

Review

# Integrative radiation carcinogenesis: interactions between cell and tissue responses to DNA damage

Mary Helen Barcellos-Hoff\*

*Department of Cancer Biology, Life Sciences Division, Lawrence Berkeley National Laboratory,  
1 Cyclotron Road, Berkeley, CA 94705, USA*

## Abstract

Tissue function requires coordinated multicellular behavior as a consequence of diverse signals integrated through the tissue microenvironment; importantly, these cell–cell and cell–microenvironment interactions also actively suppress cancer. Ionizing radiation (IR) elicits a well-defined cellular response to DNA damage that mediates the fate of the individual cell, concomitantly with a less well-characterized overarching tissue stress response that coordinates the response of multiple cell types via microenvironment signaling. We have now shown that these programs to reestablish homeostasis intersect via mutual regulation by transforming growth factor  $\beta 1$  (TGF $\beta 1$ ), which acts as an extracellular sensor and signal of stress. In this review, the concept that this type of functional integration of cell and tissue stress response programs is essential to cancer suppression will be discussed. Our experiments using IR, and several recent studies that experimentally manipulate stromal TGF $\beta$ , show that disruption of microenvironment signaling actively promotes malignant progression. Understanding the dynamic interactions between tissue and cell stress responses will be necessary for an accurate assessment of cancer risk and may also provide targets for prevention.

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*Keywords:* TGF $\beta$ ; Stromal–epithelial; Ionizing radiation; Carcinogenesis; DNA damage

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*Abbreviations:* TGF $\beta$ , transforming growth factor  $\beta$ ; IR, ionizing radiation; NMU, *n*-methylnitrosourea; rBM, reconstituted basement membrane

\* Tel.: +1 510 486 6371; fax: +1 510 486 6690.

*E-mail address:* mhbarcellos-hoff@lbl.gov.

## 1. Introduction

Multicellular dynamics between the target epithelium and cells in the stroma, vasculature, and immune and inflammatory system during carcinogenesis are critical determinants of whether neoplastic capacity is expressed, suppressed, or eliminated. The ability of neoplastic cells to subvert and recruit support from normal cells is as essential to their survival as innate programmatic disruption of signals that control growth and death [1]. Indeed, several investigators have argued that disruption of the cell interactions and tissue architecture can be a primary driver of carcinogenesis [2–6]. Even so, it is still not widely appreciated that the converse is equally true, i.e. normal tissues are very effective tumor suppressors (reviewed in [7,8]). This review will highlight studies that demonstrate how disruption of multicellular interactions, either by carcinogens or experimental manipulation, actively promotes the neoplastic process, and discuss the idea that a more complete understanding of this aspect of carcinogenesis can be used to intervene in the development of cancer.

To begin, our own studies using IR will illustrate some general features of how disruption of the microenvironment promotes carcinogenesis. In addition, recent publications by Kuperwasser et al. [9], Bhowmick et al. [10], and Maffini et al. [11] offer exciting new models and further evidence that microenvironment composition is a critical determinant of cancer suppression or promotion. These studies highlight the multicellular involvement in response to carcinogens and in cancer progression, and the need to frame a higher order view of cancer as an emergent phenomenon of tissues, rather than a property of its component cells. Lastly, this review will underscore tissue responses as a target for cancer prevention.

### 1.1. Radiation carcinogenesis

In many tissues of both humans and animals, exposure to high dose ionizing radiation represents a well-established carcinogen. Epidemiologic data demonstrates that increased risk of breast cancer in women exposed to as little as 1 Gy as a result of atomic bomb [12], therapeutic [13,14] or diagnostic [15] radiation exposures. Radiation has a well-defined physical basis for action and a statistical probability of total and specific chemical events. Radiation is generally thought to produce damage in individual exposed cells at the time of irradiation. However it has recently been recognized that non-irradiated cells respond to the presence of irradiated cells, the so-called bystander effect. Studies of carcinogenic potential of IR have frequently focused on initial DNA damage, which, if improperly repaired, can result in mutations or chromosome damage. This paradigm has been challenged by the recent recognition that cells surviving radiation can exhibit a persistent state of genomic instability [16]. Although DNA damage can cause cell death and eliminate potentially dangerous cells, misrepaired damage may result in a mutation that initiates the neoplastic cell. Consequently, DNA is commonly considered the major target of IR damage. Non-

mutagenic effects of IR however can have persistent effects that perturbs the multicellular system in a manner that clearly promotes, and may initiate, the neoplastic process.

