REVIEW

Linking the History of Radiation Biology to the Hallmarks of Cancer

Mary-Keara Boss,^a Robert Bristow^b and Mark W. Dewhirst^{c,1}

^a Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, North Carolina; ^b Department of Radiation Oncology, University of Toronto, Toronto, Ontario, Canada; and ^c Department of Radiation Oncology, Duke University, Durham, North Carolina

Boss, M-K., Bristow, R. and Dewhirst, M. W. Linking the History of Radiation Biology to the Hallmarks of Cancer. *Radiat. Res.* 181, 561–577 (2014).

Hanahan and Weinberg recently updated their conceptual framework of the “Hallmarks of Cancer”. The original article, published in 2000, is among the most highly cited reviews in the field of oncology. The goal of this review is to highlight important discoveries in radiation biology that pertain to the Hallmarks. We identified early studies that exemplified how ionizing radiation affects the hallmarks or how radiation was used experimentally to advance the understanding of key hallmarks. A literature search was performed to obtain relevant primary research, and topics were assigned to a particular hallmark to allow an organized, chronological account of the radiobiological advancements. The hallmarks are reviewed in an order that flows from cellular to microenvironmental effects. © 2014 by Radiation Research Society

INTRODUCTION

Numerous influential scientists and clinicians have contributed to the field of radiation biology. Tracing the history of radiation biology could follow several focuses and developmental pathways. In this review, we have organized the history of radiation biology according to Hanahan and Weinberg’s expanded Hallmarks of Cancer (*1*): the original six hallmarks: 1. enabling replicative immortality; 2. evading growth suppressors; 3. resisting cell death; 4. sustaining proliferative signaling; 5. inducing angiogenesis; 6. activating invasion and metastasis; — emerging hallmarks: 7. avoiding immune destruction; 8. deregulating cellular energetics, enabling characteristics; 9. genome instability and mutation; and 10. tumor promoting inflammation. Our purpose was to chronologically highlight studies that exemplified how ionizing radiation contributes

to each or how radiation was used experimentally to advance the understanding of the hallmark. This approach is intended to provide an alternative approach to discussing the history of radiation biology.

A literature search was performed using both Web of Science and PubMed to obtain primary research publications and review articles. Topics were assigned to a particular hallmark, and although we recognize that overlaps exist, this organization allowed a concise, but in no way comprehensive, account of the stepwise influences on radiation biological advancements. The hallmarks are organized in an order that allows the radiation biology topics to progress from cellular to microenvironmental effects. Because the field is very broad, we have focused on the first discoveries that relate to the Hallmarks and expand where needed to more current literature to emphasize certain points. Each section is concluded with a list of “*Current Radiobiology Challenges*” to indicate areas of active radiobiology research related to the hallmark. We cannot cover the whole field in this review and for those scientists who have contributed to this knowledge who are not highlighted here; this is not meant to reflect any lack of respect.

ENABLING REPLICATIVE IMMORTALITY

Cancer cells require the ability to replicate unlimitedly, uninhibited by normal space restrictions, to form a macroscopic tumor. Normal cells will progress through a tightly regulated, limited number of cell divisions; thereupon, they face either senescence, which is an irreversible, nonproliferative, viable state or crisis, cell death. Cancer cells do not respect the bounds of senescence or crisis and attain a state of immortality. The classic *in vitro* model of unlimited replication is the ability to continuously passage tumor cells in cell culture. Radiobiologists were the first to optimize replicative immortality of mammalian somatic cells in the laboratory; specifically, the ability to allow a single cell to grow into a clonal population. From there, many of the fundamental discoveries of radiation biology arose.

¹ Address for correspondence: Duke University, Radiation Oncology, Room 201 MSRB, Research Drive, Durham, NC 27710; e-mail: mark.dewhirst@dm.duke.edu.

In 1954, Dr. Theodore Puck wanted to study the genetics and metabolism of animal cells, however this was not possible at that time due to the lack of a simple, effective technique for large-scale colony production from single cells (2). He was searching for a way to achieve this feat, with the same high plating efficiency achieved with bacteria by microbiologists, to quantify the number of cells in a population capable of reproduction (3). At this time, Earle *et al.* had success with growing single mouse cells into colonies from within capillary tubes with conditioned nutrient media derived from mass cultures of growing cells; although, the plating efficiency for this complex process was only 4% (4). Understanding that the key to the growth of single cells in culture was the production of diffusible factors from neighboring cells, Philip Marcus, a graduate student of Puck, devised a unique design with fellow researcher Leo Szilard from the idea, "Since cells grow with high efficiency when they have many neighbors, you should not let the single cells know they are alone" (3). A colony of cervical cancer derived (HeLa) "feeder cells" was irradiated in culture to create a population of reproductively sterile, metabolically active cells, for the purpose of maintaining single cells inoculated on a microscope slide, positioned within the same culture media (Fig. 1) (2). They achieved a plating efficiency of 100% with this technique. Shortly after, it was realized that the low plating efficiency of single mammalian cells was secondary to a harsh trypsinization and washing process. Through development of a gentler technique, the necessity of the irradiated "feeder cell" population was eliminated (5).

The reproductive function of a single mammalian cell could now be assessed *in vitro* and the effects of various stressors could be quantified for cell populations by counting the formation of colonies. While it had been well established that irradiation interferes with the growth of microorganisms, it was Puck's laboratory that demonstrated for the first time the nature in which radiation inhibits the ability of mammalian cells to undergo unlimited replication. Importantly, it was discovered that mammalian cells are exquisitely more sensitive to radiation than microorganisms, with a unique survival curve comprising an initial shoulder followed by exponential cell killing (6–9). Further, it was noted that the cells exhibited a mitotic lag after irradiation, suspected to reflect repair (6). These basic characteristics of irradiated HeLa cells were determined to be shared by normal human cells, as well (7). Although the "target" concept had been demonstrated previously in various experiments, it was the development of techniques to visualize the karyotype of irradiated cells, showing that the rate of chromosomal aberrations corresponded with cell killing, which most clearly defined the main cellular target of radiation damage as DNA (8–10).

What followed was a flurry of discoveries made by assessing the unlimited replicative ability of single cells under modified experimental conditions. In regard to

FIG. 1. Enabling replicative immortality. Schematic diagram of the arrangement used to grow clones of HeLa cells. A: support for microscope slides; B: microscope slide(s) on which the clones develop; C: clone; D: Petri dish; E: level of liquid nutrient medium; F: layer of feeder cells. [Reproduced with permission. (2) Puck TT, Marcus PI. *Proc Natl Acad Sci U S A* 1955; 41, 432-37.]

irradiation of cells *in vitro*, split-dose experiments revealed sublethal damage repair (11); survival curves for varying types of ionizing radiation were developed (12); differences in survival throughout the cell cycle were discovered (13, 14); dose-rate effects were identified (15); the oxygen enhancement ratio was defined (16); and the concept of potentially lethal damage was realized (17).

Current radiobiology challenges. Intrinsic radiosensitivity may enable translation of *in vitro* radiation results to preclinical and clinical scenarios. Application of historic radiobiology fundamentals to current radiation therapy techniques (hypofractionation, stereotactic radiosurgery).

EVADING GROWTH SUPPRESSORS

The cell cycle is an organized balance of signals promoting and restricting replication and division, however, neoplastic cells exist with dysfunctional growth control mechanisms. Throughout the early experimental trials assessing the survival characteristics of irradiated mammalian cells, researchers were seeing evidence of intermittent variations in the cell growth cycle after irradiation. The basic phases of the cell cycle had been defined through the study of plants and microorganisms as S phase (DNA synthesis), M phase (mitosis) and functionally undefined

gaps, G₁ (between M and S), G₂ (between S and M), G₀ (senescence).

Autoradiography, utilizing tritiated thymidine to label DNA, allowed comparing labeled and unlabeled mitotic figures to the fraction of cells in mitosis to track the movements of cells through the phases of the cell cycle. Many researchers had documented similar findings that S phase was the most resistant to radiation and M phase was the most sensitive to radiation, but it was Yamada and Puck (18) that reported that a sublethal dose of radiation produced a temporary G₂ arrest in the HeLa cell line. This radiation-induced G₂ block was easily repeatable in HeLa cells and other transformed cell lines. Interestingly, it was not until pure normal diploid human cells in culture were studied that a delayed entry into S phase after irradiation was revealed, characterizing a G₁ block (19). The G₁ and G₂ arrests were investigated using numerous *in vitro* and *in vivo* models, greatly facilitated by advancements in recording cell cycle radiation effects with flow cytometry (13, 20–23).

It was intuitively suspected that the cause for the G₁ and G₂ blocks was to repair radiation damage done to fragile DNA prior to duplication of a faulty template, in synthesis or distribution of defective genes to a daughter cell, in mitosis. These transient delays became known as checkpoints. Both checkpoints were regarded as a safety mechanism to limit the propagation of heritable errors and promote the survival of the cell.

A breakthrough in the basic understanding of the cell cycle arose from research as to the radiation-induced effects of the tumor suppressor protein, p53. Irradiation of a myeloid leukemia cell line caused a transient increase in p53 levels in coordination with decreased DNA synthesis; further, cells with wild-type p53 displayed G₁ and G₂ blocks after irradiation, but cells with absent or mutated p53 genes only were arrested in G₂ (24). These results revealed that p53 played a critical role in the G₁ block. Additionally, transfection of wild-type p53 into malignant cells lacking p53 partially restored the postirradiation G₁ arrest, while over-expression of mutated p53 in tumor cells with wild-type p53 abrogated the G₁ block (25). Ataxia telangiectasia (AT) is a disease in humans that causes radiation hypersensitivity and genetic instability, putting patients at an increased risk for the development of cancer. In studying human AT cells, the normal cellular response of increased expression of p53 after irradiation was absent; these findings signified a link between the AT defect and the p53 pathway in triggering the G₁ checkpoint (24). In addition to regulating the G₁ arrest, p53 was implicated in inducing apoptosis (26).

