DE-FG02-08ER64654 *INTEGRATIVE RADIATION BIOLOGY*

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ABSTRACT

We plan to study tissue-level mechanisms important to human breast radiation carcinogenesis. We propose that the cell biology of irradiated tissues reveals a coordinated multicellular damage response program in which individual cell contributions are primarily directed towards suppression of carcinogenesis and reestablishment of homeostasis. We identified transforming growth factor β1 (TGFβ) as a pivotal signal. Notably, we have discovered that TGFβ suppresses genomic instability by controlling the intrinsic DNA damage response and centrosome integrity. However, TGFβ also mediates disruption of microenvironment interactions, which drive epithelial to mesenchymal transition in irradiated human mammary epithelial cells. This apparent paradox of positive and negative controls by TGFβ is the topic of the present proposal. First, we postulate that these phenotypes manifest differentially following fractionated or chronic exposures; second, that the interactions of multiple cell types in tissues modify the responses evident in this single cell type culture models. The goals are to: 1) study the effect of low dose rate and fractionated radiation exposure in combination with TGFβ on the irradiated phenotype and genomic instability of non-malignant human epithelial cells; and 2) determine whether stromal-epithelial interactions suppress the irradiated phenotype in cell culture and the humanized mammary mouse model. These data will be used to 3) develop a systems biology model that integrates radiation effects across multiple levels of tissue organization and time. Modeling multicellular radiation responses coordinated via extracellular signaling could have a significant impact on the extrapolation of human health risks from high dose to low dose/rate radiation exposure.

HISTORY

This funding was initiated in 10/01/2006 at Lawrence Berkeley National Laboratory and was transferred in 8/01/2008 upon my move from to New York University (NYU) School of Medicine (NYUSOM). Hurricane Sandy caused the flooding of NYUSOM campus on October 29, 2012, resulting in the loss of power and services to NYUSOM, Bellevue and VA Hospital buildings. As a result, biospecimens stored in our freezers and refrigerators were damaged due to temperature fluctuations. We were unable to work for four months during the relocation and our animal colony was quarantined until March. Moreover we had no access to radiation sources until June. We then relocated our laboratory and animal colony to the NYU Alexandria Building, and initiated experiments in June 2013 to continue these studies. Given an estimated carry forward amount of $382k as of 10/31/2013, we requested an extension period of one year to complete the project. These studies were completed in 2014, as summarized below.

PRODUCTS

Publications During Funded Period 2006-2014

1. Andarawewa, K.L., S.V. Costes, I. Fernandez-Garcia, W.S. Chou, S.A. Ravani, H. Park, and M.H. Barcellos-Hoff. 2011. Radiation Dose and Quality Dependence of Epithelial to Mesenchymal Transition (EMT) Mediated by Transforming Growth Factor β. *Int J Rad Onc Biol Phys*. 79:1523-1531.
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National and International Presentations from 2007-2014

1. *First International Workshop on Systems Radiation Biology,* Munich, Germany, February, 2007
2. *Workshop on Radiation and Multidrug Resistance Mediated via the Tumor Microenvironment*, Dresden, Germany, February 2007
3. *European Community Low Dose Risk Research*, Brussels, Belgium, June, 2007
4. International Congress of Radiation Research, Plenary, San Francisco, CA, July, 2007
5. American Society of Therapeutic Radiation Oncology, *Translational Research Symposium,* San Francisco, CA, September, 2007
6. Society of Toxicology, Seattle, Washington, March, 2008
7. National Council of Radiation Protection Annual Meeting, Washington, DC, April, 2008
8. 4th Pacific Rim Breast and Prostate Cancer Meeting, Whistler, Canada, August, 2008
9. Society of Medical Biochemistry and Molecular Biology, Seoul, Korea, October, 2008
10. International Workshop *Radiation and Multidrug Resistance Mediated via the Tumor Microenvironment*, Dresden, February, 2009
11. Keystone Research Conference, *Extrinsic Control of Carcinogenesis*, Vancouver, Canada, March, 2009
12. The Netherlands Radiation Research Society, Jubilee Meeting, Amsterdam, The Netherlands, April, 2009
13. American Association for Cancer Research, *Meet the Expert*, Denver, CO, April 2009
14. Gordon Research Conference, *Mammary Biology*, Newport, RI, June 2009
15. *Heavy Ions in Therapy and Space Symposium*, Cologne, Germany, July 2009
16. American Society for Therapeutic Radiology and Oncology (ASTRO) *Translational Research Symposium,* St. Louis, MO, September, 2009
17. 15th International Symposium on Microdosimetry: MICROS, Verona, Italy, October 2009
18. American Society for Therapeutic Radiology and Oncology (ASTRO), November, 2009
19. American Association for Cancer Research (AACR), *Tumor Microenvironment Working Group Panel*, April, 2010
20. *Biological Consequences and Health Risks of Low-Level Exposure to Ionizing Radiation* Workshop, Richland, WA April, 2010
21. 4th International Systems Radiation Biology Workshop, New York, NY, May, 2010
22. *Cancer and Complexity*, Symposium in Honor of Mina J. Bissell, Berkeley, CA, May 2010
23. Multidisciplinary European Low Dose Initiative (MELODI) 2nd Annual Meeting, Paris, France, October, 2010
24. American Society for Therapeutic Radiology and Oncology (ASTRO), Symposium, 52nd Annual Meeting, San Diego, CA, October, 2010
25. National Institute of Radiological Sciences, IAEA Symposia, Chiba, Japan, November, 2011

26. 9th Annual Beebe Symposium, National Academy of Sciences, Washington, DC, December 2010
27. American Association of Cancer Research, Educational Symposium, Orlando, FL, April 2011
28. Retirement Symposium in Honor of A. Begg and B. Van der Kogel, Njimengegen, The Netherlands, April 2011
29. Low Dose Radiation Workshop, Office of Biological and Environmental Research, Washington, DC, May, 2011
30. NASA Review, National Academy of Sciences, Washington, DC, June, 2011
31. International Congress of Radiation Research, Congress Lecture and Symposium Chair and Lecture, Warsaw, Poland, August, 2011
32. NASA Investigator's Meeting, League City, Texas, October 2011
33. American Association of Cancer Research, Major Symposium, DNA Damage and Repair, Chicago, IL, April 2012
34. Organization for Oncology and Translation Research, Kyoto, Japan, April, 2012
35. European Society for Radiotherapy and Oncology, Barcelona, Spain, May, 2012
36. 13th International Congress of the International Radiation Protection Association, Glasgow, Scotland, May 2012
37. 5th Annual Workshop in Systems Radiation Biology, Oxford, England, September, 2012
38. European Association for Cancer Research Tumor Microenvironment, Dublin Ireland, September, 2012
39. Gliwice Scientific Meeting, Gliwice, Poland, November 2012
40. 25th International Cancer Research Symposium, Tokyo, Japan, December 2012
41. Hiroshima University Radiation Program Symposia, Hiroshima, Japan, February, 2013
42. New York City *Tumor Microenvironment* Workshop, New York, NY, March 2013
43. *Heavy Ion in Therapy and Space Symposium*, Chiba, Japan, May 5, 2013
44. FASEB Conference on *TGF□ Superfamily: Signaling in Development and Disease*, Steamboat Springs, CO, August 29, 2013
45. European Radiation Research Society, Dublin, Ireland, September 6, 2013
46. Radiation Research Society Annual Meeting, New Orleans, September 16, 2013
47. ASTRO, American Society of Therapeutic Radiation Oncology, Atlanta, GA, September 20, 2013
48. ECCO, Amsterdam, The Netherlands, September 24, 2013
49. Institute for Environmental Sciences, Tokyo, Japan, October 21, 2013
50. American Society for Gravitational and Space Research, Orlando Florida, November 4-6, 2013
51. AACR Special Symposium Cellular Heterogeneity in the Tumor Microenvironment, San Diego, CA Feb 26-March 1, 2014

