



The Fate of a Normal Human Cell Traversed by a Single Charged Particle

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SUBJECT AREAS:
CANCER
NUCLEAR PHYSICS
BIOPHYSICS
CELL BIOLOGY

Received
18 June 2012

Accepted
23 August 2012

Published
10 September 2012

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The long-term “fate” of normal human cells after single hits of charged particles is one of the oldest unsolved issues in radiation protection and cellular radiobiology. Using a high-precision heavy-ion microbeam we could target normal human fibroblasts with exactly one or five carbon ions and measured the early cytogenetic damage and the late behaviour using single-cell cloning. Around 70% of the first cycle cells presented visible aberrations in mFISH after a single ion traversal, and about 5% of the cells were still able to form colonies. In one third of selected high-proliferative colonies we observed clonal (radiation-induced) aberrations. Terminal differentiation and markers of senescence (PCNA, p16) in the descendants of cells traversed by one carbon ion occurred earlier than in controls, but no evidence of radiation-induced chromosomal instability was found. We conclude that cells surviving single-ion traversal, often carrying clonal chromosome aberrations, undergo accelerated senescence but maintain chromosomal stability.

The “fate” of human cells after nuclear traversals of densely ionizing charged particles is one of the oldest and still unanswered problems in radiation protection and cellular radiobiology. Charged nuclei such as protons, α -particles, and heavy ions are characterized by a higher linear energy transfer (LET) than X- or γ -rays, i.e. the energy deposition pattern is non-uniform and characterized by tracks with high energy deposition (densely ionizing or high-LET radiation), unlike the energy deposition of sparsely ionizing (low-LET) photons or electrons. In radiation protection, most of the annual equivalent dose to the human population is caused by α -particles from radon gas and its short-lived progeny, but each bronchial cell has a very low probability of receiving more than one α -particle traversal in a lifetime¹. In manned spaceflight, the cells of the astronauts’ body are exposed to many low-LET protons and only a few high-LET heavy ions, but even for long travels like a mission to Mars most of the cells will only experience a single heavy ion traversal². Early experiments with radioactive isotopes on in vitro cell inactivation suggested that a single α -particle traversal was lethal for a mammalian cell³. This leads to the obvious question of whether single particle traversals represent a risk for late effects, especially for carcinogenesis: if all cells hit by the particles die, then none will carry mutations eventually leading to cancer⁴.

This problem triggered studies on the mutagenicity of densely ionizing radiation, and it became soon clear that α -particles were even more effective in the induction of mutations than for cell killing compared to X-rays⁵. Using accelerated heavy ions at very high-LET it was also possible to show that the maximum inactivation cross-section was always lower than the area of the mammalian cell nucleus⁶, suggesting that the inactivation probability by a single particle is always lower than unity. Finally, direct measurements of cell killing by single-particle nuclear traversal became possible with the construction of the microbeams at the Columbia University⁷ and Gray laboratory⁸. The results clearly showed that all mammalian cells that have been tested could survive single high-LET particle traversals in the cell nucleus.

In vitro cell transformation has also been measured in microbeam experiments at the Columbia University in New York. The probability of neoplastic transformation in the murine cell line C3H 10T1/2 after a single α -particle nuclear traversal was not higher than in controls⁹. However, transformation could be eventually caused by mutations induced by particle traversals in the cytoplasm¹⁰ or through the bystander effect¹¹, i.e. by a microenvironment-mediated mechanism involving the cells not actually hit by the ion¹².

In those experiments the cells exposed to single traversals were not followed individually for a pedigree analysis. Evidence of radiation-induced genetic instability in cells exposed to α -particles¹³, even a single nuclear traversal¹⁴, begs the question of whether cells surviving single traversals are still “normal” or harbour genetic or epigenetic changes which eventually lead to genetic instability. Genetic instability is expressed as an increased tendency of the genome to acquire alterations, when the processes involved in maintaining and replicating the genome are dysfunctional. Chromosomal aberrations are here important, since classical radiobiology predicts that surviving



cells may carry clonal chromosomal rearrangements, while genomic instability is characterized by sporadic (non-clonal) aberrations¹³. Using mFISH and a statistical analysis of the number of traversals per cell, it has been argued that the most likely result of a single α -particle traversal into a cell nucleus is a complex-type aberration¹⁵, i.e. a rearrangement involving 3 or more breaks in 2 or more chromosomes. This means that most of the cells surviving a single traversal should bring complex rearrangements, which could modify their behavior and pattern of senescence/differentiation which protects normal tissues from cancer development.

In the experiments presented here, high-LET radiation was provided by carbon ions, which are used in radiation oncology for treatment of solid radioresistant tumors¹⁶ and are relatively abundant in the galactic cosmic ray spectrum². Using the high-precision heavy ion microprobe at GSI¹⁷ and performing a pedigree analysis, we have directly measured genomic instability and senescence/differentiation in clonal descendants of normal diploid human fibroblasts AG1522D exposed to a single or 5 traversals of carbon ions (Fig. 1). Five traversals have been used to obtain a high number of chromosomal aberrations by delivering a high dose. We have also compared these long-term measurements with the initial cytogenetic damage caused by single traversals of the cell nucleus.

Results

We first measured chromosomal aberrations and cell killing following nuclear traversal of a defined number of carbon ions. Second, we measured whether the descendants of exposed cells are genetically stable and undergo a normal transition to senescence and terminal differentiation.

