

Chemical Carcinogen (Dimethyl-benzanthracene) Induced Transplantable Cancer in Fanconi Anemia (Fanca^{-/-}) Mice

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Abstract. *Background/Aim: Patients with radiation sensitive Fanconi anemia (FA) are presenting with cancers of the oral cavity, oropharynx, and other anatomic locations. Materials and Methods: Animal models for cancer in FA mice used orthotopic tumors from wild type mice. We derived a cancer cell line from Fanca^{-/-} mice by topical application of the chemical carcinogen dimethyl benzanthracene (DMBA). Results: A Fanca^{-/-} mouse rhabdomyosarcoma was derived from a Fanca^{-/-} (129/Sv) mouse. The in vitro clonogenic survival of the Fanca^{-/-} clone 6 cancer cell line was consistent with the FA genotype. Transplanted tumors demonstrated hypoxic centers surrounded by senescent cells. Conclusion: This Fanca^{-/-} mouse syngeneic cancer should provide a valuable resource for discovery and development of new normal tissue radioprotectors for patients with FA and cancer.*

Patients with Fanconi anemia (FA) demonstrate absence or functional defects in one or more of the gene products associated with the DNA-repair protein scaffold, which facilitates binding of repair enzymes to DNA double strand breaks (1, 2). Many patients with FA suffer from anemia, which results from a hyper-responsiveness of their

hematopoietic stem cells to the suppressive cytokine TGF- β (3-5) that is naturally produced by cells of the hematopoietic microenvironment. In the past decades, a significant number of patients with FA have been successfully treated with bone marrow transplantation (6-9). Successful bone marrow transplantation has been attributed in part to the availability of less toxic transplantation preparatory regimens (6-9). New preparation methods for bone marrow transplantation in patients with FA have included partial body irradiation, and delivery of chemotherapy drugs such as fludarabine (7) that are less toxic to normal tissue. Based upon the success of bone marrow transplantation, many patients with FA now live longer and have more productive lives (7, 8, 10-12).

A recent serious concern for both marrow-transplanted and non-transplanted patients with FA is the increasing frequency of cancer (13-16). Patients with FA are presenting with squamous cell carcinomas of the head and neck region, commonly at the base of tongue and oropharynx (17, 18), but also at other sites in the aerodigestive tract including those in the esophagus and vulvovaginal region (15-18). Patients with FA may be sensitive to both ionizing irradiation (19) and DNA cross-linking chemotherapy agents such as mitomycin-C or cis-platinum (10).

The management of cancer in patients with FA has been complicated not only by their sensitivity for radiotherapy, but also by the locally aggressive invasiveness of the tumors, and their propensity for both local recurrence and distant metastasis (14). At the time of initial diagnosis, cancers in patients with FA show a high frequency of microscopic invasion beyond surgical resection margins (14), significant spread to regional lymph nodes, and distant metastasis (20-22). Recent research has focused on methods to reduce the toxicity of chemoradiotherapy in patients with FA (20-27). The development of new agents to protect normal tissues, while compromising the viability of residual tumor cells has already led to the discovery of novel radioprotective agents including the mitochondrial targeted nitroxide JP4-039 (23-29).

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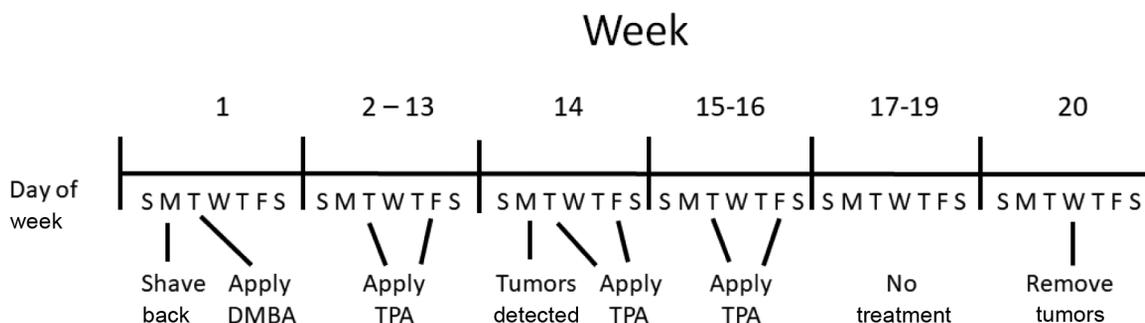


Figure 1. Time course of tumor induction. DMBA/TPA was administered for over 16 weeks and tumors appeared at week 14 for *Fanca*^{+/+} and week 15 for *Fanca*^{-/-} mice. *Fanca*^{-/-} and *Fanca*^{+/+} mice in groups of 10 were topically administered with DMBA (100 μ M) in 100 μ l saline as described in the Materials and Methods section. Beginning at week 2 and weekly for 13 weeks, mice received twice weekly skin applications of the promoter TPA as described in Materials and Methods.

One potential problem with the discovery of new radioprotective agents to manage cancer in patients with FA is that current animal models that are used to define the therapeutic ratio (tumor reduction relative to normal tissue toxicity) use orthotopic tumors of a different genotype (26). A transplantable cancer in FA mice using cells of the same genotype would facilitate a better analysis of the magnitude of tumor cyto-reduction by chemoradiotherapy relative to normal tissue toxicity. We here report a carcinogen (DMBA)-induced transplantable cancer cell line developed in *Fanca*^{-/-} mice. This cell line, when injected subcutaneously, will develop an orthotopic tumor. The initial radiobiology studies demonstrate the potential value of this cancer cell line to form orthotopic tumors, which are suitable for evaluation of the toxicity of fractionated radiotherapy.

Materials and Methods

Mice and animal care. *Fanca*^{+/-} mice on the 129/Sv background were originally obtained from Markus Grompe, PhD, Oregon Health Sciences Center. Colonies of *Fanca*^{+/+} and *Fanca*^{-/-} mice were derived by breeding *Fanca*^{+/-} mice to yield *Fanca*^{+/+} and *Fanca*^{-/-} mice. The homozygous *Fanca*^{+/+} males were bred with *Fanca*^{+/+} females, and then male *Fanca*^{-/-} with female *Fanca*^{-/-} mice to yield homozygous *Fanca*^{+/+} and *Fanca*^{-/-} colonies (27). The mouse colonies were maintained at the University of Pittsburgh. Animals were housed 4 per cage and managed according to the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) regulations. All animal protocols were approved by the University of Pittsburgh IACUC. Animals were maintained on deionized water and fed standard laboratory chow. Veterinary care was provided by the Division of Laboratory Animal Resources of the University of Pittsburgh.

