The histone methyltransferase EZH2 promotes mammary stem and luminal progenitor cell expansion, metastasis and inhibits estrogen receptor-positive cellular differentiation in a model of basal breast cancer

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Abstract. Mammary stem cells (MSCs) are the progenitor population for human breast epithelia. MSCs give rise during mammary gland development to estrogen receptor (ER)-negative basal cells and the ER^- luminal progenitor (LP) population which maintains ER^+ and ER^- luminal cells. The MSC population is expanded and tumorigenic in some mouse mammary cancer models, and these tumor-initiating cells have been isolated from human breast cancers. MSC expansion is associated with aggressive biological behavior in human breast cancer. The LP population is tumorigenic in some mouse mammary cancer models, and is the progenitor population of basal breast cancer in humans. The enhancer of zeste homolog 2 (EZH2) is a methyltransferase which catalyzes lysine 27 methylation in histone H3 resulting in suppression of target gene expression. The histone demethylase JMJD3 opposes the activity of EZH2 by demethylating histone H3 lysine 27. EZH2 is a member of the polycomb group of proteins which regulates cell type identity. EZH2 expression was found to be increased in histologically normal human breast tissue among women with high breast cancer risk, and was elevated in ductal hyperplasia and ductal carcinoma *in situ*. EZH2 overexpression is associated with poorly differentiated and aggressive breast cancer in humans. However, the mechanisms by which EZH2 results in increased breast cancer risk and aggressive tumors are not completely characterized. Using *in vivo* transplantation of mammary cancer stem cells transduced with EZH2 or JMJD3 shRNAs, we demonstrated that EZH2 promotes mammary stem and LP cell expansion, metastasis and inhibits ER-positive cellular differentiation.

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Introduction

Breast cancer accounts for 25% of all cancers in women worldwide. In the US 296,000 women were diagnosed with breast cancer and over 39,000 died of the disease in 2013 (1). Numerous treated breast cancer patients will develop incurable metastatic disease, with a median survival of 3 years. Standard adjuvant therapies have a small impact on breast cancer survival, yet more than $16 billion were spent on breast cancer clinical care in 2010 (2). Most breast cancer risk factors cannot be modified, and include age, family and reproductive history, BRCA1 status and breast density.

Mammary stem cells (MSCs) are the progenitor population for human breast epithelia (3-5). MSCs give rise during mammary gland development to estrogen receptor (ER)-negative basal cells and the ER^- luminal progenitor (LP) population which maintains ER^+ and ER^- luminal cells (6-8). MSCs have been isolated from humans and mice using cell surface markers (9-11). The MSC population is expanded and tumorigenic in some mouse mammary cancer models (12,13), and these tumor-initiating cells have been isolated from human breast cancers (14-18). MSC expansion is associated with aggressive biological behavior in human breast cancer (19,20). The LP population is tumorigenic in some mouse mammary cancer models (21), and is the progenitor population of basal breast cancer in humans (22).

The enhancer of zeste homolog 2 (EZH2) is a methyltransferase which catalyzes methylation of lysine 27 in histone H3 resulting in suppression of target gene expression (23). The histone demethylase JMJD3 opposes the activity of EZH2 by demethylating histone H3 lysine 27. EZH2 is a member of the polycomb group of proteins which regulates cell type identity (24). EZH2 overexpression in the mouse mammary gland was found to produce intraductal hyperplasia and to delay involution. EZH2 expression was increased in histologically normal human breast tissue among women at high breast cancer risk (25), and was elevated in ductal hyperplasia and ductal carcinoma *in situ* (26). EZH2 overexpression is associated with poorly differentiated and aggressive breast cancer in humans (27,28). However, the mechanisms by which EZH2 results in increased breast cancer risk and aggressive tumors are not completely characterized.

Using *in vivo* transplantation of mammary cancer stem cells transduced with EZH2 or JMJD3 shRNAs, we demonstrated that EZH2 promotes mammary stem and LP cell expansion, metastasis and inhibits ER-positive cellular differentiation.