Clonogenic survival after exposure to a defined number of carbon ions. To determine clonogenic survival, we targeted AG1522D cell nuclei with a defined number of 4.8 MeV/n carbon ions (LET in water approximately 290 keV/ μm) and performed a clonogenic assay (delayed plating 24 hours after exposure). The track structure of carbon ions at this low energy is similar to α -particles. Energy is deposited mostly along the primary track, and the track radius caused by δ -rays is less than one micrometer in radius. Hence, intertrack effects due to overlapping δ -rays are negligible. For comparison, we irradiated the cells with the same type of carbon ions using conventional broad beam or X-rays. For broad beam exposure, no nuclear staining was performed. In Fig. 2A, surviving fraction in broadbeam experiments is plotted as a function of the mean number N of particle traversals per nucleus. N is calculated as the product $F \times A$, where F is the particle fluence (in ions/ μm^2) and A the mean area of the cell nucleus (in μm^2). In contrast to targeted microbeam exposure, charged particles also hit the cell cytoplasm. The probability of surviving exactly one carbon ion traversal was 25%, whereas five nuclear traversals reduced the surviving fraction to about 5% (Fig. 2A). Survival after 1 or 5 exact traversals was significantly lower than after 1 or 5 mean traversals (compare microbeam to broad beam data in Fig. 2A). The same broad beam data set as a function of dose is shown in Figure 2B in comparison to clonogenic survival after X-ray exposure, pointing to a high relative biological effectiveness (RBE) of carbon ions. As can be derived from this Figure, the RBE (for delayed plating survival) of carbon ions producing the same effect as 2 Gy X-rays (60% survival) is about 8.

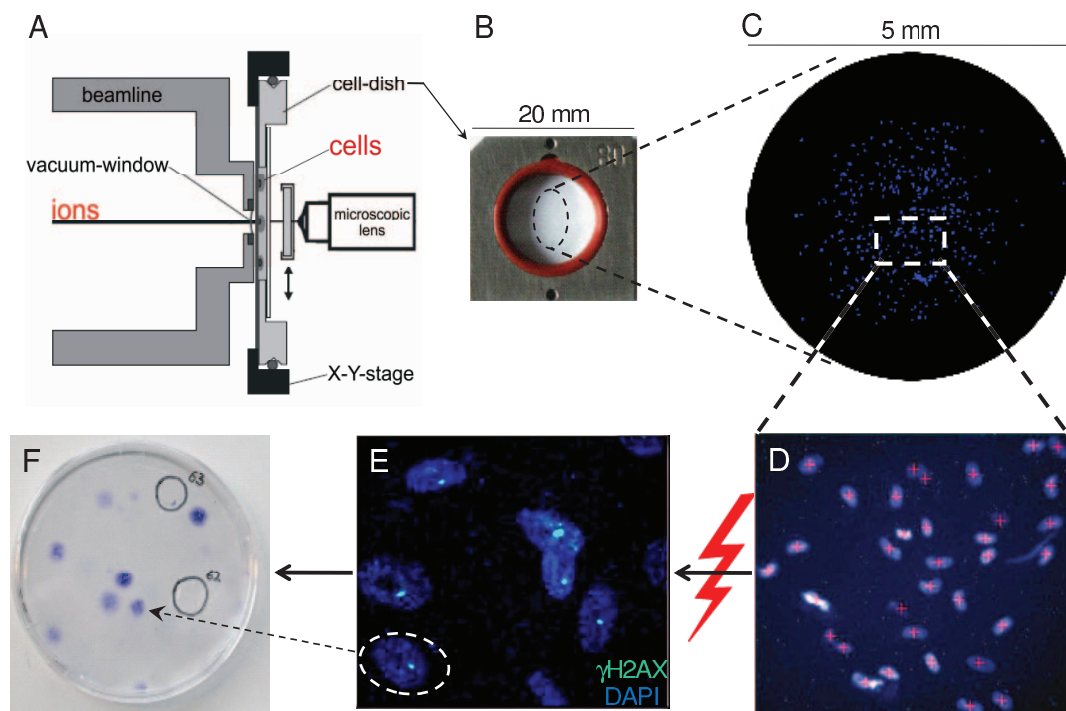


Figure 1 | Targeted irradiation of single AG1522D cells was performed with a predefined number of carbon ions at the microbeam facility.

(A) Schematic drawing of the basic elements of the microbeam end station (cortesy of M. Heiss). (B) 500 cells were seeded in the center of each microbeam irradiation chamber (dashed circle). (C) Nuclear staining (Hoechst33342, blue) was performed for fluorescence microscopic recognition prior to exposure of all cells. The irradiation area (diameter 5 mm) was virtually subdivided into overlapping irradiation fields (around 200 fields per chamber; dashed rectangle symbolizes one field). (D) View of the online microscope (20x lens) onto nuclear-stained cells; nuclei classified as targets are labeled by red crosses prior to irradiation. This assures that all cells are irradiated and that cells located within the overlapping regions of the irradiation fields are targeted only once. (E) γH2AX -immunostaining (turquoise) performed only for demonstration of the targeted exposure of nuclei (blue) to one carbon ion per nucleus. (F) Methylene blue stained colonies formed during 2 weeks of culturing by single targeted cells that have been reseeded 24 h after irradiation. From the number of these colonies clonogenic survival has been determined 14 days after irradiation. Colonies for cytogenetic or protein analysis and determination of differentiation patterns have been isolated (black circles) before staining.