DMBA carcinogen induced tumors. The intrascapular region of the dorsum of the back of each mouse of groups of 10 six- to eight-week-old *Fanca*^{+/+} and *Fanca*^{-/-} mice were shaved. Twenty-four hours later, 30 μ g of dimethyl-benzanthracene (DMBA) (Millipore Sigma, Burlington, MA, USA) was dissolved in 200 μ l of acetone

(Millipore Sigma) and applied to the backs of each animal. Beginning one week later 12.34 μ g of PMA [Phorbol 12-myristate 13-acetate, also known as tissue plasminogen activator (TPA); Millipore Sigma]. TPA was dissolved in 200 μ l of acetone and applied bi-weekly to the same area of the skin of the back for 20 weeks. Tumor nodules formed at the site of both *Fanca*^{+/+} mice and *Fanca*^{-/-} mice. The tumor nodules were removed, prepared into a single cell suspension by mincing with scissors and filtered through progressively smaller gauge needles down to 30 gauge. The cells were grown *in vitro* in Dulbecco's Modified Eagles medium containing 20% fetal calf serum and penicillin/streptomycin (5,000 U/ml). The DMBA induced *Fanca*^{-/-} tumor cells were passaged weekly at 1:2 and 1:5, then aliquots of 1×10^6 of the *Fanca*^{-/-} or *Fanca*^{+/+} tumor cells were frozen in dimethyl sulfoxide (DMSO) (Millipore Sigma) at -80° . Cells (10^6 cells in 100 μ l saline) were injected subcutaneously in the dorsum of the back of *Fanca*^{-/-} or *Fanca*^{+/+} mice. Three weeks later palpable tumors were detected on the back of the *Fanca*^{-/-} mice. By 6 weeks after injection, tumors of 1 cm diameter were removed and prepared as single cell suspensions. Aliquots of resuspended cells at 1×10^7 cells were injected subcutaneously into *Fanca*^{-/-} or *Fanca*^{+/+} mice. The remaining resuspended cells were expanded *in vitro* for cloning. A tumor from a *Fanca*^{+/+} mouse that was treated with DMBA and TPA was also established, as a cell line. The *in vitro* cell line was passaged three times. An aliquot of 1×10^7 *Fanca*^{+/+} tumor cells was injected subcutaneously in the back of each of 5 *Fanca*^{+/+} mice. The schema of the tumor induction process is shown in Figure 1.

Cloning of *Fanca*^{-/-} tumor cell line. To obtain a homogenous *Fanca*^{-/-} tumor cell line, the *Fanca*^{-/-} tumor cell line was cloned after 4 weeks passage *in vitro*. Single cell suspensions were cloned by using a flow cytometer (Beckman Coulter MoFlo Astrios High Speed Sorter, Beckman Coulter, Brea, CA, USA) to place one cell in each well of each of three 96-well plates. Clonal cell lines were grown in medium containing DMBA, 20% fetal calf serum and antibiotics. Clonal cell lines were expanded and grown in medium containing 0 to 20% fetal calf serum. Clone 6 was chosen for study due to the speed of growth in 1% fetal calf serum. Aliquots of 10^8 cells of clone 6 were centrifuged to concentrate in 100 μ l saline and injected subcutaneously into the back of each of 10 *Fanca*^{-/-} and *Fanca*^{+/+} mice.

Table I. Antibodies used in immunohistochemical analysis of *Fanca*^{-/-} tumor. Tissue sections of *Fanca*^{-/-} tumors were stained with the antibodies shown in this table to identify the classification of the *Fanca*^{-/-} tumor using the antibody conditions listed in the table.

Antibody	Immunohistochemical Staining Conditions
CD34 (hematopoietic)	Citrate retrieval solution pH6.0 Clone: ARC0219 – 1:100 dilution – Invitrogen (Waltham, MA, USA) Detection – Boost Rabbit – Cell Signaling
CD31 (epithelial)	Citrate retrieval solution pH6.0 Clone: D8V9E – 1:200 dilution – Cell Signaling Detection – Boost Rabbit – Cell Signaling
SMA	Citrate retrieval solution pH6.0 Clone: ASM-1 – 1:100 dilution – Millipore Sigma (Burlington, MA, USA) Detection – Mouse on Mouse HRP – Biocare Medical (Concord, CA, USA)
S100 (spindle cell)	Citrate retrieval solution pH6.0 Clone: EP32 – 1:200 dilution – Cell Marque (Rocklin, CA, USA) Detection – Boost Rabbit – Cell Signaling (Danvers, MA, USA)
MAC2 (dendritic/macrophages)	EDTA retrieval solution pH9.0 Clone: 1C1B2 – 1:1,000 dilution – ProteinTech (Rosemont, IL, USA) Detection – Mouse on Mouse HRP – Biocare Medical (Concord, CA, USA)
MelanA (melanoma)	EDTA retrieval solution pH9.0 Clone: EPR20380 – 1:500 dilution – Abcam (Boston, MA, USA) Detection – Boost Rabbit – Cell Signaling
Desmin (skeletal/cardiac muscle)	Citrate retrieval pH 6.0 Clone: Rabbit poly clonal (cat# ab227224)– 1:500 Dilution – Abcam Detection – Boost Rabbit – Cell Signaling

Other mouse cancer cell lines. The 3LL Lewis Lung squamous cell carcinoma cell line has been described (30, 31). The 2F8cis mouse ovarian cancer cell line has been described (32, 33).