52. 6th International Workshop in Systems Radiation Biology, Chiba, Japan, March 5-7, 2014
53. American Association of Cancer Research Annual Meeting, Major Symposium, Complexity in the Tumor Microenvironment, San Diego, CA April 5-8, 2014
54. Japanese Society of Clinical Oncology, Yokohama, Japan, August 28-30, 2014
55. 60th Annual Meeting of the Radiation Research Society, Las Vegas, NV, September 21-24, 2014

Training Under the Auspices of this Award

Postdoctoral Fellows

1. Sangeetha Vijayakumar, Ph.D., 2010-2011, *Current position*, science writer, New York
2. Christina Medina-Ramirez, Ph.D., 2011-2012, *Current position*, Educational outreach coordinator, Hunter's College, NY, NY
3. Irineu Illa Bochaca, Ph.D., 2006-2008, 2009-2012, *Current position*, Research scientist, VA, Dept of Endocrinology
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Graduate Students

1. Estafania Zapata-Rodriguez, Master's research, Cell and Molecular Biology, Sackler Graduate School, New York University, 2013-2014 Awarded M.S., 2014
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3. Haydeliz Martinez-Ruiz, Doctoral research, Cell and Molecular Biology, Sackler Graduate School, New York University, 2010-2014. Awarded Ph.D., 2014
4. Michael Gonzalez, Doctoral research, Pathobiology, Sackler Graduate School, New York University, 2011-2012
5. Allen Sun, Master's program in Cell Biology, New York University, 2010-2011. Awarded, 2011
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EXPERIMENTAL RESULTS

AIM 1. Evaluate *in vitro* the effect of low dose rate and fractionated radiation exposure in combination with TGFβ on the phenotype and genomic instability of non-malignant human epithelial cells. *We will determine the effects of low dose rate and dose fractionation in regards to susceptibility to EMT and centrosome integrity in these models. In addition, we will examine whether epithelial cells from other tissues are similarly affected. High throughput microscopy and image analysis will be used to quantify differences in the irradiated cell phenotype.*

MCF10A non-malignant human mammary epithelial cell line, which can be primed by radiation to undergo TGFβ mediated EMT (Andarawewa et al., 2011; Andarawewa et al., 2007). The human breast epithelium consists of luminal, cytokeratin 18 (CK18) epithelial cells and basal, cytokeratin 14 (CK14) epithelial cells. A rare population in which basal and luminal cytokeratins co-localize is thought to represent a pluripotent progenitor cell (Villadsen et al., 2007). MCF10A cell cultures consist of distinct CK18 and CK14 expressing cells, and reportedly contain a small population of cells expressing stem cell markers (Fillmore and Kuperwasser, 2008). We found that MCF10A exhibit a remarkable capacity to generate histiotypic ductal outgrowths in a humanized mammary gland (Figure 1A-D). Ducts formed by MCF10A consist of luminal CK18 and basal CK14 bi-layer (Figure 1B), which were confirmed as human by *in situ* hybridization for COT1 DNA (Figure 1C,D). Both single and double positive CK18 and CK14 cells are evident in MCF10A cultures (Figure 1E). Thus, we used CK18 and CK14 cytokeratin expression in MCF10A as a model to further study radiation effects on epithelial lineage commitment.

EMT, elicited by overexpression of transcription factors or exposure to TGFβ, is strongly associated with acquisition of stem cell markers and function (Mani et al., 2008). Here we tested whether this was accompanied by an increase of the CK14/18 surrogate progenitor population. The distribution of CK14, CK18 and CK14/18 positive cells in cultures arising from irradiated cells was not significantly different from sham-irradiated populations (Figure 1F). Significantly more CK14/18 cells and less CK18 cells were present in cultures exposed to TGFβ compared to control cultures or irradiated cultures. Nearly a third of the cells in irradiated cultures treated with TGFβ were CK14/18 cells, which was significantly higher than in single treatment or control cultures ($p<0.001$, Figure 1F). The dose response for EMT following either densely or sparsely ionizing radation is switch-like, i.e. doses as low as 3 cGy were sufficient and higher doses were quantitatively similar (Andarawewa et al., 2011). Thus we examined the frequency of CK14/18 cells following graded doses of either sparsely or densely (350 MeV/amu Si) ionizing radiation. The frequency of CK14/18 cells exhibited a very similar dose response (Figure 1G), which was substantiated by non-significant Pearson and linear regression coefficients. The correlation between EMT and CK14/18 cells seemed to support a dedifferentiation mechanism.

We reasoned that a dedifferentiation mechanism would mean that CK14/18 cells are the progeny of lineage restricted, single keratin positive cells, while increased self-renewal would increase symmetric division of double positive cells to produce similar double-positive daughters. To test this, we analyzed the lineage characteristics as a function of time in culture. The percentage of CK14/18 dual-labeled cells was unexpectedly greatest 24 hr after plating in control and irradiated cultures and gradually decreased over time while CK14 cells increased (Figure 3). TGFβ treated cultures began with and maintained more double-positive cells than

those cultured without TGFβ. As above, irradiated, TGFβ treated cultures maintained the highest percentage of CK14/18 positive cells.