We proposed that the ability of IR to induce changes in tissue microenvironment is a critical component of its carcinogenic potential that affects the frequency and features of neoplastic progression (reviewed in [3,8]), and thus have sought to characterize the irradiated microenvironment and determine how specific events contribute to carcinogenesis. These studies have shown that IR exposure results in a non-mutational changes in interactions with the microenvironment, stromal-epithelial, cell-cell and cell-extracellular matrix. From these and other studies in the literature, we have come to the conclusion the microenvironment and phenotype, as well as genome, are targets of IR effects that have significant and persistent ramifications in the organism. Studies described below suggest that radiation can elicit specific phenotypic alterations. Some aspects of the irradiated phenotype appear to result from intracellular signaling that culminates in a heritable phenotypic changes; others may be mediated by extracellular signaling from the irradiated microenvironment. In each model, we will discuss the role of transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) as a specific functional link between cell stress response to damage and the signaling mediated through the microenvironment.

We proposed that the cell biology of irradiated tissues is indicative of a tissue damage response program directed towards restoring tissue function in which individual cell responses are coordinated by extracellular signaling [17]. Tissue pathology and organ failure can arise from the lack of orchestrated communication between cells and among different cell types. We [18] and others [19] have argued radiation exposure ultimately compromises tissue integrity by altering the flow of information among cells. There are several general features of tissue response to ionizing radiation that support this concept summarized in Table 1.

We have used two models to ask whether radiation exposure elicits a distinct phenotype, and if such phenotypic changes can promote malignant progression. The first is the mouse mammary gland and the second is cultured HMEC. The basic biology of mammary gland is studied at many levels: gross morphology visualized in wholemount preparations, histology, molecular analysis of DNA, RNA and protein composition and abundance, and functional analysis

Table 1  
Tissue response to ionizing radiation

- 
- Microenvironment is a target of radiation
  - Tissue response to ionizing radiation is global yet innately tissue- and cell-type specific
  - Tissue responses, like cellular responses, are evident very rapidly
  - Some protein responses are secondary to others, indicative of a dynamic network
  - Tissue response can be detected after exposure to low whole body doses (0.1 Gy)
  - Radiation-induced cell phenotypes can be persistent and heritable
  - Microenvironment remodeling is radiation-quality dependent
-

that includes developmental states and responses to challenge (e.g., hormone stimulation, radiation response, chemical carcinogens). A comprehensive study of normal mammary biology integrates gross and cellular histology, functional phenotype, and informed genetic variation [20]. Inbred mouse strains with different susceptibility to mammary carcinogenesis are a platform for discovery of genetic determinants of cancer, while genetically engineered mice can be used for hypothesis testing, and generation when combined with genome-wide integration like expression microarray. HMEC on the other hand provide species-relevance and can be cultured in various configurations to determine how intrinsic cellular pathways are affected by extracellular signaling and the microenvironment [21].

### 1.2. Radiation-induced microenvironments

Ionizing radiation leads to global remodeling of the extracellular matrix and induces activity of potent modulators of cell phenotype [22–24]. Using immunofluorescence and digital microscopy we observed dynamic extracellular matrix changes in the peri-epithelial stroma, adipose stroma and epithelial basement membrane. The tissue compartment, time after irradiation and quality of radiation differentially affect extracellular matrix remodeling. In parallel, integrin extracellular matrix receptors are also differentially expressed (Tamou and Barcellos-Hoff, unpublished data). Radiation also alters the expression of endothelial and tumor cell integrins [25,26]. Recent studies have shown that cell adhesion molecules are fundamental pathways for cell signaling [27,28], and play an important role during neoplasia [29–31]. Since signaling from cell adhesion molecules also mediates cell–ECM and cell–cell adhesion, they effectively prevent cell migration and invasion into surrounding tissues. More recently, disruption of adhesion systems has been postulated to contribute a rate-limiting step to progression by modulating neoplastic processes by altering pathways that control genomic stability [32]. Mouse models in which ECM integrity is disrupted by transgenic manipulations also promote mammary tumorigenesis [33].

