+/-0.002}$	$2.14_{+/-0.04}$
1 μM AMD3100	$1.01_{+/-0.003}$	$0.61^{\$}_{+/-0.03}$
2.5 μM AMD3100	$0.75_{+/-0.004}$	$0.76_{+/-0.006}$
1 μM GSK126	$0.71^{\$\$}_{+/-0.02}$	$0.99_{+/-0.02}$
3 μM GSK126	$1.01_{+/-0.004}$	$0.46^{\$}_{+/-0.002}$
5 μM GSK126	$0.81^{\$}_{+/-0.002}$	$0.20^{\$}_{+/-0.005}$
9 μM GSK126	$1.13_{+/-0.002}$	$0.65_{+/-0.01}$
1 μM AMD3100+ 1 μM GSK126	$0.75_{+/-0.006}$	$0.88_{+/-0.08}$

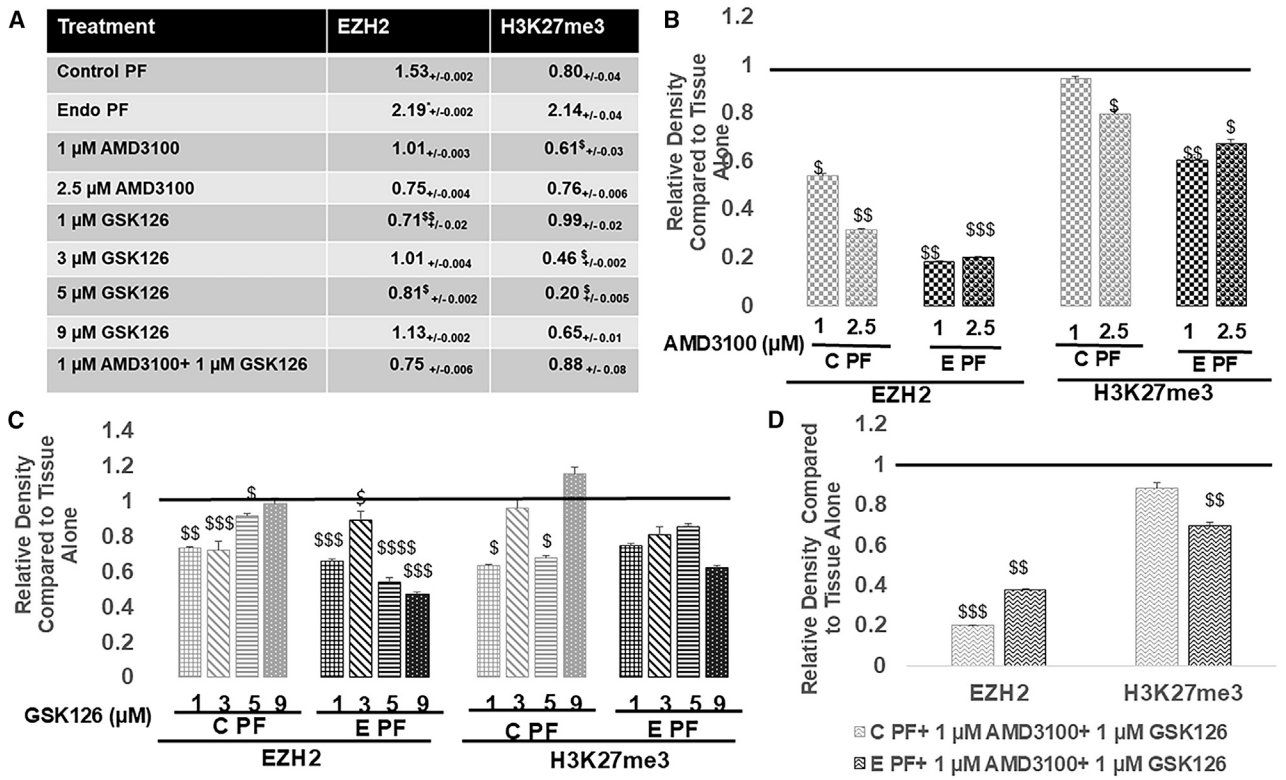


Figure 7. Protein expression of EZH2/H3k27me3 in eutopic endometrium treated with PF and/or inhibitors

(A) Densitometric analysis of the protein expression, as determined using WES, when PF alone, inhibitors alone, or their combination were added to the Eu tissues showed a significant increase in EZH2 (2.19-fold, $p = 0.0348$) in the endo PF treatments. H3K27me3 was also shown to increase but was not significant. 1 μM AMD3100 treatment decreased H3K27me3 to 0.61-fold ($p = 0.0231$). While 1 and 5 μM GSK126 treatment downregulated EZH2 (0.71, $p = 0.0082$ and 0.81-fold, $p = 0.0178$, respectively vs. endo PF alone-treated Eu tissues), a significant decrease in H3K27me3 proteins was seen at 3 and 5 μM GSK126 treatment (0.46-fold, $p = 0.0109$ and 0.20-fold, $p = 0.0495$ respectively, vs. endo PF alone-treated tissues).

