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14. ABSTRACT

We proposed that alterations in histone methylation regulate MSC fate commitment and predispose these progeny to malignant transformation. Transformed ER+ epithelial cells deregulate proliferation of MSC and luminal progenitors contributing to transformation of ER- luminal and basal cells and development of treatment resistant breast cancer. In this second progress report, we demonstrate that transformed MSC with reduced DNA repair contribute to more aggressive mammary tumors. Transformed luminal progenitor cells with altered histone methylation produce aggressive mammary tumors after long latency. The fractions of progenitor and differentiated cells in these tumors also are altered. We show that ER+ tumor cells promote proliferation and metastasis of tumor derived MSCs. The implications of the results presented in this progress report suggest that while tumor suppressor pathways can inhibit mammary tumorigenesis when DNA damage repair is inhibited, more aggressive clones may eventually evolve via genomic instability with the ability to proliferate and metastasize. Decreased DNA damage repair or altered epigenetic marks can dramatically affect the cellular composition of these tumors, thereby regulating clinical phenotype. Paracrine factors secreted by ER+ tumor cells can induce proliferation of tumorigenic MSC, leading to increased genomic instability and metastasis.

15. SUBJECT TERMS
Histone methylation, mammary stem cell, estrogen receptor, luminal progenitor, DNA repair

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INTRODUCTION

Breast cancer and its treatment impose significant physical, psychological, and economic burdens on patients, their families, and society. African-American women under age 35 have the highest age adjusted mortality rates from breast cancer. The incidence of breast cancer in this group also is higher than for Caucasian women. In several studies these disparities persisted after other variables such as socioeconomic status and access to care were controlled. These studies point to biological differences in breast cancer among these populations such as increased prevalence of the high grade, estrogen receptor negative, basal subtype of the disease in African-American women. Targeting estrogen receptor α (ER) action is a classic example of molecular therapy for breast cancer (for review see Russo et al., 2000; Crowe, 2002). ER- mammary stem cells (MSC) are believed to be the progenitor population for all breast epithelia including acinar cells and the largely ER- basal and luminal cells of the ducts from which most breast cancer arises (for review see Crowe et al., 2004; Visvader and Lindeman, 2006; Sleeman et al., 2007). The luminal epithelial population also contains a subpopulation of ER- progenitors (Asselin-Labat et al., 2007). ER is expressed by a fraction of luminal epithelial cells in normal mammary glands, but the numbers of these cells often are greatly increased in human breast cancer. MSC can reconstitute mammary glands and have been isolated from both humans and mice using cell surface markers (Gudjonsson et al., 2002; Dontu et al., 2003; Stingl et al., 2006; Shackleton et al., 2006). Some of these MSC populations are expanded in mouse mammary cancer models (Li et al., 2003; Liu et al., 2004), and tumorigenic progenitor populations have been isolated from human breast cancers (Al-Hajj et al., 2003; Ponti et al., 2005). These studies have demonstrated an important role for MSC in mammary gland development and tumorigenesis. However these models have not determined how MSC or luminal progenitors give rise to ER+ epithelial cells during mammary gland development, what predisposes ER+ cells to malignant transformation, and how these cells regulate breast tumorigenesis in conjunction with transformed ER- MSC and luminal progenitors. In clinical studies, EZH2 histone methyltransferase (HMT) expression has been associated with poorly differentiated and aggressive breast cancer in humans (Kleer et al., 2003; Raaphorst et al., 2003; Bachmann et al., 2006; Collett et al., 2006; Ding et al., 2006). The histone demethylase JMJD3 reverses the EZH2 mediated histone H3 lysine-27 methyl mark. We proposed a new mechanism of breast tumorigenesis that, during normal mammary gland development, decreased expression of DNA repair genes during HMT mediated differentiation of MSC to ER+ luminal cells makes the latter population more susceptible to transformation and expansion. These transformed cells may induce aberrant proliferation of the ER- MSC or luminal progenitors resulting in genomic instability, production of additional transformed ER+ or ER- luminal cells, or transformation of MSC giving rise to the aggressive basal subtype of mammary cancer. We hypothesized that alterations in histone methylation regulate MSC fate commitment and predispose these progeny to malignant transformation. Transformed ER+ epithelial cells deregulate proliferation of MSC and luminal progenitors contributing to transformation of ER- luminal and basal cells and development of treatment resistant breast cancer. **Specific Aim #1** will test the hypothesis that EZH2 regulates both ER and DNA repair gene expression in MSC resulting in the differentiation of ER+ but transformation sensitive mammary epithelial cells. Specific Aim #1 will test this hypothesis by comparing transformation of these cell populations following altered histone methylation. **Specific Aim #2** will test the hypothesis that transformed ER+ cells regulate proliferation and tumorigenicity of the MSC and luminal progenitor populations by determining the molecular and cellular effects of co-transplantation of these populations. Specific Aim #2 also will target histone and DNA methylation in these cells using molecular therapy to determine the effects on mammary tumorigenesis. Understanding the relationships between normal and transformed mammary epithelial cells has important ramifications for preventing ER- and basal subtype breast cancer in African-American women. The biologically aggressive basal subtype of breast cancer may result from transformation of the less differentiated MSC population. Targeting interactions between ER+ cells and MSC to prevent progression to these treatment resistant tumors will improve survival and quality of life.

