

GFP-Fluorescence-guided UVC Irradiation Inhibits Melanoma Growth and Angiogenesis in Nude Mice

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Abstract. Melanoma cell lines that stably express green fluorescent protein (GFP) and nude mice that ubiquitously express red fluorescent protein (RFP) have previously been developed to study tumor-host interaction by color-coded imaging. In the present study, the efficacy of fluorescence-guided ultraviolet C (UVC) irradiation on the growth of murine melanoma expressing GFP in the ear of RFP mice was determined using a non-invasive ear-tumor imaging model developed previously. The GFP-expressing melanoma and RFP-expressing blood vessels from the transgenic mice expressing RFP used as hosts were readily visible using non-invasive imaging. The melanoma was treated under fluorescence guidance with UVC at 650 J/m²/minute for 3 minutes. The ears of the mice were observed before and 24 hours after irradiation with UVC. UVC inhibited melanoma growth and also damaged blood vessels in the tumor. Thus, UVC irradiation has a direct effect on melanoma growth as well as an anti-angiogenesis effect. This color-coded tumor-host model is useful for evaluation of treatment efficacy on melanoma growth and angiogenesis, which are readily discernable with non-invasive color-coded fluorescent protein imaging. These results suggest that fluorescence-guided UVC irradiation is a promising therapeutic strategy for melanoma.

Photodynamic therapy has been shown to be effective for certain cancer types (1). Recently, blue light was found to be

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phototoxic for both murine and human melanoma (2). Ultraviolet (UV) light has been used for the phototherapy of cutaneous malignancies. Psoralen plus UVA (PUVA) and narrowband UVB are the most common phototherapy modalities that have been used (3-5). However, the effect of UV light on cancer cells is not well understood (6-8). UV light has been mainly used in the treatment of skin cancer, since short wavelength light does not penetrate deeply through the skin.

Hoffman *et al.* have pioneered *in vivo* imaging with fluorescent proteins (9-11). Melanoma cell lines that stably express green fluorescent protein (GFP) *in vivo* have been developed (12). It has been shown that it is possible to non-invasively image GFP-expressing B16 mouse malignant melanoma cells *in vivo* (10). Nude mice that ubiquitously express red fluorescent protein (RFP) have been developed (13) to study tumor-host interaction by color-coded imaging (14). Non-invasive subcellular imaging of cancer cells expressing GFP in the nucleus and RFP in the ear of nude mice (color-coded cells) has been demonstrated previously (15).

Recently, the cancer cell-killing efficacy of UV light on cancer cells expressing GFP in the nucleus and RFP in the cytoplasm was investigated (16). After exposure to various doses of UVA, UVB or UVC, apoptotic and viable cells were quantified under fluorescence microscopy. UV-induced cancer cell death was wave-length and dose dependent. For UVC, as little as 25 J/m² UVC irradiation killed approximately 70% of the dual-color cancer cells. This dose of UVB or UVA had almost no effect on the cancer cells. UV-induced cancer cell death varied among the cell lines. Cell death began about four hours after irradiation and continued until 10 hours after irradiation. UVC exposure also suppressed RFP-expressing cancer cell growth in nude mice in a model of minimal residual cancer (MRC). No apparent side-effects of UVC exposure were observed.

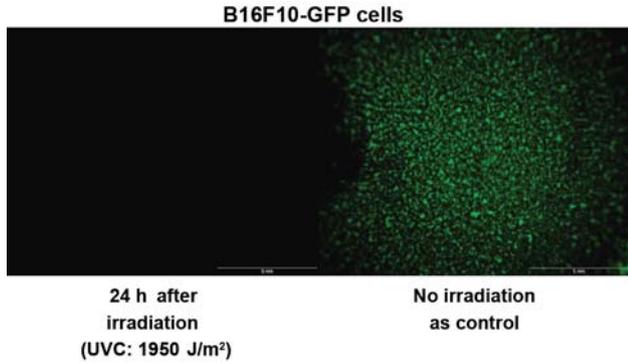


Figure 1. Cytotoxicity of UVC *in vitro*. B16F10-GFP cells in culture were treated with a total of 1950 J/m² UVC.