(B) Densitometric analysis of EZH2 and H3K27me3 in Eu tissues treated with AMD3100 in the presence of PF showed a significant decrease when compared to endo PF alone for both proteins in all treatments. The only exception was the H3K27me3 in the control PF + 1 μM AMD3100 addition, but the expression was still below 1.

(C) Densitometric analysis of EZH2 and H3K27me3 in Eu tissues treated with GSK126 in the presence of PF showed a significant decrease in the expression of EZH2 in all treatments when compared to the expression of endo PF alone Eu tissues, with all being <1. The expression of H3K27me3 significantly decreased at 1 and 5 μM GSK126 in the presence of control PF when compared to endo PF alone (0.79-fold, $p = 0.0238$ and 0.67-fold, $p = 0.0171$, respectively)-treated Eu tissues. Additions of GSK126 at all concentrations in the presence of endo PF showed decreased expression of H3K27me3 as well, but none reached significance ($p > 0.05$).

(D) Densitometric analysis of EZH2 and H3K27me3 in Eu tissues treated with 1 μM AMD3100 + 1 μM GSK126 in the presence of PF showed significant decreases in the expression of EZH2 when added in the presence of control PF (0.20-fold) and endo PF (0.40-fold) ($p = 0.0004$, 0.0031 vs. endo PF alone). H3K27me3 showed a significant decrease in expression of 0.69-fold when the combination was added in the presence of endo PF ($p = 0.0029$ vs. endo PF alone Eu tissues).

* = significant p value compared to CS-FBS media alone. $ = significant p value compared to endo PF alone.

control PF (0.20-fold) or endo PF (0.40-fold) ($p = 0.0004$ and 0.0031 compared to endo PF alone). The expression of H3K27me3 was also shown to be significantly downregulated in the Eu tissues treated with endo PF + 1 μM AMD3100 + 1 μM GSK126 when compared to the Eu tissues treated with endo PF alone (0.69-fold, $p = 0.0029$) (Figure 7D).

DISCUSSION

Endometriosis is an elusive, enigmatic disease with multiple suggested theories of its origin. One commonality among all these theories is that inflammation plays a major role in its etiology. Inflammatory molecules are shown to be highly upregulated in endometriosis, especially in the PF.[10,12,15,18,50–52] We recently showed that the PF from women with endometriosis is also able to induce epigenetic changes, specifically the expression of the PRC2 complex and its catalytic subunit, EZH2.[32] Though other studies in the literature have also seen upregulation of EZH2 in endometriosis,[41,42,44,53] the mechanism(s) by which these epigenetic pathways are modulated in endometriosis is not currently known and was the focus of this study. Knowledge of the underlying mechanisms will assist in validating the use of epigenetic inhibitors in endometriosis. The present study confirmed our previous results as the endo PF significantly increased the protein expression of EZH2 in both primary human endometrial stromal cells (hESCs, $p = 0.0221$) and eutopic

endometrium (Eu tissue, p = 0.0348). As EZH2 activates H3K27me3, its expression was also significantly upregulated in hESCs treated with endo PF (p = 0.0011).

One key inflammatory pathway that is shown to be upregulated in endometriosis is the CXCR4 axis (CXCR4-CXCL12-CXCR7), which can control migration, as well as invasion and proliferation of ovarian cancer cells.[54] Ruiz et al. showed that the nuclear levels of CXCR4 are higher in ectopic lesions when compared to proliferative endometrium from control subjects and that this was even higher in the stromal compartment and ovarian lesions. In an endometriotic cell line (12Z), a higher expression of CXCR4 leads to an increase in proliferation compared to control cells, but a higher expression of CXCL12 did not increase the proliferation rate.[31] In the present study, though the mRNA expression of CXCR4/CXCL12/CXCR7 did not significantly increase in endo PF-treated cells, the expression of *CXCR4* was increased over 2-fold in the endo PF-treated eutopic tissues showing that PF can induce this axis and/or the need for a heterogeneous cell population.