Histochemical and histopathologic staining. We analyzed the histopathology of the primary tumors and each of successive transplant generation tumors. A 2 cm tumor formed by injecting 10⁸ *Fanca*^{-/-} clone 6 cells was excised from a *Fanca*^{-/-} mouse, fixed in 10% paraformaldehyde and parafilm embedded. The tumor was sectioned and stained using hematoxylin and eosin, as well as for markers to identify the phenotype. To further characterize tumors formed by *Fanca*^{-/-} tumor cell lines, and clonal sublines, tumor cell sections were immunocytochemically stained for SMA, S100 (spindle cell markers), CD34 (hematopoietic stem cell), MAC2 (histiocytic marker), CD31 (endothelial), Melan-a (melanoma), and Desmin (smooth/cardiac muscle). Details on the antibodies such as supplier, dilution, and antigen retrieval are shown in Table I. The slides were deparaffinized and rehydrated using a standard histology protocol. Antigen retrieval was performed using a citrate retrieval solution pH 6.0 or EDTA retrieval solution pH 9.0 (ab93678 or ab93680, Abcam, Waltham, MA, USA) and a Decloaking chamber NXGEN (Biocare Medical, Pacheco, CA, USA) at 120°C. The slides were stained using an Autostainer Plus (Dako, Carpinteria, CA, USA) with TBST rinse buffer (Dako). Each antibody was applied as described in Table I. The detection system was an HRP (Horse Radish Peroxidase) labeled polymer as listed in Table I. The substrate used was 3,3, Diaminobenzidine + (Dako). The slides were then counterstained with Hematoxylin (Dako).

Assays for hypoxic regions in tumors and for senescent cells. Histopathological sections were stained for hypoxia inducible

factor-1 (34), and simultaneously for p16 or beta-galactosidase, which are biomarkers of senescence (35) as previously described.

*Absence of the *Fanca* gene and gene product in *Fanca*^{-/-} tumor cells.* To confirm that the DMBA-induced tumor cell line was derived from a *Fanca*^{-/-} mouse, DNA was extracted from the *Fanca*^{-/-} tumor cell line and a PCR reaction was performed using *Fanca*^{-/-} gene specific primers used in genotyping of *Fanca*^{-/-} mice (27).

*Clonogenic radiation survival curves of *Fanca*^{-/-} tumor cell lines.* The methods for clonogenic radiation survival curves have been published previously (24). Briefly, clonal lines and pools of cancer cells were irradiated to doses between 0 and 8 Gy using a Cesium JL Shepherd Model 68A irradiator (JL Shepherd & Associates, San Fernando, CA, USA) at 275 cGy per minute. Cells were plated at varying densities ranging from 100 to 1,000 cells per plate and stained with crystal violet (Millipore Sigma) 7 days later. Quadruplicate plates at each radiation dose were scored on day 7 for colonies containing greater than 50 cells. The radiation survival curves were plotted using a computer program that calculates the D0 and \bar{n} according to published methods using single-hit, multi-target, and linear quadratic models (24). Control cells included Lewis Lung Carcinoma (3LL) lung squamous cell carcinoma cells (30, 31). Results were calculated by pooling triplicate experiments for data presentation.

Physics. The cells were irradiated using a JL Shepherd Model 68A cesium irradiator (JL Shepherd & Associates). The dose in cGy was determined using a cesium decay chart. The dose rate was verified each year by the Center for Medical Countermeasures Against Radiation Consortium (CMCRC) Physics Core at the University of

