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QUIESCENT S-PHASE CELLS AS INDICATORS OF EXTREME PHYSIOLOGICAL CONDITIONS IN HUMAN TUMOR XENOGRAFTS

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Purpose: During the last 20 years, evidence has been accumulating for the existence in animal and human tumors of quiescent S-phase cells, i.e. cells with an S-phase DNA content that do not actively synthesize DNA. In cell culture studies, quiescent S-phase cells have been observed under physiological conditions typical for poorly vascularized regions of tumors such as reduced pH, hypoxia, and glucose deprivation. Therefore, we studied the possible correlation between the frequency of quiescent S-phase cells and the oxygenation status as determined polarographically in a number of human tumor xenografts.

Methods and Materials: Five human tumor xenografts on nude mice were used. Oxygenation was measured polarographically with an Eppendorf pO_2-Histograph in 24 to 30 individual tumors for each entity. Mice were injected intraperitoneally with 1 mg/30 g bodyweight bromodeoxyuridine (BrdU), tumors were excised 30 min later and prepared into a single-cell suspension. After immunofluorescence staining with an antibody against BrdU and staining of the DNA with propidium iodide, cells were measured in a FACScan flow cytometer and the frequency of cells in the S-phase compartment that did not incorporate BrdU was determined.

Results: In most cases, the frequency of measurements of an oxygen partial pressure < 5 mm Hg in the tumor tissue increased with tumor volume. Likewise, the frequency of quiescent S-phase cells was generally higher in larger tumors. Taking all five tumor entities together, there was a highly significant correlation between tumor oxygenation and the occurrence of quiescent S-phase cells.

Conclusions: Our data confirm earlier findings that inactive S-phase cells do exist *in vivo*. Because their frequency seems to be dependent (directly or indirectly) on the degree of oxygenation and has been shown to increase not only with hypoxia, but also with reduced pH and glucose deprivation *in vitro*, the frequency of inactive S-phase cells may be considered a summary indicator for extreme physiological conditions in tumors.

Quiescent S-phase cells, Cell proliferation, Hypoxia, Tumor micromilieu, Prognostic factors.

INTRODUCTION

The existence of quiescent cells in compartments of the cell cycle other than G_0/G_1 is not an undisputed fact. The first to discuss the possibility and present evidence for "dormant" S- and G_2-phase cells in lymphocyte populations from patients with chronic myeloid leukemia were Darzynkievicz *et al.* (1). They used an acridine orange staining technique to separate cells with different chromatin packing. Dethlefsen *et al.* (2) applied this method to human cervix carcinoma cells *in vitro* and showed that "density inhibited cells are both arrested and lost at random position about the cell cycle." At the same time, Streffer *et al.* (3) presented evidence from preimplantation mouse embryos for cells with an S-phase DNA content that did not incorporate ^{3}H-thymidine and were therefore called "silent" S-phase cells. The phenomenon was also observed in murine tumor cell spheroids by Allison *et al.* (4), who suggested that cells "may be blocked in S and G_2 as well as in the G_0/G_1 phase of the cell cycle." A similar conclusion was drawn by Drewinko *et al.* (5) when they compared S-phase fractions calculated from DNA histograms with ^{3}H-thymidine labeling indices. Since the development of two-parameter flow cytometry with bromodeoxyuridine (BrdU) immunofluorescence labeling, a number of studies have explicitly or implicitly provided further evidence for the existence of cells in the S-phase compartment that are not actively synthesizing DNA (6–10).

Detailed *in vitro* studies in our laboratory have shown that the phenomenon occurs in certain cell lines and under certain physiological conditions. Thus, up to 90% of all S-phase cells are inactive after a few days of incubation at reduced pH (6.5 instead of 7.2) in a human melanoma cell line. These cells do not seem to reenter the cell cycle when

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the pH was changed back to normal (11). Inactive S-phase cells also occur under hypoxic conditions, although here the incubation times necessary to see the effect are shorter (12). Finally, inactive S-phase cells have been observed in cell cultures lacking glucose in the medium (Zölzer and Streffer, unpublished data).