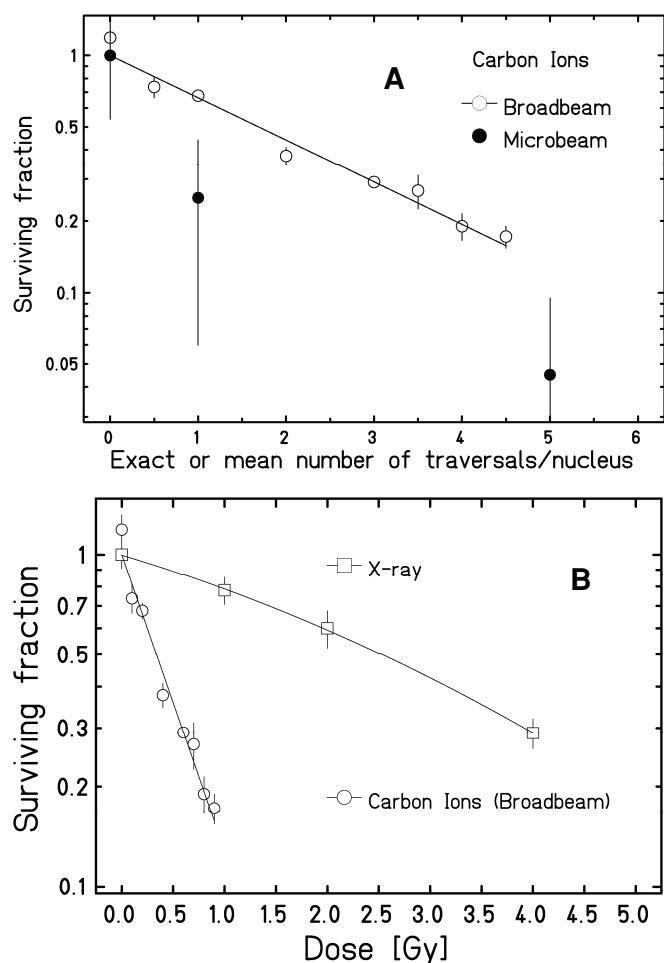


Figure 2 | Surviving fraction of AG 1522D cells after exposure to different types of irradiation (delayed plating 24 hours after exposure).

(A) Surviving fraction after exposure to carbon ions, plotted over the exact or mean number of carbon ion traversals per nucleus for microbeam or broad beam, respectively. Microbeam: error bars indicate weighted standard error of the mean of 3 experiments. Broad beam: error bars indicate typical size of fluctuations of the data points about the fit line, weighted with a statistical error obtained from triplicate plating. The broad beam experiment has been performed in parallel to one of the microbeam experiments. (B) Surviving fraction after exposure to X-rays and carbon ions (broad beam), plotted over the dose. X-rays: error bars indicate standard deviation of two independent biological samples of one experiment, performed in parallel to one of the microbeam experiments and the carbon ion broad beam experiment. Carbon ions (broad beam): redrawn from (A).

Chromosomal aberrations induced by single traversals. In order to quantify the radiation induced damage after targeted exposure of each nucleus to one carbon ion, chromosomal aberrations were analysed in cells of the first cell cycle after mFISH staining and compared to the same physical dose of X-rays (approximately 0.2 Gy, see Supplementary Information/Materials and Methods). The results are presented in Fig. 3A, showing that X-ray exposure resulted in 20% aberrant cells (almost a twofold increase compared to control cells) whereas 70% aberrant cells were detected after 1 hit of carbon ions. After traversal of one carbon ion, the major class of aberrations were simple exchanges (Fig. 3B). Again, the corresponding numbers for X-ray exposure and for control cells were significantly lower. Regarding complex-type exchanges, nearly 30 complex type aberrations per 100 cells were detected after carbon ion irradiation, whereas no complex aberrations were present in X-ray irradiated cells and in controls.

Chromosomal aberrations in the progeny of irradiated cells. As a next step we investigated the genetic stability of the progeny of the cells being traversed by one or five carbon ions. Colonies with high proliferative activity were expanded and subjected to cytogenetic analysis. Chromosomal aberrations occurring only in one metaphase per colony are in general considered as a marker for chromosomal instability. Regardless of previous radiation exposure, these spontaneous sporadic aberrations were found in each colony in 10–14% of the metaphases (Table S1 and S2; details on aberration types can be inferred from Table S2). Spontaneous aberrations are often “unstable”, which means that they cannot be transmitted to daughter cells without further changes. However, also stable aberrations (translocations) were detected and if they occurred in only one metaphase per colony they have as well been considered as a sign for chromosomal instability.

In Fig. 4 the percentage of colonies with stable, clonal or subclonal aberrations is shown for control versus descendants of exposed cells. Colonies with clonal aberrations, where in all metaphases of the colony the same aberration was detected, were distinguished from “subclonal” aberrations, where at least in two (but not in all) metaphases the same aberration was detected.

According to this classification, in 60% of the colonies (6 out of 15 colonies) descending from control cells stable aberrations were observed. All of them were subclonal, and no colonies with clonal aberrations were found. The observed subclonal rearrangements were mostly simple, but in one colony a complex-type exchange was observed.

In contrast, clonal aberrations were induced by nuclear traversal of one carbon ion in one third of the colonies (3 out of 9 colonies) which most likely were radiation-induced. They were mostly simple translocations and one colony with a complex insertion has been detected (shown in Fig. 5A). In addition, two out of these 3 colonies displayed one or multiple subclonal aberrations. Interestingly, the frequency of colonies with subclonal aberrations was lower compared to control cells, but they were exclusively observed in combination with clonal aberrations. All subclonal aberrations were simple translocations.

