A Truncated Isoform of the Protein Phosphatase 2A B56 γ Regulatory Subunit May Promote Genetic Instability and Cause Tumor Progression

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F10, a subline of the B16 mouse melanoma cell line, is itself the parent of the more metastatic BL6 line. BL6 cells differ from F10 cells by an alteration of the gene encoding the B56 γ regulatory subunit of protein phosphatase 2A (PP2A), which results in the expression of a truncated variant of the subunit ($\Delta\gamma1$). PP2A is involved in regulating the cell-cycle checkpoint and we found that the checkpoint in BL6 cells is aberrant when the $\Delta\gamma1$ protein is expressed. That is, although $\Delta\gamma1$ protein levels in cultured BL6 cells are low and these cells do not show an altered checkpoint on γ -irradiation, irradiated footpad BL6 tumor cells show both a marked increase in $\Delta\gamma1$ levels and more extensive polyploidy and less apoptosis than F10 cells. These observations were reproduced with $\Delta\gamma1$ gene-transfected F10 cells (F10 $\Delta\gamma1$). $\Delta\gamma1$ expression and an aberrant checkpoint are also associated with a higher metastatic ability because irradiated F10 $\Delta\gamma1$ tumors metastasized much more frequently than F10 tumors, which rarely metastasized whether irradiated or not. Nonirradiated F10 $\Delta\gamma1$ tumors, which do not express $\Delta\gamma1$ protein, had similarly low rates of metastasis. The greater metastatic ability of irradiated F10 $\Delta\gamma1$ tumors also correlated with the acquisition of many more genomic alterations. Thus, it seems that $\Delta\gamma1$ expression may damage the checkpoint, which may then allow the acquisition of genetic alterations that promote metastasis. These observations support the notion that mechanisms promoting the genetic instability of tumors could also aid tumor progression from the nonmetastatic to the metastatic state. (*Am J Pathol* 2003, 162:81–91)

Two well-known properties of tumor cells are that they are genetically unstable^{1,2} and tend to progress toward increasing malignancy.³ As early as 1986, Nowell⁴ drew a link between these two characteristics when he predicted

that the genetic instability of tumor cells could contribute to disease progression by generating subclones with different properties. Consistent with this hypothesis, human tumor cells often have mutations in the genes that play essential roles in the cell-cycle checkpoint, an intracellular event that protects the integrity of the genome against genotoxic stress.^{1,5} For example, the *p53* tumor suppressor gene, which plays a pivotal role in the checkpoint, is mutated in more than half of a wide variety of sporadically occurring human tumors.^{1,5} An intact cell-cycle checkpoint responds to DNA damage by either arresting cell-cycle progression, thereby facilitating DNA repair, or by initiating an apoptotic pathway that eliminates the damaged cell.⁶ However, when the checkpoint fails, the cell-cycle progresses despite the DNA damage and can occasionally result in chromosomal aberration,⁶ extensive polyploidy,⁷ and reduced apoptosis.⁸ Consequently, checkpoint failure is postulated to contribute to malignant progression by generating multiple subclones in a tumor.^{1,2} This is supported by the work of Cifone and Fidler,⁹ who showed that the increasing metastatic potential of murine fibrosarcoma cells is associated with an increased genetic instability.

Metastasis is the most malignant characteristic of a tumor and can be considered as the final stage of tumor progression.³ It is difficult to show clearly that genetic instability is directly responsible for the metastasis of clinical tumor cells, but studies with experimental tumors that can be transplanted into animals may be useful in investigating this issue. This is because it is often possible to select sublines with increased metastatic ability from a parental tumor population,³ whose heterogeneity seems to arise from its genetic instability.^{3,10} One of the best known examples of this is the selection of the F10 and BL6 sublines from B16 mouse melanoma cells.^{11,12} Although the BL6 subline was derived from F10 cells by six rounds of *in vitro* selection, it is markedly more metastatic than its parent. That is, while both cells metastasize to the lungs after being injected intravenously into mice,

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BL6 cells will metastasize to the lungs even after being injected subcutaneously.¹² According to the hypothesis proposed by Nowell,⁴ it may be that BL6 cells are genetically more unstable than F10 cells and that this has led to the accumulation of a number of genomic alterations that increased their metastatic ability.

We addressed this issue by examining the difference in gene expression between the F10 and BL6 sublines.^{13–18} We found that in BL6 cells, a type of retrotransposon had been inserted into an intronic region of the gene encoding the B56 γ regulatory subunit of protein phosphatase type 2A (PP2A).¹⁵ PP2A consists of a series of serine/threonine phosphatase holoenzymes that are composed of a common dimeric core of invariable catalytic (C) and structural (A) subunits associated with a variable regulatory (B) subunit.¹⁹ The regulatory subunit is extremely diverse because it is constituted by members from at least three unrelated families, namely, PR55 (or simply B), B56 (B'), and PR72 (B'').²⁰ Each of these families in turn consists of several subfamilies, each of which contains several proteins resembling each other structurally. For example, the B56 γ subfamily belongs to the B56 family and consists of three alternative splicing isoforms, B56 γ 1, - γ 2, and - γ 3. In BL6 cells, the rearrangement of the gene encoding the B56 γ regulatory subunit results in the abundant expression of a chimeric mRNA in which the 5' part of the original B56 γ subunit mRNA is replaced with the retrotransposon sequence.¹⁵ The chimeric mRNA encodes a mutant protein, termed $\Delta\gamma$ 1, that lacks the N-terminal 65 amino acid residues of B56 γ 1. $\Delta\gamma$ 1 protein expression is undetectable in *in vitro*-grown BL6 cells. However, when BL6 cells are transplanted into mice and undergo metastasis, $\Delta\gamma$ 1 expression is detectable in the metastatic foci but not in the transplantation site.¹⁵

The diversity of the regulatory subunit of PP2A results in different PP2A holoenzymes with distinct substrate specificities and subcellular localizations.²⁰ This allows PP2A to regulate a variety of cellular processes. Studies with yeast,²¹ *Xenopus* oocytes,²² and mammalian cells^{23–25} reveal that one of the most important functions of PP2A is to participate in the regulation of the cell-cycle checkpoint. Our previous work showed that $\Delta\gamma$ 1 prevents the B56 γ 1-containing PP2A heterotrimer from dephosphorylating its specific substrates.¹⁵ Consistent with this inhibitory effect of $\Delta\gamma$ 1, NIH3T3 cells that stably express $\Delta\gamma$ 1 are defective in cell-cycle checkpoint regulation when exposed to γ -radiation.¹⁵ This suggests that BL6 cells may also be defective in checkpoint regulation when $\Delta\gamma$ 1 is expressed.