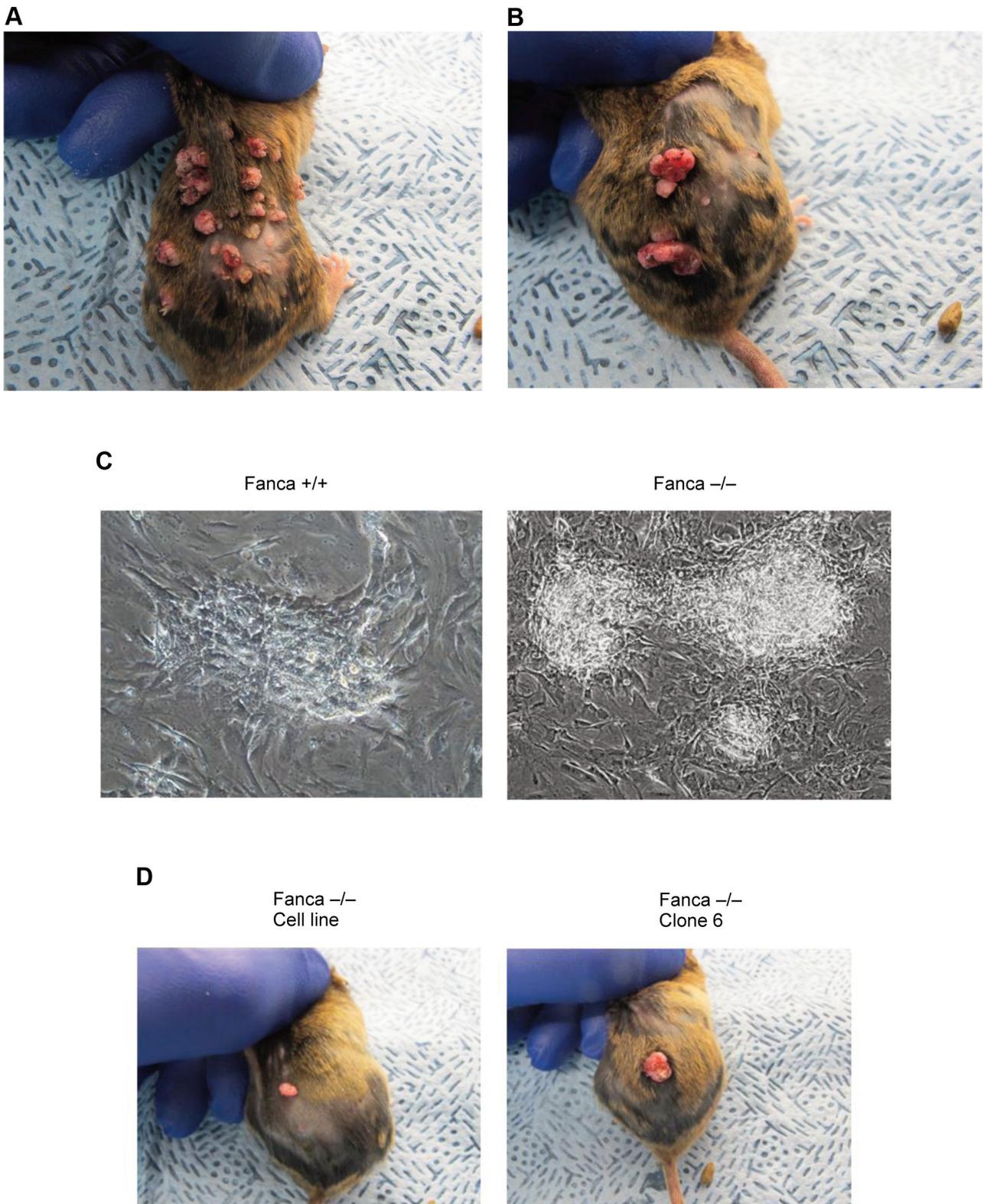


Figure 2. Tumors at the site of topical administration of DMBA/TPA in *Fanca*^{-/-} and *Fanca*^{+/+} mice (A and B). When tumors reached 1.0 cm in diameter, they were explanted to culture ($\times 100$) (C). Cells of *Fanca*^{-/-} tumors or *Fanca* clone 6 were injected into the skin of the back of a second group of *Fanca*^{-/-} and *Fanca*^{+/+} mice at 10^7 cells per 100 μ l saline (D).

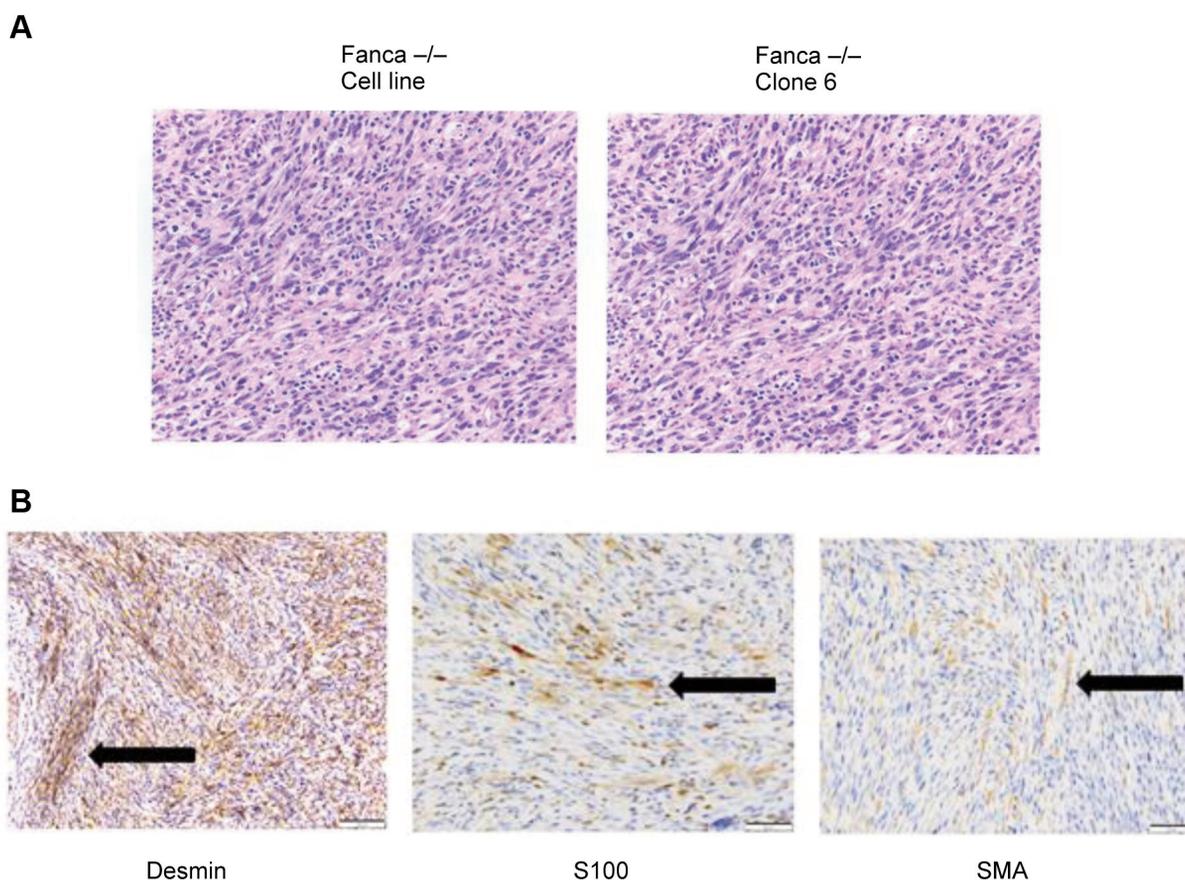


Figure 3. Histopathology of *Fanca*^{-/-} tumors. *Fanca*^{-/-} and *Fanca*^{-/-} Clone 6 tumors were excised, fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin (H & E) (A). To identify the type of tumor, sections of *Fanca*^{-/-} tumor were stained for multiple histochemical markers (B). Sections were strongly positive for Desmin and weakly positive for S100 and SMA. These are characteristics of a muscle tumor, most likely leiomyosarcoma. Black arrows point to areas of positive staining.

Wisconsin who sends out phantoms for irradiation calibration using our Cesium Irradiator (26). The phantoms were sent back to the core to calculate the dose rate.

Statistics. All statistical calculations were performed using a Student *t*-test on the Graph Pad Prism (Boston, MA, USA) (23-27).

Results

The formation of tumors at sites of DMBA injection. Tumors developed at the site of DMBA-application according to the protocol described in the Materials and Methods and are shown in Figure 1. The paradigm for DMBA and promoter application and time course are shown in Figure 1. Both *Fanca*^{-/-} and *Fanca*^{+/+} mice had tumors by 14 weeks at the site of DMBA injection (Figure 2). The tumors were removed and made into single cell suspensions and cell lines developed (Figure 2C). The *Fanca*^{-/-} cell line and clone 6

of the *Fanca*^{-/-} cell line were injected into additional C57BL/6 mice, which we refer to as second generation mice and tumors developed 2 weeks after injection (Figure 2C).

