

# At the Crossroads of Cancer Stem Cells, Radiation Biology, and Radiation Oncology

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## Abstract

Reports that a small subset of tumor cells initiate and sustain tumor growth, are resistant to radiation and drugs, and bear specific markers have led to an explosion of cancer stem cell research. These reports imply that the evaluation of therapeutic response by changes in tumor volume is misleading, as volume changes reflect the response of the sensitive rather than the resistant tumorigenic cell population. The reports further suggest that the marker-based selection of the tumor cell population will facilitate the development of radiation treatment schedules, sensitizers, and drugs that specifically target the resistant tumorigenic cells that give rise to treatment failure.

This review presents evidence that contests the observations that cancer stem cell markers reliably identify the subset of tumor cells that sustain tumor growth and that the marker-identified population is radioresistant relative to the marker-negative cells. Experimental studies show that cells and tumors that survive large radiation doses are not more radioresistant than unirradiated cells and tumors, and also show that the intrinsic radiosensitivity of unsorted colony-forming tumor cells, in combination with the fraction of unsorted tumor cells that are tumor initiating, predicts tumor radiocurability. *Cancer Res*; 76(5); 994–8. ©2016 AACR.

## Introduction

Reports that a small percentage of tumor cells are tumorigenic, bear specific markers, and are treatment resistant have stimulated and sustained cancer stem cell research for the past dozen years. The reports suggest that the assessment of treatment efficacy by changes in tumor volume is misleading, as volume changes reflect the response of the predominant sensitive non-stem tumor cell population rather than the resistant tumor initiating and sustaining population. The reports also form the basis for designing treatments that specifically target the marker-bearing tumor subpopulation. This article presents evidence that contests the notion that cancer stem cell markers identify all tumorigenic cells and that the marker identified population is resistant to radiation. Specifically, studies indicate that (i) recurrent tumors are equally or more radiocurable than parental nonirradiated tumors, (ii) cells from tumors surviving large subcurative doses of radiation are not more radioresistant than cells from unirradiated tumors, and (iii) the slopes of radiation dose versus percent tumor cure curves, do not differ from the slopes of radiation dose versus percent survival of nonselected *in vitro* colony-forming tumor cells. Furthermore, studies show that the number of tumor-initiating cells per tumor, in combination with the *in vitro* measured radiation sensitivity of nonselected colony-forming tumor cells

from the same tumor, predicts the radiation control dose of isografted murine and xenografted human tumors.

## Not All Tumor Cells Are Tumorigenic, and the Fraction That Is, Is Dynamic

In 1973, Hewitt and colleagues reported that the number of injected cells from five spontaneous murine tumors that was needed to achieve a 50% successful transplantation take rate (TD<sub>50</sub>), in recipient syngeneic mice, ranged from 21 cells to 24,000 cells (1). That is, the fraction of injected cells that was tumor initiating ranged from approximately 1 in 21 to 1 in 24,000. Additionally, and similar to previously reported *in vitro* studies (2), the study demonstrated that the expression of a cell's tumorigenic potential was influenced by its microenvironment. Specifically, when unirradiated tumor cells were mixed with lethally irradiated tumor cells immediately prior to injection, the number of unirradiated cells needed to initiate tumors decreased in 4 of the 5 tumor types. For example, the TD<sub>50</sub> decreased from 190 cells to 14 cells, and for another, from 6,900 cells to 4.4 cells. Similar to the impact of lethally irradiated cells, Matrigel, a matrix-like protein substance containing various growth factors, also reduces the number of injected tumor cells needed to initiate tumors in immunodeficient mice (3, 4). Thus, while only a fraction of tumor cells appear to be capable of initiating and sustaining tumor growth, the expression of the tumorigenic potential is dependent on microenvironmental factors.

