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14. ABSTRACT

The Wnt/b-catenin pathway has been extensively studied for its role in development and caner. It has been established that Wnt/b-catenin signaling regulates the self renewal of normal stem cells in the hematopoietic system, the epidermis, as well as many other organs, but the importance of this pathway in breast stem/progenitors has not yet been investigated. The overall objective of this study is to determine the mechanisms by which b-catenin might promote breast stem/progenitor cell survival, while inhibiting mammary differentiation. If the Wnt/b-catenin signal transduction pathway is critical for breast stem cell survival, it may be possible to sensitize these cells to chemotherapeutic agents by inhibiting this pathway. These studies may, therefore, provide new targets for understanding the etiology of, and be critical for the design of new treatments for breast cancer.

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Introduction:

The Wnt/ß-catenin pathway has been extensively studied for its role in development and cancer. It has been established that Wnt/ß-catenin signaling regulates the self renewal of normal stem cells in the hematopoietic system, the epidermis, as well as many other organs, but the importance of this pathway in breast stem/progenitors has not yet been investigated. The overall objective of this study is to determine the mechanisms by which ß-catenin might promote breast stem/progenitor cell survival, while inhibiting mammary differentiation. If the Wnt/ß-catenin signal transduction pathway is critical for breast stem cell survival, it may be possible to sensitize these cells to chemotherapeutic agents by inhibiting this pathway. These studies may, therefore, provide new targets for understanding the etiology of, and be critical for the design of new treatments for, breast cancer.

Tasks:

- ❒ Task #1
  Accomplishments have been described in the previous report and are finished.

- ❒ Task #2

Accomplishemnts have been described in the previous report and are finished. A manuscript pertaining to the task is in preparation.

- ❒ Task #3 to determine if stabilized ß-catenin protects stem cells against radiation induced cell death.

To test the hypothesis that stem-like progenitor cells in the mammary gland are resistant to radiation compared to the non-stem cells, we used the Hoechst dye effluxing Side Population (SP) and Stem Cell Antigen-1 (Sca-1) as two surrogate stem cell markers that separate these two populations of cells. As previously reported, we have observed an increase in %SP following radiation in Balb/c primary mammary epithelial cells. To determine whether the expansion observed in the SP is consistent in expansion in stem/progenitor populations, we used Sca-1, and observed a similar expansion of Sca-1^+ cells following radiation. Interestingly, the number of Sca-1^- cells decreased following radiation (Figure1).

Phosphorylation at double stranded breaks following radiation is one of the earliest responses to radiation-induced DNA damage. Using a fluorescent antibody specific for the phospho-H2AX, discrete nuclear foci can be visualized and quantitated following radiation (Rothkamm and Lobrich, 2003). To determine whether mammary epithelial stem cells may be affected by DNA damage, we used phospho-H2AX to compare DNA damage between the Sca-1^+ and Sca-1^- cells. By quantitating the number of phospho-H2AX foci in the Sca-1^+ and the Sca-1^- cells, we observed that following radiation at 2 Gy, the Sca-1^- cells displayed 3.5 fold or more foci compared to the Sca-1^+ cells. In addition, by quantitating the number of cells containing foci, we observed that following radiation the number of Sca-1^- cells increased 2 fold while the number of Sca-1^+ cells remained the same after radiation (Figure2). Taken together, this demonstrated that the Sca-1^+ cells show less DNA-damage foci following radiation than Sca-1^- cells, suggesting that this may be one mechanism the Sca-1^+ cells evade radiation-induced damage.

It is well known that rather than apoptosis or necrosis, radiation induces mitotic cell catastrophe. To determine whether radiation affects Sca-1$^+$ cells and Sca-1$^-$ cells differently, we examined the cell cycle profiles of Sca-1$^+$ and Sca-1$^-$ cells, focusing on the G0 and G1 phases. Before radiation, we observed that Sca-1$^+$ cells were mostly in G1 and S/G2/M phases of the cell cycle, whereas the Sca-1$^-$ cells were in G0. Following radiation, we observed that the Sca-1$^+$ cells were pushed towards S/G2/M phases, while the Sca-1$^-$ cells gradually increased in G1 (Figure3). This demonstrated that the Sca-1$^+$ cells were in distinctly different phases of the cell cycle than Sca-1$^-$ cells. In addition, following radiation, the Sca-1$^+$ cells were able to cycle, and seemed to be unaffected by radiation. The Sca-1$^-$ cells increased in G1, but decreased in S/G2/M, suggesting that this population may be undergoing radiation induced mitotic catastrophe.