Histopathologic diagnosis and immunohistochemistry of tumors in Fanca^{-/-} mice. Hematoxylin and Eosin staining of the *Fanca*^{-/-} tumors (Figure 3A) showed moderately pleomorphic neoplastic spindle cells. There was moderate to high cellularity and poorly defined eosinophilic cytoplasm. The mitotic rate averaged two mitoses per 40 × high powered fields (HPF) (Figure 3A). The results of immunohistochemical (IHC) staining showed a strong positivity for the skeletal/cardiac muscle marker desmin, and slight positivity for SMA and S100 (Figure 3B), which is indicative of a primitive muscle tumor, most likely a rhabdomyosarcoma. The large spindle cells and high mitotic rate were suggestive of neoplasia. There was no evidence of glandular or epithelial

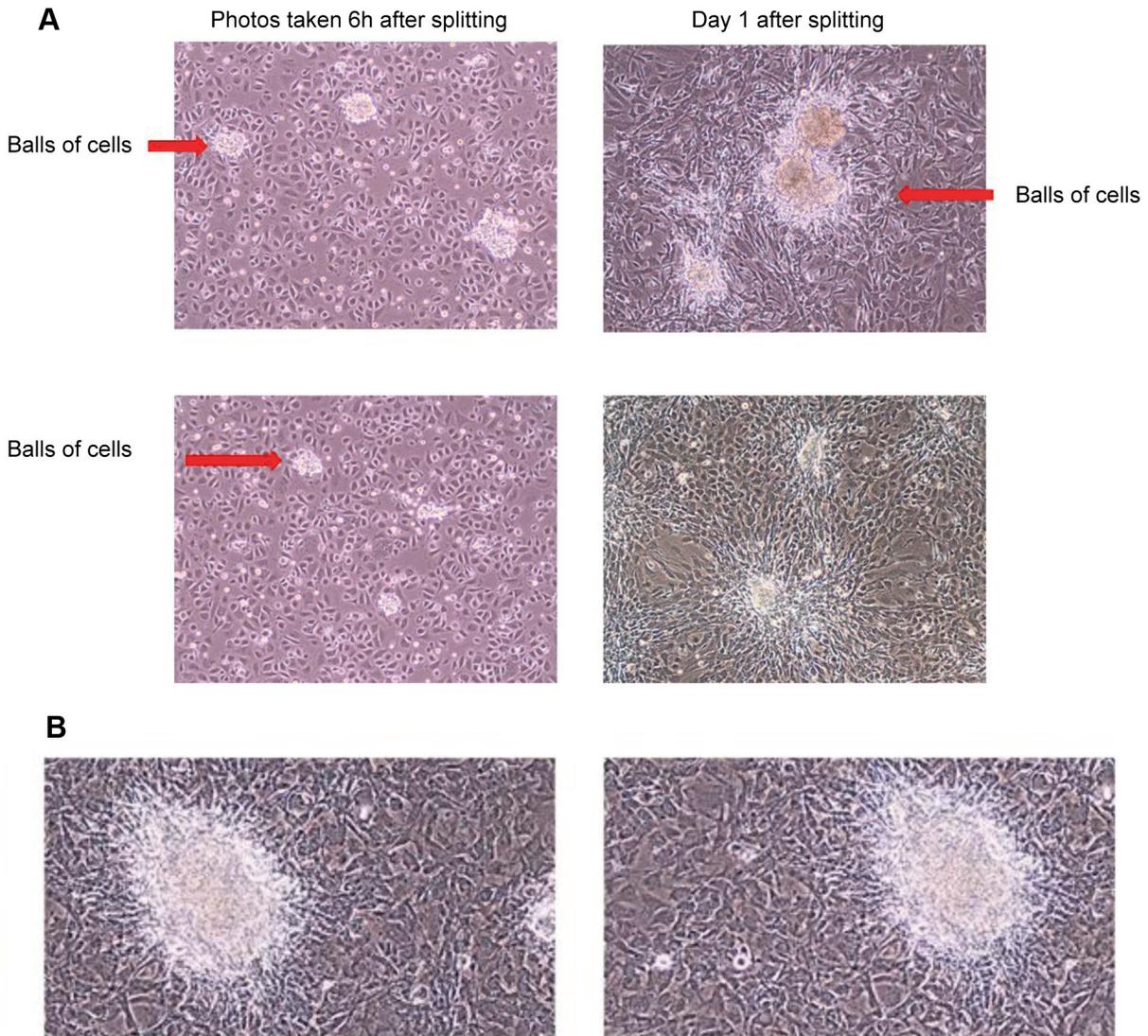


Figure 4. Morphology of tumor cell lines *in vitro* on flat surfaces. Explanted tumors were passaged weekly at 1:10 and 1:50 dilutions. When culture plates became confluent, they were photographed ($\times 100$). *Fanca*^{-/-} tumor cells grew as flattened cells with some of the cells growing in spheres of 100 cells (A) (red arrows). *Fanca*^{+/+} tumor cells do not form spheres of cells (B).

morphology. This spindle cell morphology has been seen following injection of mice with breast cancer cell lines. The spindle cell morphology combined with a strong positivity for Desmin, moderate positivity for S100, and negative for SMA defines this as a malignant smooth muscle tumor. The IHC characteristics of the *Fanca*^{-/-} clone 6 tumor were similar to those of the uncloned *Fanca*^{-/-} tumor. There was strong immunoreactivity for desmin, and moderate immunoreactivity for SMA and S100. There was no immunoreactivity for CD31 (except vessels – endothelial cells), Melan A – melanoma, MAC2 – dendritic/macrophages, and CD34 hematopoietic/

endothelial. The results of IHC staining of tumor cells were consistent with the diagnosis of rhabdomyosarcoma.