In response to damage, the flow of information both locally between cells and tissues, and distantly between organs is mediated in large part by cytokines [34]. An early and persistent event in irradiated tissues is the activation of the pluripotent cytokine, TGF $\beta$ . TGF $\beta$  is produced as a latent complex that is secreted and requires extracellular activation that permits TGF $\beta$  to bind to ubiquitous receptors [35]. This is evidenced in situ by the loss of the latent complex and unmasking of TGF $\beta$  [36]. Following radiation exposure, TGF $\beta$  activation is evident within an hour, persistent for at least a week, and detected following whole body doses of as little as 10 cGy [24]. Concomitant with, and indicative of its activation, the TGF $\beta$ -receptor phosphorylation target, Smad 2/3, is rapidly translocated to the nucleus upon irradiation in vivo [37]. We demonstrated its functional activity by showing that TGF $\beta$  mediates the induction of collagen III in the irradiated mam-

mary gland [24]. We discovered a novel mechanism of TGF $\beta$  activation occurs via exposure to reactive oxygen species that endows TGF $\beta$  with a redox sensor capability [38]. As such, TGF $\beta$  acts as an extracellular lynch pin released by radiation, and other oxidative stressors, to orchestrate multicellular response to damage [39].

Although by no means exhaustive, together, these studies have shown that the composition of irradiated microenvironment is a function of the tissue type, the dose, and the radiation quality [40,41]. Several questions arise: what is the functional significance of these events? Which are negative versus positive regulators of carcinogenesis? If the former, can they serve as targets in cancer chemoprevention? If the latter, can blocking their action/activity have therapeutic benefit for cancer intervention?

### 1.3. Interaction between tissue and cellular stress responses: p53 and TGF $\beta$ 1

The decision of a cell to undergo apoptosis or arrest in response to DNA damage is commonly attributed to the level of DNA damage and certain cellular competencies. The rapid induction of Smad 2/3 immunoreactivity that we observed in irradiated mammary tissue [37], and the observation that TGF $\beta$ 1 enhances the stress response following ultraviolet irradiation [42], suggests that there may be a direct interaction between the TGF $\beta$ 1 in tissues and cellular damage response. To test this idea, we irradiated *Tgfb1* knockout mice and discovered a surprising dependence of cellular responses to DNA damage on *Tgfb1* gene dosage [37]. Radiation-induced apoptosis is undetectable in *Tgfb1* +/- mammary gland and the apoptotic response in embryonic epithelial tissues is a function of *Tgfb1* status. Furthermore, radiation-induced cell cycle block was completely absent in *Tgfb1* null embryo skin and liver.

The mediators of cellular responses to DNA damage caused by radiation are very well characterized such that p53 is considered to be the major cellular sensor and signal of DNA damage. p53 is a classic tumor suppressor based on its major action as a transcription factor critical to determining cell fate decisions [43]. The p53 stress response pathway leads to two major cellular outcomes. Activation of p53 in damaged cells promotes apoptosis or induces cell cycle blockade. Apoptosis eliminates cells from the population that have sustained potentially carcinogenic DNA damage [44]. Cell cycle checkpoints at G1/S [43] or G2/M [45] cell cycle transition block provide time for cells to repair DNA. Since a cell's response to damage needs to be rapid, it is not surprising that the activation of the p53 stress response primarily involves post-translational changes in the p53 protein. Two major post-translational changes are: (1) a decrease in rate of p53 protein turnover and a consequent increase in the life time and total cellular content of the protein (e.g., protein stabilization), and (2) a myriad of p53 protein covalent modifications involving serine phosphorylations and de-phosphorylations.

Whereas intracellular mediators of p53 stability following radiation exposure have been the subject of intense study, little is known about the extracellular factors that affect the p53 response to ionizing radiation. We therefore examined p53 serine 18 phosphorylation using immunoblotting and immunofluorescence from irradiated *Tgfβ1* heterozygote and wildtype mammary gland [37]. Wildtype mammary epithelium showed massive induction of p53 phosphorylation, which was significantly reduced in irradiated *Tgfβ1* +/- mice and did not recover at later times. Likewise nuclear phospho-specific p53 immunofluorescence was also significantly reduced in irradiated *Tgfβ1* heterozygote compared to wild type mammary epithelium. Since chronic depletion in *Tgfβ1* +/- mice could perturb aspects of cell physiology that modify the p53 radiation response, we examined animals that had received pan-specific TGFβ1 neutralizing antibodies shortly before irradiation. Similar to the results seen in the *Tgfβ1* +/- mice, both immunoblotting of total tissue extracts and nuclear localization of phosphorylated p53 serine 18 determined by immunofluorescence staining were significantly reduced when TGFβ1 was transiently depleted prior to irradiation. Also, as seen in the knockout mice, TGFβ pan-specific neutralizing antibody treatment did not alter levels of total p53 indicating that TGFβ affected p53 post-translational modification rather than abundance. Recent experiments using primary mammary epithelial cell cultures demonstrate that this hypophosphorylation response is both epithelial cell autonomous and that the phosphorylation of p53 can be restored upon treatment with exogenous TGFβ (Jobling, Pajares and Barcellos-Hoff, unpublished data). Thus, it appears that TGFβ is necessary for the initiation of DNA damage responses in epithelial cells, which is both surprising and unprecedented.