Materials and methods

Mouse breeding and procedures. We bred MMTV-Wnt1 mice obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Mammary tumorigenesis is driven by Wnt1 oncogene expression which is a model of basal subtype breast cancer. All mice were genotyped using PCR amplification of extracted tail DNA according to The Jackson Laboratories protocols. Twenty tumors were obtained for analysis. Tumors were trypsin dissociated for cryopreservation in liquid nitrogen.

Fluorescence-activated cell sorting. Dissociated tumor cells were incubated with phycoerythrin-conjugated anti-CD24 and AlexaFluor 488 conjugated anti-CD49f antibodies (Stem Cell Technologies, Vancouver, BC, Canada), washed in phosphate-buffered saline (PBS), and the CD24$^+$/CD49f^{hi} MSC fraction was sorted by flow cytometry (MoFlo Astrios, Becton Dickinson, Franklin Lakes, NJ). The CD24$^+$/CD49f^{lo}/CD61$^+$ LP fractions were sorted in separate experiments. Dissociated tumor cells were incubated with fluorescein-conjugated estradiol to identify ER-positive cells prior to sorting.

Cell culture, lentiviral transduction and transplantation. Sorted MSCs (10^4) from MMTV-Wnt1 tumors were cultured in 3:1 Dulbecco's modified Eagle's medium:F12 medium containing 1X B27 supplement, 10 ng/ml epidermal growth factor, 25 ng/ml basic fibroblast growth factor, 0.2% heparin, 40 μg/ml gentamicin, 2.5 μg/ml amphotericin B (MSC medium) at 37˚C in a humidified atmosphere of 5% CO_2 and separately transduced with lentiviruses containing control, EZH2 or JMJD3 shRNAs with 5 μg/ml Polybrene overnight according to the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA) at 37˚C in a humidified atmosphere of 5% CO_2. MSC medium was replaced and cells were cultured for 24 h. Puromycin (2 μg/ml) was added and the cells were incubated for 48 h. MSC medium was replaced and the cells were injected into the fat pads of 2-month-old immuno-compromised NU/J mice. In separate experiments, fat pads were transplanted with 10^4 sorted MSCs with either ER$^+$ or ER$^-$ luminal cells from the MMTV-Wnt1 mammary tumors. Mice were examined weekly for tumor formation for up to 6 months. The latency and volume of tumors were recorded for each mouse. Complete necropsy was performed on each mouse. Portions of each tumor were fixed in 10% formalin, flash frozen for storage at -80˚C and trypsin dissociated for cryopreservation in liquid nitrogen.

qRT-PCR. RNA was extracted from sorted MSCs and reverse transcribed according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). PCR reactions without cDNA template were used as the negative control. cDNA was amplified using EZH2, JMJD3 and β-actin primers. PCR was performed using thermal cycling parameters of 94˚C for 25 sec, 55˚C for 1 min and 72˚C for 1 min (Stratagene, La Jolla, CA, USA) with SYBR-Green.
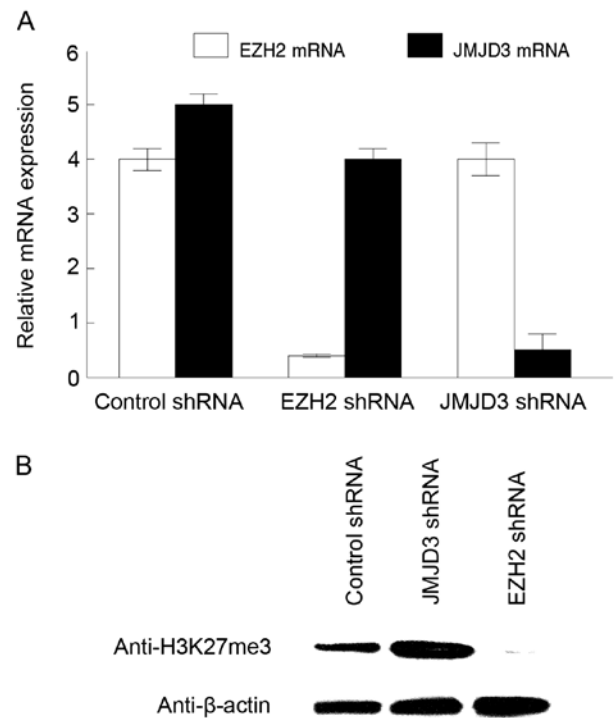