It had been determined that close to half of all human cancers are affected with mutated p53 or abrogated p53 signaling. This was the underlying reason why the G₁ checkpoint was not documented in HeLa or other transformed cell lines, and it was only revealed when freshly cultured normal fibroblasts were studied. Based on the knowledge that interfering with the G₂ checkpoint

resulted in increased radiosensitivity (27), it was hypothesized that the G₁ arrest could have significant therapeutic implications. It was soon realized, though, that the response of mutated p53 to radiation had contradictory radiation effects according to cell type; lymphoid cells had reduced radiation sensitivity due to disruption of the apoptotic response (28), fibroblasts might not show an apparent change in radiation sensitivity if they arrest in G₁ (29). Despite this variability, it was suspected that there was a common initiating factor to the p53 pathway shared by all cell types. This was determined to be the mutated AT gene (ATM). Mice with disrupted ATM genes were phenotypically similar to humans with AT, after irradiation, they displayed lymphocytic resistance to apoptosis, fibroblastic inefficiency in G₁ arrest and absent increase in p53 levels (30). With these discoveries, it is known that ATM is responsible for signaling p53 to initiate the G₁ checkpoint following DNA damage to protect the integrity of the cell; subsequent studies have implicated ATM in the S- and G₂-phase checkpoints and DNA damage signaling and repair.

Current radiobiology challenges. Understanding the benefits and risks of radiation response following manipulation of cell cycle control. Development of novel therapeutic checkpoint modulators as radiosensitizers

RESISTING CELL DEATH

The fundamental discoveries of radiation biology were developed due to the fact that ionizing radiation causes cell death. Because the formation of chromosomal aberrations correlated with cell killing, it was established early that DNA was the lethal target of radiation; however, with time, the mechanisms by which various types of cellular damage progress to lethality were defined, and it became apparent that radiation-induced cell death is a very complex process. It is thought that clonogenic cell killing represents cell kill due to multiple mechanisms that culminate in the prevention of clonogen propagation after ionizing radiation exposure.

Ionizing radiation efficiently induces lesions in DNA, the most lethal being DNA double-strand breaks, by direct hit or indirectly through damage by free radicals. Mitotic death may follow such chromosomal damage if left unrepaired due to failure of the cell to divide successfully. This may occur at the first attempted mitosis or the cell may divide several times before the inappropriate separation of DNA is fatal. As this had been witnessed microscopically, it was considered the major mechanism of radiation-induced cell death, inducing immediate tissue effects by predominantly eradicating the rapidly dividing stem cell populations.

In 1972, Dr. John Kerr defined a programmed, active method of controlled cell deletion that could be initiated or inhibited by a variety of stimuli (31). He credited James Cormak, a Greek professor, for suggesting the term “apoptosis”, which used in Greek describes the “dropping off” of petals from flowers or leaves from trees. As Dr. Kerr

reported the presence of spontaneous apoptotic cells within tumors, the challenge of understanding the regulation of apoptosis attracted a great deal of attention, particularly due to the potential implications for cancer therapy.

Shortly after the recognition that p53 controlled the G₁ checkpoint, it was shown experimentally that increased p53 expression could activate an apoptotic response after irradiation *in vitro* and *in vivo* in various tissues (32, 33). The fact that cells could die by way of apoptosis, instead of mitotic death, after irradiation was novel and exciting; however, there was a great degree of variability between cell types in regard to apoptotic susceptibility. For example, lymphoid cells, salivary gland parenchyma, and crypt cells of the small intestine were the first defined as cells that are very susceptible to radiation-induced apoptosis, while fibrous tissue was deemed resistant.

It was soon discovered that DNA damage within the nucleus, by way of activating the p53 pathway, was not the sole site to stimulate apoptosis. Haimovitz-Friedman *et al.* performed a series of experiments in bovine aortic endothelial cells and demonstrated that ionizing radiation triggered a rapid hydrolysis of sphingomyelin from the plasma membrane to produce cytoplasmic ceramide as the second messenger which activated the apoptotic pathway in these cells (34). This sphingomyelin/ceramide pathway occurred in cell preparations devoid of nuclei, which revealed that this form of apoptosis was independent of direct DNA damage. It was determined that radiation is capable of activating two independent apoptotic signaling mechanisms secondary to nuclear and/or membrane-based damage.

In addition to the variability in cell type susceptibility and the origin of signaling of the apoptotic response, it became apparent that there was a temporal difference in regard to p53-dependent apoptosis subsequent to radiation. Acute apoptosis, occurring within 4–6 h after irradiation, was induced by p53 expression, while a delayed, 24 h or more, p53-independent apoptosis exists (35, 36). Interestingly, this delayed p53-independent apoptosis, sometimes subsequent to several mitotic cycles, is mediated by *de novo* synthesis of ceramide, without the activation of the sphingomyelin pathway (37).

Multiple anti-apoptotic mechanisms exist which are intended to maintain the natural homeostasis of cellular proliferation and deletion within tissues. The most widely studied, Bcl-2, which is an inner mitochondrial membrane protein, has been shown extensively to inhibit apoptosis (38–40). However, over expression of the Bcl-2 gene (or its pro-apoptotic gene partner, Bax), does not alter final clonogenic survival in cells in which apoptosis is not the main mode of cell death (41). Further, a novel response to radiation, autophagy or “self-eating” has been described. Autophagy, which results in the development of acidic vesicular organelles to degrade cellular proteins, has been shown to contribute to cell death, as well as serve as a

protective survival mechanism, in response to radiation (42, 43).

As it is apparent that cell death can result from both mitotic and apoptotic killing induced by radiation, it is imperative to understand and exploit the differences that exist between, not only neoplastic and normal tissue, but cell type and cellular target, to maximize the efficacy of cancer therapy.

Current radiobiology challenges. Deciphering and targeting radiation-induced DNA damage signal transduction pathways; modulation of p53-induction after radiation stress.

GENOME INSTABILITY AND MUTATION

Abnormal chromosomal arrangements in cells after exposure to ionizing radiation have been described since the beginning of experimental radiobiology. The study of these chromosomal aberrations, both acute (immediately after irradiation) and heritable (in progeny several generations from the parental cell exposure) initially led to identifying components and function of genes involved in the DNA damage response (DDR) and DNA repair. Additional DDR and DNA repair information came from complementing radiosensitive Chinese Hamster Ovary (CHO) cells with exogenous DNA that led to the cloning of DSB repair genes. Ionizing radiation causes a variety of DNA damage as discrete lesions within multiply damaged localized sites. DNA double-strand breaks (DSB) are important lesions as if not repaired correctly, unrepaired DSBs can lead to cell death or the propagation of chromosomal deletions and translocations. DNA mutations within the genome during irradiation can lead to carcinogenesis in cells that have an abnormal DDR.

Dr. Barbara McClintock began in her studies of the cytogenetics of radiation-induced chromosomal aberrations in the 1940s. She examined the behavior of broken ends of chromosomes in maize and described the formation of ring chromosomes and chromatid bridges at mitosis. In her words, “The conclusion seems inescapable that cells are able to sense the presence in their nuclei of ruptured ends of chromosomes and then to activate a mechanism that will bring together and then unite these ends, one with another” (44).

Repair of DSBs occurs by at least two processes: homologous recombination (HR) and non-homologous end-joining (NHEJ). These pathways are distinct in the proteins used within each pathway, the cell cycle phases in which they act, their chromatin context and their relative fidelity of repair, and yet, they function in complementary ways (45). Homologous recombination involves repair of a DSB by producing a copy of the damaged DNA from an intact homologous DNA strand. This repair process functions in late S/G₂ phase when sister chromatids are available in close proximity. While this is the main form of

DSB repair in yeast, non-homologous end-joining (NHEJ) is the predominant mode of repair in mammals (46, 47). NHEJ does not require an undamaged homologous molecule for repair and involves ligation of broken chromosome ends. Therefore, although this process occurs in all phases of the cell cycle, it is prone to error and the creation of deletions or genetic mutations. It has been recently proposed that the NHEJ process is still the first choice of rapid DSB repair in the G₂ phase and the HR pathway facilitates repair of complex lesions that persist in heterochromatin linking chromatin biology to choice of DSB repair processes (48).

Building upon the understanding that ATM and p53 control cell cycle checkpoints and influence cell death, studies utilizing ionizing radiation contributed to the identification of the molecular components of DSB repair. It was found that ATM is central to initiating both HR and NHEJ through the direct phosphorylation of repair substrates or other kinases. In HR, this involves activating Rad51 and BRCA1/2 (49, 50), while the DNA-PK/Ku complex facilitates NHEJ (51). However, after the discovery of the MRE11-NBS1-RAD50 complex (MRN) as a key sensor of DSBs, it was determined that phosphorylation of the histone H2AX by ATM is one of the first processes after DSB induction and sensing. Activated H2AX is associated with chromatin signaling at the site of damage, which then allows the recruitment of the repair factors to the injured DNA (52, 53). It is now appreciated that initiation of a DSB leads to a series of protein post-translational modifications such as phosphorylation, ubiquitylation and acetylation in the immediate vicinity of the DSB to confine repair to specifically affected parts of the genome. This is exemplified by the ATM-dependent phosphorylation event coming together with RNF8-mediated chromatin ubiquitylation leading to an ordered accumulation of DNA repair factors (e.g., RNF168, the BRCA1-interacting RAP80 protein, 53BP1) at the break site using an ubiquitin-binding domain (54).