As can be deduced from Fig. 2A, only a low percentage of cells are able to form colonies after being traversed by 5 hits (nuclear traversals) of carbon ions. In 20% of those colonies (two out of 10 colonies analysed) also one subclonal aberration was found (Figure 4). Clonal aberrations were detected in 10% (one out of 10 colonies analysed), indicating a low transmission rate of radiation induced damage. Remarkably, this aberration was classified as highly complex, with 4 chromosomes and 5 chromosome arms involved and formed after 6 breaks have been induced. According to the C/A/B system (see Methods), this is classified as 4/5/6 (Figs. 5B and C).

As shown in Fig. 4, after exposure to 2 Gy X-rays, the proportion of colonies displaying only subclonal aberrations was comparable to control cells. In addition, in a minor fraction of colonies only clonal aberrations were detected. All clonal aberrations were simple type rearrangements. By increasing the X-ray dose (8 x 2 Gy), in about 90% of the colonies, clonal aberrations were detected, which were mostly simple exchanges, indicating a very high frequency of transmission of radiation induced chromosomal damage to the progeny. Half of the clonal aberrations occurred together with subclonal aberrations, which were all simple translocations.

Induction and transmission of stable chromosomal aberrations.

In order to determine the probability of a transmission of stable chromosomal aberrations induced by the targeted nuclear traversal of a single carbon ion, the frequency of first cycle cells carrying no or only transmissible aberrations was compared to the frequency of colonies displaying clonal aberrations. Clonal aberrations were only detected in colonies of exposed cells and therefore represent misrepair events of the initially radiation induced chromosomal

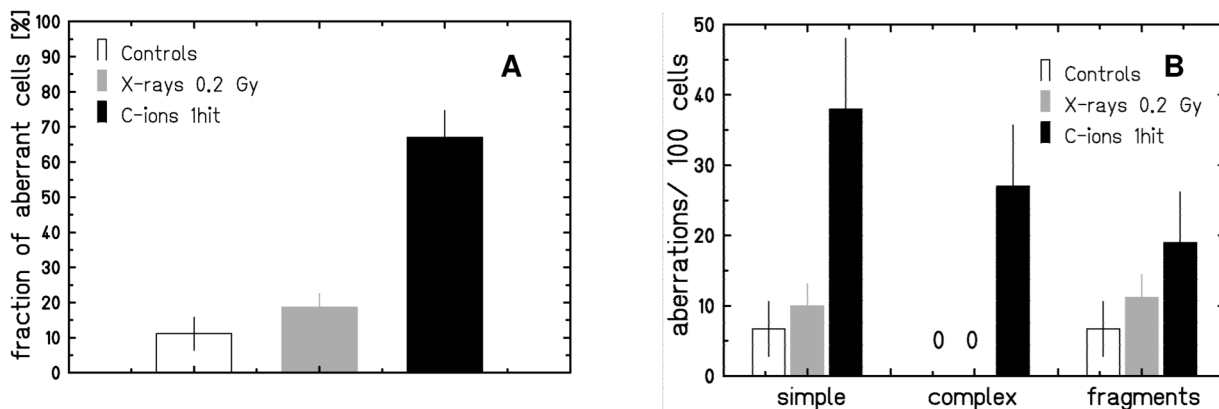


Figure 3 | Chromosomal damage (mFISH) in first cycle cells after exposure to 0.2 Gy X-rays or 1 carbon ion per nucleus (0.2 Gy). (A) Fraction of aberrant cells. (B) Number of simple or complex exchanges or fragments per 100 cells. Discrimination between simple and complex aberrations was based on the “chromosomes/arms/breaks”-system (C/A/B). A complex rearrangement has by definition $C \geq 2$, $A \geq 2$ and $B \geq 3$. Error bars were calculated as standard deviations of a binominal distribution. The number of analyzed cells were $n = 45$ for controls/ 107 for X-ray and 37 for carbon ions.

damage. For first cycle cells, only undamaged cells or cells with stable chromosomal aberrations were taken into account, referred to as “stable cells”. As shown in Fig. 6, in 50 % of the stable cells transmissible chromosomal aberrations were detected. Simple exchanges occurred in 40% of the stable cells, thus 4 times more frequently than transmissible complex exchanges (in 10% of the stable cells). The average complexity was 3/3/3 according to the C/A/B-System. Approximately 30% of the colonies contained clonal aberrations.

Differentiation pattern. The “fate” of normal fibroblasts in culture is senescence and terminal differentiation. Representative examples of colonies formed by mitotically active fibroblasts (stage MF II and III) and postmitotic cells (PMF) are shown in Figure 7B. As shown in Fig. 7A, the percentage of colonies classified as mitotic fibroblasts (MF) II is higher in control cells compared to colonies formed by irradiated cells (25% and below 5%, respectively). The percentage of colonies classified as MF III is lower for the progeny of control compared to irradiated cells (around 60% and 80%, respectively).