Figure 1. EZH2 and JMJD3 regulate histone H3 methylation in mammary cancer stem cells. EZH2, JMJD3 and control shRNAs were transduced into mammary cancer stem cells from MMTV-Wnt1 tumors and transplanted into cleared mammary fat pads of immunodeficient mice. (A) EZH2 and JMJD3 mRNA levels were determined in the transplanted tumors by qRT-PCR. Error bars indicate standard error measurement. (B) Levels of lysine 27 trimethylation of histone H3 (H3K27me3) in each group were determined by western blotting. β-actin expression was used as the control for equal loading in each lane. Experiments were performed in triplicate and representative blots are shown. EZH2, enhancer of zeste homolog 2.

Western blotting. Sorted MSCs from MMTV-Wnt1 tumors were lysed in 1X Laemmli buffer. Fifty micrograms of total cellular proteins was separated by SDS-PAGE. Proteins were electroblotted to PVDF membranes (Roche Applied Sciences, Indianapolis, IN, USA). Blots were incubated with blocking solution followed by anti-histone H3K27me3 and anti-β-actin antibodies for 16 h at 4˚C. After washing in Tris-buffered saline containing 0.1% Tween-20, blots were incubated for 30 min at room temperature with an anti-IgG secondary antibody conjugated to horseradish peroxidase. Bands were visualized by the enhanced chemiluminescence method and quantitated by densitometry. Data were analyzed by t-test.