There are numerous spontaneous and experimentally induced mutation syndromes caused by the dysfunction of various components of the DSB repair pathways that manifest as certain radiation hypersensitivity and chromosomal instability phenotypes. For example, mutation of the NHEJ process which is critical for the formation of T- and B-lymphocyte receptors was performed to create the *scid* mouse phenotype (55, 56). When *scid* mice were irradiated, it was discovered that their myeloid cells and fibroblasts were markedly more radiosensitive when compared to control mice (55).

Beginning with initial work published in 1980, the laboratory of Dr. John B. Little performed experiments over several decades which support the hypothesis that radiation can induce a genome-wide process of instability in mammalian cells that is transmitted over many generations of cell replication leading to an enhanced frequency of genetic changes occurring among the progeny of the original

irradiated cell (57, 58). A large series of experiments investigating mutagenesis was performed by studying the induction of *HPRT* mutations in CHO cells treated with X rays or α particles (Fig. 2) (59). The instability phenotype was induced in approximately 10–20% of cell populations irradiated *in vitro* with doses of 4–12 Gy, and the spectrum of DNA structural damage in mutations arising in such unstable populations differed significantly from that for direct radiation-induced mutations (58).

In addition, genetic instability can be induced in irradiated cells or develop spontaneously in cancer cells through defective or absent telomeres, the protective nucleoprotein caps at the end of chromosomes. One of the roles of telomeres is to prevent abnormal chromosomal fusion which can result in various gene mutations (60). Irradiation can induce telomeric damage at terminal and interstitial chromosomal sites, leading to abnormal chromosomal rearrangements (61).

The DDR response is enacted by accidental or chronic low-dose exposure of normal tissue. Abnormalities in these pathways can result in increased cell death due to the inability to repair DNA damage or allow the inappropriate proliferation of transformed, mutant cells. Research that elucidates the mechanisms that enable the cell to respond to and repair radiation-induced DNA damage has great implications for cancer therapy. For example, advances in radiation therapy may be possible by taking advantage of synthetic lethality. Synthetic lethality refers to a genetic interaction where the combination of mutations in two or more genes leads to cell death, while a mutation in just one of those genes would allow cell viability. Understanding the germline and somatic genetics of the DDR response in cancer cells can lead to the exploitation of genetic synthetic lethality in which DNA repair-deficient cells are sensitive to ionizing radiation and/or agents that knock out remaining DDR pathways (e.g., PARP, ATM and DNA-PKcs inhibitors) (62, 63).

Current radiobiology challenges. Understanding radiation-induced genetic instability for radiation protection purposes. Targeting the DNA-damage response to improve tumor radiosensitivity, identification of tumor specific pathways to increase synthetic lethality.

AVOIDING IMMUNE DESTRUCTION

Cancer develops in the host through deception of the immune system. Functional immune surveillance involves highly integrated cell and tissue pathways utilizing local and systemic signaling with inflammatory and immune molecules to eradicate pathogens and abnormal or damaged cells from the body. Initially, it was accepted that radiation causes immunosuppression exclusively; however, as the complexities of tumor immunity are elucidated, radiation should be considered “immunomodulatory” due to the varying effects that may follow exposure (64).

FIG. 2. Genome instability and mutation. Frequencies of *HPRT* mutations among clonal populations of CHO cells examined approximately 23 population doublings after they were derived from nontreated cells (controls) or single cells surviving irradiation with X rays (4–8 Gy) or α particles (2 Gy). The number of clones examined is shown at the bottom of the x axis. Numbers in the shaded area represent the number (%) of clones showing no mutations. Increased frequencies of mutations were observed in a significant of the clonal populations derived from irradiated cells. [Reproduced with permission. (59) Little *et al.* Radiat Res 1997; 148, 299–307.]

The roles of the immune cells are defined by their involvement in either innate or adaptive immunity. Cells of the innate pathway include phagocytic, antigen presenting cells (APC), such as macrophages and dendritic cells, and those of the adaptive pathway consist of cells with randomly generated antigen receptors, like B and T lymphocytes. Generally, APCs are activated by immunogenic stimuli, migrate to lymphoid organs to present antigen to T cells and stimulate the adaptive immune response.

Early experimental radiation studies demonstrated that, of the pathological changes that occurred after whole body irradiation, the rapid death of lymphocytes was most striking. While lymphocytes were regarded as the most radiosensitive cell in the body, this finding was puzzling due to their nonmitotic state when compared to other notably radiosensitive cells (65). Ultimately, it was determined that lymphocytes undergo apoptosis postirradiation, and the profound immunosuppression that follows whole body irradiation was credited to this effect.

In time, more details regarding additional immune effects of irradiation were discovered. Aside from the radiosensitivity of the immune cells, details were uncovered as to how radiation affects the functionality of the cells actively participating in the tumor immune response. For example, while radiation induces increased leukocyte rolling and adhesion to the endothelial cells of the vascular wall in normal blood vessels (66, 67), irradiated tumor vasculature demonstrated changes in their endothelial surface properties such that they became less adhesive to leukocytes (66). This effect, in particular, would be a case of a defect in the effector phase of tumor immunity through inadequate homing and infiltration of activated T cells. More recent work, however, demonstrates that 10 Gy irradiation can upregulate certain adhesion molecules in tumor microvasculature that can be used to increase immune cell trafficking and/or targeted therapeutics (68).

Different types of cancer cells exhibit variable sensitivity to radiation-induced death, but the understanding that

radiation causes a wide range of cellular and stromal effects, in addition to tumor cell death, has prompted investigations as to the use of radiation in an immunomodulator. The term Radiation Hormesis refers to the hypothesis that low doses of ionizing radiation are beneficial by a number of mechanisms, including stimulation of the immune response (69). Many investigators have tested the potential for cancer cells killed *in vivo* by radiation therapy to serve as a good source of antigens for APCs to present to T cells (70). Additionally, modification of tumor immunity through signal simulation has been shown to serve as a radiation sensitizer. Mason *et al.* utilized an injectable Toll-like receptor agonist coupled with radiation therapy in a mouse tumor model to demonstrate that the combination resulted in a decreased effective dose of radiation needed to induce tumor regression (71). Further, the animals cured of tumors with this protocol were more resistant months after curative therapy to autologous tumor challenge than mice that were cured with radiation therapy only.

An intriguing, although rare, phenomenon known as the “Abscopal Effect” has been reported for a number of malignancies, whereby local radiotherapy is associated with the regression of metastatic cancer at a distance from the irradiated site (72–74). While the mechanisms of this effect remain undefined, it is suggested that the immune system plays an important role (75). A recent case report described an observed abscopal effect for a patient with metastatic melanoma treated with radiotherapy and ipilimumab, a monoclonal antibody that inhibits an immunologic checkpoint on T cells, CTLA-4 (76).

As with many cellular and tissue effects, alterations in tumor immunity vary with radiation dose, tumor type and microenvironmental conditions, and many questions remain. Perhaps through adaptation of radiation dosing, with or without additional immunological manipulating agents, it could be possible to utilize the immune system to improve cancer treatment strategies.

Current radiobiology challenges. Understanding the influence of the immune system on the irradiated tissue microenvironment, distant neoplastic disease and systemic health. Developing targeted immunomodulation as an adjuvant to radiation therapy to improve radioresponse and enhance systemic anti-tumor immunity.

TUMOR PROMOTING INFLAMMATION

While it is important to understand the cellular response to ionizing radiation, we must consider that cells exist as components of a complex microenvironment. Tumor cells are intimately associated with supporting stromal cells, immune cells and microvasculature. This tissue reacts as a unit to acute radiation damage. However, response to radiation exposure is not limited to what is in the beam path; there are unique localized and systemic responses to radiation exposure that can result in perpetual effects.

Solid tumors exist in a state of active inflammation, and treatment with radiation therapy can both promote and reduce inflammation by modulation of various inflammatory pathways. The factors of the radiation-induced inflammatory response may induce beneficial anti-tumor effects, but of considerable concern are the modifications to the microenvironment that can occur that enhance tumor growth and damage normal tissues.

Immune cells play a significant role in inflammation, with macrophages considered a key contributing cell type. In the 1970s and 1980s, radiation was found to be a useful tool for studying macrophages, as exposure to radiation resulted in primed macrophage activation *in vitro* (77, 78). It was found that activated macrophages release reactive oxygen species (ROS) in a time- and dose-dependent manner. In 1987, Gallin and Green considered “it is possible that the stimulatory action of radiation on the oxidative burst of macrophages is partly responsible for the tissue damage that occurs post irradiation” (79).

It had been established that activated macrophages were the primary cell type to produce the cytokine, tumor necrosis factor (TNF), a principal mediator of inflammation and the immune response. Researchers found that TNF induces tumor cell death *in vitro* and *in vivo* (80, 81). From there, Hallahan *et al.* demonstrated that human sarcoma cells were able to generate increased TNF levels independently after irradiation *in vitro*, and that the addition of exogenous TNF enhanced the killing of these tumor cells by radiation (82). These synergistic effects of TNF and radiotherapy were exhibited for the first time *in vivo* by Sersa *et al.* in a mouse tumor model (83).

The free radical, nitric oxide (NO), is another important cellular signaling molecule involved in inflammation. Ibuki *et al.* determined that both irradiation of macrophages and low doses of ROS increased the NO production of macrophages *in vitro* (84). Tying the responses of NO and macrophages together, McKinney established that the increased NO from macrophages is mediated by the induction of TNF (85). Each of these interrelated signaling factors contributes to the complex tumor inflammatory microenvironment in which tumor cells themselves contribute to the inflammatory process.