These cell culture experiments, involving exposure to extreme physiological conditions for several days, seem to be relevant to the situation of cancer cells in poorly vascularized regions of tumors where the pH will be reduced due to lactate accumulation, oxygen partial pressure (pO_2) will be low, and glucose will be provided less efficiently. In many cases, all this is not due to a temporary occlusion of blood vessels, but to the fact that the tumor has outgrown its vasculature, so that the changes in micromilieu are chronic instead of acute. If the occurrence of quiescent cells with an S-phase DNA content is indeed related to such conditions *in vivo* as well as *in vitro*, the results of classical flow cytometry assessing tumor cell proliferation on the basis of the DNA histogram alone may be misleading. Only a two-parameter approach, including BrdU-labeling or a similar indicator of progression through the cell cycle will give reliable information on the frequencies of active and inactive cells in the S-phase. As a model system in which these questions can be studied, we chose human tumor xenografts on nude mice. Five different entities were used, three of them different kinds of sarcoma, the other two a squamous carcinoma, and a glioblastoma. As the most accessible parameter for the characterization of the tumor micromilieu we measured the pO_2 polarographically with the help of an Eppendorf pO_2-Histograph. The same individual tumors were then analyzed with respect to the frequency of quiescent S-phase cells as described above, and correlations with the degree of oxygenation were looked for.

METHODS AND MATERIALS

Animals

Nude mice [nu/nu of National Medical Research Institute (NMRI) inbred background] were used for this study. Mice were obtained from the central animal facility of Essen University Clinics, where the breeding was carried out under pathogen-free conditions. During experiments animals were housed in the laboratory of the Department of Radiation Oncology in laminar air flow units. Animals had unlimited access to water (supplemented with chlortetracycline (10 g/L) and K-sorbate (1.34 g/L) acidified to a pH of 3.0) and high caloric diet. Transplantations were carried out when animals reached an age of 6 to 7 weeks. Tumor chunks of 2 to 3 mm were transplanted into the subcutaneous tissue of the right hind leg. All experimentation had previously been approved by the regional animal ethics committee.

Tumors

Details of the tumor model have been described previously (13–16). The following tumors were used: L7—Myxoid leiomyosarcoma, Passage 43, Volume doubling time (VDT) 4.9 d; ENE2—Neuroectodermal sarcoma, Passage 70, VDT 2.7 d; ES3—Undifferentiated soft tissue sarcoma, Passage 39, VDT 2.6 d; WAN—Squamous cell carcinoma, Passage 39, VDT 3.5 d; HTZ17—Glioblastoma, Passage 97, VDT 5.3 d.

These tumors have been repeatedly characterized as to their DNA content, volume doubling time, and isoenzyme pattern of lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (GPD) (17). During the experimental period no changes in these parameters were observed, confirming the human origin of the tumors. In each experiment (except with tumor L7), there were three groups of 6–10 animals with different tumor volume: 6, 8, and 10 mm diameter, i.e., approx. 100, 250, and 500 μl, respectively. Only tumors with volumes of 6 and 10 mm diameter were studied in the case of L7.

Measurement of pO_2

Details of the experimental setting used for the pO_2 measurements have been described previously (18). Briefly, mice were positioned concentrically to the midpoint of the experimental setup, spontaneously breathing an anesthetic gas mixture through openings in the distributor. Enflurane (Ethrane) was circulated by a membrane pump and was mixed with air. For further details of the narcotic procedure see (19). A decrease in body temperature during anesthesia was avoided by surrounding the animal body gently with a perspex tube.

The invasive pO_2 measurements were performed using a computerized system (KIMOC 6650, Eppendorf, Hamburg, Germany). The details of this technique have been described previously (20). Briefly, the intratumoral pO_2 was assessed using polarographic needle electrodes with a diameter of 300 μm. The O_2-sensitive gold cathode in a steel shaft needle had a diameter of 16 μm resulting in a hemispherical measuring volume with a diameter of approximately 50 μm around the tip of the electrode. With this technique the microregional oxygenation of approximately 50 to 200 tumor cells was assessed. The needle electrode was moved through the tumor tissue by steps of 0.5 mm. Each forward movement was immediately followed by a backward step of 0.2 mm [in order to minimize tissue compression artefacts (20)] resulting in an effective step size of 0.3 mm. Tumor oxygenation measurements were performed along 3 tracks within each individual tumor so that approximately 36 individual readings were obtained per tumor. Data obtained with identical tumor volumes were pooled for analysis. Thus, between 216 and 360 individual pO_2 data points per volume group were collected for statistical evaluation. Negative readings in the range of -1 to 0 mm Hg, which may occur due to the unavoidable spread of measured values around true oxygenation values, were treated as belonging to the lowest class reading (0–2.5 mm Hg). Values below that range (i.e. < -1 mm Hg) were not observed in our investigation. The drift of the electrode was < 0.4 %/min. The raw data of the pO_2 measurements were exported from