## The Size of the Tumor-Initiating Cell Fraction Impacts Radiocurability

Hill and Milas evaluated the relationship between the fraction of tumor cells that were tumor initiating and the tumor's radiocurability (5). A significant correlation was observed between the tumorigenic fraction of 25 spontaneous murine

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tumors and the radiation dose required to achieve permanent local tumor control ( $P = 0.01$ ). Additionally, the relationship between the fraction of injected tumor cells capable of initiating tumors and the fraction of the same cells that formed colonies *in vitro* was examined in a subset of 12 spontaneous mammary carcinomas. Although the fraction of cells that formed colonies was larger than the fraction that initiated tumors, the two values significantly correlated ( $P = 0.01$ ). The study thus demonstrated a significant relationship between the fraction of tumor cells capable of forming tumors *in vivo*, the fraction that formed colonies *in vitro*, and the tumor control dose. These data generally support the cancer stem cell hypothesis.

### Do Cancer Stem Cell Markers Identify All and Only Cancer Stem Cells?

Commonly cited cancer stem cell markers include cell-surface proteins (e.g., CD24, CD44, and CD133), cells exhibiting an enhanced capacity for exclusion of dyes such as Hoechst 33342, and cells exhibiting an elevated activity of the enzyme aldehyde dehydrogenase (ALDH). A mechanistic relationship between the various markers and cancer stem cell characteristics such as indefinite self-renewal and radiation sensitivity remains under investigation but is not well established (6–8).

In seminal studies, Al-Hajj and colleagues reported that greater than 1,000 nonmarker-selected cells from xenografts were required for the initiation of orthotopic tumors in nonobese diabetic SCID (NOD/SCID) mice, whereas as few as 100 CD<sup>44+</sup>CD24<sup>-low</sup> cells were capable of initiating tumors in the same mice (9). Ponti and colleagues (10) reported that culturing breast tumor cells under conditions used to grow normal breast stem cells lead to the growth of nonadherent spheres. Ninety-five to 98% of mammosphere cells expressed the CD<sup>44+</sup>CD24<sup>-low</sup> profile. Tumors could be initiated by the injection of 3,000 mammosphere cells versus greater than 100,000 unselected cells from the MCF7 mammary tumor cell line. In contrast to these results, in estrogen receptor-negative human breast cancers, Meyer and colleagues (11) reported that both CD<sup>44+</sup>CD24<sup>-low</sup> and CD<sup>44+</sup>CD24<sup>+</sup> cells were equally tumorigenic in NOD/SCID mice, while cells selected for a three-marker combination, CD<sup>44+</sup>CD49<sup>hi</sup>CD133/2<sup>hi</sup>, exhibited even greater tumorigenicity. Additionally, cells from different xenografts, all bearing the same marker, exhibited notable differences in tumorigenicity. Ginestier and colleagues reported that 50,000 CD<sup>44+</sup>CD24<sup>-low</sup>Lineage<sup>-</sup>ALDH<sup>-</sup> breast cancer cells were not tumorigenic when injected in humanized NOD/SCID mice, whereas in the subset of CD<sup>44+</sup>CD24<sup>-low</sup>Lineage<sup>-</sup> cells exhibiting high ALDH1 activity, as few as 20 tumor cells were capable of initiating tumors (12).

Concurrent with these breast tumor studies, it was reported that as pertained to normal brain, a small fraction of cells from brain tumors cultured in low-attachment culture dishes without serum but supplemented with growth factors gave rise to the growth of cell spheres (13–16). Cells expressing the stem cell marker CD133<sup>+</sup> were reported to be capable of forming neurospheres, but not CD133<sup>-</sup> cells (14). Singh and colleagues reported that as few as 100 CD133<sup>+</sup> cells were capable of initiating brain tumors in NOD/SCID mice whereas no tumors arose from  $5 \times 10^4$  to  $10^5$  CD133<sup>-</sup> cells (17). Similarly, Bao and colleagues reported that 300 and 1,000 CD133<sup>+</sup> cells from xenografts and primary human glioblastomas were able

to initiate intracranial tumors in NOD/SCID mice, whereas  $2 \times 10^6$  CD133<sup>-</sup> cells were not tumor initiating (18). Subsequent studies, however, have reported that both CD133<sup>-</sup> and CD133<sup>+</sup> brain tumor cells are tumorigenic (19).