Next, we determined the clonogenicity of Sca-1$^+$ and Sca-1$^-$ cells. Clonogenic cells are defined as those neoplastic cells within a tumor that have the capacity to produce an expanding colony of descendants, and therefore, the capacity to regrow the tumor if left intact at the end of treatment (Pawlik and Keyomarsi, 2004). We sorted cells into Sca-1$^+$ and Sca-1$^-$ populations directly into 96well plates containing growth factor reduced Matrigel in equal numbers, and their clonogencity were assessed after 10 days. The Sca-1$^+$ cells were able to form 13.8±1.6 clonogens, and the numbers were not affected following radiation, 16±3 clonogens. The Sca-1$^-$ cells formed fewer clonogens, 4.6±1, than the Sca-1$^+$ cells, and the numbers of clonogens decreased following radiation, 3.2±0.7. This data demonstrates that the Sca-1$^+$ cells are more clonogenic than Sca-1$^-$ cells and clonogen formation is resistant to radiation.

To determine whether the Wnt/ß-catenin signaling pathway affects stem cell survival, we looked for the presence of stabilized ß-catenin in both the Sca-1$^+$ and Sca-1$^-$ cells. Using an antibody against the non-phosphorylated (stabilized) ß-catenin (Jamieson et al., 2004), we used FACS to compare the level of stabilized ß-catenin between the Sca-1$^+$ and Sca-1$^-$ populations. We found that the level of stabilized ß-catenin increased only in the Sca-1$^+$ population following radiation. To further confirm this observation, we examined activation of the downstream target survivin following radiation using real time PCR. We observed that the level of survin increased only in the Sca-1$^+$ population following radiation. Taken together, this demonstrated that the Wnt/ß-catenin pathway is involved in increased survival of stem/progenitor cells following radiation treatment.

This study demonstrates that stem-like progenitor cells in the mammary gland can be more resistant to clinically relevant doses of radiation than non-stem cells, which constitute the bulk of the mammary gland. In addition, we demonstrate that the Wnt/ß-catenin pathway contribute to the radioresistance of the stem/progenitor cells. These data are of clinical importance because radiation therapy is a valuable component of breast conserving cancer therapy, and improves overall survival in selected patients treated with mastectomy. Despite radiation, surgery, and chemotherapy, a number of patients still have disease recurrence and there remains considerable need for improvement in treatments. Our data suggest that targeting cancer stem cells may offer a new strategy for sensitizing breast cancers to radiation. It may be possible to improve locoregional control and ultimately survival in breast cancer through direct targeting of stem cell survival pathways such as Wnt/ß-catenin during radiation.

Key Research Accomplishments:

Manuscripts:
Wendy A. Woodward, Mercy S. Chen, Fariba Behbod, Maria P. Alfaro, Thomas Buchholz, and Jeffrey M. Rosen. Wnt/ß-catenin-Mediated Radiation Resistance of Mouse Mammary Stem-like/Progenitor Cells. (Manuscript in preparation.)

Mercy S. Chen, Wendy A. Woodward, Fariba Behbod, Maria P. Alfaro, and Jeffrey M. Rosen. COMMA-D: A cell line with progenitor cell characteristics. (Manuscript in preparation).

Reportable Outcomes:

Poster Presentation:
Mercy S. Chen, Wendy A. Woodward, Jeffrey M. Rosen. ß-catenin: A pivotal Role in Mammary Gland Stem Cell Survival and Differentiation. DOD Era of Hope meeting 2005.