As these connections were made, ROS were suspected to be involved in a “bystander” response described by Nagasawa and Little in 1992 (86). Chinese hamster ovary cells were exposed *in vitro* to α particles. It was estimated that less than 1% of the nuclei were traversed by the particle track, however, there was an increased number of sister-chromatid exchanges in the nonirradiated cells. Building upon this observation, studies were performed that consistently showed radiation damage in cells that were not directly irradiated (87, 88), further, the transfer of media from irradiated cells to nonirradiated cells induced the same type of bystander damage (89, 90). Also, demonstration of *in vivo* bystander injury was shown in clonal descendants of hematopoietic stem cells after bone marrow irradiation in

mice (91). These experiments have defined the phenomenon known as the bystander effect. While the mechanism of these effects remained undefined, it is strongly suspected that a chemical signaling process, involving ROS, extracellular factors and cellular gap-junctions, exists.

Mothersill emphasizes that, although characteristics of the bystander effect, described above, are suggestive of inflammatory responses, it is important to note the effects seen occur at radiation doses below those necessary to induce inflammation of the kind associated with tissue injury (92). Regardless, many biologists suspect that perpetual radiation effects after exposure to high therapeutic or accidental radiation doses could be indicative of a continual, systemic inflammatory tissue reaction to radiation injury, for example, the classic and curious, phenomenon of clastogenic factors isolated from the peripheral blood of long-ago irradiated individuals capable of inducing damage in nonirradiated cells. Inflammation is intimately involved with many of the integrated "Hallmarks of Cancer" pathways that influence tumor phenotypes and responses to therapy.

Current radiobiology challenges. Balancing the pro-inflammatory and anti-inflammatory effects of radiation exposure to optimize tumor radiosensitivity and protect normal tissue. Determining the mechanisms and biologic consequences of the radiation bystander effect to improve radiation therapy practices and radiation protection standards.

INDUCING ANGIOGENESIS

It has been recognized for over 100 years that tumor vasculature is unique and abnormal. *In vivo* imaging in the early twentieth century led to the understanding that for a developing solid tumor to grow into a macroscopic mass, it must induce the surrounding normal tissue to either share its oxygen and nutrient supply or create its own vascular network (93–95). The process by which a tumor forms new blood vessels and remodels existing vasculature involves highly integrated signaling and communication between cells of the microenvironment. The interplay of ionizing radiation effects with tumor and vascular cell death or stimulation may be exploited for improved therapeutic outcomes.

By the 1950s, it was well known that cells deficient in oxygen supply or hypoxic, at the time of exposure are much less damaged by radiation than those which are oxygenated (96). Thomlinson and Gray revealed in their histological studies of bronchogenic carcinomas that these tumors *in vivo* contain cells that were predicted to vary in degree of oxygenation; specifically, that the peripheral tumor cells, closest to vessels, were more aerobic than those at the centers of nodules because of increasing distance from a blood supply (97). They presumed that the peripheral cells would be more easily destroyed by irradiation, while the

central cells would be radioresistant. By the 1960s, Rubin and Casarett provided some of the first data suggesting that tumor stromal cells (endothelial cells) might contribute to the primary effect of radiation in the destruction of tumors. Using transplanted tumors subjected to fractionated radiation in rats, the microcirculation did not involute as rapidly as the parenchymal tumor cells, leading to a relative state of supervascularization and presumed increase of tumor tissue oxygenation (98). Thus, their theory was that radiation fractionation could increase the radiosensitivity of the mass as a result of reoxygenation. More recent studies have shown that the underlying mechanism for supervascularization is more complex.

Several noteworthy studies demonstrate the importance of inducing vascular death for effective tumor eradication by radiation therapy. Tumors stimulate persistent angiogenesis through expression of many factors, one of the most important being the family of vascular endothelial growth factor (VEGF) proteins. Building upon the observation that serum VEGF levels are increased in patients with malignant tumors after radiation therapy (99), Gorski *et al.* evaluated this paracrine relationship between the tumor and its vasculature as a potential target for radiation-induced antitumor activity (100). Treatment of tumor-bearing mice with a neutralizing antibody to VEGF before irradiation was associated with a greater than additive antitumor effect, supporting the idea that induction of VEGF by ionizing radiation contributes to the protection of tumor blood vessels from radiation-mediated cytotoxicity and tumor radioresistance (Fig. 3) (100). Further, in a series of experiments investigating high-dose radiation-induced apoptosis of endothelial cells in tumor vessels, Garcia-Barros *et al.* demonstrated that microvascular death was a key mechanism in tumor responses to radiation (101).

As numerous cytokines were discovered to be involved in vascular radioresistance, Moeller *et al.* posited that blockade of the cytokine production at the source of the common pathway, hypoxia inducible factor-1 (HIF-1), might prove effective as an antitumor treatment with radiotherapy (102). HIF-1 is capable of stimulating the expression of downstream genes involved in tumor metabolism, growth and angiogenesis, including VEGF (103). Moeller found that HIF-1 accumulated in tumors after radiation exposure; this occurred concomitantly with reoxygenation. The increase in HIF-1 was attributable to increased production of reactive oxygen species, as well as enhanced translation of HIF-1 regulated transcripts (102). The resulting increase in HIF-1 regulated cytokines enhanced endothelial radioresistance, and through inhibition of HIF-1 activation, tumor radiosensitivity was significantly increased as a result of enhanced vascular destruction (102).

In more recent studies, it has been shown that bone marrow-derived myeloid cells play an important role in modification of the tumor microenvironment following irradiation. Ahn and Brown demonstrated that bone marrow-derived myeloid cells enter irradiated tumors to

promote restoration of tumor vasculature through the process of vasculogenesis, thereby allowing growth of the tumor after irradiation (104).

An interesting therapeutic paradox exists whereby anti-angiogenic agents can improve tumor oxygenation by “normalizing” the abnormal tumor vasculature (105). Preclinical and clinical evidence to support this concept has been reviewed recently by Goel *et al.* (106). This vascular normalization may exist within the tumor micro-environment for a limited time. Treatment of solid tumors with antiangiogenic agents can create an observed “normalization window”, typically within 1–2 days of starting treatment. Irradiation of a solid tumor during this period of optimal perfusion and oxygenation could translate to improved tumor radiosensitivity and therapeutic response.

It is clear that there is a fine line that challenges the fundamental therapeutic goals of radiation therapy in regard to the importance of tumor vasculature in influencing tumor reoxygenation: (1) it increases radiosensitivity for the next dose of radiotherapy; however (2) it contributes to enhanced endothelial cell survival because of HIF-1 mediated increases in VEGF production. Which of these two phenomena contribute to improved overall tumor control remains controversial.

Current radiobiology challenges. Manipulating angiogenesis and the tumor-associated vasculature to facilitate perfusion and oxygenation of tumor cells for optimal radiosensitivity. Targeting the tumor-promoting mediators associated with radiation-induced tumor reoxygenation.

SUSTAINING PROLIFERATIVE SIGNALING

In normal tissues, growth factors regulate cellular proliferation and differentiation. Cancer cells, however, develop a reduced requirement for exogenous growth factors as they become capable of autonomous activation of autocrine and paracrine growth pathways. As the goals for radiation therapy include tumor regression and eradication, this aberrant proliferation must be inhibited to prevent resistance and recurrence.

FIG. 3. Inducing angiogenesis/sustaining proliferative signaling. Panel A: Effect of VEGF blockade before treatment with IR in human tumor xenograft (Seg-1 esophageal adenocarcinoma). Tumor-bearing mice were treated with neutralizing anti-VEGF antibody, IR or a combination of the two and tumor volumes were measured. Control (■); IR alone (◆); anti-VEGF antibody alone (▲); and IR combined with anti-VEGF antibody (●). Tumor-bearing mice were treated with 20 Gy in four 5 Gy fractions on days 0, 1, 2 and 3. There was a statistically significant difference between the IR alone and combined treatment groups for all days after day 6 ($P < 0.02$ for all days). Panel B and C: Relationship between EGFR expression and radiocurability of murine carcinomas. Panel B: Both wild-type (○) and mutant p53 (●) tumors. Panel C: wild-type p53 tumors only. The levels of EGFR are inversely correlated with radiocurability. [Panel A: Reproduced with permission. (100) Gorski *et al.* Cancer Res 1999; 59, 3374-78; and Panels B and C: Reproduced with permission. (111) Akimoto *et al.* Clin Cancer Res 1999; 5, 2884-90.]

The growth of a solid tumor is determined by cell production and cell loss. Studies performed in the 1960s using autoradiographic methods determined that the rate of cell production is a function of the fraction of proliferating cells and the cell cycle time (107). Through investigation as to how exposure to radiation induces changes in tumor growth rate, Hermens and Barendsen revealed that during the later stages of regression of irradiated rhabdomyosarcomas in rats, there is a rapid, but subclinical, increase in proliferation (108). The first clinical evidence for tumor cell repopulation came from Withers *et al.* comprehensive retrospective analysis of the relationship between radiation dose and overall irradiation time in head and neck cancer (109). The findings strongly suggested that increasing the overall duration of radiotherapy results in decreased local tumor control through accelerated repopulation (109).

Some of the key proteins regulating cell survival and proliferation are the transmembrane epidermal growth factor receptors (EGFR) and ligands epidermal growth factor (EGF) and transforming growth factor- α (TGF α). EGFR is overexpressed in many tumor types and is associated with aggressiveness and resistance to therapies. It has been shown *in vitro* and *in vivo* that tumors with high expression of EGFR are more radioresistant (Fig. 3) (110–112). Studies have reported that cell survival and repopulation are regulated by the activation and expression of EGRF/TGF α that is induced after irradiation (113, 114). Further, treatment of neoplastic cell lines *in vitro* with EGF is protective against ionizing radiation, while this effect is abrogated when the cells are exposed to monoclonal antibodies against EGFR (115, 116). With these findings, Balaban *et al.* suggested that radiation may mimic the action of a natural ligand on EGFR and trigger resistance to radiation by receptor activation (116).