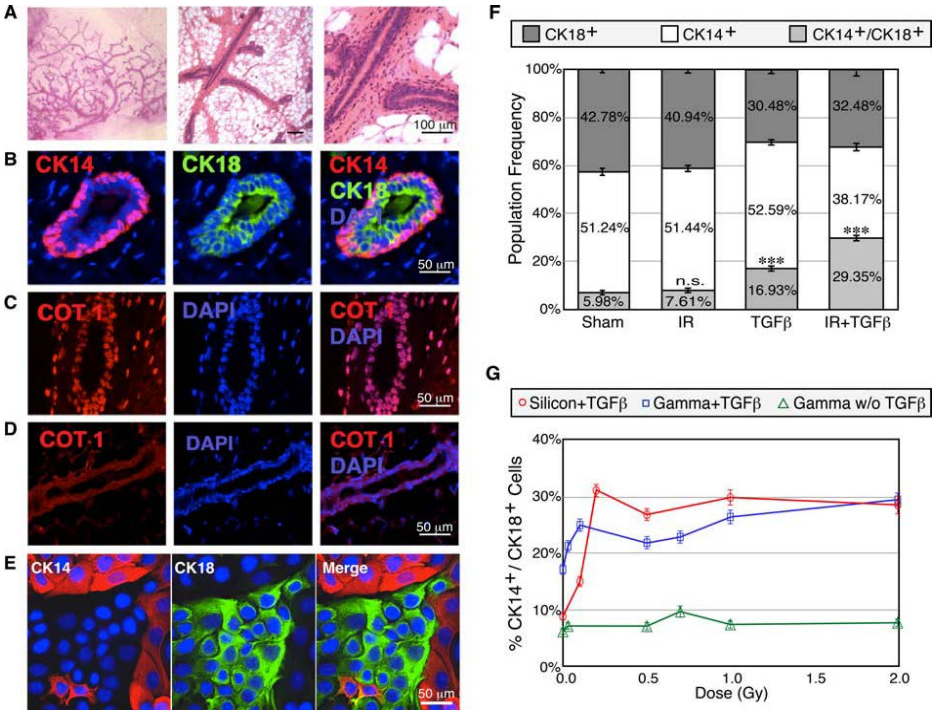


Figure 1. Lineage marker status of MCF10A treated with radiation and/or TGFβ. MCF10A outgrowths in humanized mouse fat pad consist of morphologically bi-layered epithelium. (A) Whole mounts (left) and histology (middle, 10X; right, 40X). (B) CK14 (red) and CK18 (green) dual stain. (C) Human specific DNA, COT1, in situ hybridization of humanized fat pad. Note that both fibroblasts and epithelium are human. (D) Negative control for COT1 in situ hybridization in mouse mammary gland. (E) Dual immunostaining of basal CK14 (red, left panel) and luminal CK18 (green, middle panel) in MCF10A cells. Nuclei are counterstained with DAPI (blue). Rightmost panel is merged to show the presence of double CK14/18 positive cells. Scale bar represents 50 µm. Cells in which both markers simultaneously co-localize appear yellow in the merged image. (F) Quantitation of basal CK14, luminal CK18 and double positive populations under four treatment conditions (sham, treatment with TGFβ and/or 200 cGy g-radiation). Frequencies are calculated as the total number of cells expressing each or both of the cytokeratin markers over the total number of cells in the population. Three biological replicates where randomly imaged for a total analysis of 6935 cells. The frequency of cells expressing both cytokeratins were significantly increased ($p<0.001$, Chi square) after treatment with TGFβ or radiation and TGFβ compared to the sham irradiated controls. ***$p<0.001$ (G) Quantitation (mean±S.D.) of MCF10A cells positive for both basal CK14 and luminal CK18 cultured with TGFβ as a function of graded doses of g-radiation (blue) or Si particle radiation (red) compared to g-irradiated cells cultured without TGFβ.

To further test whether TGFβ is a necessary signal for self-renewal *in vivo*, *Tgfb1* heterozygote and wildtype littermates were irradiated with 10 cGy at 4 weeks of age. Although null genotypes are embryonic or perinatal lethal, *Tgfb1* heterozygote mammary gland has 70-90% less TGFβ than wildtype tissue (Ewan et al., 2002). As previously reported (Nguyen et al., 2011), mammary repopulation frequency doubled in the mammary gland of irradiated wildtype mice. In contrast, the mammary repopulating activity of irradiated *Tgfb1* heterozygote mice was similar to unirradiated mice (data not shown). These data that provide functional validation of radiation-induced stem/progenitor expansion (Tang et al., 2013).

Radiation-induced Notch and TGFβ promote self-renewal

Stem cells down regulate Let-7 miRNA(Yu et al., 2007). Ibarra et al. constructed a reporter in which Let-7c miRNA silences Ds-Red expression in differentiated cells. Therefore, the lack of Let-7c in stem/progenitor cells leads to Ds-Red cells (Ibarra et al., 2007). MCF10A were stably transduced with the retrovirus Let7c-reporter (Figure 2A). As evident in dual phase-fluorescence micrographs, monolayer cultures contain few Ds-Red cells (Figure 2B) and mammospheres grown in non-adherent growth restricted conditions contained only 1 or 2 Ds-Red cells (Figure 2C). To test whether Ds-Red positive progenitor cells underwent self-renewal, we viably sorted the population using flow cytometry into Ds-Red positive and negative populations and cultured

them independently. The Ds-Red positive sorted population gave rise to cultures containing both Ds-Red positive cells and negative cells but the Ds-Red negative sorted population did not yield Ds-Red cells. The percentage of Ds-Red positive cells in cultures initiated from sorted Ds-Red cell increased from 1.7±0.08% to 3.7±0.18%, indicative of self-renewal.