One might postulate that sensors of damage have also evolved outside the cell that are capable of registering certain types of damage and producing a signal that recruit non-damaged cell to facilitate reestablishment of homeostasis. A number of striking similarities exist between p53 and TGFβ: both regulate complex cellular decisions regarding fate by mediating cell proliferation and apoptosis, both are induced by a variety of damage and specifically ionizing radiation, both exist in latent forms, both exhibit redox modulation of protein activity, both are very rapidly activated (within minutes of exposure), and both are translationally and transcriptionally controlled to moderate later events. Recent studies using transgenic knockout animals have also demonstrated that each protein is auto-regulating as evidenced by striking phenotypes of haploid genotype. In addition, their respective intracellular signaling pathways intersect such that p53 status affects responses to TGFβ. However, p53 is intracellular and mediates individual cell fate, while TGFβ is extracellular and orchestrates diverse multicellular fates.

Recent data from normal epithelial cells indicate that signaling events often attributed to p53 may be induced directly by TGFβ. Both GADD-45 and WAF/p21 are induced

by TGFβ treatment in primary p53 wildtype keratinocytes and in transformed cells that have non-functional p53 [46]. Furthermore, TGFβ activates c-Jun amino-terminal kinases within 5 min of exposure; this kinase pathway is involved in UV-mediated apoptosis and phosphorylation of c-Jun, all of which are part of the cellular stress response [42]. More importantly, p53 itself is increased during TGFβ1 induced apoptosis in rat liver epithelial cells [47]. Recent studies have demonstrated that p53 is involved in mammary gland involution as well [48]. Both p53 mRNA and protein were detected in the mammary epithelium within 48 h following weaning and resulted in an 8-fold increase in levels of WAF/p21 mRNA, which was absent in BALB/c-p53null mice [48]. Elevated TGFβ gene expression is an early event during mammary involution [49].

A variety of studies have linked p53 status and TGFβ responsiveness in cancer cells. Mutant p53 correlates with reduced TGFβ responsiveness in human bronchial epithelial cells [50], murine keratinocytes [51] and thyroid epithelial cells [52]. A few reports have concluded that they are independent [53], or that mutant p53 is not required for loss of TGFβ response [54,55]. However, in HaCaT cell line, which have mutant p53, TGFβ exposure induces p53 nuclear relocalization [46]. Of course, transformed and cancer cells have a very high incidence of p53 mutations and disrupted TGFβ signaling [56], therefore these data may be influenced by such perturbations. On the other hand, fibroblasts, which are growth-stimulated by TGFβ in the presence of a functional p53, convert to a growth inhibited TGFβ response when transfected with mutant p53 or when p53 is abrogated by SV40 [57].

Interestingly, there is mounting evidence that TGFβ itself may signal certain events through the generation of ROS [58–63]. TGFβ induces the production of hydrogen peroxide in bovine endothelial cells [64], mouse osteoblastic cells, where it has been shown to be necessary for the transcriptional activation of the *egr-1* gene [60], and human lung fibroblasts, where it is generated by the activation of NADH oxidase [61]. Recent studies have implicated ROS as an important signal for TGFβ induced apoptosis [62,63]. TGFβ may also be involved in the ROS mediated bystander effect following α-particle irradiation according to recent meeting reports [65,66].

Finally, the importance of a role for TGFβ in p53 response gains support from *Tgfβ1* gene knockout animals. Cultured keratinocytes from these animals were shown to have greatly increased levels of gene amplification, as evidenced by PALA-induced CAD resistance, an index of genomic instability [67]. These cells also lack the typical PALA-induced p53 dependent G1 arrest, but instability could be decreased by low levels of exogenous TGFβ without inducing G1 arrest. Notably PALA treatment also induced TGFβ activation in heterozygotes and wildtype cells. Other DNA damaging treatments such as *cis*-platinum [68] or alkylating agents [69] also induce TGFβ activity that contributes to therapeutic outcome. Such studies provide strong motivation



for testing the hypothesis that TGF $\beta$  modulates the type and degree of p53 responses in situ.