In the present study, we found that the ability of the cell-cycle checkpoint in BL6 cells to respond appropriately to γ -radiation is indeed inferior to that in F10 cells when the cells are grown *in vivo* and express $\Delta\gamma$ 1. These characteristics of BL6 cells were reproduced in F10 cells transfected with $\Delta\gamma$ 1 (F10 $\Delta\gamma$ 1). F10 cells transfected with $\Delta\gamma$ 1 also showed an increased metastatic ability after being grown in mouse footpads and then irradiated. The metastatic colonies of these cells were found to have acquired considerable numbers of genomic alterations relative to the genomes of the primary lesions. Such

alterations were infrequently detected in the more rarely occurring metastatic colonies of F10 cells. These observations suggest that $\Delta\gamma$ 1 expression could have caused the checkpoint of BL6 cells to degenerate, thereby helping BL6 cells to acquire multiple genetic alterations that promote their metastatic ability.

Materials and Methods

Cell Culture and Transfection

B16 melanoma sublines F10 and BL6 were kindly provided by Dr. I. J. Fidler (University of Texas). NIH3T3 mouse fibroblastic cells were purchased from the American Type Culture Collection (Manassas, VA). All cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Construction of the plasmid vector encoding $\Delta\gamma$ 1 and transfection of F10 and NIH3T3 cells with this plasmid or the empty vector have been described previously.¹⁵ After the transfection, subclones of F10 and NIH3T3 cells were selected with 1.5 and 1.0 mg/ml of G-418, respectively. The cell-doubling time was determined by counting cells in triplicate samples at 24-, 48-, and 72-hour intervals as described previously.¹⁸

In Vitro γ -Irradiation and Cell-Cycle Analysis

Cells growing exponentially in culture dishes were γ -irradiated using a ¹³⁷Cs AECL Gamma cell 40 γ -irradiator (Nordion, Ottawa, Ontario, Canada) at a dose rate of 1.1 Gy/minute. Cells were subsequently cultured for the indicated time periods, fixed in 70% ethanol at -20°C, treated with RNase, stained with propidium iodide (PI; Sigma, St. Louis, MO), and analyzed by FACScan (Becton Dickinson, Franklin Lakes, NJ).

Tumor Growth, in Vivo γ -Irradiation, and Cell-Cycle Analysis

Cells (1×10^5) were injected subcutaneously into the right footpads of 4-week-old male C57BL/6 mice. Tumor size was monitored by measuring the anteroposterior diameter with calipers three or more times a week. Tumor-bearing mice were anesthetized, placed in a lead box that shields the entire trunk and head, and the right foot was extended outside through a hole in the side of the box. Radiation was delivered over the box with a collimated beam in a Radioflex 350 irradiator (Rigaku Co. Ltd., Osaka, Japan) at a dose rate of 1.5 Gy/minute. Control mice were anesthetized and placed in a lead box but received no γ -irradiation. Tumor cell masses were removed from the footpads by cutting out the skin tissue and single tumor-cell suspensions were obtained as described previously.^{26,27} Briefly, the tumor mass was minced with sharp scissors and passed first through a 23-gauge needle and then a nylon net (pore size 40 μ m). During the latter procedure, phosphate-buffered saline (PBS) was poured continuously over the net. The tumor-cell suspension was washed twice with PBS by centrifugation.

gation and resuspension. The cells were fixed in 70% ethanol at -20°C , treated with RNase, stained with PI (Sigma) and analyzed by FACScan (Becton Dickinson).

Spontaneous Metastasis Assay Combined with In Vivo γ -Irradiation

Mice were inoculated subcutaneously with F10, $10^{\Delta\gamma 1}$, F10^{V_{ec}}, or BL6 cells, and half received γ -irradiation locally to the footpads. On the day the footpad tumors reached a diameter of 6 mm, they were excised along with the popliteal lymph node. The mice were allowed to survive an additional 4 weeks after which autopsies were performed to macroscopically count the metastatic colonies that had formed in the bilateral lungs. All experiments included five to seven mice per group and were reproduced twice.

In Situ Detection of DNA Fragmentation

Tumor cell masses were removed from the footpads by cutting out the skin tissue which was then fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4- μm -thick slices. DNA fragmentation was detected with the fluorescein-based terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, sections were stained with the TUNEL reagent and PI and observed through a $\times 40$ objective of a confocal laser-scanning microscope (LSM510; Carl Zeiss, OberKochen, Germany). The high-power field contained ~ 100 tumor cells. After images were created on the computer monitor of the LSM510 system, sections were washed with distilled water and stained with hematoxylin and eosin (H&E). To quantify apoptosis of tumor cells, the areas where tumor cells grew compact were observed. Tumor samples were obtained from two individual mice before and after irradiation, and 10 randomly selected high-power fields were examined in each sample. The proportions of TUNEL-positive cells to the total cell number were pooled and the mean and SE were calculated. All experiments were reproduced once.

Western Blot Analysis

The production and specificity of the anti-B56 γ antibody has been described previously.¹⁵ Tumor cells growing in culture, in the footpads and in the lungs were lysed in a buffer containing 10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid, 0.5% Nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride. The resulting lysates were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, transferred to Immobilon (Millipore, Bedford, MA), and reacted with the anti-B56 γ antibody. After washing, the blots were incubated with peroxidase-labeled anti-rabbit IgG antibody (MBL Co. Ltd., Nagoya, Japan) and then reacted with Renaissance reagents (NEN, Boston, MA) before expo-

sure. After stripping, the blot was reacted with the anti- α -tubulin monoclonal antibody (Sigma), whose binding was detected with a peroxidase-labeled anti-mouse IgG antibody (MBL Co. Ltd.).