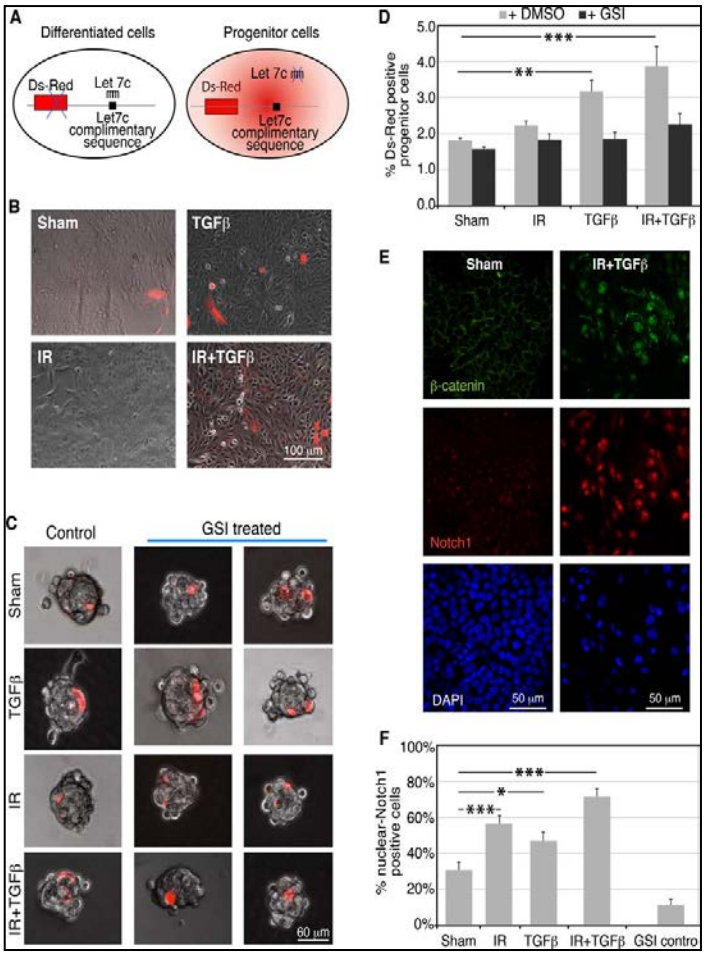


Figure 2. Radiation and TGFβ increase the frequency of cells expressing the Let-7 reporter in a Notch-dependent fashion. (A) Diagram of the assay in which Let-7c expression in differentiated cells represses Ds-Red reporter. Cells lacking Let-7c are red. (B) Phase/fluorescence merged images of MCF10A expressing the Let-7c reporter after radiation and/or TGFß treatment. Scale bar represents 100 μm. (C) Representative images of MCF10A mammospheres expressing the Let-7c reporter after radiation and/or TGFß treatment. (D) FACS analysis of Ds-Red cell frequency of cells from sham, after radiation and/or TGFß treatment (grey) compared to those treated with γ-secretase inhibitor (black). Cells were increased when cultures were treated with TGFβ or radiation and TGFβ compared to the sham controls (mean±S.E. from three biological replicates). Notch inhibition blocked this increase. Ds-Red positive sorted population continued to give rise to both Ds-Red positive cells and negative cells, whereas the Ds-Red negative cells did not yield Ds-Red progenitor cells under any conditions. (E) Representative images of Notch and β-catenin immunostaining illustrating the nuclear localization of Notch induced by 200 cGy γ-radiation and TGFβ. Scale bars represent 50 μm. (F) The frequency of cells that exhibit nuclear Notch staining is significantly increased by either single or double treatments. Cells treated for four hours with γ-secretase inhibitor (GSI) alone exhibit reduced Notch immunoreactivity, evidence of antibody specificity. *p<0.05; **p<0.01; ***p<0.001 Chi-square.

Radiation alone did not affect the frequency of Ds-Red cells as measured by flow cytometry (Figure 2D). In contrast, TGFβ treatment significantly increased the Ds-Red population ($p<0.01$) which was further enhanced by irradiation ($p<0.001$). When Ds-Red cells were sorted from irradiated cultures treated with TGFβ, 39.6±0.48% of the subsequent population was Ds-Red, consistent with increased self-renewal. In contrast, sorting Ds-Red negative cells from sham, TGFβ, radiation or irradiated, TGFβ cultures generated cultures without Ds-Red positive cells, which support the conclusion that dedifferentiation does not contribute to the increase in Ds-Red cells that we observed after exposure to radiation and TGFβ. We then modeled the self-renewal capacity of Ds-Red positive progenitor cells and found that, in accord with the results for the CK14/18 double positive population, the probability of symmetric divisions of these cells increased after radiation and TGFβ exposure (0.68 in sham cultures; 0.99 in radiation and TGFβ).

Stem cells, radiation and EMT can be linked through their common association with the Notch pathway(Bouras et al., 2008). Notch activation precedes increased mammary repopulating activity in irradiated tissue based on expression profiling, expression of Notch target genes, and protein localization (Nguyen et al., 2011). To test whether Notch activation could be elicited by radiation, we localized nuclear Notch in MCF10A cells (Figure 2E). Interestingly, either single

or double treatments induced nuclear Notch (Figure 2F) even though only double treatments induce EMT or CK14/18 dual labeled cells. Given the efficacy of radiation to increase Notch but not CK14/18 double positive cells or Let-7c reporter cells, we next tested whether this was functionally required by using a γ-secretase inhibitor to prevent Notch activation (Figure 2F). Notch inhibition blocked the increase in the frequency of DsRed cells in TGFβ treated cultures (Figure 2D), which indicates that concurrent Notch and TGFβ induced by radiation cooperate to stimulate self-renewal.

TGFβ Regulates the Mammary Epithelial Hierarchy via BRCA1

Delineation of the mammary epithelial hierarchy is essential for understanding the complex development and secretory maturation of the breast. In addition, the cell of origin hypothesis posits that long-lived mammary stem cell (MaSC) and progenitors underlie breast tumor heterogeneity. It is therefore important to understand the consequences of stem and progenitor cell misregulation during development and pathogenesis.

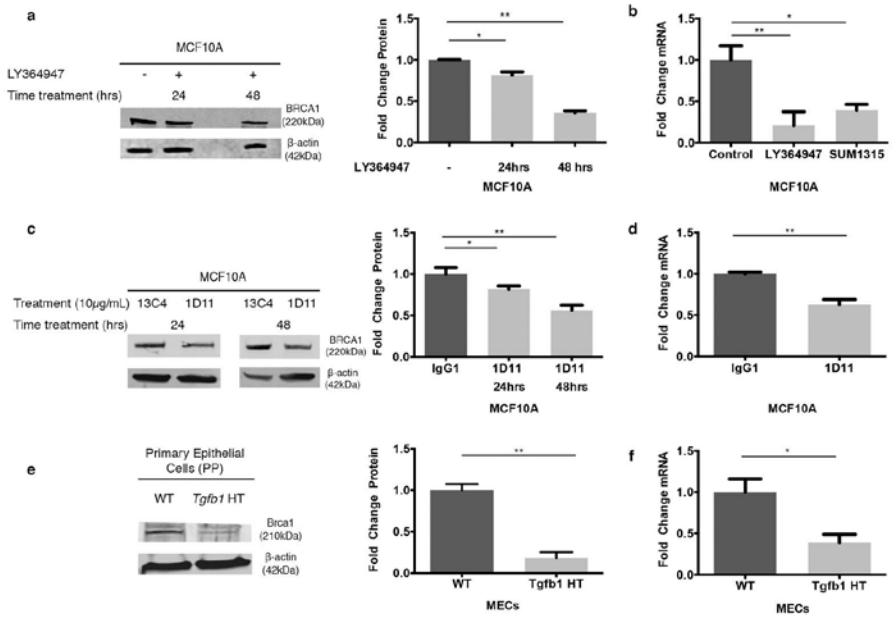


Figure 3. TGFβ1 regulates BRCA1 protein and mRNA expression. (**a,b**) MCF10A cells treated with a small molecule LY364947. (**a**) Western Blot showing Brca1 protein levels and quantification. TGFβ inhibition for 24 hours results in a decrease of BRCA1 protein (**a**) and mRNA (**b**) and 48 hours inhibition resulted in a greater response. (**c,d**) MCF10A treated with 1D11 TGFβ neutralizing antibody showed a significant decrease of BRCA1 protein (**c**) and mRNA (**d**) when treating for 24 hours and decreased further when treated for 48 hours. (**e**) *Tgfb1* HT

We investigated the contribution of TGFβ to regulation of the murine mammary epithelial hierarchy. These studies reveal that homeostatic TGFβ suppresses MaSC and controls mammary hierarchy via regulation of the tumor suppressor gene, *BRCA1* (breast cancer early-onset 1). *BRCA1* was the first genetic locus associated with familial breast cancer risk (Bowcock et al., 1993). Women with germline *BRCA1* mutations have an extremely high risk of developing breast cancer, specifically carcinomas negative for estrogen receptor, progesterone receptor and HER2 amplification, so called triple-negative breast cancer, that generally have a very poor prognosis. The high risk of breast cancer in *BRCA1* mutation carriers is attributed to a constellation of cellular defects in DNA damage repair(Deng, 2006; Konishi et al., 2011), centrosome replication (Deng, 2002), and lineage commitment (Lim et al., 2009; Liu et al., 2008; Proia et al., 2011). While much of current research focuses on identifying discrete cell populations, the function of the tissue depends on the appropriate composition as a result of lineage commitment decisions. Here we show that TGFβ depletion affects MaSC self-renewal and lineage commitment by regulating BRCA1 via transcriptional control and mRNA stability mediated by miR-182, which provides both a novel and detailed mechanism by which TGFβ

coordinates epithelial hierarchy to maintain homeostasis.