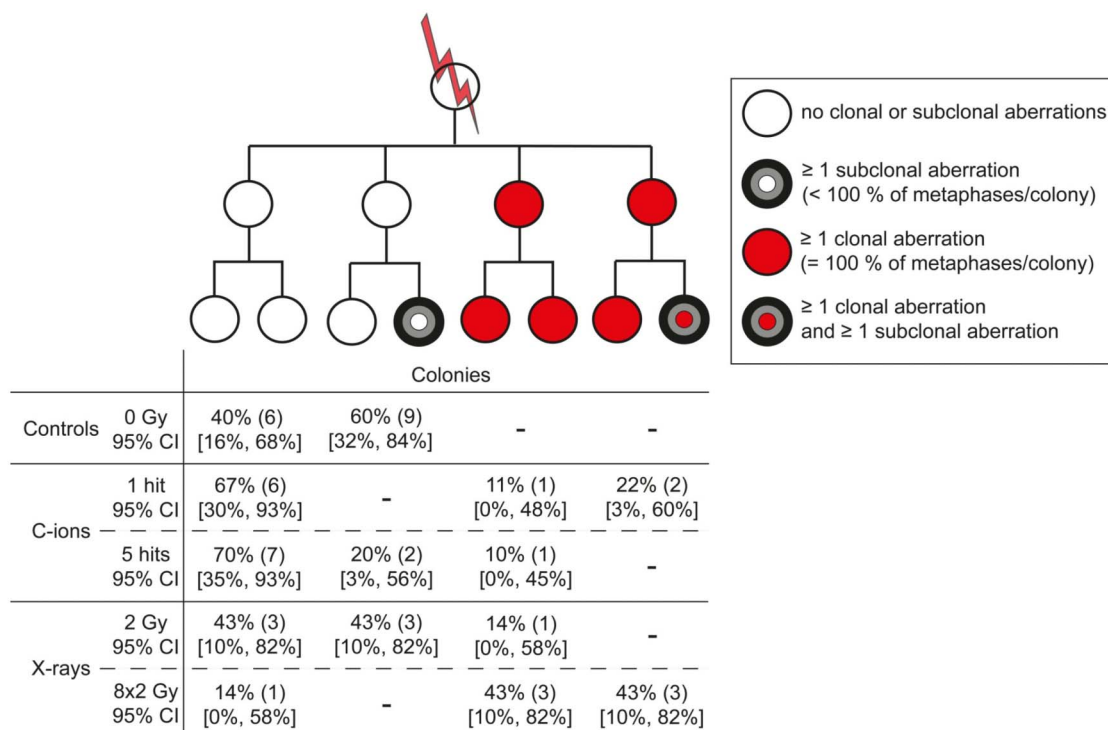


Figure 4 | Frequencies of clonal or subclonal chromosomal aberrations in the progeny of control cells and irradiated cells. Irradiation was performed with X-rays (2 Gy or 8 daily fractions à 2Gy) or carbon ions (1 or 5 hits per nucleus). Identical chromosomal aberrations were classified as “subclonal” if they were present in at least two metaphases of one colony and “clonal” if they were present in all cells. Clonal or subclonal aberrations were mostly simple translocations. Additionally, in all colonies and regardless of previous radiation exposure, spontaneously occurring aberrations were observed, e.g. breaks, dicentrics and translocations. Statistical confidence intervals were calculated assuming binomial distribution. Data points are based on results from 2 or 3 experiments. Number of analyzed colonies were $n = 15$ (controls)/ 7 (2 and 8x2 Gy X-ray)/ 9 (1 hit of carbon ions)/ 10 (5 hits of carbon ions). Median/ maximum/ minimum number of metaphases analyzed per colony were 91/ 100/ 55 for controls, 58/ 100/ 15 and 84/ 100/ 36 for 2 and 8x2 Gy X-rays, 84/ 101/ 21 for 1 hit of carbon ions and 56/ 139/ 36 for 5 hits of carbon ions.

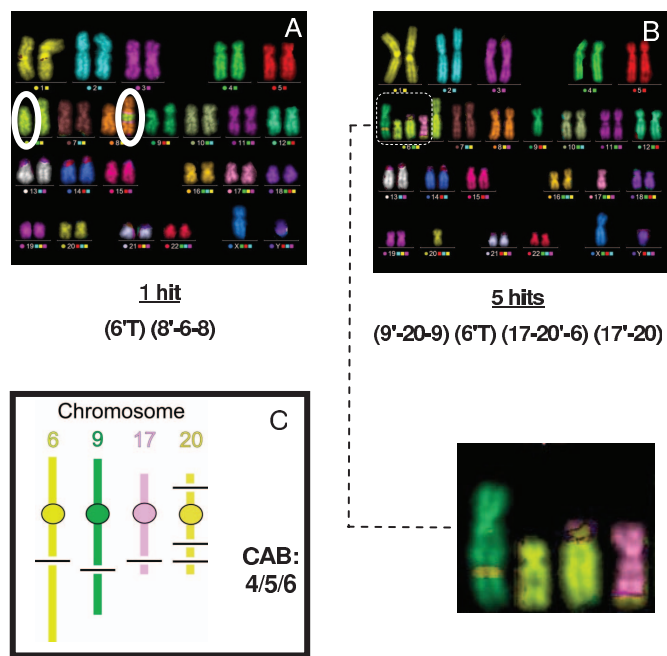


Figure 5 | Examples of karyotypes (mFISH) with clonal complex aberrations occurring in the progeny of cells exposed to carbon ions. (A): 1 hit or (B): 5 hits per nucleus. The aberration types are indicated below the pictures; aberrant chromosomes are shown indicated. T: truncated chromosome; ': contains a centromere. (C) Level of complexity for the aberration shown in (B), according to the CAB system (number of chromosomes, chromosome arms and breaks involved), is 4/5/6. This indicates a highly complex aberration, while the CAB number for (A) is 2/2/3 representing the lowest level of complexity capable to produce a complex exchange. The complexity of clonal aberrations in the progeny of exposed cells increased with the number of hits.

The same accounts for single postmitotic cells (PMF, up to below 5% and 15%, respectively). The higher proportion of MF III colonies and PMF indicates a higher degree of differentiation in survivors of single or multiple traversals. These cells undergo the same “fate” as controls, but earlier.