Thus, we propose extracellular signaling is an important determinant of the tissue and cellular response to DNA damage. The functional intersection of TGF $\beta$  and p53 supports the hypothesis that genomic stability can be significantly affected by the character and activity of the microenvironment. This conclusion gains support from recent studies from the Moses lab in which TGF $\beta$  signaling was genetically abrogated by floxing the Tgf $\beta$ RI receptor specifically in stromal cells [10]. These mice developed gastric and prostate tumors without further insult, which the authors propose is due to dysregulation of another growth factor, hepatocyte growth factor. These data support the view that signaling from the stroma is as critical to tumor control as are oncogenes to driving malignant cell behaviors. Like the stromelysin over-expressing mammary gland that spontaneously develops tumors [70], these mice are an exciting new model in which to test the evolution of epithelial genomic instability resulting from stromal disruption.

#### 1.4. Contributions of irradiated microenvironment to neoplastic progression

Our previous studies have shown that radiation alters the environment in which mammary epithelial cells reside. We believe that these radiation-induced changes in the mammary tissue can under certain circumstances contribute to the known action of radiation as a carcinogen. To test this hypothesis we created radiation chimeric tissue by transplanting unirradiated preneoplastic mammary cells to an irradiated mammary gland [71]. The female mammary gland is unique among all glands in that the epithelium develops postnatally from a rudiment that can be removed from the inguinal glands at approximately 3 weeks of age [72]. Surgical removal of the parenchyma results in a gland-free mammary fat pad, referred to as a cleared fat pad, suitable for receiving donor tissue at the time of clearing or later [72]. Transplantation of normal mammary epithelial cells produces ductal outgrowths that fill the fat pad and are nearly indistinguishable in whole-mounts or histologically from intact gland [72]. In addition, an occasional mouse mammary epithelial cell line, like the COMMA-D, retains the ability to proliferate in vivo [73]. COMMA-D cells are non-tumorigenic if injected into the cleared fat pads of 3-week-old mice or subcutaneously of immature or adult mice, or into nude mice. Although clonal in origin, COMMA-D cells exhibit morphological and phenotypic diversity in culture [74]. However, the cell line harbors two mutant p53 alleles that confer neoplastic potential [75].

We hypothesized that radiation effects on the tissue microenvironment is evidence of an additional, previously unrecognized action of carcinogens in general [71]. We found that the irradiated stroma dramatically promoted the ability of the cells to progress to tumors. COMMA-D tumor incidence was 2–4-fold greater when cells were injected into the cleared fat pads of irradiated hosts. Animals irradiated from 1 to 14

days prior to transplantation showed a significant increase in tumor incidence, ranging from a peak of 100% at 3 days and was still twice sham-levels at 14 days post-irradiation. Furthermore, the mean size of tumors from irradiated animals were nearly five times larger than the few tumors that arose in sham-irradiated hosts, indicating that tumor features, as well as frequency, were affected.

It is difficult to ascertain from these experiments whether the neoplastic population is preexisting in the cells injected or induced during outgrowth. We tested the former possibility by subculturing the two morphological variants that occur in this cell line. One is cuboidal and predominantly keratin-positive and the other is spindle-shaped and vimentin positive. Experiments using clonal isolation suggest that the former give rise to the latter [76]. Both formed tumors in irradiated hosts but the spindle, vimentin-positive cells had greater neoplastic potential in both sham and irradiated hosts, whereas the cuboidal, keratin-positive cells were less tumorigenic. These data suggested that the mixture of cells in the parent population was interacting in a way that suppressed the tumorigenic potential of the spindle clones and supported mammary ductal outgrowth, perhaps from the keratin-positive, cuboidal cells. Interestingly, neither cell type alone formed normal ductal outgrowths in the fat pad, suggesting that their contributions were interdependent in maintaining mammary “stemness”.

These studies indicate that the microenvironment created by the irradiated stroma can promote neoplastic progression in unirradiated epithelial cells, which is evidence that events “outside of the box”, in terms of a widely held paradigm in which mutations alone drive carcinogenesis, can significantly increase cancer risk. We attribute this adverse “by-stander effect” of irradiated cells on unirradiated cells to the fact that either the dose or total body irradiation corrupted the extracellular signaling from the microenvironment that suppresses abnormal cells. The effect of the irradiated microenvironment on neoplastic is persistent for several weeks and appears to be independent of systemic radiation effects (as tested by hemi-body irradiation), which support the hypothesis that non-mutagenic effects of radiation can contribute significantly to radiation carcinogenesis in vivo. Greenberger et al. have shown that irradiated bone marrow stroma actively contributes to leukemiogenesis [77]. The effect of radiation on the microenvironment affected the frequency of neoplastic progression, and it also affected the features (e.g. tumor size) of the resulting cancer. However, it is also important not to lose sight that the normal microenvironment was very effective in suppressing the tumorigenic behavior of these cells, which has been recently reviewed [78].