BODY

Since the submission of the first progress report, we have characterized tumors derived from transformed mammary stem cells (MSC) with reduced Rad50 and NBS1 expression and CD61+ luminal progenitors with reduced EZH2 expression. These transplanted tumors were characterized as poorly differentiated adenocarcinoma by histopathology (Fig. 1A-C). Tumors with reduced Rad50/NBS1 or EZH2 expression demonstrated markedly increased latency (mean 72 and 78 weeks respectively; $P<0.01$). However these tumors were highly metastatic compared to those derived from control clones (4 fold increase in lung tumors; Fig. 2). These tumors were analyzed for cellular composition by flow cytometry. Compared to tumors derived from control clones, those with decreased Rad50/NBS1 expression exhibited a 2 fold increase in the percentage of MSCs ($P<0.05$; Fig. 3). The number of estrogen receptor (ER) positive cells in these tumors was reduced by 9 fold ($P<0.03$). In tumors derived from luminal progenitor cells with reduced EZH2 expression, the CD61+ population was expanded by 7 fold ($P<0.02$) and the percentage of ER+ cells was reduced 10 fold ($P<0.01$). These results indicate that mammary tumors derived from transformed MSC with reduced DNA repair and luminal progenitors with decreased EZH2 expression have increased latency and metastasis and reduced ER+ differentiation.

In Specific Aim 2, we characterized mammary tumors derived from MMTV-Wnt1 MSC transplanted with ER+ or ER- tumor cells to cleared mammary fat pads of nude mice. These transplanted tumors were characterized as poorly differentiated adenocarcinoma by histopathology (Fig. 4A, B). Transplanted tumors derived from MMTV-Wnt1 MSC and ER+ tumor cells exhibited decreased latency compared to those transplanted with MSC and ER- tumor cells (4 vs. 12 weeks respectively; $P<0.03$; Fig. 5A). MSC/ER+ transplants developed a significantly higher number of tumors than MSC/ER- transplants (19 versus 11 tumors; $P<0.05$; Fig. 5B). MSC/ER+ tumors exhibited a higher percentage of proliferating cells (52% versus 21%; $P<0.01$; Fig. 5C). MSC/ER+ tumors were highly metastatic compared to MSC/ER- tumors (35% versus 12% metastatic tumors; $P<0.004$; Fig. 5D). MSC/ER+ tumors exhibited a higher percentage of MSC (40% versus 21%; $P<0.04$; Fig. 6). These results indicate that tumor derived ER+ cells enhance proliferation and tumorigenicity of transplanted tumorigenic MSC. We have transplanted MSC with ER+ tumor cells expressing reduced JMJD3 levels to determine how alterations in histone methylation in ER+ cells affect the tumorigenic MSC phenotype. We are currently evaluating tumor formation in these groups with an approved one year no cost extension of the award. The specific aims of the project will be complete following characterization of these tumors.