Due to initial evidence for its role in endometriotic lesion formation, it was suggested that targeting CXCR4 in women with endometriosis might provide a new nonhormonal treatment.[31] Another study used a bone marrow transplantation mouse model and showed that CXCR4 and CXCR7 are highly expressed in the bone-marrow-derived cells engrafting endometriosis and that when either of those receptors was targeted, lesion size was decreased compared to untreated controls.[55] Since in our studies, the PF alone treatment modulated the CXCR4 expression, we tested if the CXCR4 inhibitor, AMD3100, would inhibit or reverse these PF-mediated effects in both hESCs and eutopic endometrium (Eu tissues). We found that AMD3100 lowered the mRNA expression of *CXCR4*, *CXCL12*, and *CXCR7* and was able to decrease the expression of EZH2 and H3K27me3. Contrary to our expectations, though AMD3100 decreased CXCR4 expression, it increased the proliferation of hESCs in the presence of endo PF while it lowered migration, but not significantly. These results differed from the study by Pluchino et al. where AMD3100 alone treatment had no significant effect on the proliferation of endometrial cells.[55] This suggests that factors in the PF are blocking the effects of AMD3100.

Since our recent study showed that endo PF can induce the EZH2 pathway,[32] we wanted to determine if blocking EZH2 would also block the CXCR4 axis and hence prevent PF influence on the CXCR4-mediated effects. To our surprise, we found that GSK126, though inhibited the mRNA and protein expression of EZH2 and protein expression of H3K27me3, upregulated the CXCR4 axis in the presence of PF in both the hESCs and Eu tissues. Like AMD3100, GSK126 treatment also had dual effects on the proliferation and migration of hESCs. Though not significant from the media alone-treated cells, GSK126 lowered the proliferation of hESCs. However, interestingly, GSK126 alone treatment significantly increased the migration rate of hESCs, and this was more pronounced in the presence of PF again suggesting that the PF is modulating the GSK126 drug effects. Both control and endo PF increased the proliferation and migration of hESCs though at differing intensities. This similarity in responses between using the two types of PF may be related to the source of the samples (patients having similar inflammatory molecules in the PF, due to underlying clinical conditions). Our findings correlated with other studies where GSK126 does decrease the levels of EZH2/H3K27me3, as observed here, but it increased the levels of CXCR4 and other inflammatory markers due to increasing signaling through nuclear factor κB and genes residing in PRC2-regulated chromatin.[49,56]

Due to the opposing influence that AMD3100 and GSK126 had on the PF-mediated effects on the CXCR4 axis and EZH2, as well as on the proliferation and migration of hESCs, we tested a combination approach (1 μM AMD3100 + 1 μM GSK1216), by targeting both inflammation and epigenetics. Overall, this combination approach resulted in a more positive outcome where the combined use of these drugs in the presence of endo PF significantly decreased the expression of all genes in the CXCR4 axis and the protein expression of both EZH2 and H3K27me3. Additionally, the combination of the drugs significantly lowered hESCs proliferation, and while it initially increased migration, the rate fell below the 1% CS-FBS media alone cells by hour 12.

Overall, our study showed that endo PF increased the expression of EZH2/H3K27me3 but did not increase the expression of CXCR4 as much as expected. This might be attributed to the presence of repressive marks for H3K27me3 on the CXCR4 promoter thereby inhibiting its expression.[49] The increase in expression of H3K27me3 in endo PF-treated hESCs and Eu tissue inhibited the CXCR4 expression in those same treatments. There are no supporting studies in the literature on the CXCR4 inhibitor AMD3100 effects on EZH2/H3K27me3 expression. However, studies have shown that the use of AMD3100 decreased the components of the PRC1 complex.[57] It is known that during PRC recruitment, methylation of H3K27me3 due to PRC2 can recruit PRC1, which in turn facilitates the formation of heterochromatin and repression of genes.[58] While the effects of AMD3100 have not been directly studied on the PRC2 complex, it may be working through similar processes as that seen with PRC1, as both PRC2 and PRC1 have similar functions of repressing gene transcription. In our studies, it can only be speculated that AMD3100 by inhibiting the CXCR4 axis lowers inflammation in the PF, thereby lowering EZH2/H3K27me3 expression, as observed here. Pluchino et al. showed that AMD3100 effects on lowering endometrial lesions were probably due to blocking bone-marrow-derived stem cell recruitment and not due to its direct effects on endometrial cells.[55] Additionally, with our studies showing that AMD3100 increased the proliferation of hESCs in the presence of PF, the use of this drug alone to reduce lesions is cautioned.