Clonogenic Survival Assay

Cells ($n = 3000$) were plated in a 10-cm culture dish. The following day, after the cells had attached to the plate, they were γ -irradiated with single doses ranging from 2 to 10 Gy. The cells were grown for another 10 days without changing the medium and then stained with Giemsa solution (Sigma). Colonies containing >50 cells were counted. The number of colonies in the plate receiving γ -irradiation was divided by the number not receiving γ -irradiation and expressed as survival fraction. Each experiment was done in triplicate and repeated three times.

Cytogenetic and Genomic DNA Analysis of Mouse Tumors

Mice were treated with the protocol of the spontaneous metastasis assay combined with *in vivo* γ -irradiation. Footpad tumors were isolated aseptically by amputation from the mice who were not γ -irradiated, and lung metastatic colonies were isolated at autopsy from the mice who were γ -irradiated. A single metastatic colony was isolated from each mouse. Tumor cell masses were minced with sharp scissors in Dulbecco's modified Eagle's medium/10% fetal calf serum supplemented with G-418 (1.5 mg/ml) and proliferating adherent cells from these preparations were used for cytogenetic chromosome analysis and DNA extraction. For cytogenetic analysis, the chromosomes were stained with Giemsa solution as described previously.^{14,17} To extract genomic DNA, cells were digested with 1 mg/ml of proteinase K for 3 hours at 55°C , followed by 0.5 mg/ml of RNase treatment for 1 hour and then phenol:chloroform extraction and ethanol precipitation. DNA was subjected to inter-simple sequence repeat polymerase chain reaction (inter-SSR PCR) analysis according to the procedure of Basik and colleagues²⁸ but with the minor modification described by Jackson and Matthews.²⁹ (CA)₈AG and (CA)₈GG primers were synthesized and the 5' ends of a proportion of the primers were labeled with an infrared fluorescence dye (IRD 800; Nissinbo, Chiba, Japan). PCR amplification was performed in 20- μL volumes containing 200 nmol/L of labeled primer [a 1:1 mixture of (CA)₈AG and (CA)₈GG] and 800 nmol/L of unlabeled primer of the same mixture. In addition, the reaction contained 50 ng of genomic DNA and 0.2 μL of Ex Taq polymerase (Takara, Ohtsu, Japan) in PCR buffer (10 mmol/L Tris-HCl, pH 9.0, 2% formamide, 50 mmol/L KCl, 0.2 mmol/L dNTPs, 1.5 mmol/L MgCl_2 , 0.01% gelatin, and 0.01% Triton X-100). The thermal profile consisted of an initial denaturation for 3 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C , 45 seconds at 52°C , and 2 minutes at 72°C , and a final extension for 7 minutes at 72°C . After denaturing the samples, a 2- μL aliquot of each

PCR mixture was loaded onto a 3% polyacrylamide gel containing 5.2 mol/L of urea and visualized on the Base ImagIR DNA analyzer (model 4000L; Li-Cor, Lincoln, NE). Experiments were reproduced at least twice. Four primary tumors were examined for each cell type and all yielded a similar electrophoretic band pattern. Electrophoretic bands of the primary tumor and metastatic colony of the same cell type were compared. A genomic alteration index²⁸ was determined by dividing the number of altered and lost bands in the metastatic colony DNA by the total number of bands in the primary tumor DNA.

Statistical Analysis

The *t*-test, the Mann-Whitney *U*-test, and the chi-square test were performed using StatView (Abacus Concepts Inc., Cary, NC) software on a Macintosh computer. Data are given as mean $\pm$ SE. A *P* value of <0.05 was considered to be significant.

Results

The DNA Damage Checkpoint in BL6 Cells Is Defective When the Cells Are Grown in Vivo

To examine the integrity of the DNA damage checkpoints in F10 and BL6 cells, we monitored the cell-cycle profile of both cells after they were cultured and then γ -irradiated (Figure 1). Before γ -irradiation, most of the cells had a 2N DNA content (G_1 phase) while a smaller but significant fraction contained 4N DNA (G_2/M phase). A few cells had a DNA content greater than 2N but less than 4N (S phase). A day (24 hours) after γ -irradiation, both the F10 and BL6 cells showed a decrease in 2N DNA cells and an increase in 4N DNA cells, indicating cell-cycle arrest in the G_2/M phase but not in G_1 . The next day (48 hours), considerable numbers of F10 and BL6 cells had an 8N DNA content. These polyploid cells had probably exited mitosis and initiated a new round of DNA replication without cytokinesis. After 2 more days, the polyploid fraction decreased and cells with a sub- G_1 DNA content appeared, indicating that the polyploid cells were proceeding to apoptosis. By 7 days after irradiation, the 8N- and sub- G_1 DNA-containing cells had become much less frequent and both F10 and BL6 cells had returned to their original diploid state. Obvious differences between F10 and BL6 cells in this pattern were not observed.

We next examined the response to γ -irradiation by F10 and BL6 cells grown *in vivo*. Two weeks after both cell types were inoculated into mouse footpads, tumors grew up to ~ 4 mm in diameter and were γ -irradiated by a single 12 Gy dose. At various times throughout the following week, cells from the F10 and BL6 footpad tumor masses were excised and their DNA content examined (Figure 2). F10 cells exhibited a cell-cycle profile quite similar to that observed after *in vitro* γ -irradiation. In contrast, BL6 cells responded to *in vivo* γ -irradiation by extending the degree of polyploidy and reducing the rate of apoptosis. Two and 4 days after γ -irradiation, the BL6 cells were highly polyploid, having a 16N or 32N DNA

Figure 1. Cell-cycle response of F10, F10 $\Delta\gamma 1$, and BL6 cells to *in vitro* γ -irradiation. Cells were grown *in vitro* and γ -irradiated by a single 8-Gy dose. On the days indicated after irradiation, cells were harvested, stained with PI, and examined by FACS. The proportion (%) of cells with a sub- G_1 DNA content is shown at the **top** of some histograms.

content (Figure 2). In addition, there was only a small accumulation of BL6 cells in a sub- G_1 fraction compared to the F10 cells (Figure 2). Finally, by 7 days after irradiation, while both F10 and BL6 cells were primarily diploid, a small proportion of the BL6 cells remained polyploid with an 8N DNA content. These observations indicate that the DNA damage checkpoint in BL6 cells is less capable of responding appropriately to γ -irradiation than the checkpoint in F10 cells. However, this difference manifests itself only when the cells are γ -irradiated in *in vivo* growing conditions.