Wendy A. Woodward, Mercy S. Chen, Fariba Behbod, Maria P. Alfaro, Thomas Buchholz, and Jeffrey M. Rosen. Wnt/ß-catenin-Mediated Radiation Resistance of Mouse Mammary Stem-like/Progenitor Cells. Keystone Symposium Stem Cells, Senescence and Cancer, 2005.

Oral Presentation:
Mercy S. Chen, Wendy A. Woodward, Jeffrey M. Rosen. ß-catenin: A pivotal Role in Mammary Gland Stem Cell Survival and Differentiation. DOD Era of Hope meeting 2005.

Conclusions:

We are on the right track with respect to the assigned tasks, we have reported a number of fascinating observations here. We are pleased with the progress we have made in the past year, and we feel confident that we will complete the remaining tasks on schedule.

References:

Jamieson, C. H., Ailles, L. E., Dylla, S. J., Muijtjens, M., Jones, C., Zehnder, J. L., Gotlib, J., Li, K., Manz, M. G., Keating, A. et al. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med* **351**, 657-67.

Pawlik, T. M. and Keyomarsi, K. (2004). Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys* **59**, 928-42.

Rothkamm, K. and Lobrich, M. (2003). Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc Natl Acad Sci U S A* **100**, 5057-62.

Figure1.

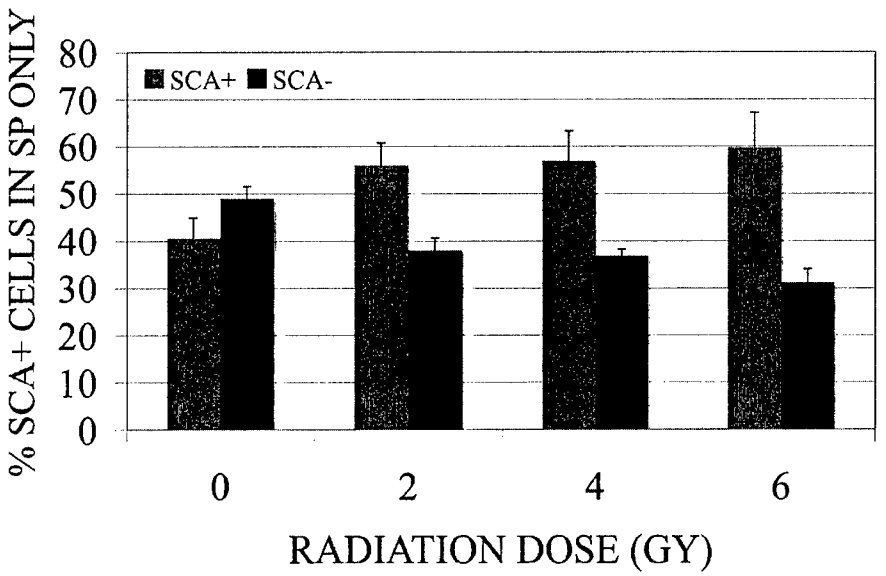


Figure1. Mammary epithelial cells were isolated from Balb/c mice, cultured 3 days, irradiated, and analyzed for %Sca in SP 24 hrs after radiation.

Figure2.