Strategies to counteract tumor proliferation with radiation therapy have included modifications to treatment protocols and biological inhibitors. Alternative radiotherapy regimens, particularly used for treatment of head and neck squamous cell carcinomas (HNSCC) have been assessed. Such schedules as hyperfractionation and/or acceleration have been designed to increase the dose intensity, and reducing the total treatment time is meant to reduce the repopulation of tumor cells between treatments, resulting in improved locoregional control (117). In meta-analysis of HNSCC, alternative fractionation showed a significant benefit for locoregional control when compared to conventional protocols (117).

Biological antiproliferative agents have been shown to alter tumor radiosensitivity in experimental models and clinically. Blockade of EGFR through anti-EGFR monoclonal antibodies (118, 119) or EGFR-selective tyrosine kinase inhibitors (120, 121) have been proven to be effective adjunct treatments to radiotherapy *in vitro* and *in vivo*, as well as in multinational, randomized, phase 3 trials (122).

Despite the success of these new treatment strategies, at most 15% of patients with HNSCC will have increased locoregional control or survival (117, 122). Alternative treatment modalities, such as adjuvant therapies targeting proliferative pathways downstream to EGFR, are being intensely investigated to counter radioresistance more effectively and improve patient outcomes (123).

New information as to the initial driving events responsible for tumor repopulation after radiotherapy has been provided by Huang *et al.* They describe that dying, irradiated tumor cells use the apoptotic process to generate potent growth-stimulating signals through activated caspase-3 to stimulate the repopulation of tumors (124). This recent discovery may lead to enhanced tumor control and curability through the development of novel caspase-3 inhibiting agents.

Current radiobiology challenges. Interfering with tumor proliferation and repopulation through manipulation of radiation treatment protocols and novel targeted therapeutics.

DEREGULATING CELLULAR ENERGETICS

It has been discussed with the preceding hallmarks that tumors adapt and proliferate despite harsh conditions of inflammatory stress and disorganized, ineffective microcirculation. The tumor microenvironment is not only characterized by varying degrees of hypoxia, but also nutrient deprivation and low extracellular pH. The unusual metabolism of cancer cells is intimately involved in creating such an abnormal physiologic state and the intricacy of how ionizing radiation alters this cancer hallmark is an active area of research.

Normal tissues are able to sustain routine cellular functions through the oxidation of nutrients to provide energy in the form of adenosine triphosphate (ATP). Glucose metabolism is regulated in response to changes in cellular oxygen levels. Initially, glucose is converted to pyruvate by glycolytic enzymes. Under well-oxygenated conditions, pyruvate is oxidized in the tricarboxylic acid cycle to form water and a substantial amount of ATP (oxidative phosphorylation), however, under hypoxic conditions, lactate dehydrogenase A converts pyruvate to lactate and generates very little ATP (glycolysis).

German biochemist and physiologist, Dr. Otto Warburg, was the first to define the difference in glucose metabolism between normal and neoplastic cells. In 1924, Warburg analyzed the ratio of oxidative phosphorylation to glycolysis in different tissues of cancer cells and normal cells. He found that glycolysis, under aerobic conditions, was particularly high in aggressive tumors when compared with benign tumors and normal tissues (125). This discovery of tumor-specific, oxygen-independent glycolysis was labeled the Warburg Effect. This peculiar metabolic preference of tumor cells drives a significantly inefficient breakdown of glucose for ATP and creates an acidic microenvironment through the build-up of the byproduct lactic acid.

As has been mentioned in previous sections, HIF expression drives many neoplastic pathways. In the case of tumor metabolism, crosstalk between HIF and the proto-oncogene c-MYC drives the exceptionally high rate of glycolytic activity of cancer cells to maintain sufficient ATP for tumor growth and progression (126). HIF-1 has been shown to regulate glycolysis (127), as well as expression of the glucose transporters, GLUT1 and GLUT3, which mediate cellular glucose uptake (128). Determined to be upregulated after irradiation (102), HIF-1 can enhance tumor radiosensitivity by increasing ATP metabolism and proliferation rates (Fig. 4); however, this is countered as HIF-1 also promotes radioresistance through vascular protection (129). Alternatively, activation of oncogenes, such as c-Myc or inactivation of p53 can contribute to the Warburg effect in a HIF-1 independent fashion (130, 131).

The increased dependence of cancer cells on the glycolytic pathway provides a biochemical basis for the design of therapeutic strategies to preferentially kill cancer cells by pharmacological inhibition of glycolysis (132). Inhibitors of the glycolytic pathway have been tested for their ability to cause radiosensitization, such as 2-deoxyglucose (2-DG) (133, 134). 2-DG is a glucose analog that acts as a competitive inhibitor of glucose metabolism. In 1996, Mohanti *et al.* reported the results of their clinical trial combining 2-DG with radiotherapy in cerebral glioma patients (133). Their study showed that administration of 2-DG in combination with radiation is safe and could be tolerated without acute toxicity and late radiation damage to the brain. Further clinical studies combining radiation therapy with glycolytic inhibitors will determine if this is an effective approach to tumor control.

While there remains a gap in our understanding of how energy inefficient cancer metabolism benefits the progressing neoplasm, a key to enhanced radiosensitivity may lie in exploiting this tumor-specific state.

Current radiobiology challenges. Determining the influence of tumor cell metabolism on the radiation response. Investigations of glycolytic inhibitors as an adjuvant to radiotherapy

ACTIVATING INVASION AND METASTASIS

The final Hallmark to be reviewed is invasion and metastasis, the processes of which are particularly convoluted and intricate. The properties required to attain the phenotype of an invasive and metastatic neoplasm involve the multistep, coordinated foundation of the previously discussed hallmarks. As such, this concluding section will highlight the influence of several cancer-promoting genes, proteins and molecules, unique to or complimentary to invasion and metastasis, and how they pertain to radiation biology.

Radiation therapy is a principal treatment modality in clinical oncology because it is an effective means of local tumor control and can be highly curative for many cancer

types. However, there were observations in the earliest stages of radiation oncology that ineffective irradiation of solid tumors could ultimately result in the enhancement of metastasis. The first major experimental investigation of the effects of local tumor irradiation on metastasis was reported by Drs. Kaplan and Murphy in 1949 (135), and then the first prospective, randomized trial performed by Strong *et al.* in 1978 provided significant evidence that there is a correlation between radiation exposure of a primary tumor and the development of metastasis (136). Additional clinical studies revealed that patients with local failure after radiation therapy have a higher incidence of distant metastases than those who maintain local control (137, 138).

It has now been realized that interactions of the complex tumor microenvironment can drive the development of an invasive, metastatic tumor. Several noteworthy clinical trials were performed in the 1990s, which investigated the influence of various tumor parameters on outcome after radiation therapy. Significant positive correlations between low tumor oxygenation or high lactate concentration and poor radiation treatment outcomes, particularly the development of regional or distant metastasis, have been repeatedly reported for numerous tumor types (139–145).

Experimental molecular oncology studies suggest that the progression of a solid tumor to a metastatic phenotype is not simply the result of dysregulated signal transduction pathways, but instead is achieved through a stepwise selection process driven by hypoxia (146). The adaptation of populations of neoplastic cells to hypoxia facilitates cancer cell dissemination through the up- or down-regulation of critical metastasis-associated genes, such as E-/N-cadherin for epithelial-mesenchymal transition (147, 148), uPAR for degradation of the basement membrane and extracellular matrix (149), HGF/MET for cell motility (150, 151) and VEGF for stromal interactions, intra/extravasation and angiogenesis (152). The systematic alteration of these phenotypic regulators allows cells to escape the hostile microenvironment of the primary tumor and colonize a secondary location.

Considered separately from the influence of hypoxia on tumor aggression, radiation exposure may promote invasion and metastasis, as well. It has been shown that exposure to ionizing radiation can cause significant tumor reoxygenation and elicit a stress response in cells (102, 129). Further, this response induces the expression of HIF-1, known to activate many genes known to be important in cancer metastasis (153).

The standard radiotherapeutic goals are effective and efficient eradication of the primary tumor for long-term control or curability. Certain physiologic conditions are known to be associated with a more advanced, malignant tumor, likely to be resistant to therapy and to metastasize. Repeated exposure of such tumors to radiation may exacerbate these traits if not countered by novel, targeted therapies against the mechanisms of neoplastic adaptation and progression.

FIG. 4. Deregulating cellular energetics. HIF-1 maintains tumor bioenergetics. Panel A: *Ex vivo* ATP bioluminescence. ATP is significantly depleted in 4T1 and HCT116 tumors in response to HIF-1 blockade ($P < 0.05$). Panel B: PC-3 cells were cultured under hypoxia with high glucose (HG; 5g/l)- or low glucose (LG; 0.1g/l)-containing media, with or without doxycycline stimulation. Low glucose concentrations caused a significant right shift in the survival curve and HIF-1 inhibition further enhanced this effect ($P < 0.05$). [Reproduced with permission. (129) Moeller *et al.* Cancer Cell 2005; 8, 99–110.]

Current radiobiology challenges. Understanding the local and systemic mechanisms of radiation-induced or radiation-influenced tumor cell invasion and metastasis. Identification of targeted inhibitors of tumor cell invasion, colonization and/or metastatic development as adjuvants to radiotherapy.

MOVING FORWARD

The field of radiation biology continues to evolve. While the Hallmarks of Cancer describe the intricacies of the development and progression of neoplasms, we have reviewed the original discoveries, which have led to our understanding of how the tumor cells and microenviron-

FIG. 5. Hallmarks of cancer with associated radiation biology concepts. The featured examples of biological molecules, mechanisms, and effects reviewed as they pertain to radiation biology and therapy are summarized according to the expanded categorization of the hallmarks. [Reproduced and modified with permission. (1) Hanahan D, Weinberg RA. Cell 2011; 144, 646-74.]

ment react and change after exposure to ionizing radiation. Exciting new radiobiology research featured at the 59th annual meeting of the Radiation Research Society included presentations in the topics of radiation DNA damage and repair, the roles of miRNA in radiation response, effects of oxidative stress, radiation immune responses, inflammatory responses, vascular effects, the radiation responses of stem cells, radiation effects of normal tissue and microenvironmental radiation responses. While the laboratories of established, expert radiobiologists continue to make late-breaking discoveries, the next generation of radiation researchers is active, enthusiastic and eager to contribute meaningfully to the field.