Poor Checkpoint Integrity Is Associated with Radiation-Induced Expression of $\Delta\gamma 1$ Protein

The observations described above may relate to our previous finding that $\Delta\gamma 1$ protein is expressed in BL6 cells only when they are grown in metastatic foci.¹⁵ When we measured the levels of $\Delta\gamma 1$ protein, we found that γ -irradiation of *in vitro*-grown BL6 cells did not alter the negligible levels of $\Delta\gamma 1$ expression (Figure 3A) but that

Figure 2. Cell-cycle response of F10, F10 $\Delta\gamma1$, and BL6 cells to *in vivo* γ -irradiation. Cells were injected into mouse footpads and locally γ -irradiated by a single 12-Gy dose 2 weeks after the injection. On the days indicated after irradiation, tumor cell masses were excised from the footpad tumors and passed through a mesh. The resulting tumor cell suspension was stained with PI and examined by FACS. The proportion (%) of cells with a sub-G₁ DNA content is shown at the **top** of some histograms. The single peaks marked by **vertical arrows** represent mouse stromal cells.

irradiation of *in vivo*-grown BL6 cells strikingly increased $\Delta\gamma1$ protein levels 2 and 3 days after the irradiation (Figure 3B). The protein level of the B56 $\gamma1$ isoform was concomitantly slightly decreased but the level of the B56 $\gamma2$ isoform was unaltered. Two days after the emergence of $\Delta\gamma1$ expression, $\Delta\gamma1$ expression fell below the detection limit. The expression levels of the B56 $\gamma1$ and $\gamma2$ isoforms in irradiated *in vivo*-grown F10 cells were primarily unchanged in the week after the irradiation (Figure 3B).

Figure 3. Protein expression of the B56 γ subunit isoforms and $\Delta\gamma1$ in F10, F10 $\Delta\gamma1$, and BL6 cells after *in vitro* (A) and *in vivo* (B) γ -irradiation. Cells were grown in culture or in mouse footpads and γ -irradiated by single 8- and 12-Gy doses, respectively. On the days indicated after irradiation, cell lysates were extracted from cultured cells and footpad tumors, electrophoresed, and blotted with anti-B56 γ antibody. **Arrowheads** indicate the normal B56 γ isoforms and $\Delta\gamma1$.

To further examine the possible involvement of $\Delta\gamma1$ in the defective checkpoint of BL6 cells, we transfected F10 cells with $\Delta\gamma1$ cDNA. We isolated >10 F10 transfectants but all had a very low or undetectable expression of the $\Delta\gamma1$ protein when they were grown *in vitro* regardless of whether they were irradiated or not (data not shown). This suggested that the transfected F10 cells may express $\Delta\gamma1$ protein only when grown *in vivo*, as is the case with BL6 cells. We thus tested the F10 transfectants for $\Delta\gamma1$ protein expression when grown in mice and found that $\Delta\gamma1$ protein levels were markedly increased from their negligible baseline level when the tumor site was γ -irradiated (Figure 3B). The $\Delta\gamma1$ expression profile in the F10 transfectants throughout the week after γ -irradiation was essentially similar to that in BL6 cells. Furthermore, as seen in BL6 cells, the protein level of the B56 $\Delta\gamma1$ isoform was decreased when the exogenous $\Delta\gamma1$ was expressed while the B56 $\gamma2$ isoform levels remained primarily unaltered (Figure 3B). It thus appears that $\Delta\gamma1$ may down-regulate the expression of wild-type B56 $\gamma1$, although the molecular basis for this is presently unclear. In this study, the results of only one $\Delta\gamma1$ -transfected F10 subclone, denoted F10 $\Delta\gamma1$, are shown because the other two subclones examined showed essentially identical results. A vector-transfected F10 subclone, denoted F10 $\Delta\gamma1^{vec}$, was used as a negative control.

F10 $\Delta\gamma1$ Cells Show Poor Checkpoint Response to Irradiation

We monitored the cell-cycle profile of F10 $\Delta\gamma1$ and F10 $\Delta\gamma1^{vec}$ cells after *in vitro* γ -irradiation and found that the profiles of both cells were quantitatively identical to that of F10 cells (Figure 1; data not shown). In contrast, when they were grown *in vivo* and irradiated, there was a significant difference in the response because F10 $\Delta\gamma1^{vec}$ cells responded similarly to F10 cells while F10 $\Delta\gamma1$ cells responded similarly to BL6 cells (Figure 2; data not shown). The extensive polyploidy and reduction in apoptosis were somewhat more profound in F10 $\Delta\gamma1$ cells than in BL6 cells. These results suggest that the poor response to irradiation by the checkpoint in BL6 cells compared to F10 cells is because of the expression of $\Delta\gamma1$.

The differential apoptotic responses in irradiated F10 and F10 $\Delta\gamma1$ tumors were examined by detecting DNA fragmentation *in situ*. Before irradiation and 4 days later, tumor tissues were excised and stained with TUNEL and PI. As PI stains all nuclei red and TUNEL stains apoptotic nuclei green, apoptotic cells were identified as those with yellow nuclei. Few or no apoptotic cells were detectable in the nonirradiated tumors derived from either F10 or F10 $\Delta\gamma1$ cells (data not shown). Four days after irradiation, apoptotic cells were occasionally observed in F10 tumors (Figure 4) and H&E staining confirmed that these apoptotic cells were pyknotic F10 cells (Figure 4). In F10 tumors, the proportion of TUNEL-positive apoptotic cells to the total cell number was 14.4%. In contrast, there were few apoptotic cells in the irradiated F10 $\Delta\gamma1$ tumors (Figure 4).