KEY RESEARCH ACCOMPLISHMENTS

- Tumors derived from MSC with reduced Rad50 and NBS1 expression and CD61+ luminal progenitors with reduced EZH2 expression were characterized as poorly differentiated adenocarcinoma by histopathology.
- Tumors with reduced Rad50/NBS1 or EZH2 expression demonstrated markedly increased latency.
- Tumors with reduced Rad50/NBS1 or EZH2 expression were highly metastatic compared to those derived from control clones.
- Tumors with decreased Rad50/NBS1 expression exhibited a 2 fold increase in the percentage of MSCs. The number of estrogen receptor (ER) positive cells in these tumors was reduced by 9 fold.
- In tumors derived from luminal progenitor cells with reduced EZH2 expression, the CD61+ population was expanded by 7 fold and the percentage of ER+ cells was reduced 10 fold.
- Transplanted tumors derived from MMTV-Wnt1 MSC and ER+ or ER- tumor cells were characterized as poorly differentiated adenocarcinoma by histopathology.
- Transplanted tumors derived from MMTV-Wnt1 MSC and ER+ tumor cells exhibited decreased latency compared to those transplanted with MSC and ER- tumor cells.
- MSC/ER+ transplants developed a significantly higher number of tumors than MSC/ER- transplants.
- MSC/ER+ tumors exhibited a higher percentage of proliferating cells.
- MSC/ER+ tumors were highly metastatic compared to MSC/ER- tumors.

- MSC/ER+ tumors exhibited a higher percentage of MSC.

REPORTABLE OUTCOMES

An abstract describing studies which led to the current award was presented at the 2011 Era of Hope Meeting in Orlando, FL. Two manuscripts are in review which describe the detailed results summarized in the abstract. These manuscripts will be provided when final versions are accepted by the journals.

CONCLUSION

The implications of the results presented in this progress report suggest that while tumor suppressor pathways can inhibit mammary tumorigenesis when DNA damage repair is inhibited, more aggressive clones may eventually evolve via genomic instability with the ability to proliferate and metastasize. Decreased DNA damage repair or altered epigenetic marks can dramatically affect the cellular composition of these tumors, thereby regulating clinical phenotype. Paracrine factors secreted by ER+ tumor cells can induce proliferation of tumorigenic MSC, leading to increased genomic instability and metastasis.

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APPENDICES

Abstract presented at 2011 Era of Hope Meeting:

COORDINATE ACTIVATION OF THE NBS1 GENE REGULATES ESTRADIOL-MEDIATED PROTECTION FROM DOUBLE-STRAND DNA BREAKS

David Crowe, Rowena Wan, and Kaitrin Baloue

University of Illinois, Chicago

Introduction: Double-strand break repair is mediated by two major repair pathways, homologous recombination (HR) or nonhomologous end joining (NHEJ). In mammalian cells more than 90% of double-strand breaks are repaired by NHEJ. Impairment of these pathways is associated with cell cycle arrest, cell death, genomic instability, and cancer. Human diseases such as Nijmegen breakage syndrome, due to mutations in the NBS1

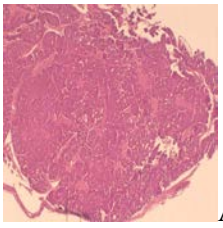
gene, produce defects in resection of double-strand breaks. NBS1 polymorphisms have been associated with increased risk of breast cancer. We previously demonstrated that estradiol protected estrogen receptor (ER)-positive breast cancer cell lines against double-strand breaks and cell death.

Objective: To determine the mechanism by which ER-positive breast cancer cells are protected from double-strand breaks and cell death.