Expression of cell cycle regulating proteins. To assess if the survivors of a targeted nuclear irradiation with a single carbon ion undergo a transition to replicative senescence (irreversible inhibition of the cell cycle progression) similar to controls, the expression-pattern of the cell cycle regulating and senescence-associated protein p16 was measured in individual colonies of controls and exposed cells using the so called “In Cell Western”-assay. Because of the limited number of cells available due to the special conditions of the microbeam-irradiation procedure, only very few colonies could be analyzed. Fig. 8 shows the cellular amounts of p16, relative to the level of young unexposed cells, detected in colonies consisting of proliferating cells (differentiation stages MF II and III) or in postmitotic and senescent cell-clusters (PMF), respectively. The amount of p16 protein measured for mitotically active colonies of controls was comparable to the level of young unexposed cells, whereas in a single colony of an exposed cell the level of p16 was 2.5-fold elevated. In postmitotic, senescent cell-clusters of unexposed and exposed cells we observed comparable levels of p16, each, which were around 4-fold elevated compared to young unexposed cells. This result has been confirmed by Western Blot analysis of a higher number of cells obtained after bulk expansion of irradiated cells (Fig. S1). In these experiments we also assessed the expression of PCNA, a processivity factor of DNA polymerase. Regardless of previous radiation exposure, a decline of the cellular level PCNA

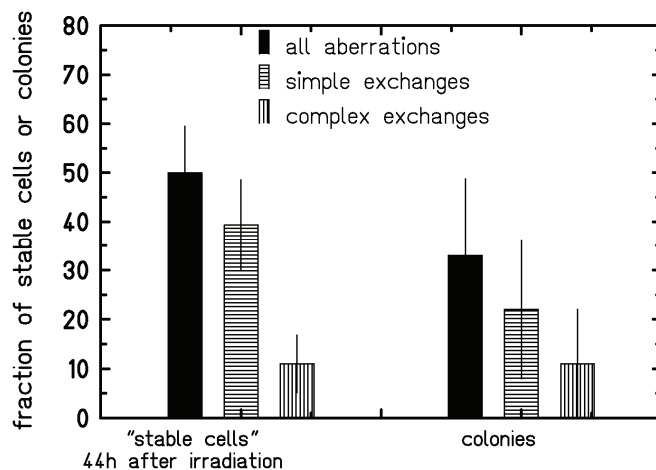


Figure 6 | Fraction of cytogenetically aberrant first cycle “stable” cells (44 hours after exposure) after exposure to one carbon ion per nucleus or colonies of descendants from “stable” cells harboring clonal aberrations as an indicator of the transmission of radiation induced damage. Aberrant cells are considered to be “stable”, if the aberration(s) can be transmitted to the progeny. “Stable” aberrations were simple translocations and complex exchanges. Error bars were calculated as standard deviations of a binominal distribution. The number of analyzed cells is given in Figure 3 and the number of analyzed colonies in Figure 4.

protein was observed in senescent cells due to cessation of proliferation.

Discussion

Erstwhile believed to be almost invariably lethal, it became soon clear that mammalian cells can survive single particle traversals through the cell nucleus⁵⁻⁷. We tried here to elucidate the late behaviour of the survivors. Previous experiments in human lymphocytes suggested that single α -particle traversals induce a very high fraction of complex-type chromosomal rearrangements¹⁵, and can lead to genomic instability¹⁴. Human lymphocytes grow in suspension and are therefore not ideal for studies with microbeams and low-energy charged particles in general, as well for clonal expansion experiments. We have instead concentrated on normal diploid human fibroblasts, which can be clonally expanded and end their life in senescence and terminal differentiation, thus resembling the in vivo cellular behaviour¹⁸.

About 1 out of 4 cells survives a single carbon ion traversal through the cell nucleus and can form colonies (Fig. 2A). The value is lower than expected from the broad beam experiments, and this effect, as previously reported¹⁹, is caused by the mathematical conversion from a Poisson-distributed fluence to an exact number of traversals (see Supplementary Material, Results). About 70% of these cells had visible aberrations at the first cell-cycle, and 40% of these aberrations were complex-type (Fig. 3). DNA lesions induced by heavy ions have low mobility within the cell nucleus^{20,21}, and the formation of complex-type aberrations has been attributed to cumulative rearrangements of chromosomes along the track with their respective neighbours¹⁵. The yields of simple and complex exchanges in AG1522D cells are consistent with previous experiments using broad beam exposure to high-LET heavy ions²², also pointing to a reduced yield of aberrations in fibroblasts compared to lymphocytes.

The clonal analysis (Fig. 4) made it possible to determine the “fate” of individual survivors. It is known that AG1522 cells undergo spontaneous genomic instability at the onset of senescence, but no low-dose radiation-induced instability had been previously measured in mass cultures²³⁻²⁵. The clonal analysis reported here supports those