Figure 4. *In situ* detection of DNA fragmentation in footpad tumors derived from F10 and F10 $\Delta\gamma 1$ cells. Sections of the tumor tissues 4 days after irradiation were stained with the fluorescein-based TUNEL reagent and PI and observed with the LSM510 microscope (**top**). TUNEL-positive cells contain yellow nuclei because the nuclei of all cells were also stained red with PI. After washing, the same sections were stained with H&E (**bottom**). **Arrowheads** indicate TUNEL-positive cells (**top**). The mean proportions of TUNEL-positive cells appear under the histological images. *, $P < 0.05$ by *t*-test when compared with the value of F10 tumors. Original magnifications, $\times 400$.

Effects of $\Delta\gamma 1$ on Cell-Cycle Checkpoint of NIH3T3 Cells

We previously established an NIH3T3 cell clone (NIH3T3 $\Delta\gamma 1$) that stably expresses $\Delta\gamma 1$ at a level equivalent to that of the endogenous B56 $\gamma 1$.¹⁵ Before irradiation, the cell-cycle profile of NIH3T3 $\Delta\gamma 1$ cells was similar to that of original NIH3T3 cell line or vector-control (NIH3T3^{Vec}) cells (Figure 5). Consistent with this is that the cell-doubling time was comparable among the three cell types [NIH3T3, 17.5 ± 2.9 ; NIH3T3^{Vec}, 17.9 ± 2.9 ; NIH3T3 $\Delta\gamma 1$, 18.0 ± 3.0 (hour)]. Cultured cells were then γ -irradiated and their cell-cycle profile examined. In the 12 hours after γ -irradiation, the 4N DNA cell fractions of the NIH3T3 and NIH3T3^{Vec} lines expanded, indicating cell-cycle arrest in the G₂/M phase. Half of the NIH3T3 and NIH3T3^{Vec} cells still contained 4N DNA after 24 hours of γ -irradiation and it took another 24 hours for both cell types to primarily revert to their original state. In contrast, 12 hours after γ -irradiation, the 2N and 4N DNA cell fractions of the NIH3T3 $\Delta\gamma 1$ line expanded and the line returned to its original state as early as 24 hours after irradiation (Figure 5). This indicates that the arrest of NIH3T3 $\Delta\gamma 1$ cells in the G₂/M phase after γ -irradiation was only transient.

Growth Response of F10 and Its Subclone Cells to *In Vitro* and *In Vivo* γ -Irradiation

In vitro radiation sensitivity was assessed by the standard clonogenic survival assay (Figure 6A). In this assay, the

Figure 5. Cell-cycle response of NIH3T3, NIH3T3^{Vec}, and NIH3T3 $\Delta\gamma 1$ cells to *in vitro* γ -irradiation. Cells were grown *in vitro* and γ -irradiated by a single 8-Gy dose. On the days indicated after irradiation, cells were harvested, stained with PI, and examined by FACS.

plating efficacy of F10 and its transfected subclones was $>70\%$ and did not differ between the cell lines. When the cells were γ -irradiated by single doses ranging from 2 to 10 Gy and their survival measured, there was little difference in the *in vitro* radiation sensitivity among F10 and its transfected subclones. The sensitivity of BL6 cells was also similar to that of F10 cells.

Nathanson and colleagues³⁰ have studied the *in vivo* growth kinetics and metastatic properties of irradiated F10 cells in detail, and revealed that single doses ranging from 7.5 to 15 Gy are subcurative. We followed the protocol in their report and γ -irradiated the F10 footpad tumor with a single 12-Gy dose 7 days after inoculation. No tumor growth was detectable for the next 10 days (Figure 6B) but in the fourth week after inoculation the γ -irradiated tumors began to grow and by the sixth week after inoculation had reached a diameter of 6 mm. Tumors that had not been γ -irradiated reached this size around the end of the third week (data not shown). Although F10 $\Delta\gamma 1$ cells did not apoptose as frequently as

Figure 6. Growth response of F10 and F10 $\Delta\gamma 1$ to *in vitro* and *in vivo* γ -irradiation. **A:** Clonogenic survival of F10, F10 $\Delta\gamma 1$, and BL6 cells after γ -irradiation. The day after plating, the cells were γ -irradiated with single doses ranging from 2 to 10 Gy. After culturing for 10 days, the numbers of colonies were counted and expressed as survival fractions. The mean values are plotted with bars indicating the SE when larger than the symbols. Experiments were repeated three times with essentially identical results. **B:** Growth of footpad tumors of F10 and F10 $\Delta\gamma 1$ cells after γ -irradiation. Cells (1×10^5) were injected subcutaneously into the mouse footpad. After 7 days, the footpads were γ -irradiated by a single 12-Gy dose. The anteroposterior diameters of the tumors were measured by a caliper. The mean values are plotted with bars indicating the SE when larger than the symbols. Experiments were repeated three times with essentially identical results.

F10 cells after *in vivo* γ -irradiation, the footpad tumors of F10 $\Delta\gamma 1$ and F10^{Vec} cells still showed primarily the same growth kinetics as the F10 cells in this experiment. Possible reasons for this are mentioned in the Discussion.

In Vivo Irradiated F10 $\Delta\gamma 1$ Cells Show Enhanced Lung Metastatic Ability

After mice were treated according to Nathanson's protocol,^{30,31} the footpad tumors were excised when they reached a diameter of 6 mm. It took 39.0 ± 3.5 days for F10 cells to reach this diameter, 36.8 ± 1.5 days for F10 $\Delta\gamma 1$ cells, and 37.8 ± 2.3 days for F10^{Vec} cells. This indicates that the length of days that mice were exposed to a tumor was comparable among three groups of mice inoculated with tumor cells. The mice were subsequently maintained for another 4 weeks and then autopsied. Few or no metastatic colonies were generated in the lungs of nonirradiated mice regardless of the type of F10 cell that had been inoculated (Table 1). This observation is consistent with our previous report.¹⁵ In contrast, when mice had been inoculated with F10 $\Delta\gamma 1$ cells and were then irradiated, the majority of the mice suffered from lung metastasis consisting of an average of 4.9 metastatic colonies (Table 1). Mice inoculated with F10 or F10^{Vec} cells did not exhibit this enhancement of lung metastasis on γ -irradiation. *In vivo* γ -irradiation also did not render BL6 cells more metastatic (data not shown).