Results of serial passage of tumor cell line. Cell lines were grown *in vitro* as nonadherent cells mixed with adherent cells and piled up in clusters of cells on plates (Figure 4). The cells formed colonies when grown in agar. The results of serial *in vitro* passage of *Fanca*^{-/-} and *Fanca*^{+/+} tumor cell lines revealed a highly reproducible morphology and growth pattern of adherent and clumped cells. The *Fanca*^{-/-} tumor cells grew as adherent cells as well as spheres of 50

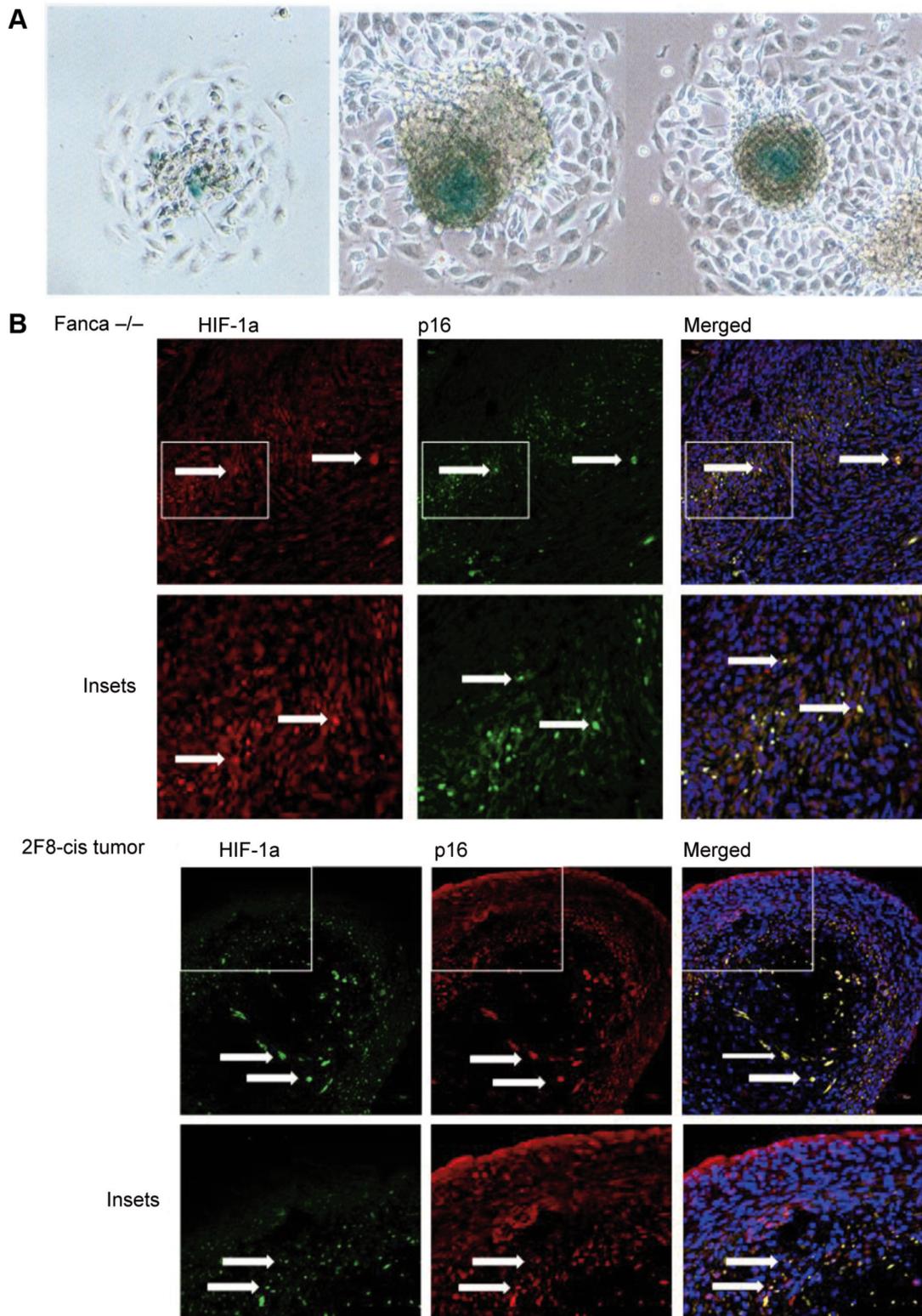


Figure 5. Hypoxia and senescent cells in Fanca $-/-$ tumors. HIF-1 positive cells and senescent cells in Fanca $-/-$ spheres in vitro. Cell cultures were stained for biomarkers of hypoxia (HIF-1) and senescence (Beta-galactosidase) as described in the Materials and Methods ($\times 100$) (A): Tumor sections from Fanca $-/-$ and 2F8cis ovarian cancer cell lines demonstrating senescent cells (p16 cells positive), which are also positive for HIF-1a. Arrows point to p16 positive, HIF-1a positive, or positive for both p16 and HIF-1a cells (B).

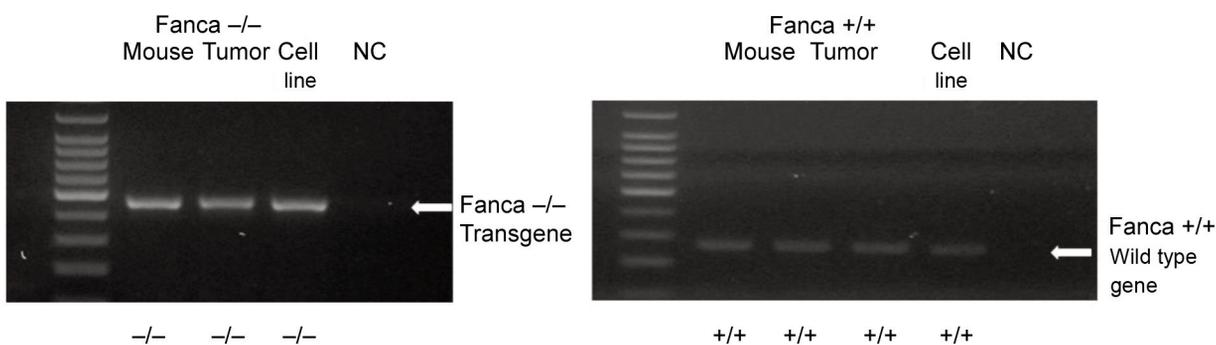


Figure 6. Absence of *Fanca* gene in *Fanca*^{-/-} clone 6 tumor cell line. DNA extracted from *Fanca*^{-/-} clone 6 and control *Fanca*^{+/+} tumor cell lines was subjected to polymerase chain reaction (PCR) using primers specific for *Fanca*^{-/-} transgene or *Fanca*^{+/+} gene. The PCR products were electrophoresed, gels stained with Ethidium Bromide, and observed under a UV light. *Fanca*^{-/-} PCR reaction is shown on the left and *Fanca*^{+/+} PCR reaction shown on the right. *Fanca*^{-/-} clone 6 cells had the *Fanca*^{-/-} transgene, but not the *Fanca*^{+/+} gene, while the *Fanca*^{+/+} cells contained only the *Fanca*^{+/+} gene. NC: Negative control.

to 100 cells (Figure 4A). The *Fanca*^{+/+} cells grew as adherent cells; however did not produce spheres, but formed nodules when they became confluent (Figure 4B). Clonal lines displayed the same pattern.