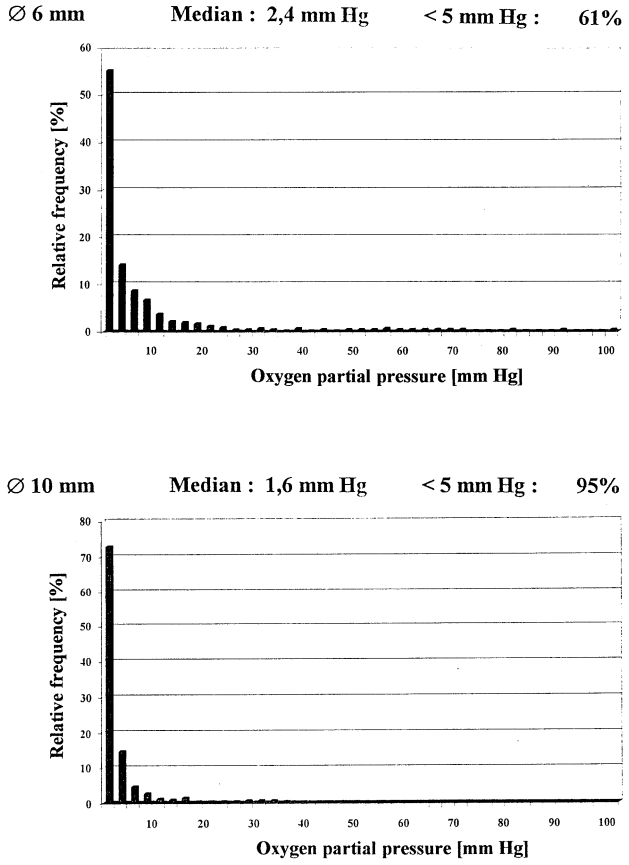

Fig. 1. Examples of polarographical oxygen measurements (tumor L7). Each histogram contains the summed data from 10 individual tumors of similar volume, i.e., the total number of data points included is 360.

the KIMOC device to a personal computer and plotted in histograms either for individual tumors or as summed data from several tumors of similar volume as shown in Fig. 1. Standard statistical software was used for data analysis. The oxygenation status of each individual tumor was described by the fraction of hypoxic pO_2 values, i.e. measurements < 5 mm Hg. The results for the different groups of tumors were described by the mean $\pm$ standard error of the mean from the 6–10 tumors in a particular volume group.

Determination of quiescent S-phase cells

After the oxygen measurements had been carried out, the animals were injected intraperitoneally with 1 mg/30 g bodyweight BrdU (0.3 ml of a 10 mM solution of BrdU in 0.9% NaCl). It is to be noted here that invasive oxygen measurements obviously do not have any influence on tumor growth and other endpoints (15). We therefore assumed that the oxygen measurement had no effect on BrdU labeling either. Thirty minutes after injection, the animals were sacrificed and the tumors were excised. Single-cell suspensions were prepared by first cutting the tissue into small pieces with scissors and then mincing it through a 50-μm mesh, while rinsing with 0.9% NaCl. Finally, the cells were centrifuged, fixed in 96% ethanol and stored for at least 24 h, usually a few days.

For flow cytometric analysis cells were centrifuged, washed with 0.9% NaCl and incubated in pepsin solution (0.5% pepsin [1000 U/g] in 0.055 N HCl, pH 1.8) for 10 min at 37°C to isolate nuclei. After washing again with 0.9% NaCl, 2 N HCl was added for 30 min at room temperature to partially denaturate DNA. The nuclei were then washed once in 0.9% NaCl and once in phosphate-buffered saline (PBS)–Tween (0.05%), and incubated with anti-BrdU mouse IgG (Becton Dickinson, 1:20 in PBS–Tween) for 30 min at 4°C in the dark. After washing twice with PBS–Tween (0.05%)–bovine serum albumin (BSA) (1%), they were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (DuPont, 1:100 in PBS–Tween–BSA), again for 30 min at 4°C in the dark. Finally, the nuclei were washed in PBS–Tween, resuspended in PBS, and stained with 2.5×10^{-5} M propidium iodide (in 0.1 M Tris, 0.1 M NaCl, pH 7.5).