We examined whether $\Delta\gamma 1$ was expressed in metastatic colonies of F10, BL6, and F10 $\Delta\gamma 1$ cells and found detectable $\Delta\gamma 1$ protein expression in the metastatic colonies of BL6 and F10 $\Delta\gamma 1$ cells but not in the few F10 metastatic colonies (Figure 7).

F10 $\Delta\gamma 1$ Cells Have More Genomic Alterations after in Vivo γ -Irradiation than F10^{Vec} Cells

It is possible that γ -irradiation allowed F10 $\Delta\gamma 1$ cells to produce subclones endowed with increased metastatic potential. We therefore performed cytogenetic chromosome analysis to assess whether the chromosomes of the primary lesion tumor cells differed from those of the tumor cells from metastatic lesions. However, we found that the tumor cells from both lesions were cytogenetically indistinguishable from one another, regardless of the F10 cell type being examined (data not shown). We then used inter-SSR PCR analysis to detect genomic alterations in

Table 1. Spontaneous Metastasis after *in Vivo* γ -Irradiation of Footpad Tumors with a Single 12-Gy Dose

Cell	<i>In vivo</i> γ -irradiation	No. of mice	No. of metastatic colonies [†]	No. of mice bearing lung metastasis
F10	No	12	0, 0, 0, 0, 0, 0, 0, 0, 0, 3, 3, 6	3/12
	Yes*	14	0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 2, 8	4/14
F10 $\Delta\gamma 1$	No	13	0, 0, 0, 0, 0, 0, 0, 0, 0, 3, 4, 7	3/13
	Yes	11	0, 0, 0, 4, 4, 4, 5, 6, 6, 7, 18 [‡]	8/11 [§]
F10 ^{Vec}	No	10	0, 0, 0, 0, 0, 0, 0, 0, 2, 9	2/10
	Yes	10	0, 0, 0, 0, 0, 0, 0, 0, 4, 8	2/10

Data are pooled from two independent experiments.

*, The footpad tumors were irradiated on the 7th day after-inoculation.

[†], Numbers in parentheses are the mean $\pm$ SE.

[‡], $P < 0.05$ by Mann-Whitney *U*-test when compared to the values of the nonirradiated F10 $\Delta\gamma 1$ tumors or the irradiated and nonirradiated F10 and F10^{Vec} tumors.

[§], $P < 0.05$ by *t*-test when compared to the mean $\pm$ SE of the nonirradiated F10 $\Delta\gamma 1$ tumors or the irradiated and nonirradiated F10 and F10^{Vec} tumors.

[¶], $P < 0.05$ by chi-square test when compared to the proportions of the nonirradiated F10 $\Delta\gamma 1$ tumors or the irradiated and nonirradiated F10 and F10^{Vec} tumors.

Figure 7. Expression of the $\Delta\gamma 1$ protein in metastatic tumors derived from *in vivo* γ -irradiated BL6 and F10 $\Delta\gamma 1$ cells. Mice were inoculated with F10, BL6, and F10 $\Delta\gamma 1$ cells and treated with the spontaneous metastasis assay combined with *in vivo* γ -irradiation. Protein samples of the footpad primary tumors (P) were obtained from the mice who had not been γ -irradiated. Protein samples of lung metastatic tumors (M) were obtained from mice who had been γ -irradiated. Samples were electrophoresed and blotted with anti-B56 γ antibody. **Arrowheads** indicate the normal B56 γ isoforms and $\Delta\gamma 1$. After stripping, the blot was probed with the anti- α -tubulin antibody to indicate the total amount of protein loaded per lane. Representative results of more than five metastatic tumors are shown.

the metastatic lesions relative to the genome of the primary lesion (Figure 8). To avoid contamination with genomes from stromal cells, we first made single cell suspensions from the excised tumor lesions of F10 $\Delta\gamma 1$ and F10^{Vec} cells and cultured them with G-418. The survivors were then used for genome extraction. To calculate the genomic alteration index,²⁸ we compared the electrophoretic bands of the PCR products from the primary lesion with those from three metastatic lesions (Figure 8). Each genomic alteration index was calculated with lesions originating from the same mouse. F10 $\Delta\gamma 1$ - and F10^{Vec}-inoculated mice that were not irradiated showed genomic alteration indices <1.0% (data not shown). Irradiation of F10^{Vec}-inoculated mice did not alter this low index (Table 2) but in contrast, irradiated F10 $\Delta\gamma 1$ -inoculated mice had a higher index of 3.7% (Table 2).

Discussion

In this study, we have examined the link between the genomic instability of tumor cells and their increasing metastatic ability by comparing the mouse melanoma cell line F10 and its more metastatic subline BL6. We revealed previously that the BL6 subline differs from F10 by a rearrangement in the gene encoding PP2A,¹⁵ a protein known to be involved in the cell-cycle checkpoint. The rearrangement generates a chimeric protein denoted as $\Delta\gamma 1$. We hypothesized that $\Delta\gamma 1$ may have helped BL6 cells acquire its increased metastatic ability by inducing an aberrant cell-cycle checkpoint and thereby promoting its acquisition of genomic alterations that aid metastasis. Here we tested this hypothesis.