Prior studies have shown that BRCA1 knockdown in primary breast epithelial cells appears to increase stem/undifferentiated cells and decrease the proportion of cells expressing luminal epithelial markers and ER (Liu et al., 2008). The breast tissue of *BRCA1* mutation carriers contains a disproportionate increase in luminal progenitor cells (Lim et al., 2009) and exhibits defects in progenitor cell lineage commitment before cancer onset (Proia et al., 2011). Because we found that that BRCA1 protein levels decrease in human epithelial cells treated with a TGFβ small molecule inhibitor of the type I receptor kinase (Maxwell et al., 2008), we considered whether TGFβ depletion affect MaSC and mammary lineage commitment by affecting BRCA1 levels.

We initially confirmed and expanded the analysis of BRCA1 in the non-tumorigenic human mammary epithelial cell line, MCF10A, treated with a small molecule TGFβ type I receptor kinase inhibitor, LY346947. Inhibition of the TGFβ signaling for 24 hours resulted in a significant decrease of both BRCA1 protein and mRNA levels (Figure 3a,b). The response was greater after 48 hours of inhibition. The decrease in mRNA levels of *BRCA1* was comparable to BRCA1 levels in SUM1315, a human *BRCA1* haploinsufficient breast cancer cell line (Figure 3b). BRCA1 expression is tightly regulated during cell cycle progression in many cell types and its expression is increased during S-phase (Ruffner and Verma, 1997; Vaughn et al., 1996). Inhibition of TGFβ signaling in MCF10A increases proliferation and thus BRCA1 would be expected to increase, rather than decrease as observed. We confirmed this in cell cycle synchronized MCF10A; BRCA1 levels were still decreased after TGFβ inhibition, supporting the conclusion that TGFβ regulation of BRCA1 expression was not dependent on cell cycle.

Small molecules often affect similar kinases, thus to further test whether BRCA1 regulation was due to TGFβ canonical pathway activation, MCF10A were treated with 1D11 TGFβ neutralizing antibodies. BRCA1 expression significantly decreased with 24-48 exposure to TGFβ neutralizing antibody (Figure 3 c,d). Consistent with these results, Brca1 protein and mRNA were significantly decreased in *Tgfb1* HT MEC compared to WT MEC (Figure 3 e,f). Furthermore, microarray data analysis from *Tgfb1* HT mammary glands showed that *Brca1* expression was significantly decreased compared to WT glands.

Demonstration that Stroma Mediates Carcinogenesis

Ionizing radiation is an unequivocal human carcinogen. The prevailing paradigm in radiation carcinogenesis is that radiation exposure increases mutations from misrepaired DNA damage. Certain epidemiological studies suggest that additional mechanisms may contribute to cancer risk in certain tissues. While exposure during childhood confers a 300% increase in breast cancer relative risk, exposure *in utero* does not increase breast cancer risk, and radiotherapy for breast cancer after the age of 40 increases relative risk by only 20%. The cumulative incidence of breast cancer for women who were treated with radiation for childhood cancer is 13–20% by 40–45 years of age, similar to the 10–19% by age 40 in women with a *BRCA* gene mutation, and substantially higher than the 1% cumulative incidence of general population by age 45. Radiation-preceded human cancer is genomically characterized as aggressive (Broeks et al., 2010). Moreover, early radiation exposure also affects the cancer molecular subtype. Women treated with radiation as children are significantly more likely than age-matched controls to develop “triple-negative” breast cancer (TNBC), which is negative for estrogen receptor (ER) and progesterone receptor (PR) and lack HER2 amplification (Castiglioni et al., 2007; Horst et al., 2014). Both the age dependence and change in tumor types are poorly understood. Notably, we replicated the effect of radiation on tumor type in a murine model of mammary carcinogenesis. Our experiments using a novel radiation-genetic mammary chimera show that radiation exposure during puberty acts via the microenvironment

to increase murine TNBC (Nguyen et al., 2011; Tang et al., 2013). The resulting tumors are biologically and genomically diverse in a manner very like that of human breast cancer (Herschkowitz et al., 2011; Nguyen et al., 2013).

Bioinformatic analysis identified two processes, stem cell and chronic inflammation, associated with radiation and TNBC frequency. The signature of tumors arising in irradiated mice murine is strongly associated with both radiation-preceded human tumors and sporadic TNBC (Nguyen et al., 2013). Stem cell self-renewal during puberty expands the pool of cells most susceptible to transformation, while inflammation creates the critical context to promote malignant progression. These studies have led us to propose that extrinsic, rather than cell intrinsic, processes underlie the window-of-susceptibility to radiation carcinogenesis.

Host age at Trp53 null transplantation affects tumor biology. We have conducted preliminary experiments to determine whether the age of the host at transplantation affects *Trp53* null tumorigenesis. The mammary glands of 3 week old recipient mice were cleared of endogenous epithelial and transplanted with 10-week old *Trp53* null epithelium when the recipients reached 5, 10 or 70 weeks of age (juvenile, adult and mature). The time to first tumor (~275 days) was similar as a function of age at transplantation (data not shown) but the type of tumors arising from transplantation into juvenile versus adult vs mature mice are biologically distinct. For example, tumors arising over the course of a year from *Trp53* null outgrowths in adult hosts (AH) (n=26) were compared to those arising from outgrowths initiated in juvenile hosts (JH) (n=55). Once detected, JH tumors grew significantly faster, contained larger blood vessels (p=0.015) and had a higher mitotic index (p=0.009) compared to AH tumors (**Figure 4**). Tumors transplanted at either age were predominantly ER+ (80% JH vs. 60% AH) as measured by the Allred score but many more ER immunoreactive tumor cells were present in JH tumors compared to AH tumors (p=0.0001). Microarray analysis of 15 JH and 12 AH tumors identified 273 genes that were differentially modulated by at least 1.5-fold (juvenile vs adult, JvA). Ingenuity Pathway Analysis of 243 JvA gene profile reflected embryonic development, organ development, and organ morphology (IPA score 43); and cellular growth & proliferation, cancer, and tumor morphology (IPA score 38). Consistent with the high ER protein expression, there