Histopathology and immunohistochemistry. Formalin-fixed tumor tissue was dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections were deparaffinized and stained with hematoxylin and eosin. For immunohistochemical studies, the sections were rehydrated in PBS (pH 7.4) and blocked with 10% normal serum. For immunohistochemistry studies, the sections were incubated with PCNA primary antibody overnight at room temperature. Following washing in PBS, the sections were incubated with biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase. Antigen-antibody complexes were detected by incubation with peroxide substrate solution containing aminoethylcarbazole chromogen followed by hematoxylin counterstaining. The
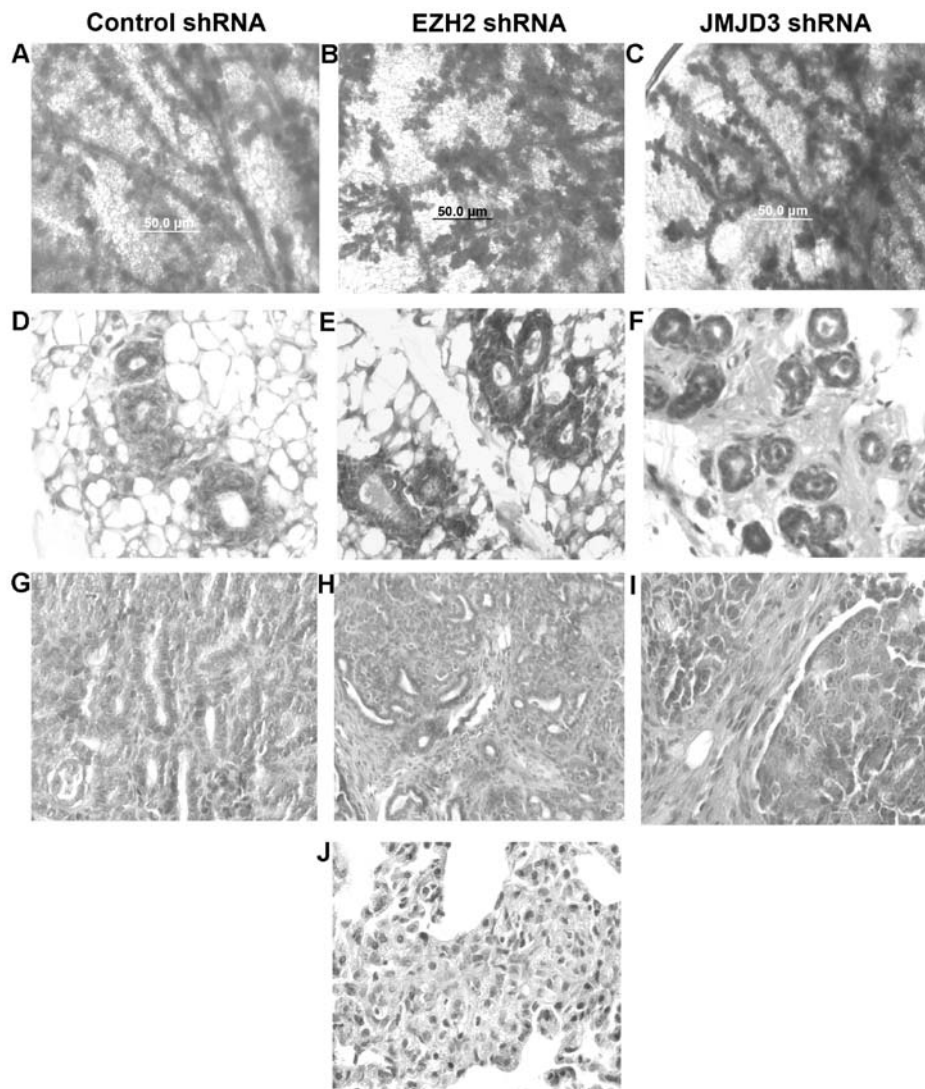
Figure 2. Increased EZH2 activity promotes lung metastasis in the MMTV-Wnt1 model of mammary cancer. (A-C) Whole mount preparations of cleared mammary fat pads transplanted with mammary cancer stem cells transduced with the control, EZH2 or JMJD3 shRNAs. (D-F) H&E staining of histopathologic sections of cleared mammary fat pads transplanted with mammary cancer stem cells transduced with the control, EZH2 or JMJD3 shRNAs. (G-I) H&E staining of histopathologic sections of mammary tumors derived from transplanted mammary cancer stem cells transduced with the control, EZH2 or JMJD3 shRNAs. (J) H&E staining of histopathologic sections of lung metastasis from mammary tumors derived from transplanted mammary cancer stem cells transduced with the JMJD3 shRNAs. A minimum of five transplants was performed for each group. Representative sections are shown. EZH2, enhancer of zeste homolog 2; H&E, hematoxylin and eosin.

percentage of PCNA cells in 10 random high power fields was determined by counting. Data were analyzed by t-test.

Cell death analysis. Tumor tissue sections were incubated with terminal deoxynucleotidyl transferase and dUTP-fluorescein for 1 h at 37˚C according to the manufacturer's recommendations (Roche Applied Sciences). After washing, apoptotic cells were visualized by fluorescence microscopy following coverslipping with anti-fade mounting medium containing DAPI. The percentage of fluorescent cells in 10 random high power fields was determined by counting. Data were analyzed by t-test.

Results

To determine the role of EZH2 in mammary cancer stem cell function, we transduced MSCs from MMTV-Wnt1

mammary tumors with EZH2, JMJD3 or control lentiviral shRNAs. JMJD3 is a histone demethylase which counteracts EZH2 activity by removing methyl groups from lysine 27 of histone H3. EZH2 and JMJD3 shRNAs reduced expression of these mRNAs in the tumorigenic MSCs by 80% compared to the control shRNAs (Fig. 1A). We examined trimethylation of lysine 27 in histone H3 in the transduced tumorigenic MSCs by western blotting. JMJD3 inhibition produced a 3-fold upregulation of H3K27me3 levels in the tumorigenic MSCs, while EZH2 shRNAs resulted in >95% reduction of H3K27me3 (Fig. 1B). We concluded that EZH2 and JMJD3 regulate histone H3K27me3 levels in tumorigenic MSCs.