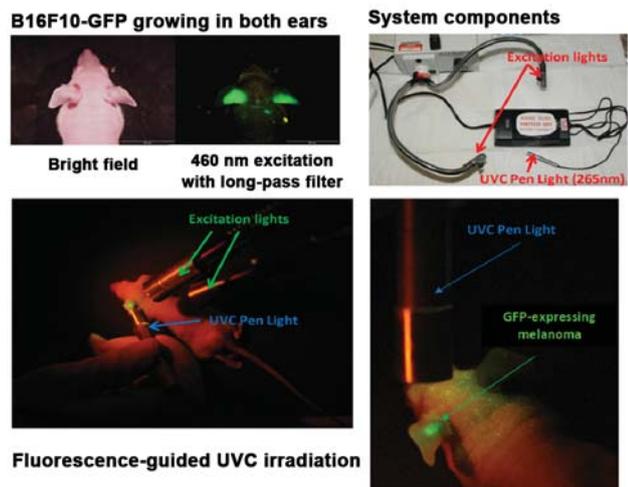


Figure 2. Fluorescence-guided UVC pen light treatment of melanoma. To determine accurate tumor location, fluorescence-guided navigation was used. Subcutaneous ear melanoma was exposed to UVC (650 J/m²/min) for 3 min with a customized UV pen light (UVP LLC, Upland, CA, USA) under anesthesia. Imaging was carried out 1 and 24 hours after irradiation using the Olympus OV100 imaging system.

The precise identification of malignant tissue margins, including the smallest disseminated lesions, can greatly enhance cancer treatment. GFP fluorescence can intensely illuminate even single cells but requires *GFP* sequence transcription within the cell. Introducing and selectively activating the *GFP* gene in malignant tissue *in vivo* was made possible by the development of OBP-401, a telomerase-dependent, replication-competent adenovirus expressing GFP. Only the malignant tissue fluoresced after OBP-401 infection *in vivo* (17, 18).

In the present study, the color-coded fluorescent tumor-host interaction model described above was utilized for fluorescence-guided UVC-treatment of melanoma in the ear of nude mice.

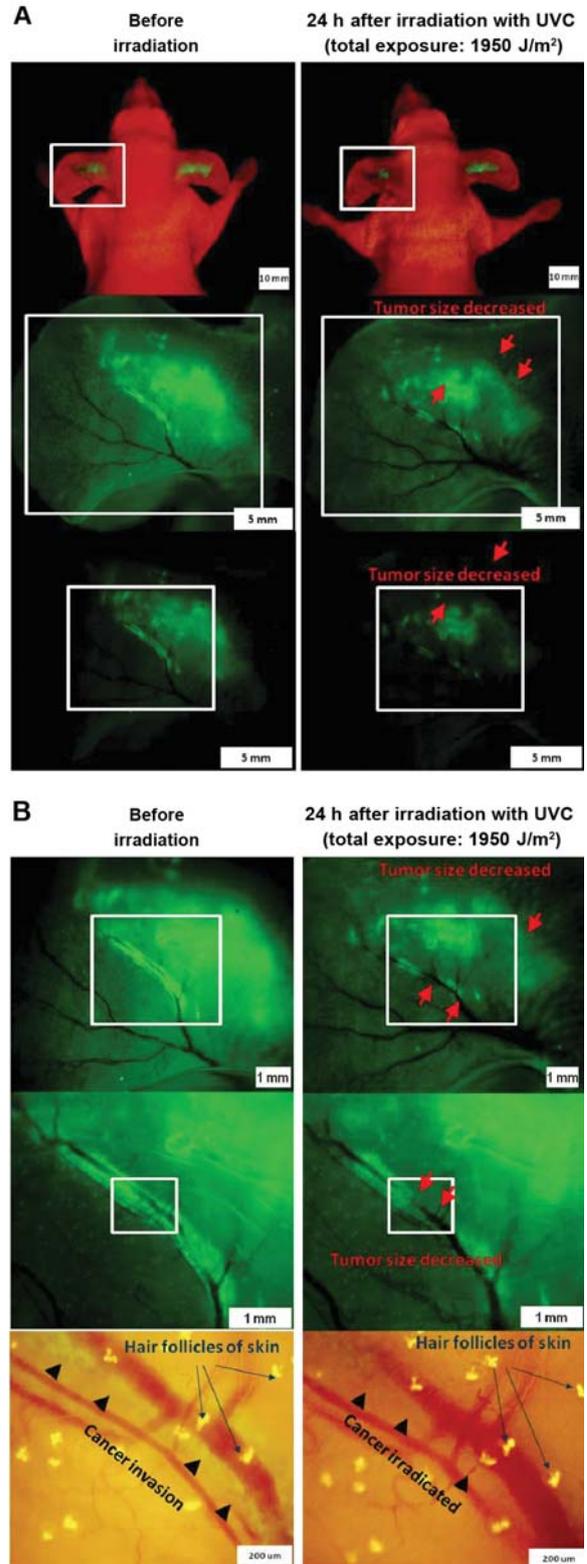


Figure 3. Efficacy of fluorescence-guided UVC irradiation on melanoma. A: Before irradiation, B16F10-GFP cells spread and infiltrated perivascularly; B: 24 h after irradiation with UVC, the B16F10-GFP ear tumor decreased.