We found that compared to F10 cells, the checkpoint in BL6 cells is aberrant when the cells were grown *in vivo* and then irradiated, and that this aberration may primarily be attributed to the presence of $\Delta\gamma 1$. This notion is supported by several observations. First, in response to *in vitro* γ -irradiation, F10 and BL6 cells display an equivalent defect in their checkpoints because both exhibit a transient arrest at the G₂/M phase and a subsequent accumulation of a polyploid cell fraction, followed by apoptosis. This type of defect is known as the "uncou-

Figure 8. Incidence of genetic alterations in spontaneous metastases of *in vivo* γ -irradiated F10^{Vec} or F10 $\Delta\gamma 1$ cells. Mice were inoculated with F10^{Vec} or F10 $\Delta\gamma 1$ cells and treated with the spontaneous metastasis assay combined with *in vivo* γ -irradiation. Tumor cell masses of the footpad primary lesions (P) obtained from the mice were not γ -irradiated. Tumor cell masses of lung metastatic tumors (M) were obtained from γ -irradiated mice. Tumor cells were cultured in the presence of G-418 and survivors were subjected to DNA extraction. DNA was amplified by inter-SSR PCR and visualized on the Base Imager DNA analyzer. Regions of the electrophoretic gels encompassing 200 to 500 bp are shown. **Arrows** and **arrowheads** placed alongside the lanes indicate the bands from the metastatic lesions that are altered or lost compared to the bands from the primary lesions, respectively. The primary tumors from different mice gave essentially similar results and a representative result is shown (P). Results of the metastatic lesions from three mice (M1, M2, and M3) inoculated with F10 $\Delta\gamma 1$ cells and one mouse (M1) inoculated with F10^{Vec} cells are also shown.

pling" of the S and M phases.^{32,33} However, when BL6 and F10 cells are grown and irradiated *in vivo*, the BL6 cell-cycle checkpoint becomes more aberrant compared to F10 because the BL6 cells display a high degree of polyploidy that is evident for a longer period and the rate of apoptosis is reduced. In contrast, the cell-cycle profile of F10 cells is the same as that after *in vitro* γ -irradiation. Thus, the checkpoint of BL6 cells becomes aberrant only in certain conditions, namely, when the cells are grown *in vivo* and then irradiated. These are exactly the conditions in which the $\Delta\gamma 1$ protein is expressed as the $\Delta\gamma 1$ protein is not detected in *in vitro*-grown BL6 cells, whether irradiated or not, or in irradiated *in vivo*-grown F10 cells. Furthermore, the extension of the polyploid period in irradiated *in vivo*-grown BL6 cells peaks on the fourth day after irradiation and 1 or 2 days before this, $\Delta\gamma 1$ protein levels become markedly increased in BL6 cells. Thus, the emergence of the aberrant cell-cycle checkpoint in irradiated *in vivo*-grown BL6 cells correlates with the synthesis of the $\Delta\gamma 1$ protein. Supporting this correlation is that the disparate cell-cycle phenotypes of BL6 cells grown *in vitro* and *in vivo* were completely reproducible in F10 $\Delta\gamma 1$ cells, which, like BL6 cells, only express $\Delta\gamma 1$ after *in vivo* γ -irradiation. Expression of $\Delta\gamma 1$ thus appears to cripple the ability of the DNA damage checkpoint to respond appropriately to irradiation.

To further clarify the role of $\Delta\gamma 1$ on the cell-cycle checkpoint, we used NIH3T3 cells that stably expressed exogenous $\Delta\gamma 1$ protein at a level comparable with that of the endogenous B56 $\gamma 1$. As shown previously,¹⁵ NIH3T3 $\Delta\gamma 1$ cells are defective in G₂/M cell-cycle arrest after γ -irradi-

Table 2. Incidence of Genomic Alterations in the Lung Metastases Relative to the Primary Tumors of *in Vivo* Irradiated F10 $\Delta\gamma 1$ and F10^{Vec} Cells

Cell	No. of metastatic colonies examined by inter-SSR PCR*	Genomic alteration index (%) [†]
F10 $\Delta\gamma 1$	6	3.1, 3.1, 3.4, 4.2, 4.2, 4.2 (3.7 $\pm$ 0.23) [‡]
F10 ^{Vec}	4	0.8, 0.8, 1.0, 1.0 (0.90 $\pm$ 0.058)

*, Parts of the results are shown in Figure 8.

[†], Numbers in parentheses are the mean $\pm$ SE.[‡], $P < 0.05$ by *t*-test when the mean was compared with the mean of F10^{Vec} cells.

ation. $\Delta\gamma 1$ expression thus appears to shorten the duration that NIH3T3 cells are arrested in the G₂/M phase in response to γ -irradiation. This is a somewhat different pattern to that observed in irradiated *in vivo*-grown BL6 and F10 $\Delta\gamma 1$ cells because these cells had an extended period of polyploidy and underwent less apoptosis. This difference can be explained by the observation that the F10 and BL6 cells not expressing $\Delta\gamma 1$ already suffer a checkpoint defect because S/M uncoupling is observed in these cells regardless of whether or not they are irradiated. However, once grown *in vivo* and irradiated, BL6 and F10 $\Delta\gamma 1$ express $\Delta\gamma 1$, which inflicts an additional checkpoint fault that causes the cells to undergo multiple rounds of DNA synthesis without arresting long enough in the G₂/M phase. The extensive polyploidy exhibited by BL6 and F10 $\Delta\gamma 1$ cells may thus be attributable to a combination of S/M uncoupling and defective G₂/M arrest.

Notably, in both BL6 and F10 $\Delta\gamma 1$ cells, $\Delta\gamma 1$ protein expression occurred only when the cells were grown *in vivo* and irradiated. When the cells were grown and irradiated *in vitro*, no expression was observed. The levels of mRNA encoding $\Delta\gamma 1$ do not differ when the cells are grown under *in vivo* or *in vitro* conditions and thus it appears that $\Delta\gamma 1$ protein expression depends on a post-transcriptional regulation in F10 $\Delta\gamma 1$ and BL6 cells that operates only in *in vivo* irradiated tumors and metastatic foci. It may be that the *in vivo* environment enhances the translation of $\Delta\gamma 1$ or increases its stability. Similarly to this notion, certain microenvironments in which tumor cells grow are known to change the levels of transcription of some genes.³⁴ For example, in colon cancer cells, several metastasis-related genes such as *MDR1* and *CEA* are expressed differently depending not only on whether the cells are grown *in vitro* or *in vivo* but also on whether they are grown in orthotopic or ectopic environments.³⁴ These observations clearly indicate that performing *in vivo* experiments and in particular orthotopically implanting tumor cells into recipient animals are important approaches in the study of metastasis and its related fields.^{35,36}