We transplanted tumorigenic MSCs transduced with control, EZH2 or JMJD3 shRNAs to the cleared fat pads of immunodeficient mice. We examined mammary gland reconstitution by these MSCs using whole mount staining. As shown in Fig. 2A-C, transplanted mammary glands exhibited
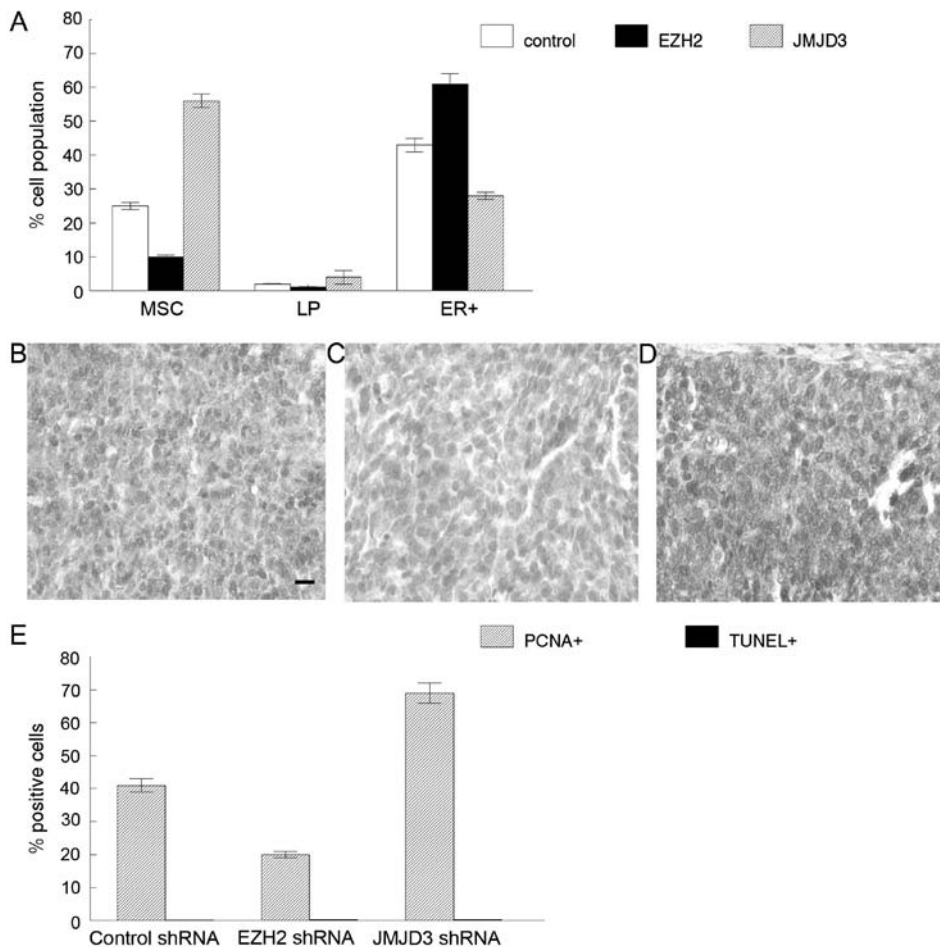
Figure 3. Increased EZH2 activity expands the tumorigenic mammary cancer stem cell and luminal progenitor populations yet inhibits ER[+] differentiation. (A) FACS analysis of mammary tumors derived from cancer stem cells transduced with the control, EZH2 or JMJD3 shRNAs. Mammary stem cell (MSC), luminal progenitor and ER[+] cell populations were sorted by flow cytometry from mammary tumors in each group. Error bars indicate standard error measurement. Elevated EZH2 activity increases proliferation in the MMTV-Wnt1 mammary tumors. PCNA immunohistochemistry was performed on tumor sections derived from the MSCs transduced with (B) the control, (C) EZH2 or (D) JMJD3 shRNAs. Representative sections are shown. (E) Quantization of PCNA[+] and TUNEL^{+} cells in the tumor sections derived from the MSCs transduced with the control, EZH2 and JMJD3 shRNAs. Error bars indicate standard error measurement. EZH2, enhancer of zeste homolog 2; FACS fluorescence-activated cell sorting.