A recent study from the laboratory of Soto and Sonnenschein expands the critical role of stroma to chemical carcinogenesis in rats [11]. In this study, primary cultures of rat mammary epithelial cells were treated with *n*-methylnitrosourea (NMU). These cells retained the ability to form normal ductal outgrowths when placed in a non-treated cleared mammary fat pad. However when the same cells were placed in

NMU-treated hosts, tumors formed in almost all the out-growths. The authors conclude that the stroma itself is a 'target' of chemical carcinogenesis since untreated epithelial cells also form tumors in treated hosts. This interpretation may be confounded by the high frequency of spontaneous transformation by rodent cells during culture; nevertheless, the data clearly support the hypothesis generated by our radiation studies, i.e. that the stroma is a target of carcinogens, and such activity is distinct from those actions affecting genomic change and proliferation [71]. And, as observed in our studies of the mouse, this rat model also demonstrate that the normal stroma is extremely effective in suppressing tumorigenesis.

#### *1.4.1. Radiation exposure induces a heritable malignant HMEC phenotype*

To evaluate whether IR exposure also perturbs epithelial cell behavior, we asked whether irradiated HMEC undergo tissue-specific morphogenesis in a three-dimensional culture model in which cells are grown suspended within a reconstituted basement membrane (rBM). These three-dimensional colonies recapitulate acinar morphology typical of functional mammary gland, i.e. a hollow sphere consisting of highly polarized cells. Furthermore, three-dimensional morphogenesis in rBM readily distinguishes between the behaviors of tumorigenic and non-tumorigenic mammary epithelial cells, which are nearly indistinguishable when cultured as monolayers. While tumor cells remain proliferative and fail to establish appropriate cell–cell and cell–ECM connections [79], non-malignant mammary epithelial cells growth arrest and form acini similar to those found in situ [80]. Mammary acinar-like structures form upon establishment of epithelial polarity characterized by appropriately localized cell adhesion molecules, e.g. intercellular E-cadherin, basal-lateral  $\beta$ 1-integrin and basal  $\alpha$ 6-integrin [29].

Using this ability to organize into acini as a functional endpoint of cell–cell and cell–ECM interactions, we evaluated the response of HMEC to IR [81]. Single cells from the non-malignant cell line, HMT-3522, were irradiated with low doses (10–200 cGy) at the time of plating in the rBM assay. TGF $\beta$  was added to some cultures to mimic the presence of an irradiated stroma. The multicellular organization of colonies arising from irradiated, TGF $\beta$ -treated cells displayed pronounced disorganization in comparison to colonies from sham controls or following single treatments, which was quantified using confocal microscopy and analysis of the mathematical fit of an ellipse to the center of the segmented nuclei. Surprisingly, we also found that the number of cells per colony was significantly increased in double-treated specimens, suggesting that growth regulation was also altered. Since radiation causes apoptosis and TGF $\beta$  inhibits mammary epithelial proliferation, one concern is that the colonies surviving treatment were selected from previously existing heterogeneity within the population. To address this possibility we examined each treatment as a function of individual colonies. This analysis indicated that the dual-treated

colonies form a distinct population that is not present in the sham cultures.

The assembly of cells into tissue-specific structures requires the interaction of different cell adhesion systems. E-cadherin is a crucial epithelial adhesion molecule that links cells via an homophilic extracellular domain and is anchored intracellularly to the cytoskeleton via dynamic interactions with the catenins [82]. Low E-cadherin immunoreactivity in breast cancer is associated with poor prognosis [83], while restoration of E-cadherin reverts the invasive phenotype of cancer cells [84]. E-cadherin was localized using immunofluorescence, confocal microscopy and image analysis. Colonies from irradiated cells cultured in the presence of TGF $\beta$  showed a dramatic loss of E-cadherin immunoreactivity. E-cadherin protein levels were reduced compared to controls to similar levels by both IR and TGF $\beta$  but double-treatment resulted in no greater reduction. E-cadherin localization, and immunoreactivity, can be modified by the degree of association with cytoskeleton via the catenins. Preliminary data suggest that the loss of E-cadherin at the cell junctions in the dual treated colonies reflects a change in complex formation such that E-cadherin is not linked appropriately to the cytoskeleton (Erickson and Barcellos-Hoff, unpublished data). These observations suggest that low doses of radiation could dispose preneoplastic cells, which may already lack or have less E-cadherin [85,86], to further compromise this essential mediator of normal cell–cell adhesion. Likewise, the number of connexin-43 aggregates per colony was significantly decreased following radiation exposure, regardless of TGF $\beta$  exposure. Connexins are a family of proteins associated with gap junctions that modulate the transfer of molecules between cells. Breakdown of gap junctional complexes correlate with breast cancer metastatic potential [87].