Cell-cycle analysis indicated that the apoptosis rate of F10 $\Delta\gamma 1$ tumor cells after *in vivo* γ -irradiation is slower than that of F10 cells. A similar observations was noted when irradiated F10 or F10 $\Delta\gamma 1$ tumors were stained with the TUNEL method, as the proportion of TUNEL-positive cells was larger in F10 $\Delta\gamma 1$ tumors than in F10 tumors 4 days after γ -irradiation. Curiously, however, the footpad tumors of the two cells did not differ significantly in their growth kinetics after γ -irradiation (Figure 6B). This discordance

suggests that the apoptosis rate of tumor cells when their colonies are still microscopic does not influence subsequent tumor growth in a macroscopic level. Alternatively, the F10 tumor cells that survive apoptosis may recover their growth more rapidly than F10 $\Delta\gamma 1$ tumor cells. This latter notion is supported by the previous work comparing tumor growth of p53-wild-type cells and p53-deficient cells.⁸ p53-wild-type cells are more sensitive to irradiation and thus tumors of these cells shrink more severely and rapidly after exposure to radiation. However, once the tumors begin to grow again, their growth rate is higher than that of p53-deficient tumors. As a result, the tumors of p53-wild-type cells reach their original size as quickly as the tumors of p53-deficient cells, and thereafter both tumors show similar growth kinetics.⁸ The extent of apoptosis after irradiation may thus not necessarily reduce the final tumor size or growth rate.

Given that $\Delta\gamma 1$ appears to disrupt the proper functioning of the cell-cycle checkpoint, we wondered whether the expression of $\Delta\gamma 1$ could also be associated with the high metastatic ability of BL6 cells. We consequently combined the spontaneous metastasis assay with *in vivo* γ -irradiation. In these experiments, the duration that a host animal was exposed to a tumor and the final tumor size were equivalent among the various cell types examined. These are both parameters that often affect subsequent metastasis.^{30,37} In addition, tumors were γ -irradiated when they were so small that metastasis might not yet have occurred.³⁰ Under these experimental conditions, *in vivo* irradiated F10 $\Delta\gamma 1$ cells had an increased metastatic ability. Moreover, $\Delta\gamma 1$ expression was detectable in the metastatic tumors derived from irradiated F10 $\Delta\gamma 1$ cells, but not from irradiated F10 cells. These observations support the notion that the expression of $\Delta\gamma 1$ correlates with the acquisition of greater metastatic ability.

It has been shown that local γ -irradiation at subcurative doses often increases the rate of the subsequent metastasis of various tumors in experimental models.^{30,31,37} The two most likely mechanisms driving this phenomenon are that either the irradiation directly alters the cell's genome, leading to the development of subclones with increased metastatic properties, or the irradiation facilitates the entry of tumor cells into the circulation, possibly by affecting the permeability of blood vessels, the so-called "tumor bed effect."³⁷ The tumor bed effect does not appear to explain our observations because *in vivo* γ -irradiation does not enhance the metastasis of F10 cells. However, the comparison of the

genomes between the primary and metastatic lesions supports the former possibility. Few alterations occurred in the genomes of the rare F10^{Vec} metastatic colonies whether the primary lesions had received *in vivo* γ -irradiation or not. In contrast, genetic alterations were detected in the metastatic colonies of F10 $\Delta\gamma$ 1 cells at an increased frequency, albeit only when the primary tumors had received *in vivo* γ -irradiation. The metastatic colonies of F10 $\Delta\gamma$ 1 cells were not clonally identical to each other because the altered bands were not always located at the same positions in the inter-SSR PCR analysis. Thus, $\Delta\gamma$ 1-induced defect in the cell-cycle checkpoint may have contributed directly to increased mutation rates in *in vivo* irradiated F10 $\Delta\gamma$ 1 cells. As a result, these F10 $\Delta\gamma$ 1 cells appeared to allow the development of multiple subclones, some of which might be endowed with increased metastatic potential.

BL6 cells did not become more metastatic after *in vivo* γ -irradiation despite the fact that *in vivo* γ -irradiation increased $\Delta\gamma$ 1 protein levels in BL6 cells to the same degree as observed in F10 $\Delta\gamma$ 1 cells. This may be simply because BL6 cells already possess as many genomic alterations as they can tolerate. Alternatively, the defect in the cell-cycle checkpoint induced by $\Delta\gamma$ 1 expression may only allow a specific type and number of genomic alterations to pass unedited into the genome, and BL6 cells may have already fully acquired this complement. It is possible that during the six rounds of *in vitro* selection of F10 cells that generated the BL6 line, an early event was the retrotransposon insertion into the B56 γ gene that allows $\Delta\gamma$ 1 expression. This rearrangement then allowed the cells to efficiently accumulate multiple mutations in their genomes, some of which promoted metastatic ability. Supporting this is that BL6 cells are still more metastatic than *in vivo* irradiated F10 $\Delta\gamma$ 1 cells, whether irradiated or not, because they form >20 metastatic colonies in the lungs when injected subcutaneously into the footpads.^{15,16} Thus, it is likely that BL6 cells have accumulated more genomic alterations than *in vivo* irradiated F10 $\Delta\gamma$ 1 cells when compared to F10 cells. Probably, BL6 cells either cannot tolerate any additional changes or may have acquired the full range of changes permitted by the $\Delta\gamma$ 1-induced defect in cell-cycle checkpoint.

In summary, $\Delta\gamma$ 1 expression in response to irradiation is associated with three effects: first, the degradation of checkpoint integrity; second, the acquisition of multiple genomic alterations; and third, the development of greater metastatic ability. Although the direct links between these three events remain to be proved, our observations thus suggest that $\Delta\gamma$ 1 expression, which disrupts proper PP2A functioning in the cell-cycle checkpoint, can cause all three effects. This is also supported by the observation that okadaic acid, a PP2A inhibitor, is known to promote genetic instability and tumorigenicity, probably by inhibiting the activities of PP2A,³⁸ although it remains unclear which of the PP2A holoenzymes are targeted by okadaic acid. Nevertheless, our current observations suggest that the B56 γ -containing PP2A holoenzymes may be important in guarding genome integrity and that impairment of the function of these holoenzymes may result in malignant progression. Our observations also support the more general hypothesis

that genetic instability could promote tumor progression from the nonmetastatic to the metastatic state.

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