Methods: Human mammary epithelial and breast cancer cell lines were cultured in DMEM and 10% fetal bovine serum. Primary cultures of NBS1+/-;neu and NBS1+/+;neu mouse mammary tumor cells were established by trypsinization of minced tumor tissue and cultured in this medium. Human breast cancer cell lines were treated with estradiol (E2), ionizing radiation (IR), combined E2 and radiation, or vehicle. Cells were transfected with c-myc, CBP, SRC1, or neomycin resistance plasmids. For gene knockdown experiments, cells were transfected with shRNA. DNA damage was quantitated by single cell gel electrophoresis. Apoptotic cells were determined by TUNEL assay. Gene expression was determined by quantitative reverse transcriptase-polymerase chain reaction and western blot. Occupancy of the NBS1 intron 1 by p53, c-myc, coactivators, or histones was determined by chromatin immunoprecipitation and nucleosomal mapping. Intron activity and double-strand break repair was determined by reporter gene analysis. Tumor development in NBS1+/-;neu mice was compared to that in NBS1+/+;neu control mice. Mammary tumor tissue was processed for histopathologic and molecular analysis.

Results: The combination of E2 and IR was required to induce NBS1 expression in ER-positive breast cancer cell lines. The protective effect of E2 against double-strand break damage was dependent on ER expression. NBS1 mediated the E2 protective effects against ionizing radiation-induced double-strand break damage and apoptosis. E2 and IR were required to activate the NBS1 intron 1 via cooperative c-myc and p53 binding to their cognate binding sites. E2 and IR recruited coactivators SRC1 and CBP to the myc and p53 binding sites of the NBS1 intron 1. Coactivator induction of NBS1 gene expression was dependent on these sites, and c-myc functionally substituted for E2 treatment in ER-positive cells. CBP and SRC1 functionally substituted for both E2 and IR induction of NBS1 gene expression. ER-positive cells in oncogene driven mammary tumors exhibited fewer double-strand breaks than ER-negative cells. NBS1 haploinsufficiency produced increased double-strand breaks and inhibited oncogene driven mammary tumorigenesis via induction of apoptotic cell death. However, tumors arising in this model were highly metastatic as the result of increased genetic alterations in transformed mammary epithelial cells.

SUPPORTING DATA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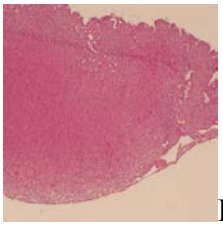
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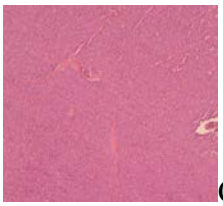


C

Fig. 1. Mammary tumor formation in control shRNA transduced mammary stem cells (A), mammary stem cells transduced with Rad50 and NBS1 shRNAs (B), and luminal progenitors transduced with EZH2 shRNA (C) transplanted to cleared mammary fat pads of nude mice. Tumors were dissected and stained with hematoxylin and eosin.

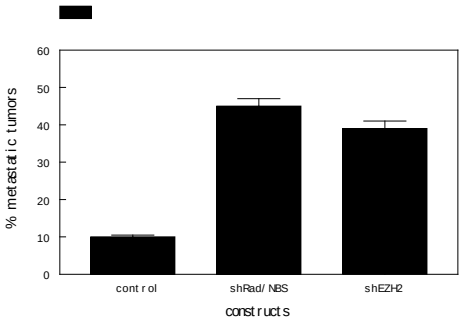


Fig. 2. Increased metastatic tumors in Rad50/NBS1 shRNA transduced mammary stem cells, EZH2 shRNA transduced luminal progenitors, and control shRNA transduced mammary stem cells transplanted to cleared mammary fat pads of nude mice. Error bars indicate SEM.