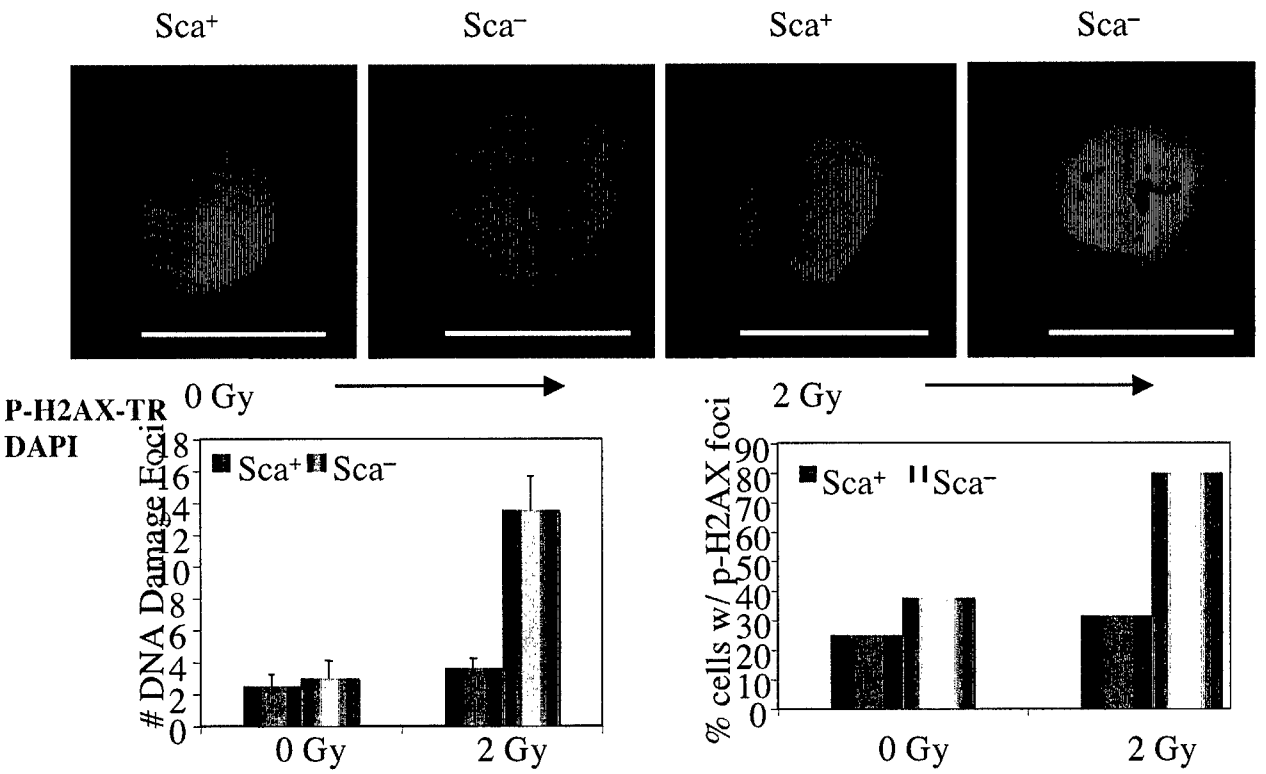
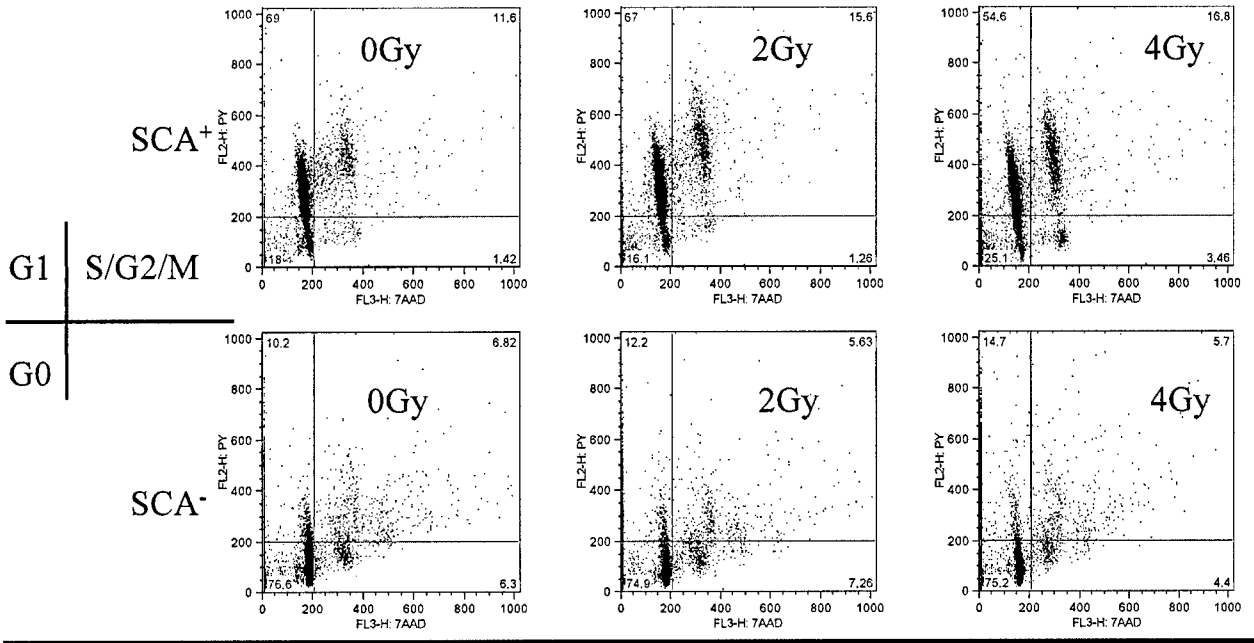