To summarize, the percentage of tumorigenic cells in a marker selected population is generally substantially higher than in a nonselected or marker-negative population. Nevertheless, the majority of marker selected cells are not tumorigenic, and marker-negative cells may also exhibit tumorigenicity. Additionally, cells from different tumors of the same tumor type, although bearing the same marker or marker combination, are not equally tumorigenic. References (6, 7, 20) further examine the distribution of proposed cancer stem cell markers in tumors and normal tissues as well as marker specificity and the tumorigenicity of marker-bearing cells.

### Are Tumors and Tumor Cells Surviving Large Doses of Radiation Radioresistant?

The small percentage of marker-bearing tumorigenic cells within a much larger population of marker-bearing nontumorigenic cells confounds an assessment of the radiation sensitivity of the tumorigenic cells based on marker expression. This pertains regardless of the response metric. While an increase in the fraction of cells exhibiting a marker following irradiation may be indicative of greater resistance of the marker-bearing cells, the assay requires the passage of several hours to days between radiation and marker quantification, to clear the radiation sterilized cells from the analyzed population. The findings are also contingent on the absence of radiation-induced reprogramming of marker-negative tumor cells into marker-positive cells, as has been reported previously (21, 22). In short, increased marker expression following irradiation is not equivalent to an evaluation of the radiation sensitivity of demonstrably tumorigenic cells that survive irradiation.

Suit (23) exposed isotransplants of a spontaneous murine mammary tumor to various dose levels. In tumors receiving a TCD<sub>95</sub> dose (the dose that on average achieved permanent local control of 95% of treated tumors), a recurrent tumor was excised and isotransplanted into syngeneic mice. The TCD<sub>50</sub> of the recurrent transplanted tumor was lower than pertained to unirradiated tumors, i.e., 51.3 versus 59.9 Gy. Ando and colleagues (24) reported that the TCD<sub>50</sub> of a recurrent isotransplanted spontaneous fibrosarcoma was substantially lower than the parental tumor, i.e., 58.0 versus 78.9 Gy. The tumors' stem cell fractions, resolved by the TD<sub>50</sub> transplantation assay, were similar in the nonirradiated control and recurrent tumors. Majima and colleagues (25) also found that the radiosensitivity of tumor clones, isolated from recurrent murine tumors following large subcurative doses of radiation, were not more radioresistant than clones from unirradiated tumors. The enhanced radiocurability of heavily irradiated recurrent tumors is consistent with earlier *in vitro* studies first reported by Sinclair (26). An increasing fraction of cells surviving increasingly large single radiation doses exhibited increasing radiosensitivity, i.e., "heritable non-lethal damage."

Studies reporting a similar or greater sensitivity of tumors and cells surviving large single-fraction irradiation do not preclude the possibility that multiple smaller doses of radiation administered over days to weeks may select for a preexisting relatively resistant tumorigenic subpopulation. This possibility