CONCLUSION

Hanahan and Weinberg's Hallmarks of Cancer provides a useful conceptual framework for understanding the complex biology of cancer (1) and it is evident that intimate associations link each. The categorical focus of these cancer principles allows the specialized refinement of molecular mechanisms, however, the appreciation that each hallmark operates as part of a greater circuitry will promote discoveries that further advance radiation biology and therapy. The achievements of radiation biologists and clinicians featured in this review article mark particular

eras in interpretation of oncologic concepts (Fig. 5). It is stimulating and motivating to envision what current breakthroughs and innovations will be the radiation biology history of tomorrow.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH (CA40355-28) and T32 RR024394. The authors thank Gustavo S. Montana for his inspiration to prepare this review, which follows from a lecture on this same topic delivered at the 2011 ASTRO meeting.

Received: January 14, 2014; accepted: March 12, 2014; published online: May 8, 2014

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell 2011; 144:646-74.
2. Puck TT, Marcus PI. A rapid method for viable cell titration and clone production with hela cells in tissue culture - the use of x-irradiated cells to supply conditioning factors. Proc Natl Acad Sci U S A 1955; 41:432-37.
3. Marcus PI. A tribute to Dr. Theodore T. Puck - (September 24, 1916–November 6, 2005) - The genesis of "feeder cells": Concept and practice. In Vitro Cell Dev Biol Anim 2006; 42:235-37.
4. Earle WR, Bryan JC, Schilling EL. Certain factors limiting the size of the tissue culture and the development of massive cultures. Ann NY Acad Sci 1954; 58:1000.

5. Puck TT, Marcus PI, Cieciura SJ. Clonal growth of mammalian cells invitro - growth characteristics of colonies from single hela cells with and without a feeder layer. *J Exp Med.* 1956; 103:273-83.
6. Puck TT, Marcus PI. Action of x-rays on mammalian cells. *J Exp Med.* 1956; 103:653-66.
7. Puck TT, Morkovin D, Marcus PI, Cieciura SJ. Action of x-rays on mammalian cells. 2. survival curves of cells from normal human tissues. *J Exp Med.* 1957; 106:485-500.
8. Puck TT. Action of radiation on mammalian cells. 3. Relationship between Reproductive death and induction of chromosome anomalies by x-irradiation of euploid human cells invitro. *Proc Natl Acad Sci U S A* 1958; 44:772-80.
9. Puck TT. The action of radiation on mammalian cells. *Am Naturalist* 1960; 94:95-109.
10. Tjio JH, Puck TT. Genetics of somatic mammalian cells. 2. Chromosomal constitution of cells in tissue culture. *J Exp Med* 1958; 108:259-68.
11. Elkind MM, Sutton H. X-Ray damage and recovery in mammalian cells in culture. *Nature* 1959; 184:1293-95.
12. Barendsen GW, Beusker TLJ, Vergroesen AJ, Budke L. Effect of different ionizing radiations on human cells in tissue culture. 2. Biological experiments. *Radiat Res* 1960; 13:841-49.
13. Terasima T, Tolmach LJ. Variations in several responses of Hela cells to x-irradiation during division cycle. *Biophys J* 1963; 3:11-33.
14. Sinclair WK, Morton RA. Variations in X-ray response during division cycle of partially synchronized chinese hamster cells in culture. *Nature* 1963; 199:1158-60.
15. Hall EJ, Bedford JS. Dose rate - its effect on survival of hela cells irradiated with gamma rays. *Radiat Res* 1964; 22:305-15.
16. Barendse GW, Koot CJ, Vankerse GR, Bewley DK, Field SB, Parnell CJ. Effect of oxygen on impairment of proliferative capacity of human cells in culture by ionizing radiations of different let. *Int J Radiat Biol Relat Stud Phys Chem Med* 1966; 10:317-27.
17. Phillips RA, Tolmach LJ. Repair of potentially lethal damage in X-irradiated HeLa cells. *Radiat Res* 1966; 29:413-32.
18. Yamada M, Puck TT. Action of radiation on mammalian cells. 4. Reversible mitotic lag in S3 Hela cell produced by low doses of x-rays. *Proc Natl Acad Sci U S A* 1961; 47:1181-91.
19. Little JB. Delayed initiation of dna synthesis in irradiated human diploid cells. *Nature* 1968; 218:1064-65.
20. Painter RB, Robertson JS. Effect of irradiation and theory of role of mitotic delay on the time course of labeling of hela-s3-cells with tritiated thymidine. *Radiat Res* 1959; 11:206-17.
21. Whitmore GF, Gulyas S, Stanners CP, Till JE. Nucleic acid synthesis and division cycle in x-irradiated l-strain mouse cells. *Biochimica et biophysica acta* 1961; 47:66-77.
22. Leshner J, Leshner S. Effects of Single-dose partial-body x-irradiation on cell-proliferation in mouse small intestinal epithelium. *Radiat Res* 1974; 57:148-57.
23. Laasonen A, Pyrhonen S, Kouri M, Raty J, Holsti LR. The effect of fractionated-irradiation on cell-kinetics. *Acta Oncologica* 1991; 30:51-56.
24. Kastan MB, Zhan QM, Eldeiry WS, Carrier F, Jacks T, Walsh WV, et al. A mammalian-cell cycle checkpoint pathway utilizing p53 and gadd45 is defective in ataxia-telangiectasia. *Rid C-3063-2008 Rid A-7407-2008. Cell* 1992; 71:587-97.
25. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell-cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci U S A* 1992; 89:7491-95.
26. Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, et al. P53 status and the efficacy of cancer-therapy in-vivo. *Science* 1994; 266:807-10.
27. Russell KJ, Wiens LW, Demers GW, Galloway DA, Plon SE, Groudine M. Abrogation of the G(2) checkpoint results in differential radiosensitization of G(1) checkpoint-deficient and G(1) checkpoint-competent cells. *Cancer Res* 1995; 55:1639-42.
28. Oconnor PM, Jackman J, Jondle D, Bhatia K, Magrath I, Kohn KW. Role of the P53 tumor-suppressor gene in cell-cycle arrest and radiosensitivity of burkitts-lymphoma cell-lines. *Cancer Res* 1993; 53:4776-80.
29. Slichenmyer WJ, Nelson WG, Slebos RJ, Kastan MB. Loss of a P53-associated G1 checkpoint does not decrease cell-survival following dna-damage. *Cancer Res* 1993; 53:4164-68.
30. Xu Y, Baltimore D. Dual roles of ATM in the cellular response to radiation and in cell growth control. *Genes Development* 1996; 10:2401-10.
31. Kerr JFR, Wyllie AH, Currie AR. Apoptosis - basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26:239-57.
32. Lowe SW, Ruley HE, Jacks T, Housman DE. P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993; 74:957-67.
33. Merritt AJ, Potten CS, Kemp CJ, Hickman JA, Balmain A, Lane DP, et al. The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal-tract of normal and p53-deficient mice. *Rid C-4920-2008. Cancer Res* 1994; 54:614-17.
34. Haimovitz-Friedman A, Kan C, Ehleiter D, Persaud R, McLoughlin M, Fuks Z, et al. Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J Exp Med* 1994; 180:525.
35. HaasKogan DA, Yount G, Haas M, Levi D, Kogan SS, Hu L, et al. p53-Dependent G(1) arrest and p53-independent apoptosis influence the radiobiologic response of glioblastoma. *Int J Radiat Oncol Biol Phys* 1996; 36:95-103.
36. Merritt AJ, Allen TD, Potten CS, Hickman JA. Apoptosis in small intestinal epithelia from p53-null mice: Evidence for a delayed, p53-independent G2/M-associated cell death after gamma-irradiation. *Oncogene* 1997; 14:2759-66.
37. Liao WC, Haimovitz-Friedman A, Persaud RS, McLoughlin M, Ehleiter D, Zhang N, et al. Ataxia telangiectasia-mutated gene product inhibits DNA damage-induced apoptosis via ceramide synthase. *J Biol Chem* 1999; 274:17908-17.
38. Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ. Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* 1991; 67:879-88.
39. Strasser A, Harris AW, Cory S. Bcl-2 Transgene inhibits t-cell death and perturbs thymic self-censorship. *Cell* 1991; 67:889-99.
40. Strasser A, Harris AW, Jacks T, Cory S. Dna-damage can induce apoptosis in proliferating lymphoid-cells via p53-independent mechanisms inhibitable by bcl-2. *Cell* 1994; 79:329-39.
41. Tannock IF, Lee C. Evidence against apoptosis as a major mechanism for reproductive cell death following treatment of cell lines with anti-cancer drugs. *Br J Cancer* 2001; 84:100-05.
42. Paglin S, Hollister T, Delohery T, Hackett N, McMahon M, Sphicas E, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001; 61:439-44.
43. Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. *Cancer Res* 2008; 68:1485-94.
44. McClintock B. The significance of responses of the genome to challenge. *Science* 1984; 226:792-801.
45. Liang F, Romanienko PJ, Weaver DT, Jeggo PA, Jasin M. Chromosomal double-strand break repair in Ku80-deficient cells. *Proc Natl Acad Sci U S A* 1996; 93:8929-33.
46. Wang C, Lees-Miller SP. Detection and repair of ionizing radiation-induced dna double strand breaks: new developments in nonhomologous end joining. *Int J Radiat Oncol Biol Phys* 2013; 86:440-49.

47. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, et al. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells RID B-6568-2008. *Embo J* 1998; 17:5497–508.
48. Kakarougkas A, Jeggo P. DNA DSB repair pathway choice: an orchestrated handover mechanism. *The Br J Radiol* 2014; 87.
49. Liu N, Lamerdin JE, Tebbs RS, Schild D, Tucker JD, Shen MR, et al. XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages RID D-1997-2010. *Mol Cell* 1998; 1:783–93.
50. Chen J, Silver D, Walpita D, Cantor S, Gazdar A, Tomlinson G, et al. Stable interaction between the products of the BCRA1 and BCRA2 tumor suppressor genes in mitotic and meiotic cells. *Mol Cell* 1998; 2:317.
51. Jackson SP, Jeggo PA. DNA double-strand break repair and v(d)j recombination - involvement of dna-pk. *Trends Biochem Sci* 1995; 20:412–15.
52. Bassing CH, Chua KF, Sekiguchi J, Suh H, Whitlow SR, Fleming JC, et al. Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX. *Proc Natl Acad Sci U S A* 2002; 99:8173–78.
53. Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, et al. Genomic instability in mice lacking histone H2AX. *Science* 2002; 296:922–27.
54. Panier S, Ichijima Y, Fradet-Turcotte A, Leung CCY, Kaustov L, Arrowsmith CH, et al. Tandem protein interaction modules organize the ubiquitin-dependent response to dna double-strand breaks. *Molecular cell* 2012; 47:383–95.
55. Fulop GM, Phillips RA. The scid mutation in mice causes a general defect in dna-repair. *Nature* 1990; 347:479–82.
56. Biedermann KA, Sun JR, Giaccia AJ, Tosto LM, Brown JM. Scid mutation in mice confers hypersensitivity to ionizing-radiation and a deficiency in dna double-strand break repair. *Proc Natl Acad Sci U S A* 1991; 88:1394–97.
57. Kennedy AR, Fox M, Murphy G, Little JB. Relationship between x-ray-exposure and malignant transformation in c3h 10t1/2 cells. *Proc Natl Acad Sci U S A-Biological Sciences* 1980; 77:7262–66.
58. Little JB. Radiation-induced genomic instability. *Int J Radiat Biol* 1998; 74:663–71.
59. Little JB, Nagasawa H, Pfenning T, Vetrovs H. Radiation-induced genomic instability: Delayed mutagenic and cytogenetic effects of X rays and alpha particles. *Radiat Res* 1997; 148:299–307.
60. Smith KA, Stark MB, Gorman PA, Stark GR. Fusions near telomeres occur very early in the amplification of cad genes in syrian-hamster cells. *Proc Natl Acad Sci U S A* 1992; 89:5427–31.
61. Alvarez L, Evans JW, Wilks R, Lucas JN, Brown JM, Giaccia AJ. Chromosomal radiosensitivity at intrachromosomal telomeric sites. *Genes Chrom Cancer* 1993; 8:8–14.
62. Chalmers AJ, Lakshman M, Chan N, Bristow RG. Poly(ADP-Ribose) Polymerase inhibition as a model for synthetic lethality in developing radiation oncology targets. *Sem Radiat Oncol* 2010; 20:274–81.
63. Thoms J, Bristow RG. DNA Repair Targeting and radiotherapy: a focus on the therapeutic Ratio. *Sem Radiat Oncol* 2010; 20:217–22.
64. McBride WH, Chiang CS, Olson JL, Wang CC, Hong JH, Pajonk F, et al. Failla Memorial Lecture - A sense of danger from radiation RID A-9562-2008 RID E-4247-2010. *Radiat Res* 2004; 162:1–19.
65. Trowell OA. The sensitivity of lymphocytes to ionising radiation. *J Pathol Bacteriol* 1952; 64:687–704.
66. Wu NZ, Ross BA, Gullledge C, Klitzman B, Dodge R, Dewhirst MW. Differences in leukocyte-endothelium interactions between normal and adenocarcinoma bearing tissues in response to radiation. *Br J Cancer* 1994; 69:883–89.
67. Hallahan D, Kuchibhotla J, Wyble C. Cell adhesion molecules mediate radiation-induced leukocyte adhesion to the vascular endothelium. *Cancer Res* 1996; 56:5150–55.
68. Hallahan DE, Geng L, Cmelak AJ, Chakravarthy AB, Martin W, Scarfone C, et al. Targeting drug delivery to radiation-induced neoantigens in tumor microvasculature. *J Controlled Release* 2001; 74:183–91.
69. Liu SZ. Radiation Hormesis - a New Concept in Radiological Science. *Chinese Med J* 1989; 102:750–55.
70. Demaria S, Formenti SC. Sensors of ionizing radiation effects on the immunological microenvironment of cancer RID A-8959-2008. *Int J Radiat Biol* 2007; 83:819–25.
71. Mason KA, Ariga H, Neal R, Valdecanas D, Hunter N, Krieg AM, et al. Targeting Toll-like receptor 9 with CpG oligodeoxynucleotides enhances tumor response to fractionated radiotherapy. *Clin Cancer Res* 2005; 11:361–69.
72. Kingsley D. An interesting case of possible abscopal effect in malignant melanoma. *Br J Radiol* 1975; 48:863–66.
73. Robins HI, Buchon JA, Varanasi V, Weinstein AB. The abscopal effect: demonstration in lymphomatous involvement of kidneys. *Med Pediatric Oncol* 1981; 9:473–76.
74. Wersäll PJ, Blomgren H, Pisa P, Lax I, Kalkner K-M, Svedman C. Regression of non-irradiated metastases after extracranial stereotactic radiotherapy in metastatic renal cell carcinoma. *Acta Oncologica* 2006; 45:493–97.
75. Drake CG. Radiation-induced immune modulation. Molecular determinants of radiation response. DeWeese, T. and Laiho M, ed. Springer; 2011.
76. Postow MA, Callahan MK, Barker CA, Yamada Y, Yuan J, Kitano S, et al. Immunologic correlates of the abscopal effect in a patient with melanoma. *New Eng J Med* 2012; 366:925–31.
77. Cheers C, Waller R. Activated Macrophages in congenitally athymic nude mice and in lethally irradiated mice. *J Immunology* 1975; 115:844–47.
78. Gallin EK, Green SW, Sheehy PA. Enhanced Activity of the macrophage-like cell-line j7741 following exposure to gamma-radiation. *J Leukocyte Biol* 1985; 38:369–81.
79. Gallin EK, Green SW. Exposure to gamma-irradiation increases phorbol-myristate acetate induced h2o2 production in human macrophages. *Blood* 1987; 70:694–701.
80. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Shepard HM. Recombinant human-tumor necrosis factor-alpha - effects on proliferation of normal and transformed-cells in vitro. *Science* 1985; 230:943–45.
81. Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, et al. Human-tumor necrosis factor - precursor structure, expression and homology to lymphotoxin. *Nature* 1984; 312:724–29.
82. Hallahan DE, Spriggs DR, Beckett MA, Kufe DW, Weichselbaum RR. Increased tumor necrosis factor-alpha messenger-rna after cellular exposure to ionizing-radiation. *Proc Natl Acad Sci U S A* 1989; 86:10104–07.
83. Sersa G, Willingham V, Milas L. Anti-tumor effects of tumor necrosis factor alone or combined with radiotherapy. *Int J Cancer* 1988; 42:129–34.
84. Ibuki Y, Goto R. Enhancement of NO production from resident peritoneal macrophages by in vitro gamma-irradiation and its relationship to reactive oxygen intermediates. *Free Radic Biol Med* 1997; 22:1029–35.
85. McKinney LC, Aquilla EM, Coffin D, Wink DA, Vodovotz Y. Ionizing radiation potentiates the induction of nitric oxide synthase by IFN-gamma and/or LPS in murine macrophage cell lines: role of TNF-alpha. *J Leukocyte Biol* 1998; 64:459–66.

86. Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low-doses of alpha-particles. *Cancer Res* 1992; 52:6394-96.
87. Prise KM, Belyakov OV, Folkard M, Michael BD. Studies of bystander effects in human fibroblasts using a charged particle microbeam. *Int J Radiat Biol* 1998; 74:793-98.
88. Wu LJ, Randers-Pehrson G, Xu A, Waldren CA, Geard CR, Yu ZL, et al. Targeted cytoplasmic irradiation with alpha particles induces mutations in mammalian cells. *Proc Natl Acad Sci U S A* 1999; 96:4959-64.
89. Mothersill C, Seymour C. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *Int J Radiat Biol* 1997; 71:421-27.
90. Lehnert BE, Goodwin EH. Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells. *Cancer Res* 1997; 57:2164-71.
91. Wright EG. Radiation-induced genomic instability in haemopoietic cells. *Int J Radiat Biol* 1998; 74:681-87.
92. Mothersill C, Seymour CB. Radiation-induced bystander effects - implications for cancer. *Nature Rev Cancer* 2004; 4:158-64.
93. Sandison JC. Observations on the growth of blood vessels as seen in the transparent chamber introduced into the rabbit's ear. *Am J Anatomy* 1928; 41:475-96.
94. Ide Ag BNHWSL. Vascularization of the brown-pearce rabbit epithelioma transplant as seen in the transplant ear chamber. *Am J Roentgenol* 1939; 42:891-99.
95. Algire GH, Chalkley HW, Earle WR, Legallais FY, Park HD, Shelton E, et al. Vascular reactions of normal and malignant tissues in vivo. 3. Vascular reactions of mice to fibroblasts treated invitro with methylcholanthrene. *J National Cancer Ins* 1950; 11:555-79.
96. Gray LH, Conger AD, Ebert M, Homsey S, Scott OCA. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol* 1953; 26:638-48.
97. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer* 1955; 9:539-49.
98. Rubin P, Casarett G. Microcirculation of tumors part II: the supervascularized state of irradiated regressing tumors. *Clin Radiol* 1966; 17:356-55.
99. Gridley DS, Loreda LN, Slater JD, Archambeau JO, Bedros AA, Andres ML, et al. Pilot evaluation of cytokine levels in patients undergoing radiotherapy for brain tumor. *Cancer detection and prevention* 1998; 22:20-29.
100. Gorski DH, Beckett MA, Jaskowiak NT, Calvin DP, Mauceri HJ, Salloum RM, et al. Blockade of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Res* 1999; 59:3374-78.
101. Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S, Haimovitz-Friedman A, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 2003; 300:1155-59.
102. Moeller BJ, Cao YT, Li CY, Dewhirst MW. Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: Role of reoxygenation, free radicals, and stress granules. *Cancer Cell* 2004; 5:429-41.
103. Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends in molecular medicine* 2002; 8:S62-S67.
104. Ahn GO, Brown JM. Influence of bone marrow-derived hematopoietic cells on the tumor response to radiotherapy. Experimental models and clinical perspectives. *Cell Cycle* 2009; 8:970-76.
105. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005; 307:58-62.
106. Goel S, Wong AH-K, Jain RK. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. *Cold Spring Harbor perspectives in medicine* 2012; 2. (<http://perspectivesinmedicine.org/content/2/3/a006486.full.pdf+html>)
107. Lamerton LF. Radiation biology and cell population kinetics. *Phys Med Biol* 1968; 13:1.
108. Hermens AF, Barendse GW. Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after x-irradiation. *Eur J Cancer* 1969; 5:173-89.
109. Withers H, Taylor J, Maciejewski B. The hazard of accelerated tumor clonogen repopulation during radiotherapy. *Acta Oncologica* 1988; 27:131.
110. Kwok TT, Sutherland RM. Differences in Egf related radiosensitization of human squamous carcinoma-cells with high and low numbers of Egf receptors. *Br J Cancer* 1991; 64:251-54.
111. Akimoto T, Hunter NR, Buchmiller L, Mason K, Ang KK, Milas L. Inverse relationship between epidermal growth factor receptor expression and radiocurability of murine carcinomas. *Clin Cancer Res* 1999; 5:2884-90.
112. Ang KK, Berkey BA, Tu XY, Zhang HZ, Katz R, Hammond EH, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res* 2002; 62:7350-56.
113. Schmidt-Ullrich R, Valerie K, Chan W, McWilliams D. Altered expression of epidermal growth factor receptor and estrogen receptor in MCF-7 cells after single and repeated radiation exposures. *Int J Radiat Oncol Biol Phys* 1994; 29:813.
114. Schmidt-Ullrich R, Mikkelsen R, Dent P, Todd D, Valerie K, Kavanagh B, et al. Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. *Oncogene* 1997; 15:1191.
115. Wollman R, Yahalom J, Maxy R, Pinto J, Fuks Z. Effect of epidermal growth-factor on the growth and radiation sensitivity of human breast-cancer cells in-vitro. *Int J Radiat Oncol Biol Phys* 1994; 30:91-98.
116. Balaban N, Moni J, Shannon M, Dang LO, Murphy E, Goldkorn T. The effect of ionizing radiation on signal transduction: Antibodies to EGF receptor sensitize A431 cells to radiation. *Biochimica Et Biophysica Acta-Molecular Cell Research* 1996; 1314:147-56.
117. Bourhis J, Overgaard J, Audry H, Ang KK, Saunders M, Bernier J, et al. Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis. *Lancet* 2006; 368:843-54.
118. Huang SM, Bock JM, Harari PM. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* 1999; 59:1935-40.
119. Milas L, Mason K, Hunter N, Petersen S, Yamakawa M, Ang K, et al. In vivo enhancement of tumor radioresponse by C225 anti-epidermal growth factor receptor antibody. *Clin Cancer Res* 2000; 6:701-08.
120. Huang SM, Li J, Armstrong EA, Harari PM. Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). *Cancer Res* 2002; 62:4300-06.
121. Bianco C, Tortora G, Bianco R, Caputo R, Veneziani BM, Caputo R, et al. Enhancement of antitumor activity of ionizing radiation by combined treatment with the selective epidermal growth factor receptor-tyrosine kinase inhibitor ZD1839 (Iressa). *Clin Cancer Res* 2002; 8:3250-58.
122. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *New Eng J Med* 2006; 354:567-78.
123. Bussink J, van der Kogel AJ, Kaanders JHAM. Activation of the PI3-K/AKT pathway and implications for radioresistance mechanisms in head and neck cancer. *Lancet Oncol* 2008; 9:288-96.
124. Huang Q, Li F, Liu X, Li W, Shi W, Liu F-F, et al. Caspase 3-

- mediated stimulation of tumor cell repopulation during cancer radiotherapy. *Nat Med* 2011; 17:860–U231.
125. Demetrius LA, Coy JF, Tuszynski JA. Cancer proliferation and therapy: the Warburg effect and quantum metabolism. *Theoretical Biol Med Modelling* 2010; 7:2–2.
 126. Gordan JD, Thompson CB, Simon MC. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell* 2007; 12:108–13.
 127. Griffiths JR, McSheehy PMJ, Robinson SP, Troy H, Chung YL, Leek RD, et al. Metabolic changes detected by in vivo magnetic resonance studies of HEPA-1 wild-type tumors and tumors deficient in hypoxia-inducible factor-1 beta (HIF-1 beta): Evidence of an anabolic role for the HIF-1 pathway. *Cancer Res* 2002; 62:688–95.
 128. Chen CH, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of *glut1* mRNA by hypoxia-inducible factor-1 - Interaction between H-ras and hypoxia. *J Biol Chem* 2001; 276:9519–25.
 129. Moeller BJ, Dreher MR, Rabbani ZN, Schroeder T, Cao YT, Li CY, et al. Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. *Cancer Cell* 2005; 8:99–110.
 130. Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: Implications for tumor metabolism and growth. *Proc Natl Acad Sci U S A* 1997; 94:6658–63.
 131. Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science* 2010; 330:1340–44.
 132. Pelicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. *Oncogene* 2006; 25:4633–46.
 133. Mohanti BK, Rath GK, Anantha N, Kannan V, Das BS, Chandramouli BAR, et al. Improving cancer radiotherapy with 2-deoxy-D-glucose: Phase I/II clinical trials on human cerebral gliomas. *Int J Radiat Oncol Biol Phys* 1996; 35:103–11.
 134. Singh D, Banerji AK, Dwarakanath BS, Tripathi RP, Gupta JP, Mathew TZ, et al. Optimizing cancer radiotherapy with 2-deoxy-D-glucose. *Strahlenther Onkol* 2005; 181:507.
 135. Kaplan HS, Murphy ED. The effect of local roentgen irradiation on the biological behavior of a transplantable mouse carcinoma. 1. Increased frequency of pulmonary metastasis. *J National Cancer Inst* 1949; 9:407–13.
 136. Strong MS, Vaughan CW, Kayne HL, Aral IM, Ucmakli A, Feldman M, et al. Randomized trial of preoperative radiotherapy in cancer of oropharynx and hypopharynx. *Am J Surgery* 1978; 136:494–500.
 137. Anderson P, Dische S. Local tumor-control and the subsequent incidence of distant metastatic disease. *Int J Radiat Oncol Biol Phys* 1981; 7:1645–48.
 138. Fagundes H, Perez CA, Grigsby PW, Lockett MA. Distant metastases after irradiation alone in carcinoma of the uterine cervix. *Int J Radiat Oncol Biol Phys* 1992; 24:197–204.
 139. Hockel M, Knoop C, Schlenger K, Vorndran B, Baussmann E, Mitze M, et al. Intratumoral Po₂ predicts survival in advanced cancer of the uterine cervix. *Radiother Oncol* 1993; 26:45–50.
 140. Schwickert G, Walenta S, Sundfor K, Rofstad EK, Muellerklieser W. Correlation of high lactate levels in human cervical-cancer with incidence of metastasis. *Cancer Res* 1995; 55:4757–59.
 141. Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, et al. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. *Cancer Res* 1996; 56:941–43.
 142. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996; 56:4509–15.
 143. Brizel DM, Sibley GS, Prosnitz LR, Scher RL, Dewhirst MW. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int J Radiat Oncol Biol Phys* 1997; 38:285–89.
 144. Brizel DM, Dodge RK, Clough RW, Dewhirst MW. Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. *Radiother Oncol* 1999; 53:113–17.
 145. Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW, et al. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. *Int J Radiat Oncol Biol Phys* 2001; 51:349–53.
 146. Sullivan R, Graham CH. Hypoxia-driven selection of the metastatic phenotype. *Cancer Metastasis Rev* 2007; 26:319–31.
 147. Krishnamachary B, Zagzag D, Nagasawa H, Rainey K, Okuyama H, Baek JH, et al. Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFX1A, and ZFX1B. *Cancer Res* 2006; 66:2725–31.
 148. Kuphal S, Bosserhoff AK. Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma. *Oncogene* 2006; 25:248–59.
 149. Graham CH, Fitzpatrick TE, McCrae KR. Hypoxia stimulates urokinase receptor expression through a heme protein-dependent pathway. *Blood* 1998; 91:3300–07.
 150. Koochekpour S, Jeffers M, Wang PH, Gong CN, Taylor GA, Roessler LM, et al. The von Hippel-Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. *Molecular Cell Biol* 1999; 19:5902–12.
 151. Oh RR, Park JY, Lee JH, Shin MS, Kim HS, Lee SK, et al. Expression of HGF/SF and Met protein is associated with genetic alterations of VHL gene in primary renal cell carcinomas. *Apmis* 2002; 110:229–38.
 152. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth-factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992; 359:843–45.
 153. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* 2010; 29:625–34.