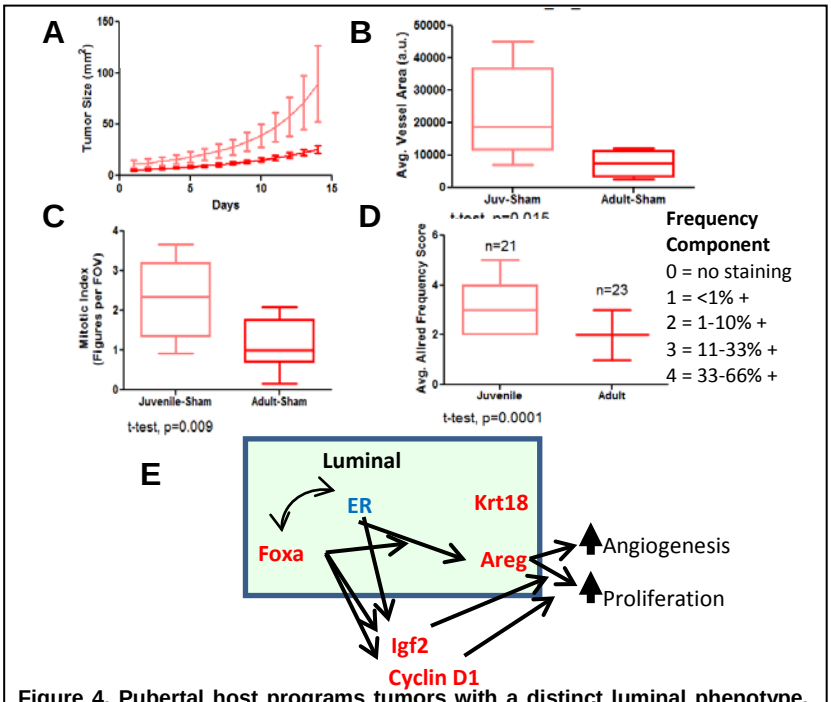


Figure 4. Pubertal host programs tumors with a distinct luminal phenotype. (A) Transplants into juvenile-hosts formed tumors one year later that grew much faster than those from adult-hosts (Pink, juvenile-host; red, adult-host). (B) Juvenile-host tumors exhibited a 2-fold higher mitotic index. Mitotic figures in H&E-stained tumor sections were scored as proliferative index in 10 independent fields of view (FOV) per tumor. (C) Juvenile-host tumors exhibited 2-fold larger perfused blood vessels. Auto-fluorescent red blood cell clusters in H&E-stained tumor sections were quantified as a perfusion index. (**D**) The Allred Score was used determine ER-status of tumors. 80% of juvenile-host tumors were ER+, compared to adult-host tumors of which 60% were positive. Among ER+ tumors, ones from juvenile-hosts are more highly positive than ones from adult-hosts (pink, juvenile-host; red, adult-host). Legend shows the frequency component of Allred Score. (**E**) Lumina tumorl transcriptional program within the 243 JvsA gene profile.

is a clear luminal gene expression program induced in JH tumors exemplified by *Foxa1, Areg*, and *Krt18*. Up regulation of *Igf2* and *Ccnd1* (Cyclin D1) transcripts is consistent with the increased mitotic index in JH tumors. *Areg* is also associated with pro-angiogenic properties, which is consistent with the increased perfusion observed in JH tumors. The distinct biology of tumors arising from *Trp53* null tissue undergoing morphogenesis during puberty suggests that tumor intrinsic subtype is strongly influenced by physiological status, which supports our overarching hypothesis that TGFβ mediated inflammation and stem cell deregulation are primary mediators of the high risk of TNBC in women treated with radiation for childhood cancer.

The research we have conducted is highly relevant to assessing the accuracy of the over-arching cancer paradigm that is the basis for predicting human cancer risk. First, ionizing radiation is one of very few environmental exposures strongly associated with breast cancer risk (Boice Jr., 2001). Thus our experimental studies concern a very real risk factor for humans, in which we have mechanistically separated radiation effects that promote cancer by genetic damage from those acting via the microenvironment. Second, preventing radiation associated cancer is an unmet need in children and young people irradiated for cancer treatment. Our data strongly support the notion that radiation acts through the stroma to increase breast cancer risk, which provides a new prevention target (Barcellos-Hoff, 2013b). Third, our published bioinformatics analyses strongly support the utility of our mouse model as a means to derive significant insight into the mechanisms of radiation-preceded human cancer (Barcellos-Hoff, 2013a; Nguyen et al., 2013).

AIM 2. To test the hypothesis that stromal-epithelial interactions suppress the irradiated phenotype. *We will determine to what extent centrosome abnormalities are expressed in irradiated HMEC and whether they can undergo EMT when influenced by normal or irradiated stromal cells in vitro and in vivo. Two models will be used. First, fibroblasts will be incorporated in the three-dimensional rBM assay (Barcellos-Hoff et al., 1989; Petersen et al., 1992). The second model is the "humanized" mouse model, which allows HMEC to grow and undergo tissue-specific morphogenesis in the context of human fibroblasts (Kuperwasser et al., 2004). We will test TGFβ's mechanistic role in the process by engineering both stromal and epithelial cells used in these models to be resistant or sensitive. In addition, we will examine other extracellular signaling involving hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF).*

Multicellular organization and cell to cell junctions suppresses radiation-induced EMT

We have evaluated the capacity of IR to induce EMT in epithelial cells that have already established cell to cell junction at the time of the exposure. MCF10A cultures were irradiated at different degrees of confluence by exposing them to IR and TGFβ at several time points after plating. We observed that, as long as the cells became partially confluent and established cell to cell interactions, the radiation induced phenotype was prevented. In contrast, if confluent cells are re-plated after IR exposure and cultured in the presence of TGFβ, they undergo the characteristic EMT-associated disrupted morphogenesis as evidenced by the dramatically decreased expression of E-cadherin. Accordingly, in a scratch assay on confluent cultures, the cells that repopulate the scratch still retain the epithelial properties. However, if the culture is irradiated prior to the scratch and cultured in the presence of TGFβ, the cells that colonize the culture gap exhibit the loss of E-cadherin and cell morphology characteristic of the EMT.

We then evaluated the effects of radiation exposure in combination with TGFβ in epithelial cells co-cultured in the organotopic 3D model that resembles the acinar and ductal morphology of the mammary gland to test the hypothesis that the established cell junctions and the stromal-epithelial interactions (characterized in this model by the interactions between fibroblast and

MCF10As) can prevent the radiation induced EMT phenotype. When the MCF10A in established organotopic co-cultures together human fibroblasts over a surface coated with 1:1, collagen type I and Matrigel in 2% matrigel-media were irradiated with 2 Gy in the presence of TGFβ, they maintain acinar-like morphology and expression of the epithelial marker, E-cadherin.