Cytofluorometric measurements were carried out with a FACScan instrument (Becton Dickinson). Green (515–545 nm) and red (> 650 nm) fluorescence after blue (488 nm) excitation was determined from 5000 cells. Data were plotted as green vs. red fluorescence scattergrams and analyzed with the help of windows set on the computer screen. As will be seen from Fig. 2, the cut-off line for the BrdU-positive cells was placed comparatively high to exclude unspecifically labeled cells. This was checked by analyzing some of the samples under the fluorescence microscope. The setting of the windows was the same as in earlier studies from our group (10–12).

Cell cycle fractions were determined from the scattergrams of individual tumors and the mean $\pm$ standard error of the mean was calculated for each volume group. The fraction of quiescent S-phase cells given is always the percentage of all cells that are BrdU-negative and have an S-phase DNA content, i.e., not the percentage of cells with an S-phase DNA content that are BrdU-negative.

Relationship between tumor oxygenation and occurrence of quiescent S-phase cells

The fraction of quiescent S-phase cells was plotted against the fraction of oxygen measurements < 5 mm Hg and a linear least squares fit was obtained with standard statistical software. Correlation coefficients and p-values were calculated.

RESULTS

Measurement of pO_2

Typical results of polarographical oxygen measurements are shown in Fig. 1. As in these examples, the frequency of measurements with a partial pressure < 5 mm Hg was always higher than 60% and increased with tumor volume (Table 1). If all five tumors were taken together, there was a highly significant correlation between the frequency of

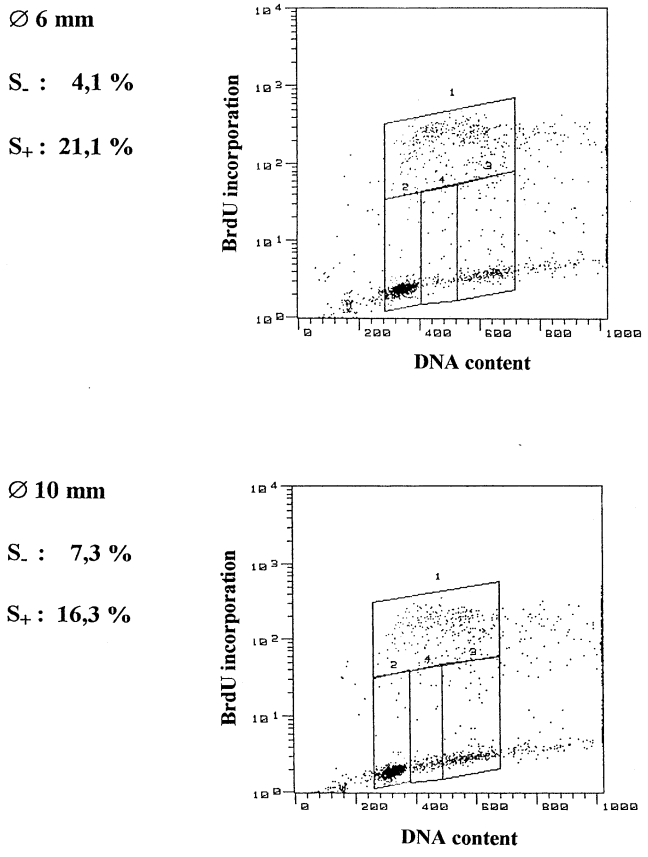

Fig. 2. Examples of flow cytometrical cell cycle analyses (tumor L7). (Window 1: labeled S-phase cells, S_+; window 2: G_1-phase cells; window 3: G_2-phase cells; window 4: unlabeled S-phase cells, S_-). The frequencies of quiescent S-phase cells in these individual tumors are close to the mean of their respective volume group.

measurements < 5 mm Hg and the tumor volume ($r = 0.753$, $p < 0.002$).