Presence of hypoxic centers in tumors from *Fanca*^{-/-} Clone 6 transplantation. We next quantified the presence of senescent cells in the tumors produced by the transplanted *Fanca*^{-/-} and *Fanca*^{+/+} tumor cell lines. The tumor cells demonstrated β -Gal positive tumor spheroids or tumor nodules when grown *in vitro* (Figure 5A). The tumors derived from *Fanca*^{-/-} clone 6 cells contained hypoxic areas stained for HIF-1 and senescent cells that were stained for p16 (Figure 5B). As shown in Figure 5B, tumors demonstrated hypoxic centers with positive cells stained for hypoxia-inducible factor (HIF-1). The appearance of senescent cells in areas of hypoxia was uniformly observed in each transplant generation. Staining of tumor sections for senescent biomarkers, p16 and HIF-1, revealed senescent cells in areas of hypoxia (Figure 5B). These results are consistent with the rapid growth of cancer cells in orthotopic tumors. The results were compared with a tumor developed by a cell line derived from mouse ovarian cancer, 2F8cis (which also showed hypoxic centers stained for HIF-1 and senescent cells stained for p16) (Figure 5B).

Genotype of *Fanca*^{-/-} carcinogen-induced cancer cells. To confirm the genotype of cancer cell lines, RT-PCR performed for the detection of the *Fanca* gene revealed the absence of *Fanca* gene in the *Fanca*^{-/-} mouse derived cell lines (Figure 6). These results indicate that the cancer cell line maintained the genotype of the original mouse from which the tumor was derived. In contrast, cells from the *Fanca*^{+/+} mouse derived tumor showed an intact *Fanca* gene (Figure 6).

Clonogenic radiation survival of *Fanca*^{-/-} tumor cell lines. As shown in Figure 7, the clonogenic radiation survival curve for *Fanca*^{-/-} clone 6 revealed $D_0=1.57\pm 0.11$ and $\bar{n}=3.3\pm 1.3$ similar to those of the 3LL (Lewis Lung Carcinoma) cell line: $D_0=1.56\pm 0.08$ and $\bar{n}=3.6\pm 1.6$. The clonogenic radiation survival curves of *Fanca*^{-/-} clone 6 tumor cells in culture were similar to those of wild type 3LL tumor cells.

Discussion

The success of bone marrow transplantation in patients with FA has resulted in improved survival and quality of life for most children and young adults presenting with FA (6-8, 11, 18-19, 36). The increased number of patients with FA, worldwide, is the result of the earlier diagnosis of many patients with anemia of unknown origin and also of the discovery of now over 26 genes associated with FA (1, 2). The first reported patients with FA presented as children with short stature, absent thumbs, and anemia (10). The first gene associated with FA was *Fanca*, which was followed by the discovery of multiple other genes in the FA pathway (1). The anemia in FA has been shown to be the result of the hypersensitivity of bone marrow stem cells to TGF- β , which is a suppressor of hematopoiesis (4). The complexity of the evolution of FA during embryogenesis, growth, and development after birth have been elucidated with studies of double knockout FA mice that have simultaneous deletion of TGF- β (5). Currently, patients with FA live into their 40s and 50s. They reveal sensitivity to DNA crosslinking agents that are required for management of other diseases. The sensitivity of adults as well as children to DNA crosslinking agents remains a primary diagnostic criterion for FA (10).

With the success of bone marrow transplantation for patients with FA, a new clinical problem has arisen. This is

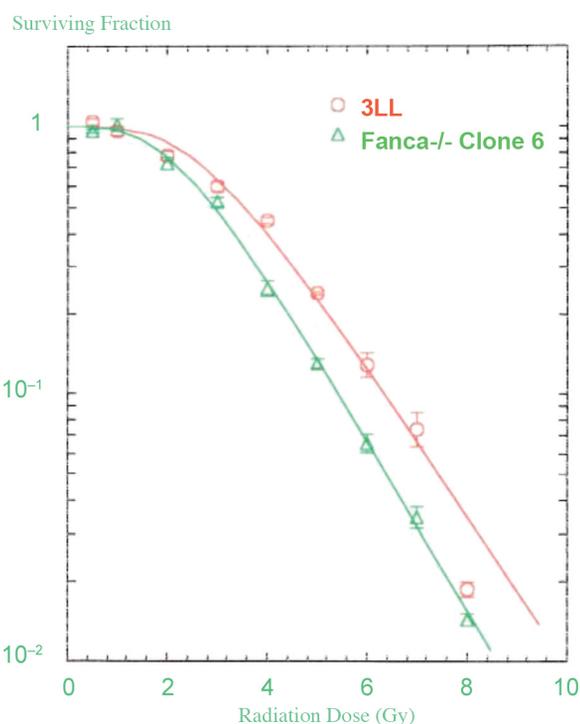


Figure 7. Radiation survival curves *in vitro*. Clonogenic radiation survival curves were carried out as published (24) and are described in Materials and Methods. The *Fanca*^{-/-} and 3LL mouse lung cancer cell lines were irradiated at doses ranging from 0 to 8 Gy and plated at 100, 500, or 1000 cells per culture dish. Seven days after irradiation, the cells were stained with crystal violet and colonies greater than 50 cells were counted. The data was analyzed using a single-hit, multi-target, and linear quadratic models. The data are the results of at least 3 separate experiments. The survival curve for *Fanca*^{-/-} and 3LL cells is shown.