hyperplastic branching characteristic of the MMTV-Wnt1 mice in all three transduced groups. There were no statistically significant differences in the number of branches or terminal end buds between the three groups. We also examined these reconstituted mammary glands by histopathologic sectioning, and hematoxylin and eosin (H&E) staining. Reconstituted mammary glands exhibited ductal and basal cell hyperplasia characteristic of MMTV-Wnt1 mice (Fig. 2D-F). Some glands exhibited early signs of stromal cell hyperplasia and fibrosis. However, we did not detect significant differences in the histopathology of reconstituted mammary glands transduced with the control, EZH2 nor JMJD3 shRNAs. Mammary glands transplanted with the tumorigenic MSCs transduced with the control, EZH2 or JMJD3 shRNAs developed mammary tumors. We did not detect significant differences in tumor latency or growth rate between the three groups. Tumors from each group were classified as poorly differentiated adenocarcinoma exhibiting ductal and stromal hyperplasia (Fig. 2G-I). Metastatic tumors were not detected in cancers arising from the MSCs transduced with the control or EZH2 shRNAs. However, lung metastasis was detected in all immunodeficient mice bearing tumors derived from the MSCs transduced

with the JMJD3 shRNAs. Lung metastases were classified as poorly differentiated adenocarcinoma (Fig. 2J). We concluded that increased EZH2 activity promotes lung metastasis in MMTV-Wnt1 mammary cancer.

To determine whether EZH2 activity alters tumorigenic MSC and LP cell fractions in MMTV-Wnt1 mammary tumors, we sorted these populations by fluorescence-activated cell sorting (FACS) from cancers derived from the MSCs transduced with the control, EZH2 or JMJD3 shRNAs. As shown in Fig. 3A, the MSC fraction in the mammary tumors transduced with the EZH2 shRNAs was reduced by 2.5-fold (P<0.01). Similarly the tumorigenic LP fraction in the tumors transduced with the EZH2 shRNAs was reduced by 2-fold (P<0.03). However, the ER[+] luminal cell fraction increased from 42 to 61% (P<0.05). We also sorted the tumorigenic MSC and LP cell fractions from tumors derived from the MSCs transduced with JMJD3 shRNAs. The MSC fraction in mammary tumors transduced with JMJD3 shRNAs increased from 25 to 56% (P<0.002), and the LP cell fraction also was elevated by 2-fold (P<0.04). However the ER[+] luminal cell fraction was reduced from 42 to 28% (P<0.02). We concluded that EZH2 activity promotes tumorigenic MSC and LP cell
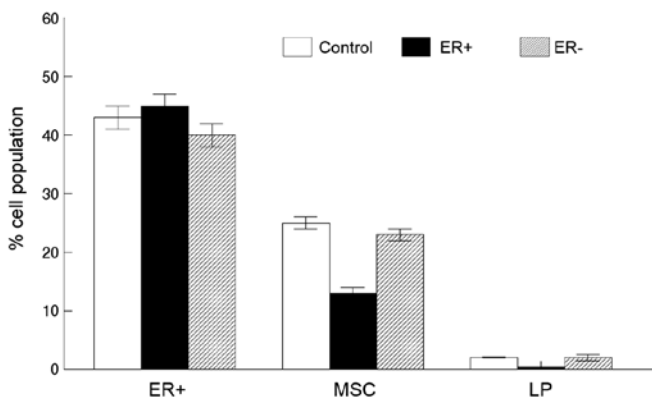
Figure 4. ER+ cells inhibit the tumorigenic mammary stem cell and luminal progenitor populations. MSCs were transplanted alone (control) or with ER+ or ER- luminal cells from MMTV-Wnt1 mammary tumors to cleared mammary fat pads of immunodeficient mice. Transplanted tumors were sorted by flow cytometry to determine the ER+, MSC and LP fractions. At least five transplants were performed in each group. Error bars indicate standard error measurement. MSCs, mammary stem cells; LP, luminal progenitor.

expansion in mammary tumors yet represses ER+ cellular differentiation.