The use of EZH2 inhibitor, GSK126 on the other hand decreased the expression of H3K27me3, thereby releasing those repressive histone marks on CXCR4, allowing for the full expression of CXCR4 as observed with endo PF treatment. This also now allows CXCR4 to activate other inflammatory pathways in the peritoneal cavity, thus releasing factors such as vascular endothelial growth factor and matrix metalloproteinase-9, both of which are upregulated and involved in the pathology of endometriosis.[59–63] Due to its ability to inhibit hESC proliferation, the use of GSK126 to lower endometriotic lesions is tempting, but its ability to increase the CXCR4 axis would not make it useful as a drug targeting inflammation and pain in these women. While the use of GSK126 as a treatment for endometriosis has begun to

be examined in other laboratories, our findings serve as a caution for its use as a solo drug.

Hence, the best alternative is the combination approach of targeting both CXCR4 (AMD3100) and EZH2 (GSK126). By hitting these mechanisms from both angles, we were able to restrict both the inflammatory and epigenetic pathways and not result in any cross-modulatory effects (graphical abstract). Our results suggest that the interplay between inflammation and epigenetic pathways in endometriosis is more complex than what is currently known. Therapeutics that simultaneously target both these pathways might be more optimal in endometriosis treatment.

Limitations of the study

Only *ex vivo* studies were performed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be filled by the lead contact, Dr. Nalini Santanam (santanam@marshall.edu).

Materials availability

No new materials were generated.

Data and code availability

All data associated with this study are presented in this paper or supplementary files. No codes were generated.

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AUTHOR CONTRIBUTIONS

S.B.: conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review and editing, visualization, and funding acquisition; K.W.: formal analysis, investigation, writing – review and editing, and visualization; T.F.: formal analysis, investigation, writing – review and editing, and visualization; M.R.: resources and writing – review and editing; B.M.: resources, writing – review and editing, and supervision; N.S.: conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review and editing, visualization, supervision, and funding acquisition.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
EZH2	Cell Signaling	5426S; RRID: AB_10694683
H3K27me3	Cell Signaling	9733C: RRID: AB_2616029
β-actin	Cell Signaling	4970S; RRID: AB_2223172
H3	Active Motif	39451; RRID: AB_2793242
Biological samples		
Peritoneal Fluid	OBGYN Dept at Cabell Huntington Hospital	
Eutopic endometrium tissue	OBGYN Dept at Cabell Huntington Hospital	
Chemicals, peptides, and recombinant proteins		
DMEM/F12	Gibco	11320033
FBS	Gibco	A5670701
Pen/Strep	Gibco	15070063
Glutamine	Gibco	A2916801
GSK126	Cayman Chemical	1346574-57-9
AMD3100	155148-31-5	Sigma-Aldrich
HBSS	HyClone	SH 30268.02
TRI reagent	Sigma-Aldrich	T9424
Protease inhibitor cocktail	Sigma-Aldrich	P2714
SYBR Green	Biorad	1725270
Critical commercial assays		
iScript cDNA synthesis kit	Biorad	1708890
E-plates	Aglient	5469813001
CIM16 plates	Aglient	5665825001
Experimental models: Cell lines		
Primary stromal endometrial cells (hESCs)	Emory University	
Oligonucleotides		
Primers for mRNA expression. See Table S1		
Software and algorithms		
Compass for WES software	Protein Simple	
Prism	GraphPad	Version 9.0.0
Other		
xCELLigence Technology	Agilent	05469759001
Bullet Blender homogenizer	Next Advance	BBX24
WES automated western blotting system	Protein Simple	004-600

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human subject participants

Women ages 18 to 60 years, undergoing tubal ligation or having non-endometriosis disorders (controls) or patients with endometriosis (“endo”, laparoscopically diagnosed followed by pathological confirmation and/or patients with symptoms) were recruited from the Obstetrics-Gynecology clinic at Cabell Huntington Hospital, Joan C Edwards School of Medicine, Marshall University (Huntington, WV). This HIPAA compliant study was approved by the Institutional Review Board of the Marshall University School of Medicine and was carried out per the principles of the Declaration of Helsinki. All patients were consented prior to the study. The inclusion criteria included women ages 18–60 years old, with regular menstrual cycles who have not been on any hormonal medication for

at least one month before sample collection. Exclusion criteria included subjects with current medical illnesses such as systemic lupus erythematosus or rheumatologic disease, positive HIV/AIDS, and active infection. Subjects were asked to stop multivitamins that contain high levels of antioxidants and anti-inflammatory medications a month prior to sample collection.