the increasing incidence of cancers, primarily squamous cell carcinomas of the aerodigestive tract and vulvovaginal region in both marrow transplanted and non-transplanted patients with FA (13-14, 17, 18). Cancer in patients with FA represents a major challenge for combined modality management due to several characteristics of the cancers and the patient's sensitivity to both chemotherapy and radiotherapy (19). Patients with FA demonstrate cancers with marked invasiveness, a propensity for regional node metastasis, and local and distant recurrence (21). Radiotherapy in the management of cancer in patients with FA has been difficult due to the radiosensitivity of some patients with FA. Novel approaches have been designed to identify the radiosensitive patients with FA and, thereby, minimize the toxicity of radiotherapy (20-22). One such strategy is to apply a low dose per fraction to small tissue volume for the first weeks of a multiweek program and examine patients daily for signs of radiation toxicity: skin redness, mucositis, and neutropenia (7-9). Radiation

protective drugs have been developed to increase the therapeutic ratio of irradiation in animal models using transplantation of orthotopic tumors. Orthotopic tumors have been derived from wild type mice that do not have the FA genotype (25-29). Furthermore, the radiobiology of a mouse FA cancer cell line has not been well characterized.

In the present study, we established a transplantable cancer cell line produced by treating *Fanca*^{-/-} mice with a chemical carcinogen (DMBA). We demonstrated the skeletal/cardiac sarcoma histopathology, transplantability *in vivo*, and radiobiology *in vitro*. The cancer cell line that was maintained in the *Fanca*^{-/-} mice had the *Fanca*^{-/-} genotype and both the parent cell line and a clonal subline *Fanca*^{-/-} clone 6 formed tumors *in vivo* that were characterized by a skeletal/cardiac sarcoma morphology. The tumor cells were histochemically positive for biomarkers of sarcomas. The radiobiology revealed a clonogenic survival of $D_0=1.57\pm 0.11$ Gy and $\bar{n}=3.3\pm 1.3$, which was similar to that of the squamous cell lung carcinoma 3LL Lewis Lung Carcinoma, which was developed in C57BL/6 mice ($D_0=1.56\pm 0.08$; $\bar{n}=3.6\pm 1.6$). That the *Fanca*^{-/-} clone 6 mouse cancer cell line was not relatively radiosensitive may not translate to a relative radiation responsiveness of tumors *in vivo*. Further studies are required to establish the radiation response *in vivo*, and potential different responsiveness to added chemotherapy (30). Furthermore, the *Fanca*^{-/-} clone 6 tumor cell line did not form tumors in unirradiated *Fanca*^{+/+} mice. Whether novel histocompatibility antigens that prompted immune rejection were induced in the tumors in *Fanca*^{-/-} mice by DMBA treatment requires further investigation. At present we cannot explain the mechanism of tumor cell rejection of *Fanca*^{-/-} clone 6 following transplantation into *Fanca*^{+/+} mice.

The presence of hypoxic cells in tumors that are formed by the *Fanca*^{-/-} clone 6 tumor cell line presents an opportunity to test new, therapeutic agents, which target hypoxic cells in patients with FA. Furthermore, the detection of senescent cells in *Fanca*^{-/-} clone 6 cell line derived tumors *in vivo* presents an opportunity to evaluate the therapeutic effect of new senolytic agents for potential therapeutic effect on tumor growth *in vivo*. Comparison of the senescence associated secretory proteins (SASP) produced by *Fanca*^{-/-} clone 6 senescent cells with the SASP proteins produced by senescent cells in *Fanca*^{+/+} tumors may reveal mouse genotype specific differences. Such differences could facilitate identification of novel SASP (35) in *Fanca*^{-/-} tumors; perhaps, explaining their radiosensitivity and tumor aggressiveness *in vivo*.

The present *Fanca*^{-/-} cancer cell line and subclonal line could be valuable for studies of the genes associated with tumor invasiveness, regional lymph node metastasis, and propensity for local recurrence of cancers in patients with FA. These cell lines could also be valuable for studies of early activation of novel carcinogen-induced oncogenes (37).

There is an additional reason to study tumors formed by Fanca^{-/-} clone 6. Patients with FA present with cancers that are known to be more invasive, aggressive, and have a propensity for local recurrence and metastasis, which appears to be greater than that in similar stage cancers in non-FA patients.

There is new interest in the role of senescent cells in tumors. Fanca^{-/-} clone 6 transplanted tumors have been shown to have areas of HIF-1-positive cells and surrounding areas of senescent cells, which are p16⁺. Cell sorting and separation techniques are now available to remove senescent cells from normal tissues and cancer cells. The Fanca^{-/-} clone 6 tumors may present an opportunity to sort and study the senescence secretory proteins (SASP) produced by the different cell types found in the tumor. The SASP components of senescent cells that are specific to cancer may be valuable to elucidate the aggressiveness (invasiveness beyond resection margins) of the cancers, the propensity for local recurrence and metastasis. Senescent cells are known to increase in numbers following exposure to ionizing irradiation in both normal tissues and in cancer. If irradiation induced senescent cells within Fanca^{-/-} clone 6 tumors are producing novel SASP the new data may provide an opportunity to isolate and evaluate those particular SASP components that are associated with aggressiveness, local recurrence, and metastasis.

The present method of induction of cancer by DMBA administration may be applicable to other FA mouse models including Fancd2^{-/-} (C57BL/6 and 129/Sv background genetic strains), and Fancg^{-/-} mice. Common elements of the SASP in senescent cells in control and irradiated tumors of other FA genotypes may provide valuable information that may lead to an understanding of the factors that are associated with the aggressiveness, propensity for local recurrence, and metastasis of squamous cell cancers in patients with FA.

Conflicts of Interest

None of the Authors have any conflicts of interest to declare in relation to this study.

Authors' Contributions

JG designed experiments; ME and RF performed the animal experiments; DS made the cell lines, performed all *in vitro* experiments, including radiation survival experiments; RF passaged tumors; WH, LR and AG carried out animal tumor histopathology evaluations; SH assessed radiation dosimetry for *in vitro* survival curves; and HW carried out statistical analysis for all experiments.

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