To determine the mechanism of increased MSC and LP cell fractions in these tumors, we examined proliferation and programmed cell death in cancers from each group. The PCNA+ cell fraction was reduced in the mammary tumors derived from the MSCs transduced with EZH2 shRNAs compared to the control transplants (20 vs. 41%; P<0.01; Fig. 3B, C and E). The PCNA+ cell fraction was significantly increased in the mammary tumors derived from the MSCs transduced with the JMJD3 shRNAs compared to the control transplants (69 vs. 41%; P<0.003; Fig. 3B, D and E). There were no significant differences in the TUNEL+ cell fractions in the mammary tumors derived from the MSCs transduced with the EZH2 or JMJD3 shRNAs compared to the control transplants (Fig. 3E). We concluded that EZH2 promotes tumor cell proliferation in our model of basal mammary cancer.

Our results demonstrated that EZH2 promoted MSC and LP expansion and inhibited ER+ cellular differentiation (Fig. 3). To determine whether ER+ cells inhibited MSC expansion as a possible mechanism for these observations, we transplanted tumor-derived ER+ or ER- luminal cells with the MSCs into the cleared mammary fat pads of immunodeficient mice. Tumors resulting from these transplants were sorted by FACS to determine the MSC and LP cell fractions. As shown in Fig. 4, the mammary tumors derived from transplants of the MSCs and ER+ cells exhibited significantly reduced MSC fractions compared to those derived from the MSCs transplanted with the ER- cells (13 vs. 24%; P<0.002). Similarly, mammary tumors derived from transplants of the MSCs and ER+ cells exhibited significantly reduced LP fractions compared to those derived from the MSCs transplanted with the ER- cells (0.4 vs. 2%; P<0.04). We conclude that ER+ cells inhibit MSC and LP expansion in a model of basal mammary cancer.

Discussion

EZH2 is associated with younger age at breast cancer diagnosis (29), increased tumor size, high histopathologic grade,

negative hormone receptor status, epidermal growth factor receptor and Her2 overexpression, p53 mutations, lymphatic invasion, poor survival and metastasis (30-32). Our results demonstrated that EZH2 activity was associated with lung metastasis in a model of basal breast cancer. Future studies will determine which of these genetic changes are the direct result of EZH2-mediated regulation of target genes.

Our results demonstrated that EZH2 results in expansion of tumorigenic MSCs and LP cells. Previous studies demonstrated that expansion of breast tumor-initiating cells resulted from Raf1 or Notch gene expression in cancers overexpressing EZH2 (33,34). The present study was the first to determine that EZH2 also induced expansion of the tumorigenic LP population in basal mammary tumors. Our results also demonstrated that EZH2 suppresses ER+ cellular differentiation. Previous retrospective studies using human breast cancer tissue demonstrated that patients with high tumoral EZH2 expression had the least clinical benefit from anti-estrogen therapy (35). We also demonstrated that EZH2 activity resulted in increased cell proliferation which was observed in human breast cancers (36). A previous *in vitro* study demonstrated that EZH2 inhibition resulted in decreased proliferation of human breast cancer cell lines (37). The present study demonstrated a novel mechanism by which tumor-derived ER+ cells repress tumorigenic MSC and LP expansion.

EZH2 overexpression was shown to induce ductal hyperplasia in the mouse mammary gland (24). MMTV-Wnt1 mammary glands exhibit ductal hyperplasia (13), and we did not detect changes in glandular hyperplasia resulting from transplantation of the Wnt1 tumor-derived MSCs transduced with the EZH2 or JMJD3 shRNAs. Similarly, mammary tumors derived from these transplants did not exhibit significant differences in histopathologic appearance. These results are likely due to Wnt1-induced hyperplasia which overrides the effects of EZH2.

In summary, EZH2 promotes tumorigenic MSC and LP cell expansion and metastasis while suppressing ER+ cellular differentiation. Future studies will determine how EZH2 induces mammary tumor cell proliferation, expansion of the tumorigenic LP cell population, and suppresses ER+ cellular differentiation.

Acknowledgements

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