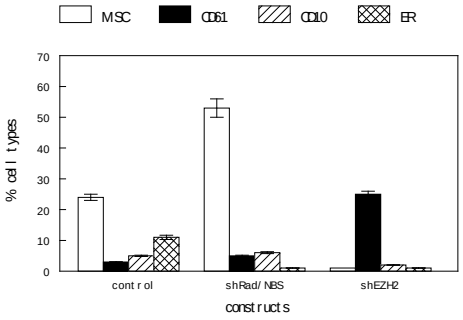
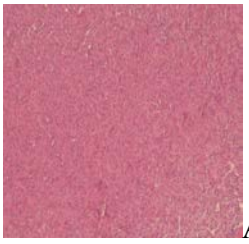


Fig. 3. Cellular composition of mammary tumors derived from control shRNA transduced mammary stem cells, Rad50/NBS1 shRNA transduced stem cells, and EZH2 shRNA transduced luminal progenitor cells. The fraction of MSC, CD61+ luminal progenitors, CD10+ basal cells, and ER+ luminal cells was determined by flow cytometry. Error bars indicate SEM.



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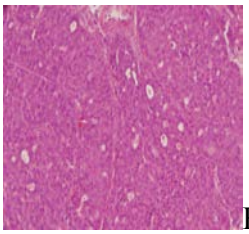
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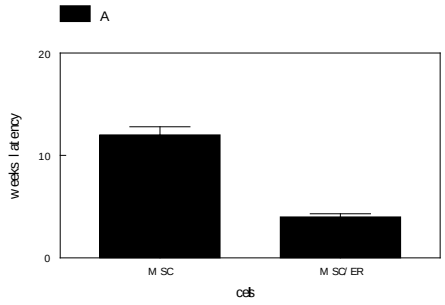
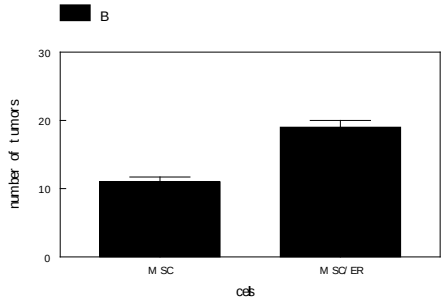
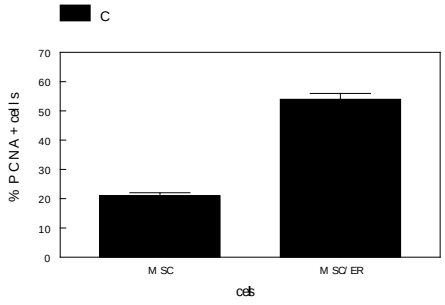
Fig. 4. Mammary tumors derived from MMTV-Wnt1 mammary stem cells transplanted with estrogen receptor positive (A) or estrogen receptor negative (B) tumor cells. T umors were dissected and stained with hematoxylin and eosin.







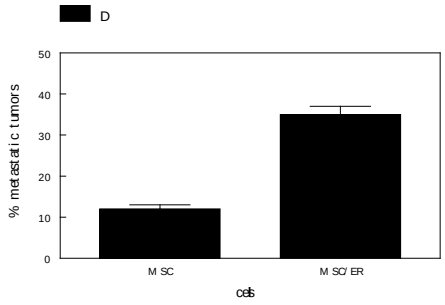


Fig. 5. Decreased tumor latency (A) correlated with increased tumorigenicity (B), proliferation (C), and metastasis (D) by MMTV-Wnt1 mammary tumor stem cells (MSC) transplanted with estrogen receptor positive (ER+) tumor cells (MSC/ER). Tumorigenic MSC also were transplanted with ER negative tumor cells. Error bars indicate SEM.

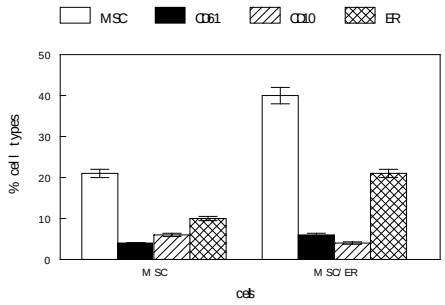


Fig. 6. Cellular composition of mammary tumors derived from MMTV-Wnt1 mammary tumor stem cells (MSC) transplanted with estrogen receptor negative or positive (ER) tumor cells. The fraction of MSC, CD61+ luminal progenitors, CD10+ basal cells, and ER+ luminal cells was determined by flow cytometry. Error bars indicate SEM.