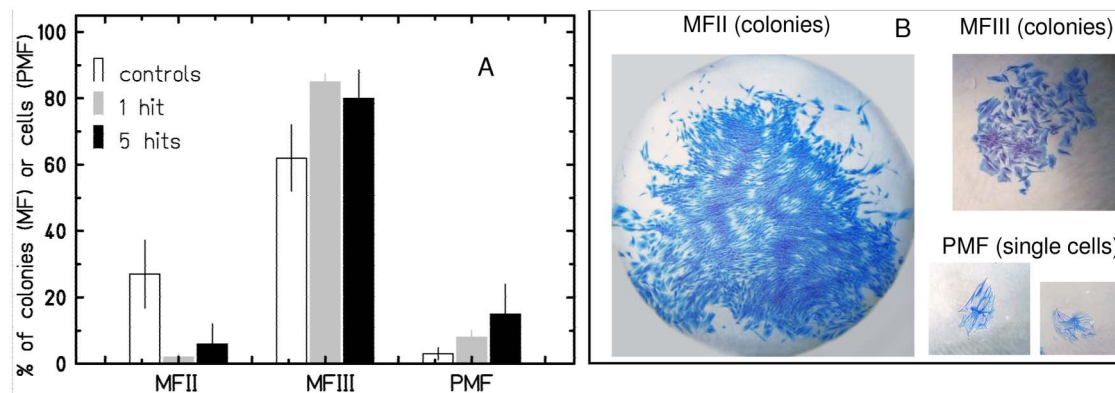


Figure 7 | (A) Differentiation patterns of the descendants of cells exposed to carbon ions (1 hit or 5 hits per nucleus). Colonies of descendants with high proliferative capacity have been isolated, expanded and reseeded at low densities. In these populations the fraction of mitotically active, colony forming cells (MF II and MF III) and postmitotic cells (PMF) has been determined by morphological features (4 weeks after exposure). Error bars indicate SEM. Number of colonies $n = 9$ (controls)/ 14 (1 hit of carbon ions)/ 6 (5 hits of carbon ions); data from one experiment. (B) Representative examples for MFII and MFIII colonies and for PMF are shown.

previous experiments and demonstrates that a single particle traversal in these cells does not induce instability exceeding the level in control cells. Approximately 30% of the survivors carry clonal aberrations, a feature not found in controls. Interestingly, after 5 nuclear traversals the fraction of cells producing only clonal aberrations is the same as after 1 traversal. This is caused by the increased clonogenic mortality at high doses. Data on differentiation (Fig. 7) and p16 expression (Fig. 8) point to slight but significant differences between controls and survivors. The increase in PMF (with a corresponding decrease of MFII) and higher expression of p16 in mitotic cultures reflects an earlier terminal differentiation in irradiated cells compared to controls.

The results demonstrate that human cells surviving a single high-LET particle traversal, i.e. those that have maintained a high proliferative potential and are therefore highly relevant cells with respect to the genomic stability of the population, form colonies which behave similarly to unirradiated cells, but they carry clonal aberrations and

reach senescence earlier. The presence of clonal aberrations is not surprising and is often observed in humans exposed to high doses of ionizing radiation, e.g. in atomic bomb (A-bomb) survivors²⁶. The lack of radiation-induced chromosomal instability confirms that this phenomenon is elusive when normal human cells are used *in vitro*²³. Studies in A-bomb survivors²⁶ and radiation workers²⁷ also failed to demonstrate *in vivo* radiation-induced instability. Genomic instability is certainly a driver for carcinogenesis, but our results show that it is induced in normal human fibroblasts by other factors beyond radiation. This direct measurement strongly supports the hypothesis that genomic instability is linked to non-targeted effects²⁸. High-LET radiation-induced chromosomal instability in mouse hematopoietic stem cells can indeed be induced through a non-targeted mechanism in non-hit cells²⁹, and this pathway may be the leading cause of genomic instability in mice³⁰.

Methods

X-ray and carbon ion exposures. AG1522D cells (see Supplementary information for details on the cell culture) were exposed to X-rays (250 kV, 16 mA) or carbon ions. X-ray irradiations were delivered either as single dose (0.2 or 2 Gy) or in daily fractions (8×2 Gy). Carbon ions (4.8 MeV/nucleon on target, corresponding to an LET of 290 keV/ μm) were obtained at the UNILAC facility at GSI Helmholtz Center for Heavy Ion Research, Darmstadt, Germany. The mean size of the cell nuclei was $190 \pm 50 \mu\text{m}^2$ as determined following immunofluorescence staining. According to this measurement and the stochastic dispersion of the number of particle traversals per nucleus (Poisson distribution), a fluence of 5.3×10^5 particles/ cm^2 results in a mean number of one ion traversal per nucleus.

In both types of irradiation experiments, comparable culture conditions were maintained. Cells were seeded in cell culture dishes at a density of 10^4 cells/ cm^2 10 days prior to irradiation. After irradiation, cells were kept under standard culture conditions for 24 hours before reseeding for further analysis.

Microbeam. A flow chart of the experiment is shown in Fig. 1. Targeted irradiation (carbon ions, 4.8 MeV/nucleon on target, LET 290 keV/ μm) with accuracy of 1.3 μm in the center of the nucleus was performed using the microbeam facility at GSI, Darmstadt, Germany [Fig. 1A; technical details¹⁷]. Further details are given as Supplementary Material.

Chromosomal aberrations. To determine chromosomal damage induced by targeted exposure to one carbon ion per nucleus, chromosome preparations were performed 40 and 44 hours after irradiation for controls and exposed cells, respectively. The time points of analysis were chosen according to the expected maximum fraction of controls and exposed cells entering the first cell cycle 35–45 hours after irradiation as determined after conventional broad beam irradiation of AG1522-fibroblasts with low energy carbon-ions³¹. For collection of mitotic cells, colcemid (0.1 $\mu\text{g}/\text{ml}$, Roche Diagnostics GmbH) was added to the samples 4 hours before chromosome preparation, followed by calyculin A (50 nM, Alomone Labs) 45 minutes prior to chromosome preparation to induce premature chromosome condensation³². Cells of 5 to 6 mock-irradiated or irradiated microbeam chambers were pooled and chromosome preparation was performed according to standard techniques, and mitotic and G₂-M phase cells (referred to as first cycle cells) were analyzed by means of the mFISH-technique. For the preparation of chromosome