Adhesion of cells to the ECM was evaluated by assessing the localization of several integrins, which are a class of ECM receptors. Integrins form heterodimers consisting of an  $\alpha$  and  $\beta$  subunit, to bind to ECM proteins.  $\beta$ 1-Integrin is critical for normal mammary gland development [88,89]. HMT-3522 colonies exhibit basolateral  $\beta$ 1 integrin but colonies arising from irradiated cells showed significantly increased  $\beta$ 1-integrin immunoreactivity that was distributed throughout the cytoplasm. TGF $\beta$  treatment did not affect  $\beta$ 1 integrin in the absence of prior irradiation. In contrast, the immunoreactivity of  $\alpha$ 6- and  $\alpha$ 3-integrin, which partner with  $\beta$ 4 integrin, decreased in colonies generated from irradiated cells or cultured in the presence of TGF $\beta$ . Since these integrins are dispensable for mammary alveolar morphogenesis [90], their loss may be a correlate rather than a driver of disrupted morphogenesis. A distinct collagen IV containing basement membrane was observed in all treatment groups, indicating that the changes in integrin expression was not due to the lack of appropriate ligand.

Together, these data demonstrate that colonies arising from irradiated cells exhibit a consistent phenotype consisting of inappropriate intercellular adhesion, deranged extracellular adhesion molecules, loss of gap junction proteins,

and disorganized tissue-specific organization. This phenotype is augmented by the presence of TGF $\beta$ , which itself is rapidly and persistently activated in irradiated tissue [23]. Since the phenotype is exhibited by the daughters of individually irradiated cells, radiation exposure appears to induce a heritable derangement of pathways affecting cell adhesion, ECM interactions, epithelial polarity and cell–cell communication.

The significance of the HMEC irradiated phenotype is suggested by a variety of studies showing that loss of microenvironment constraints has profound consequences on tumorigenesis, progression and metastasis. Experimentally induced loss of E-cadherin leads to an invasive phenotype while restitution of E-cadherin impedes malignant behavior [84,91]. Expression of constitutively active stromelysin that locally degrades the mammary epithelial basement membrane results in invasive tumors [70]. If disruption of the cell interactions can promote neoplastic behavior, then it is also possible to consider the potential therapeutic applications of whether restoration of appropriate extracellular signaling can control cancer. Studies from Bissell and colleagues have shown that treating tumorigenic HMECs with  $\beta$ 1 integrin function-blocking antibodies causes disorganized colonies to revert to organized acinar-like colonies [29]. This study suggests that, in terms of morphogenesis, appropriate signaling from the microenvironment can override signaling from an aberrant cancer cell genome. There is some evidence that restoration of microenvironmental controls may contribute to therapeutic responses. Bhatia et al. have suggested that the basis of interferon- $\gamma$  induced remission in chronic myelogenous leukemia is the induction of  $\beta$ 1 integrin in leukemic cells, which causes them to re-attach to the stroma [31]. It is promising that such mechanisms can be specifically exploited in the future.

An exciting new human mammary model recently developed by Kuperwasser underscores both the requirement for the appropriate microenvironment in the ability of epithelial cells to perform in a tissue-appropriate manner and a critical role of abnormal stroma in cancer promotion [9]. The model employs the mouse mammary gland as the host for human fibroblasts, which, when irradiated *in vitro*, take up permanent residence in the cleared fat pad. This humanized stroma supports the growth and morphogenesis of subsequently transplanted human mammary epithelial organoids. Proper ductal morphogenesis depends on the admixture of primary normal breast fibroblasts to these organoids prior to engraftment into humanized fat pads. Interestingly, specimens from most individuals gave rise to apparently normal ductal outgrowths but one specimen gave rise to hyperplastic growth, suggesting the presence of neoplastically initiated, but dormant, cells. When that preparation was transplanted in the absence of normal human stromal fibroblasts into a murine stroma humanized with stromal cells engineered to express either human hepatocyte growth factor or human TGF $\beta$ 1, the organoids developed into growths that closely resembled human comedo- and basal-type invasive carcinomas. The authors conclude

that these observations indicate that an altered stromal environment can promote human breast cancer formation by abnormal epithelial cells present, but dormant, in the normal human breast. This model promises to provide an excellent venue for investigation of specific mechanisms by which the microenvironment influences the neoplastic process.