Determination of quiescent S-phase cells

Figure 2 shows examples of flow cytometric measurements. The frequency of quiescent S-phase cells (fraction of all cells in the BrdU-negative S-phase compartment) was not particularly high in these cases, other tumors containing

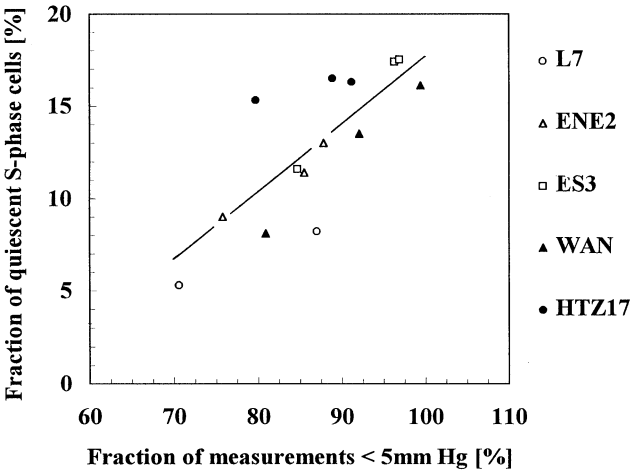

Fig. 3. Relationship between tumor oxygenation and the occurrence of quiescent S-phase cells in five tumor entities. The complete data are given in Table 1. The line drawn through the data points was obtained by a linear least squares fit (correlation coefficient $r = 0.768$, $p < 0.002$).

up to 25% of such cells (Table 1). For each individual tumor entity, the frequency of quiescent S-phase cells increased with tumor volume, but there was no significant overall correlation ($p = 0.10$).

The frequency of BrdU-positive cells decreased with tumor volume in four of the five entities, whereas one (ENE2) showed the opposite trend. However, no overall correlation was seen even if ENE2 was excluded from the analysis ($p = 0.39$; data not shown). Moreover, the frequency of quiescent S-phase cells tended to increase with decreasing frequency of BrdU-positive cells for each individual entity except ENE2, but again the two parameters were not significantly correlated even among the four more promising cases ($p = 0.06$; data not shown).

Relationship between tumor oxygenation and the occurrence of quiescent S-phase cells

When the frequency of quiescent S-phase cells was plotted against the frequency of oxygen measurements < 5 mm Hg (Fig. 3), a highly significant correlation became apparent ($r = 0.768$, $p < 0.002$). A separate analysis of tumors

Table 1. Fraction of measurements < 5 mm Hg (hypoxic areas) and fraction of quiescent S-phase cells or S_0 cells in five human tumor xenografts*

	∅ 6 mm		∅ 8 mm		∅ 10 mm	
	< 5 mm Hg	S_0	< 5 mm Hg	S_0	< 5 mm Hg	S_0
L7	70.6 ± 5.1	5.3 ± 0.3	—	—	87.0 ± 2.0	8.2 ± 0.7
ENE2	75.8 ± 5.8	10.8 ± 0.5	85.5 ± 4.6	11.4 ± 0.9	87.8 ± 5.7	13.0 ± 0.8
ES3	84.7 ± 5.9	11.6 ± 0.6	84.7 ± 5.9	17.4 ± 0.7	96.9 ± 4.1	17.5 ± 0.9
WAN	80.9 ± 6.5	8.1 ± 0.7	92.1 ± 3.5	13.5 ± 1.4	99.4 ± 0.5	16.1 ± 0.4
HTZ17	79.7 ± 4.7	15.3 ± 1.8	88.9 ± 5.8	16.5 ± 2.8	91.2 ± 3.7	16.3 ± 1.1

* Two or three groups of tumors with similar volume were investigated; the figures given are means and standard errors of the mean from 6–10 animals per group.

with similar volume (6, 8, or 10 mm diameter) revealed the same relationship, but because of the limited number of data points significance was not achieved ($p = 0.31$, $p = 0.28$, and $p = 0.13$, respectively).

When the two parameters were plotted for the three voume groups of each individual tumor entity, significant correlations were found in two cases (ES3: $r = 0.9996$, $p < 0.02$; WAN: $r = 0.9969$, $p < 0.05$), but not in the remaining two (ENE2: $p = 0.38$; HTZ17: $p = 0.22$). L7 could not be analyzed in this way, because there were only two volume groups.