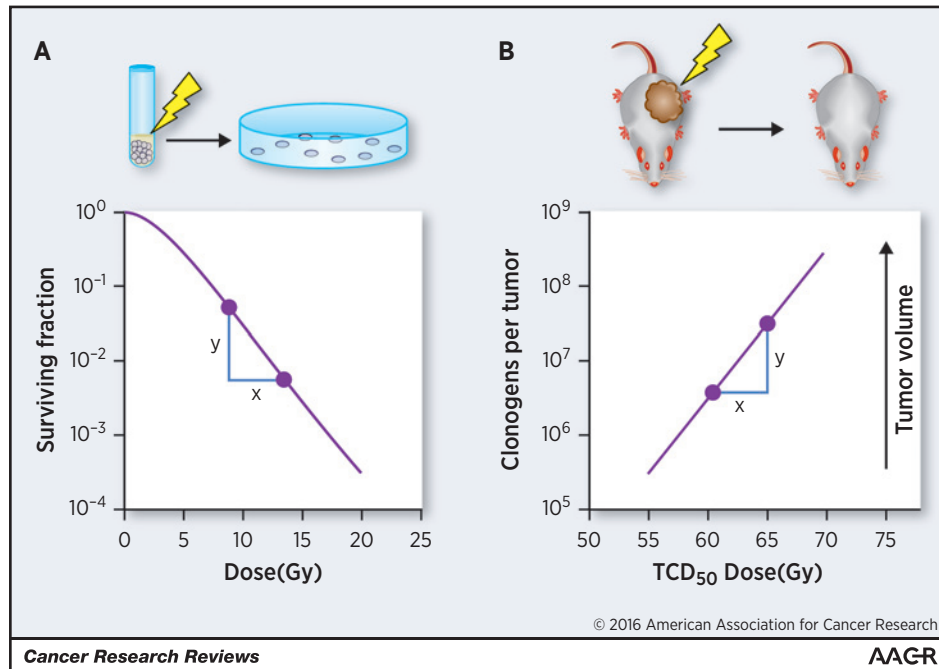
warrants investigation; however, it is informative to note that Yaromina and colleagues reported a highly significant correlation between the single-dose TCD<sub>50</sub> of 8 human squamous cell carcinomas and their 30 fraction TCD<sub>50</sub> values ( $R = 0.91$ ;  $P = 0.002$ ; ref. 27).

### **In Vitro Colony-Forming Tumor Cells and Tumorigenic Cells In Situ Are Equally Sensitive to Radiation**

In 2006, Bao and colleagues reported that CD133<sup>+</sup> brain tumor-initiating cells were radioresistant compared with CD133<sup>-</sup> cells (18). Several subsequent studies reported greater resistance of marker-positive versus marker-negative cells from brain, breast, and other tumor types (28–35), with various mechanisms being proposed as the cause of radioresistance (29). However, other investigators have not found CD133<sup>+</sup> or CD44<sup>+</sup>CD24<sup>-</sup> tumor cells to be more resistant than their marker-negative counterparts (36–41). For example, in early passaged xenografts, Zielske and colleagues reported the enrichment of the CD44<sup>+</sup>CD24<sup>-</sup>Lin<sup>-</sup>ALDH1<sup>+</sup> breast tumor stem cell population 2 weeks following irradiation, suggesting resistance and survival of the marker-identified population (36). In contrast, radiation led to the depletion of CD44<sup>+</sup>CD24<sup>-</sup>Lin<sup>-</sup>ALDH1<sup>+</sup> cells in xenografts initiated from a different patient.

The colony formation assay has long been considered the gold-standard *in vitro* assay for assessing the intrinsic radiosens-

sitivity of tumor cells. Few, if any, of the thousands of published *in vitro* radiation survival curves exhibit a decrease in slope indicative of a small resistant subpopulation of cells at high radiation doses. In addition to *in vitro* colony formation studies, the TCD<sub>50</sub> assay provides a powerful quantitative tool for directly assessing the sensitivity of tumorigenic cells in their *in situ* environment. Rofstad and colleagues exposed human melanoma xenografts to a range of doses and measured the slope of a curve fit to percent tumor cure versus dose. The slopes of the curves did not differ from the slopes of curves fit to percent surviving fraction of cells from the same tumors that were exposed to various doses of radiation *in vivo*, and then plated for *in vitro* colony formation (42). Baumann and colleagues examined the *in situ* cell sensitivity of the human squamous cell carcinoma FaDu by evaluation of the TCD<sub>50</sub> of tumors of increasing size and number of colony-forming cells (43). After adjusting for differences in the sensitivity of cells irradiated under oxygenated conditions *in vitro*, and hypoxic conditions *in vivo*, their study showed that the slope of a curve fit to the dose to achieve 50% tumor control versus clonogens per tumor did not differ from the survival curve slope of FaDu cells irradiated *in vitro*. Interrogation and validation of the equivalent sensitivity of colony-forming tumor cells and the *in situ* sensitivity of tumorigenic cells is illustrated in Fig. 1. Studies by both Rofstad and colleagues and Baumann and colleagues demonstrated that the radiation sensitivity of colony-forming tumor cells and cells that lead to treatment failure if not sterilized did not differ.