Extracellular matrix interactions alter the epithelial phenotype and the response to IR

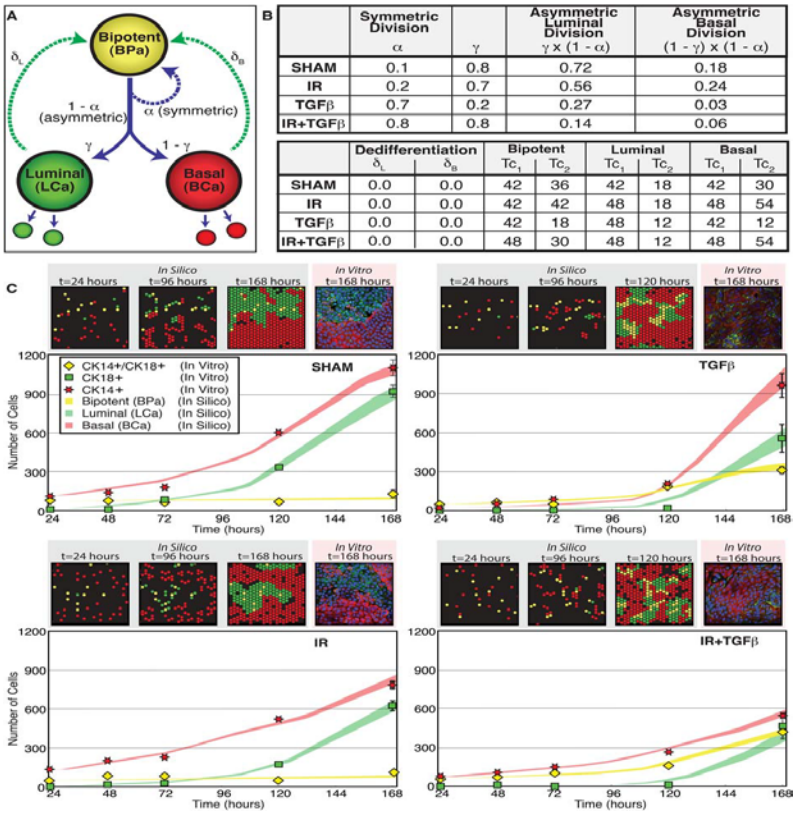
We next performed the experiments in the cells cultured in the presence of low concentrations of exogenous extracellular matrix (ECM), a situation in which those cells still grow in monolayer without generating 3D structures. Surprisingly, under those conditions, we found no reduction of E-cadherin expression in the samples treated with TGFβ and different doses of IR. Interestingly, even the introduction of the ECM in the growth media 24 h after IR was able to protect the cells from the phenotypic change. To examine epithelial lineages, we analyzed the expression of both cytokeratins in the cells cultured with low concentrations of ECM. First we observed that all those cells were positive for CK14 and negative for CK18. Then we evaluated the expression of the basal marker p63 in the same cells. Again, we found that all of the cells cultured with low concentrations of ECM were positive for p63. These results indicate that the epithelial interactions with the surrounding microenvironment play a pivotal role in the cell fate and in the cellular response to radiation.

We repeated the humanized chimera experiment (shown in Figure 1) by injecting MCF10A cells previously exposed to IR and treated with TGFβ to induce EMT. We generated MCF10A EMT cells one week prior to their injection into the humanized fat pads. Two months after the co-injection, we collected the mammary glands and analyzed the new outgrowths. Interestingly, the MCF10A EMT did not generate fully differentiated outgrowths as observed previously. The only histological structures observed were round shaped spheres near the point of injection resembling cysts. By immunofluorescence, this new generated structures did not express the epithelial markers E-cadherin, CK14, CK18 or p63 but instead, they were positive for the mesenchymal markers fibronectin, vimentin and α-smooth muscle actin. These results indicate that the radiation induced EMT phenotype of the MCF10A non malignant cell line, lacks the capacity to recapitulate the fully differentiated mammary gland epithelial ductal tree. Furthermore, it points at the process of radiation induced EMT as a mechanism of induced aberrant morphogenesis in vivo. These data provide functional validation of the requirement for radiation-induced TGFβ in vivo (Tang et al., 2013).

SPECIFIC AIM 3 DEVELOP A SYSTEMS BIOLOGY MODEL THAT INTEGRATES MULTICELLULAR RESPONSES TO RADIATION EXPOSURE ACROSS DIFFERENT LEVELS OF TISSUE ORGANIZATION AND TIME. *There have been few systematic studies in radiation biology of signaling and cellular crosstalk in radiation biology, which appear to lie at the heart of carcinogenesis. Therefore, we will develop an integrated model of radiation response based on the systems biology principles of network interconnectivity and spatial organization of cellular phenotypes within the higher order multicellular structure. Measurements from Aims 1 and 2 of HMEC responses to radiation in the in vitro model and humanized stroma model will be integrated into formal models that describes how dose-dependent interactions between cells and between tissue compartments affect genomic instability.*

Collaboration with Dr. Syvlain Costes and Jon Tang at Lawrence Berkeley National Laboratory under the auspices of NASA NSCOR program project developed a computational model of the processes we had described in vivo and in vitro. To test whether symmetric division of pre-existing progenitors could account for the population distribution over time, we formulated an ABM consisting of bipotent cell agents (BPa), representing CK14/18 positive cells, and defined by their ability to undergo symmetric self-renewal or asymmetric lineage commitment to either a basal (BCa) or luminal (LCa) cell agent type (Figure 5A). Monolayer culture lag and log phase

were simulated by assigning each agent with one cell cycle time for the first three simulated days and a second cell cycle time for the remainder of the week-long *in vitro* experiments. Contact inhibition was included such that a cell agent can divide only if there is neighboring space. Dedifferentiation of BCa or LCa to BPa was also included (Figure 5A). A parameter sweep determined that turning off dedifferentiation led to the best fit of the simulated treatment conditions (Figure 5B, 5C), suggesting that this mechanism is the least likely to explain the *in vitro* results. Moreover, the best-fit simulation results using dedifferentiation are noticeably worse than the fit using increased symmetric division. The fitted parameters indicated that the probability of symmetric self-renewal increased in TGFβ treated cells and the greatest difference was observed between controls and irradiated, TGFβ treated cells (Figure 5B). As the *in vivo* ABM predicted that self-renewal would only be effective during morphogenesis when there is considerable proliferation, MCF10A were grown to confluence (7 days) to reduce proliferation, then irradiated and cultured for additional 5 days with and without TGFβ. Under these conditions of low proliferation, neither radiation nor treatment with TGFβ affected the proportion of CK14/18 cells compared to control cultures ($p=0.323$). Together, ABM of *in vivo* and *in vitro* mammary cell fate decisions and experimental data support the hypothesis that radiation stimulates self-renewal, which requires both TGFβ and active proliferation.