Materials and Methods

GFP expression vector. The pLEIN retroviral vector (CLONTECH, Palo Alto, CA, USA) expressing enhanced GFP and the neomycin-resistance gene on the same bicistronic message, which contains an internal ribosome entry site (19) was used to transduce tumor cells (20).

Packaging cell culture, vector production, transfection, and subcloning. PT67, a NIH 3T3-derived packaging cell line expressing the 10 A1 viral envelope, was purchased from CLONTECH. PT67 cells were cultured in DMEM (Irvine Scientific, Irvine, CA, USA) supplemented with 10% heat-inactivated FBS (Gemini Biological Products, Calabasas, CA, USA). For vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N* trimethylammoniummethylsulfate reagent (Roche Molecular Biochemicals, Mannheim, Germany) and saturating amounts of pLEIN plasmid for 18 hours. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 hours after transfection. For selection of high GFP expression, the cells were cultured in the presence of 500-2000 mg/ml of G418 (Life Technologies, Grand Island, NY, USA) for 7 days (19, 20).

Retroviral GFP transduction of B16F10 cells. For GFP gene transduction, 70% confluent rodent B16F10 melanoma cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI-1640 or other culture media (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA, USA) for 72 hours. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium, which contained 50 μ g/ml G418. To select brightly fluorescent cells, the level of G418 was increased to 800 μ g/ml in a stepwise manner. Clones expressing GFP (Figure 1) were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent (21, 22).

Transgenic red fluorescent protein nude mice. C57/B6-RFP mice expressed RFP (DsREDT3) under the control of a chicken β -actin promoter and cytomegalovirus enhancer (23). All of the tissues from this transgenic line, with the exception of erythrocytes, were red under blue excitation light. Six-week-old transgenic RFP female mice were crossed with both 6-8-week-old BALB/c *nu/nu* and NCR *nu/nu* male mice (Harlan, Indianapolis, IN, USA), respectively. Male F1 fluorescent nude mice were crossed with female F1 immunocompetent RFP mice. When female F2 immunocompetent RFP mice were crossed with male RFP nude or using F2 RFP nude male to back cross with female F1 immunocompetent RFP mice, approximately 50% of their offspring were RFP nude mice. RFP nude mice were then consistently produced using the methods described above (13).

Fluorescence-guided irradiation of subcutaneous ear tumor model with UVC pen light. Subcutaneous tumors in the ear were produced by injection of 1×10^5 B16F10-GFP murine melanoma cells in 5-week old RFP-nude mice. Mice were examined for GFP and RFP fluorescence with a long-pass filter (a filter for simultaneous observation of both GFP and RFP) (Figure 1) using the Olympus OV100 Small Animal Imaging System (Olympus Corp, Tokyo, Japan). To determine accurate tumor location, fluorescence-guided