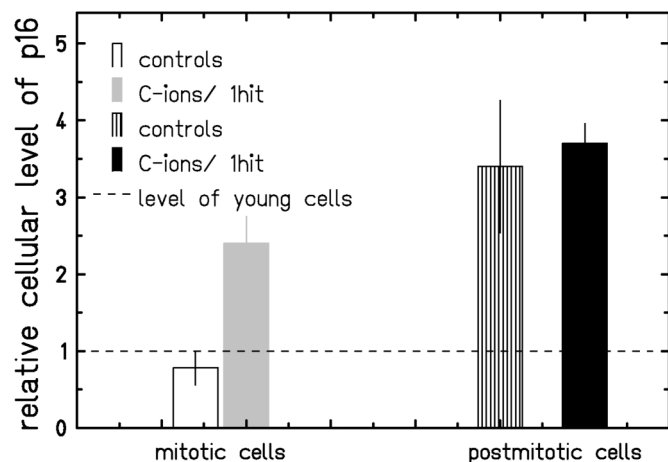


Figure 8 | Relative protein expression levels of the senescence-related protein p16 in colonies of mitotically active cells (MFII and III) or clusters of postmitotic cells formed by the descendants of controls or cells exposed to carbon ions (1 hit per nucleus), compared to the expression level in young cells (dashed line). Error bars in controls indicate the SEM, calculated for the number of colonies (MFI-III)/ PMF clusters analyzed (5/ 3); data from one experiment. Due to the low occurrence of mitotically active colonies or clusters in the progeny of irradiated cells only one sample for each data point could be analyzed, and error bars indicate the standard deviation of technical triplicates of one sample and one experiment.



spreads early after exposure to X-rays (0.2 Gy), the cells were cultured in flasks and treated as described above. To determine chromosomal damage in the progeny of sham-irradiated and irradiated cells, cells were reseeded and single colonies formed after 2 weeks were isolated and expanded as described in Supplementary Material. Chromosomes were stained by mFISH, using a 24XCyte mFISH kit according to the protocol recommended by the manufacturer (Metasystems). Image capturing and processing was performed as described elsewhere³³. Generally, 100 metaphases containing 45 or 46 chromosomes were scored. In samples with a very low mitotic index at least 30 metaphases were analyzed. Structural chromosomal aberrations were recorded according to the mPAINT-system and then grouped into the categories of excess acentric fragments not associated with an exchange and simple or complex exchanges³⁴. Complex exchanges were classified following the “chromosomes/arms/breaks”-system (C/A/B)³⁵, where a complex rearrangement is defined by $C \geq 2$, $A \geq 2$ and $B \geq 3$. In addition, Giemsa staining was performed for the analysis of spontaneous aberrations in the progeny of irradiated cells (see Table S2).

Differentiation pattern. The differentiation pattern of clonal descendants of irradiated cells was analyzed based on morphological features¹⁸. Cells from individual colonies (see Supplementary information) were reseeded at low density (10 cells/cm²) and stained after 14 days. The differentiation pattern was determined by discrimination of mitotically active cells (differentiation stages MF I-II-III) and postmitotic cells (PMF).

Senescence-related protein expression. Quantification of cellular protein levels in clonal isolates was performed applying the “In Cell Western” assay (LI-COR Biosciences), an immunocytochemical technique with cultured cells in microtiter plates using target-specific antibodies to quantify protein levels in fixed cells. Clonal descendants of sham-irradiated cells or cells traversed by 1 carbon ion per nucleus were seeded in triplicates for the determination of individual proteins per colony in 96 well microtiter plates at a density of $5-10 \times 10^3$ cells per well and cultured under standard conditions until reaching at least 70% of confluency. Fixation, permeabilisation and immunocytochemical staining was performed according to the manufacturers protocol (LI-COR Biosciences), using the primary antibody anti-INK4a (anti-p16, Neomarkers) and a fluorochrome-conjugated secondary antibody (IRDye 800CW conjugated anti-mouse, LI-COR Biosciences). For normalization of the fluorescence-signals of the protein of interest (p16) to the referring cell densities per well, cells were additionally stained with a cell-permeable DNA-intercalating agent (DRAQ5, Biostatus Limited) and a non-specific cell stain accumulating in the nucleus and cytoplasm of dead cells (Sapphire700, LI-COR Biosciences). The respective fluorescence-intensities emitted in the infrared-range (IRDye 800CW $\lambda = 800$ nm, DRAQ5 and Sapphire700 $\lambda = 680$ nm) were detected using the Odyssey® Infrared Imaging System (LI-COR Biosciences) equipped with a two diode infrared laser exciting at a wavelength of 685 nm and 785 nm. All measurements are compared to the relative protein levels detected in young (PD 20-25) and unexposed AG1522D-fibroblasts.

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Acknowledgements

This work was partly supported by BMBF [02S8497], ESA (IBER grant), and Beilstein Stiftung (Nano-BIC). We thank Christian Sieben and Jan Knoop for technical assistance, and Bernd Fischer and Bruno Merk for excellent technical support at the microbeam device.

Author contributions

CF, SR and MD designed the experiment and wrote the paper; SZ and SR made the chromosome analysis; DK and CF made the molecular studies; TF and MD analysed the data; KOV performed the microbeam irradiations; all authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Fournier, C. *et al.* The Fate of a Normal Human Cell Traversed by a Single Charged Particle. *Sci. Rep.* **2**, 643; DOI:10.1038/srep00643 (2012).