### 1.5. Integrative radiation carcinogenesis

Our studies using HMEC have two important implications for radiation carcinogenesis. The first is that radiation exposure of epithelial cells leads to a high probability of a persistently altered phenotype in daughter cells. This epithelial phenotype lacks critical controls imposed via receptors for microenvironment proteins that are necessary for maintenance of tissue architecture, cell polarity and growth control. This epigenetic event occurs as a high frequency that could promote neoplastic potential, albeit it may occur in a subset of genetically predisposed cells. The frequency of carcinogenic initiation by radiation exceeds the mutation potential by several logs in rat mammary gland [92]. Second is that the loss of cell–cell and cell–ECM adhesion as a result of this phenotype, or due to altered signaling from the microenvironment, could disrupt genomic integrity. It is well documented that the frequency of chromosome aberrations increases many cell generations after irradiation by an as yet unknown mechanism, e.g. in the progeny of irradiated bone marrow [93,94] and epithelial cells [95]. The loss tissue-specific architecture and cell–cell interactions, which are themselves also characteristic of malignant progression, precedes, and could augment, destabilization of the genome. Indeed, we have recently found that the daughters of irradiated cells show a dose dependent increase in abnormal centrosomes (Erickson and Barcellos-Hoff, unpublished data). Centrosomes are tiny organelles that contain discrete protein aggregates that nucleate microtubule growth, organize spindle functions, and provide docking sites for protein complexes involved in cell cycle progression, checkpoint control and epithelial cell polarization (reviewed in [96]). Abnormal centrosomes number, size and distribution are found in many solid tumors [97], but precede morphological changes in transformation by HPV E7 oncoprotein [98]. Overexpression of pericentrin, a component of centrosomes, induces chromosome instability and aneuploidy in prostate cancer cells [97]. We are investigating whether the aberrant polarity in the progeny of irradiated HMEC disrupts the linkage to centrosomes, or vice versa, either of which would provide a means of generating instability through chromosomal mis-segregation.

## 2. Conclusion

Despite many attempts to derive the sum from the parts in classical radiation biology, it is now evident that integrative radiation carcinogenesis must take into account complexity in which cellular events are governed by tissue-level processes.

Tools to analyze the organizing principles that operate beyond the single cell and outside the nucleus are beginning to be evaluated and developed under systems biology, which defines organisms in terms of problems of network organization and emergent phenomena that are not resolvable into local events. A higher order view of cancer as an emergent phenomenon of tissues, rather than a property of the component cells would provide a framework for integrating its complexity. If cancer is considered a failure of the tissue, rather than a disease of cells, then the difficulty of predicting the fate of individual cells is significantly increased, but the ability to predict the behavior of the tissue should be improved. Rather than attempting to explain observable phenomena (e.g. function of a given gene, consequences of a mutation) independently of each other by reducing them to interplay between elementary units so that they can be investigated, systems biology defines biological functions as dynamic networks [99,100]. The conceptual difference is exemplified in the description of a phenotype as the result of selective expression of the genotype in response to the external signals, while the *phenome* is the sum of biological components, responses and signaling pathways of cells studied in context, i.e., within a proper tissue structure. The former is a dictionary while the latter is an encyclopedia. Systems biology approaches based on extensive component analysis should be used for integrating the heterogeneity of biological responses at the molecular, cellular and organismal level physiologically, in response to external stimuli, e.g. radiation or other carcinogens, and during the pathogenesis of cancer [101].

In normal tissues, extracellular signaling via a network of soluble and insoluble signals inhibits carcinogenesis by eliminating abnormal cells and suppressing neoplastic behavior. The loss of cell polarity and multicellular organization exhibited by the progeny of irradiated cells and the contribution of irradiated stroma are evidence that radiation, the prototypic DNA damage agent, mutagen and carcinogen, can promote malignant carcinogenesis progression by pathways other than mutational. If so, perhaps we should look more closely at alternative mechanisms of spontaneous carcinogenesis. Our future plans are directed to defining the molecular mechanisms underlying leading to the irradiated phenotype and toward testing whether and how the irradiated phenotype is a precursor to genomic instability. Data from new mouse and human models provide further imperative to focus attention on microenvironmental control of the initiated cell [102]. The multicellular nature of tissue response to carcinogens may prove to be the Achilles heel suitable for targeting in cancer prevention.

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