Figure2. DNA-damage foci following radiation, scored by phospho-H2AX positive foci. Sca+ and Sca– cells were sorted onto glass slides following radiation, and immunostained with anti-phospho-H2AX (scale bar: 10um).

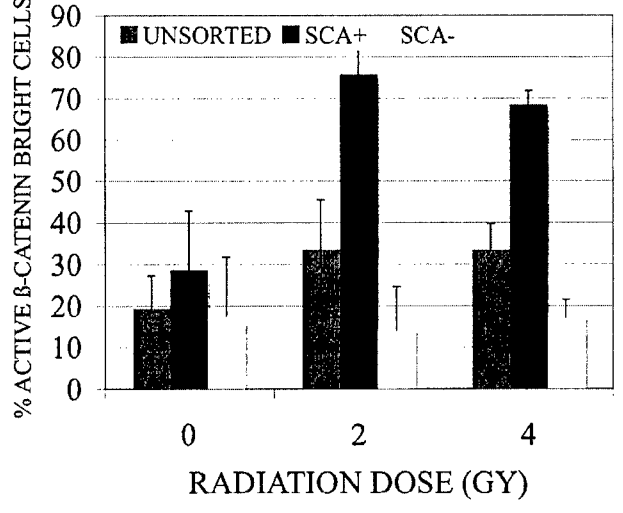
Figure3.



	0GY SCA+	0GY SCA-	2GY SCA+	2GY SCA-	4GY SCA+	4GY SCA-
G0	18	76.6	16.1	74.9	25.1	75.2
G1	69	10.2	67	12.2	54.6	14.7
S/G2/M	11.6	6.82	15.6	5.63	16.8	5.7

Figure3. Primary mammary epithelial cells were sorted into Sca+ and Sca- populations. Cell cycle analysis was performed using 7AAD (DNA) -Pyronin Y (RNA) to distinguish between G0 and G1, respectively.

Figure4 (a)



(b)

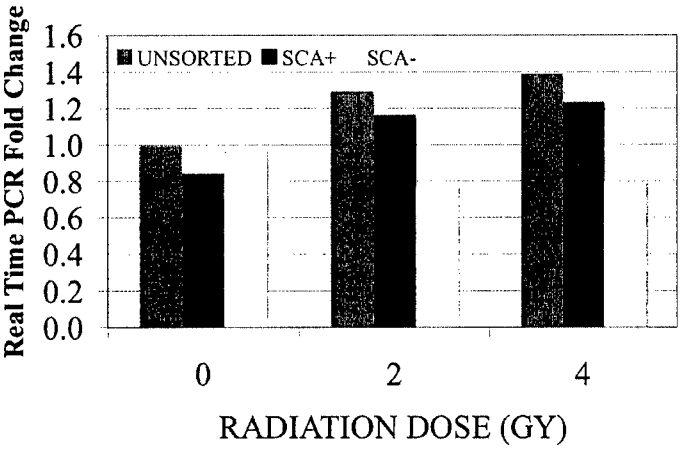


Figure4. The level of active ß-catenin increases in only the Sca+ population following radiation (a). After 48 hrs, real time PCR for survivin, a downstream target, were performed using the ABI real time PCR system (b).