All women completed a gynecologic/infertility history form, a pre-operative quality of life questionnaire, and an assessment of pain using a visual analog scale for assessment of endometriosis-associated pain (dysmenorrhea, non-menstrual pelvic pain, dyspareunia, and dyschezia) (adapted from the validated International Pelvic Pain Society's Pelvic Assessment Form). The date of their last menstrual period was used to assess their cycle time.

Of the 17 different endometrial PF samples that were used for this study, 8 were stage I/II and 4 were stage IV, for the remaining 5, the stage was not indicated. PF was used as individual samples and not pooled. Pathological confirmation for endo patients revealed that most of the patients belonged to the peritoneal, uterine serosa, or ovarian pathology. Only 3 of the patients with endometriosis indicated that they did not experience pain symptoms. PF (devoid of blood contamination) was collected on ice from all women during laparoscopic surgery. PF was spun at 2000 x *g* to remove any cellular debris. The supernatant was used immediately for studies or stored in a −80°C freezer for future use. Twelve different (age and cycle-matched) control PF samples were used in this study; 5 of them were collected from women who indicated that they did experience pain. All patients were undergoing laparoscopic hysterectomy or tubal ligation of which 2 patients had an adhesion or fibroid, while, 3 others were noted for uterine serosa or adenomyosis pathology but were not shown to be positive for endometriosis.

The eutopic endometrium (Eu tissue) was obtained from women without endometriosis undergoing a laparoscopic hysterectomy. Five different tissue samples were used (3 fresh and 2 frozen). The excised tissues were either used immediately or placed in a −80° freezer for future use.

Cell lines

Primary stromal endometrial cells (hESCs) (a gift from Emory University isolated from eutopic endometrium obtained from a 34-year-old woman without endometriosis undergoing surgery for tubal ligation), were cultured in T75 flasks in complete media (DMEM/F12, 10% FBS, 1% Pen/Strep, 1% glutamine). hESCs were stained with vimentin to confirm that they were of stomal origin.

METHOD DETAILS

PF treatment of endometrial stromal cells and eutopic (endometrium) tissues

When hESCs were approximately 80% confluent, media was changed to a DMEM/F12 containing 1% charcoal-stripped FBS (1% CS-FBS media) before being treated for 48 hours with either 1% PF from women with (endo PF) or without (control PF) endometriosis diluted in cell culture media (1 in 100), 1 or 2.5 μM GSK126, 1 μM AMD3100, or 1 μM AMD3100+1 μM GSK126 alone for 48 hours, or 1% control or endo PF for 24 hours before adding 1 or 2.5 μM GSK126, 1 μM AMD3100, or 1 μM AMD3100+1 μM GSK126 for an additional 48 hours. Cells were collected for protein and RNA analysis at the end of the treatment time points. hESCs grown in 1% CS-FBS media alone were used as the media-alone group for data comparison. Concentrations of AMD3100 and GSK126 used in this study were taken from studies published studies[64,65] as well as MTT assays performed in our laboratory (data not shown).

Eutopic endometrium (Eu tissue) from women without endometriosis (n=5) was washed twice using Hanks' Balanced Salt Solution (HBSS) prior to use. 50 mg aliquots of Eu tissue were placed into each well of a 24-well plate. 500 μL of media (DMEM/F12, 10% FBS, 1% Pen/Step, 1% Glutamine) was added to each well and then placed in the 37°C incubator for 4 hours. After 4 hours, Eu tissues were treated with 1% control or endo PF (n=6 each) alone, 1, 3, 5 or 9 μM GSK126 alone, or 1% PF plus 1, 3, 5 or 9 μM GSK126. Another subset of tissues was treated with 1 or 2.5 μM AMD3100, or 1 μM AMD3100+ 1 μM GSK126 alone or in combination with 1% PF (control or endo PF). For PF or drug(s) alone treatments, the compounds were added for 48 hours before collection. For combination treatments, PF was added to the tissues for 48 hours before drug(s) were added for an additional 48 hours and then collected. All treatments were performed in duplicates, with one set of treatments used for protein analysis and the other for RNA isolation. Eu tissue treated with 1% CS-FBS media alone was used as the media-control for comparison.