	Symmetric Division α	γ	Asymmetric Luminal Division γ x (1 - α)	Asymmetric Basal Division (1 - γ) x (1 - α)
SHAM	0.1	0.8	0.72	0.18
IR	0.2	0.7	0.56	0.24
TGFβ	0.7	0.2	0.27	0.03
IR+TGFβ	0.8	0.8	0.14	0.06

	Dedifferentiation		Bipotent		Luminal		Basal	
	δ_L	δ_B	Tc_1	Tc_2	Tc_1	Tc_2	Tc_1	Tc_2
SHAM	0.0	0.0	42	36	42	18	42	30
IR	0.0	0.0	42	42	48	18	48	54
TGFβ	0.0	0.0	42	18	48	12	42	12
IR+TGFβ	0.0	0.0	48	30	48	12	48	54

Figure 5. Comparison of *in vitro* lineage distribution with *in silico* modeling after radiation and/or TGFβ exposure. (A) The *in silico* model containing bipotent progenitor agents (BPa), luminal (LCa) and basal (BCa) cell agents. (B) The best fit parameters for the model. The parameter α is the probability for BPa symmetric division. The parameter g dictates the probability for asymmetric luminal division. The parameters d_L and d_B determine the probability for dedifferentiation of LCa or BCa to BPa, respectively. Tc_1 and Tc_2 are cell agent cycle times for the first three days and the remaining days in culture, respectively. The best fit parameters indicate that both TGFβ-treatment alone and radiation with TGFβ-treatment increases self-renewal, but the greatest difference in the probability of symmetric self-renewal is observed between sham and cells that were irradiated and TGFβ-treated. (C) Experimental data points indicating the number of cells (mean±S.E.) counted for a set surface area randomly scanned (n=3). Red stars: CK14+ cells; green squares: CK18+ cells; yellow diamonds: double positive cells. Shaded areas indicate simulation results (red: BCa, green: LCa, yellow: BPa, mean±S.D., n=50) using the model with the best fit parameters yielding the lowest squared residual error for all three cell type growth kinetics and for all four experimental conditions. Inserts show example simulations at 24 hr, 96 hr and 168 hr time points (red: BCa, green: LCa, yellow: BPa) along with an in vitro image at 168 hr for each treatment (CK14: red, CK18: green, Nuclear counter stain: DAPI blue).

These data that provide functional validation of the requirement for radiation-induced TGFβ *in vivo* were published in collaboration with Dr. Sylvain Costes (LBNL) with partial support from NASA in 2013 (Tang et al., 2013).

Conceptual Modeling

I published a conceptual framework with two oncology collaborators, David Lyden of Cornell and Timothy Wang of Columbia in *Nature Cancer Reviews* that integrates the duality of malignant cell progression with the evolution of the tumor microenvironment. To quote from this perspective:

"The paradigm that cancer is a cellular disease defined only by events within the genome of cancer cells has given way in recent years to one in which cancer is viewed as a ecological disease involving a dynamic interplay between malignant and non-malignant cells. This shift redirects attention to the tumor microenvironment — signals, proteins and cells (such as immune cells and fibroblasts) present in the tumor mass that are necessary for tumor growth and progression. The contribution of the tumor microenvironment (TME) to malignant behaviors, treatment response and metastasis is an area of active research. The importance of the TME has been further underlined by the identification of the pre-metastatic niche(Kaplan et al., 2006). This concept is based on evidence from mouse models that established tumors release factors that can act on cells within distant organs to recruit bone marrow derived cells that create an environment conducive to the survival and proliferation of newly arrived metastatic cells(Peinado et al., 2011). But is the generation of a hospitable environment restricted to metastasis? In this Perspective, we propose a broader concept: that the construction of a 'cancer niche' is

a necessary and early step required for neoplastic cells to evolve towards a clinically relevant cancer. We are not suggesting that such niches pre-exist to facilitate cancer development and are somehow dormant or inactive in healthy individuals; rather, we propose that *de novo* cancer niche formation is the earliest stage at which non-malignant cells can be stimulated by the initiating carcinogenic insult and can support the survival of an initiated clone. In short, the development of a cancer niche is a prerequisite for tumorigenesis.

This Perspective uses the classical framework of initiation, promotion and progression to divide the stages of carcinogenesis (Barrett, 1993). We propose that the evolution of the cancer niche can be divided in three phases: construction, expansion and maturation (Figure 1). In brief, niche construction is a spontaneous interaction between activated stromal cells and normal cells that enables initiated (or transformed) clone survival; niche expansion, which could be viewed as a 'micro-microenvironment', generates secreted factors (such as chemokines, cytokines and exosomes) that remodel local tissue concurrent with initiated clone expansion and parallels promotion (that is the stage prior to tumor invasion). Recruitment of bone marrow-derived cells (BMDC) as well as resident cells, fibroblasts in particular, drives niche maturation from a nascent to an established TME, whose composition is currently under intense research. "

The schematic shown below (Figure 5), provides a systems biology modeling framework in which the cell and tissue are both represented for analyzing multiple scales of organization and time during carcinogenesis.

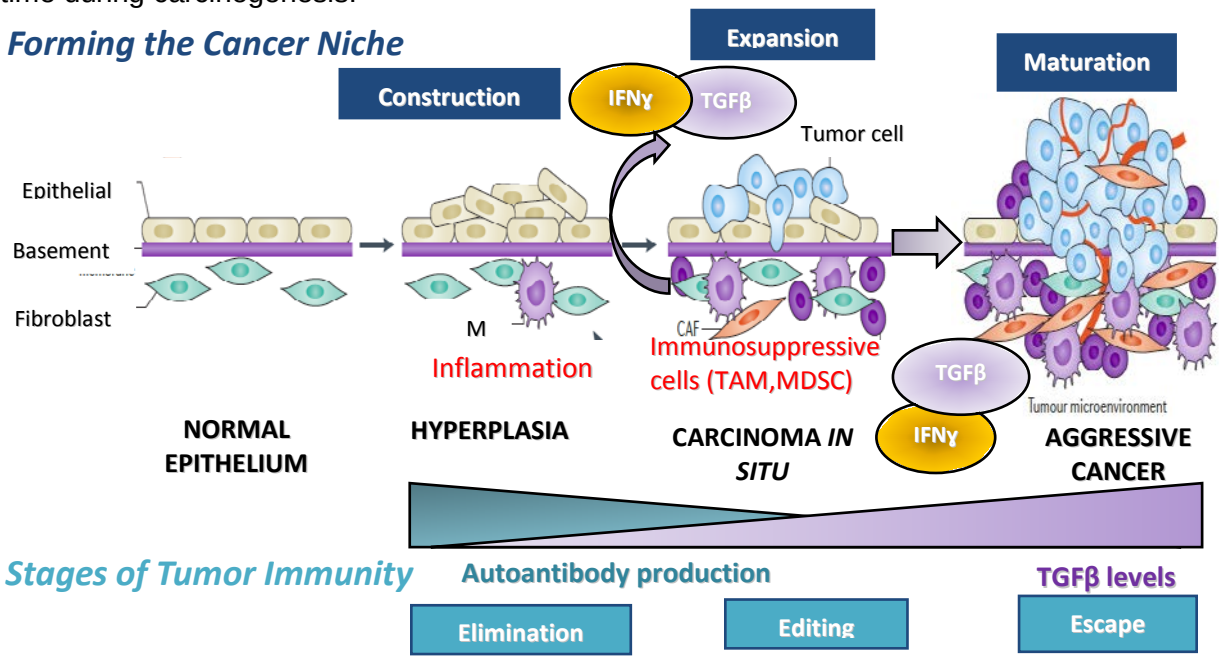


Figure 5. Requirement for coopting the tissue microenvironment to form a cancer niche while evolving to escape anti-tumor immunity. Adapted from Barcellos-Hoff et al. Nat Cancer Reviews, 2012.

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