BrdU-positive cells tended to be less frequent in tumors with low oxygenation. When all tumors were taken together, a significant correlation resulted ($r = 0.613$, $p < 0.02$; data not shown). Whereas the data presented so far were mean values from 6–10 individual tumors of similar volume, we also compared individual oxygenation measurements with individual quiescent S-phase fractions. However, a significant correlation between the two parameters was found only in one subgroup of tumors, namely ENE2 with a diameter of 10 mm ($r = 0.749$, $p < 0.05$); in all the other cases, there was no such correlation.

DISCUSSION

Our data support the assumption that inactive S-phase cells found in animal and human tumors by a number of authors (see Introduction) are not an artifact of the labeling method, but a phenomenon related to the specific physiological conditions prevailing in tumors. It could be argued that the BrdU used as label does not reach cells in poorly vascularized areas and that as a consequence an indirect relationship between oxygenation and the frequency of unlabeled cells exists. This explanation seems unlikely, however, for the following reasons:

1) As described above, inactive S-phase cells have been observed *in vitro* under exactly those physiological conditions which are known to occur in tumors: hypoxia, reduced pH, and glucose deprivation. In this kind of experiment, the accessibility of the cells to BrdU certainly does not play a role. It may be speculated that the breakdown of the cellular energy metabolism under the conditions mentioned above leads to a general halt in cell cycle progression, so that at least some of the cells that are in S-phase do not continue to synthesize DNA.
2) In human colorectal tumors, samples of which were obtained after surgical resection, quiescent S- and G_2M-phase cells could be detected without using the BrdU labeling method (21). To distinguish between cycling and noncycling cells, we simultaneously measured DNA content and Ki-67 immunofluorescence. The latter antibody is known to recognize a nuclear antigen expressed in proliferating cells only, but not in quiescent cells. Ki-67-negative cells in the S- and G_2M-compartments of the cell cycle were quite abundant in some of the tumors (up to 80% of all cells in these compartments), and their frequency was significantly correlated with tumor size as reflected in the T category (21). This argument, of course, depends on the assumption that the expression of Ki-67 in cells exposed to hypoxia is the same as that in cells not so exposed, which is not known yet.
3) There is preliminary evidence from microscopical sections of human tumor xenografts that BrdU-labeled cells do occur in hypoxic areas (Stüben and Knühmann, unpublished data). These were distinguished with the help of pimonidazole injected into the mice at the same time as BrdU. Because the label obviously reached the hypoxic areas, the lack of its incorporation by cells in the S-phase compartment does not seem to be a question of BrdU accessibility. We do not yet know, of course, whether the hypoxia in those microscopically investigated tumors was of a chronic or acute nature, but this is irrelevant in the present context, because any short-term occlusion of blood vessels or general lack of vascularization would have affected BrdU and pimonidazole in the same way.

It is thus most probable that the inactive S-phase cells observed in human tumor xenografts are not artifacts, and that the correlation between their frequency and the degree of oxygenation is due to a direct influence of the tumor micromilieu on cell cycle regulation. From a practical perspective, the frequency of inactive cells in the S-compartment may turn out to be a summary indicator of certain extreme physiological conditions in tumors, not only of hypoxia, but also of reduced pH and glucose deprivation. An increased lactate/pyruvate ratio may be another common characteristic of these conditions (22).

Interestingly, hypoxia as well as reduced pH and glucose deprivation have all been shown to increase radiation sensitivity if prevailing for more than a few hours. This is in seeming contrast to the general assumption that hypoxia protects cells from radiation damage. With chronically low oxygenation, however, this protection may be cancelled out by an increased radiosensitivity (23–26). Also, prolonged exposure to reduced pH as well as glucose deprivation sensitize cells to radiation (11) (and Zölzer and Streffer, unpublished data). It is exactly these chronic irregularities in tumor micromilieu that favor the occurrence of quiescent S-phase cells. The latter may therefore be indicators not only of the specific conditions mentioned, but moreover of an altered tumor radiation response. This speculation, of course, needs to be corroborated in further *in vitro* and *in vivo* studies.

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