Cell proliferation using xCELLigence

hESCs were used to test cell proliferation under various conditions using xCELLigence technology. This technology uses modified 16-well plates in which microelectrodes are attached at the bottom of the wells and cell impedance or cell index (CI) can be measured. When more cells adhere to the bottom of the wells and divide, the CI measurement is higher. 5,000 hESCs per well were plated in 100 μL of complete media and placed on the xCELLigence machine for 24 hours; readings were taken every hour. After 24 hours, media was removed from all wells and 1% CS-FBS media was added before beginning various cell treatments. Cell treatments included 1% control or endo PF alone (n=8 for control and n=7 for endo), 1 or 2.5 μM GSK126 or AMD3100 alone, 1% PF plus 1 or 2.5 μM GSK126 or AMD3100, or a combination of 1 μM AMD3100 and 1 μM GSK126 alone and in the presence of 1% PF. Both compounds were added at the same time for the combination treatments. Once all treatments were added to appropriate wells, the plates were left in the xCELLigence machine within a 37°C incubator, and continuous readings were taken every hour for a total of 100 hours. All treatments were performed in triplicates and the CI averages were compared to 1% CS-FBS media treated wells as a percentage of cell growth.

Cell migration using xCELLigence

xCELLigence technology was also used to study how the migration potential of hESCs could change under various conditions. We used 16-well CIM16 plates in which two different chambers were present. Electrodes attached to the bottom of the wells of the bottom chamber measure the number of cells that had passed from the top chamber to the bottom (migrated) providing a cell impedance (CI) number. First, 160 μL of 1% CS-FBS media was added to each well of the bottom chamber making sure a meniscus had formed. The top chamber was placed on top of the bottom chamber before adding 30 μL of 1% CS-FBS media to each well. Plates were placed in the incubator for one hour before a background scan was taken. 20,000 hESCs per well were added to the top plate along with various cell treatments, which were similar to the cell proliferation studies. Once hESCs and treatments were added, scans were taken by the xCELLigence machine every 15 minutes for 48 hours. All treatments were performed in triplicate and the number of hESCs that had passed between chambers (migrated hESCs) was compared to 1% CS-FBS media only wells and given as a percentage.

mRNA isolation and expression

PF or drug-treated hESCs were suspended in TRI reagent and total RNA was isolated following the manufacturer's instructions. PF or drug-treated Eu tissues were homogenized in TRI reagent using zirconium oxide beads in a Bullet Blender® homogenizer. The supernatant was removed, and RNA was isolated following the manufacturer's instructions. The quantity and quality of RNA were measured using the NanoDrop 2000 spectrophotometer. cDNA synthesis from 1 μg RNA of each sample was performed using iScript cDNA synthesis kit. mRNA expression was analyzed in the cDNA samples using the respective primers listed in Table S1 by the SYBR Green method. The mRNA levels of the respective genes in the treated hESCs or Eu tissues were determined using the Pfaffl method considering the expression in the 1% CS-FBS media treated samples as 1.

Protein expression using automated western blotting (WES)

Protein lysates of the PF and drug-treated hESCs were suspended in RIPA buffer containing protease inhibitors. Eu tissues were homogenized using zirconium oxide beads in a Bullet Blender® homogenizer. Protein concentrations were determined using a modified Lowry protocol.[66] 7 μg of protein from either cells or tissues was run on the automated western blotting system, WES. The primary anti-rabbit antibodies for EZH2 (1:50), H3K27me3 (1:25), β-actin (1:100), and H3 (1:100) were used. HRP-conjugated rabbit secondary antibody provided in the WES kit was used. WES plates (12-250 kDa and 25 capillaries) were run using default settings and results were analyzed using the Compass for WES software. The band area corresponding to the molecular weight of the protein being studied as detected by the Compass software was used and expressed as their relative ratio to β-actin or H3. Results were expressed as a ratio in which 1% CS-FBS media alone was considered 1. It is important to note that proteins determined using the automated Western Blotting system, WES, have different observed molecular weights compared to traditional Western blotting, due to differences in its technology.

QUANTIFICATION AND STATISTICAL ANALYSIS

Prism software was used to perform statistics for all protein and mRNA analyses using one-way ANOVA. P-values less than 0.05 were considered significant.