**Figure 1.**

Key features of the relationship between radiation dose and the loss of cell reproductive capacity *in vitro* and *in vivo*. A, *in vitro*, a linear increase in dose results in an exponential decrease in the surviving fraction of colony-forming tumor cells. The presence of a small radiation-resistant subpopulation of cancer stem cells in the unsorted tumor cell population would result in a decrease in the slope of the survival curve at high doses. This is not observed. B, the same nonselected tumor cells are used to initiate tumors and the dose required for permanent local tumor control in 50% of treated mice (TCD<sub>50</sub>) is determined. The tumor control dose increases with increasing tumor size and number of colony-forming cells per tumor. For similarly oxygenated tumors and cells *in vitro*, the slope of the change in dose needed to compensate for an increase in the number of clonogens per tumor ( $y/x$  in B) does not differ from the change in dose needed to sterilize the same fraction of tumor cells *in vitro* ( $y/x$  in A). Thus, the radiation sensitivity of unsorted colony-forming tumor cells *in vitro* is the same as that of cells that give rise to treatment failure if not sterilized *in vivo*.

## The Number of Tumorigenic Cells per Tumor and Their *In Vitro*-Measured Radiation Sensitivity Determine the Tumor Control Dose

Treatment failure occurs secondary to the survival and growth of one or more tumor-initiating cells. If the radiation sensitivity of unsorted *in vitro* colony-forming tumor cells does not differ from the sensitivity of the tumorigenic cells *in situ*, with knowledge of tumor volume, approximate number of tumor cells per unit tumor volume, and the fraction of tumor cells that are tumorigenic, an equation based on standard radiobiologic principles should predict a tumor's 50% tumor control dose (44). To assess this possibility, the tumorigenic fraction of four spontaneous murine tumors in syngeneic mice and two human tumors in nude mice was evaluated by the TD<sub>50</sub> assay. These data were combined with the *in vitro* radiation sensitivity of the unsorted tumor cells from the tumors, to predict the tumors' 50% tumor control doses. The number of tumor-initiating cells per tumor and their intrinsic radiosensitivity predicted the measured 50% tumor control doses with a rank-order correlation coefficient of 1.0,  $P = < 0.01$ . Adjusting for the oxygen-dependent difference in radiosensitivity between uniformly oxygenated cells *in vitro* and uniformly hypoxic tumors, the average difference between the predicted and measured TCD<sub>50</sub> values was approximately 3 Gy (44). These results provide evidence that elimination of the tumorigenic cell fraction is required for the successful radiotherapy of cancer, and these tumorigenic cells are not more resistant than the bulk colony-forming tumor cells.

### Summary

- The size of the tumorigenic cell fraction is tumor specific and is significantly influenced by the tumor cells' microenvironment.
- Only a small subset of marker-positive cells initiate tumors upon injection into immunodeficient mice.

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- A pan tumor-marker or marker combination identifying all and only tumorigenic cells in individual tumors of the same type or across tumor types has not been identified.
- A positive correlation exists between the fraction of unselected tumor cells capable of initiating tumors, the fraction of the same cells that form colonies *in vitro*, and the radiation dose required to sterilize tumors.
- Recurrent tumors surviving large single-fraction doses of radiation are equally or more curable by radiation than their parental nonirradiated tumor.
- The slope of the percentage of tumor cure versus dose curves measured *in vivo* does not differ from the surviving fraction versus dose curves of unselected colony-forming cells measured *in vitro*.
- The number of tumor-initiating cells per tumor and their radiation sensitivity predicts the radiation dose required to achieve permanent local tumor control.

These results indicate that colony-forming tumor cells constitute an appropriate cell population for targeting, enhancing, and quantifying the radiation sensitivity of tumor cells, which give rise to treatment failure if not sterilized. They caution against over-reliance on "markers" for the identification of the tumorigenic cell population and its radiosensitivity.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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