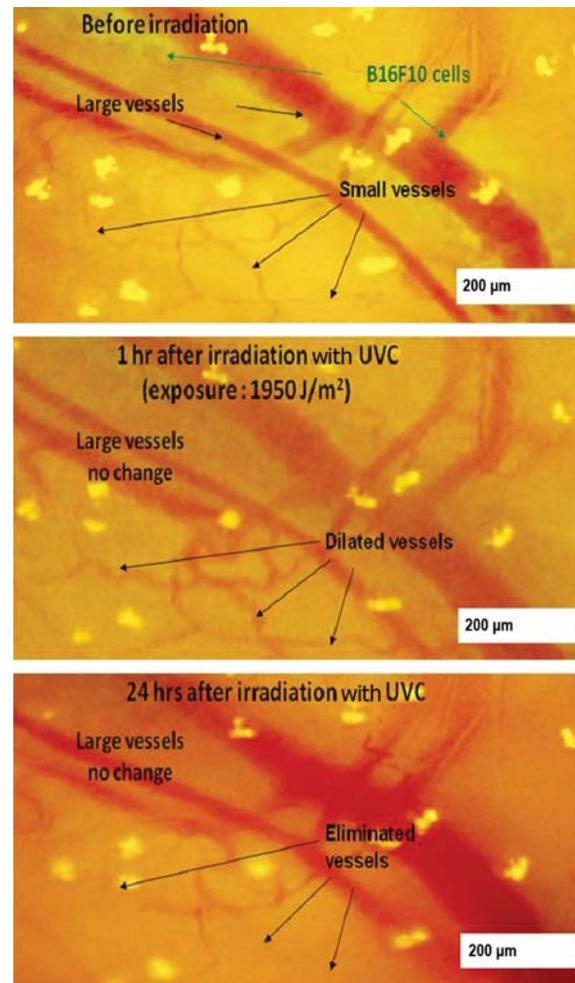


Figure 4. Efficacy of fluorescence-guided UVC irradiation on melanoma angiogenesis. Before irradiation, small vessels, expressing RFP, in the ear melanoma in the RFP-nude mouse were observed. One hour after irradiation with UVC, the small vessels were dilated; 24 hours after irradiation of UVC, small vessels were eliminated. Large vessels did not appear to change.

navigation was used. Subcutaneous ear melanoma were exposed to UVC (650 J/m²/min) for 3 min with a customized UV pen light (UVP LLC, Upland, CA, USA) under anesthesia. Imaging was carried out 1 and 24 hours after irradiation (Figure 2).

Results and Discussion

Efficacy of UVC on B16F10-GFP in vitro. The B16F10-GFP cells were very sensitive to UVC and could be eliminated from culture (Figure 1).

Efficacy of UVC-irradiation on melanoma growth in vivo. Before irradiation, B16F10-GFP cells spread and infiltrated perivascularly. 24 hours after irradiation with UVC (Figure 2), the B16F10-GFP ear tumor size decreased (Figure 3). These results show UVC inhibits melanoma growth.

Effect of UVC-irradiation on melanoma blood vessels. Before irradiation, RFP-expressing small vessels in the ear tumor in the RFP-nude mouse could be observed. One hour after irradiation with UVC, the small vessels became dilated. By 24 hours after irradiation with UVC, the small vessels were eliminated. Large vessels did not appear to change. These results demonstrated that UVC damaged the small tumor vessels (Figure 4).

The killing efficacy of UV light on cancer cells expressing fluorescent proteins has been demonstrated previously *in vitro* and *in vivo* (16). However, in the model in the previous study, only the cancer cells were fluorescent so that the effect on the blood vessels could not be observed. In the present study, a color-coded dual-color model was used with the tumor expressing GFP and blood vessels expressing RFP. Therefore, the effect of UVC irradiation on both the blood vessels as well as the cancer cells could be observed. The current results indicate that UVC damages both cancer cells and tumor blood vessels and suggest the clinical potential of UVC treatment on melanoma. Fluorescence-guided-treatment using UVC radiation suggests the possibility of highly-targeted tumor treatment in combination with fluorescent-guided surgery (17, 18).

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References

- Castano AP, Liu Q and Hamblin MR: A green fluorescent protein-expressing murine tumour but not its wild-type counterpart is cured by photodynamic therapy. *Br J Cancer* 94: 391-397, 2006.
- Sparsa A, Faucher K, Sol V, Durox H, Boulinguez S, Doffoel-Hantz V, Calliste CA, Cook-Moreau J, Krausz P, Sturtz FG, Bedane C, Jauberteau-Marchan MO, Ratinaud MH and Bonnetblanc JM: Blue light is phototoxic for B16F10 murine melanoma and bovine endothelial cell lines by direct cytotoxic effect. *Anticancer Res* 30: 143-147, 2010.
- Gilchrest BA, Parrish JA, Tanenbaum L, Haynes HA and Fitzpatrick TB: Oral methoxsalen photochemotherapy of mycosis fungoides. *Cancer* 38: 683-689, 1976.
- Hofer A, Cerroni L, Kerl H and Wolf P: Narrowband (311-nm) UV-B therapy for small plaque parapsoriasis and early-stage mycosis fungoides. *Arch Dermatol* 135: 1377-1380, 1999.
- Carter J and Zug KA: Phototherapy for cutaneous T-cell lymphoma: online survey and literature review. *J Am Acad Dermatol* 60: 39-50, 2009.
- Benencia F, Courrèges MC and Coukos G: Whole tumor antigen vaccination using dendritic cells: comparison of RNA electroporation and pulsing with UV-irradiated tumor cells. *J Transl Med* 6: 21, 2008.
- Zacal N and Rainbow AJ: Photodynamic therapy resistant human colon carcinoma HT29 cells show cross-resistance to UVA but not UVC Light. *Photochem Photobiol* 83: 730-737, 2007.
- Kim SC, Park S-S and Lee YJ: Effect of UV irradiation on colorectal cancer cells with acquired TRAIL resistance. *J Cell Biochem* 104: 1172-1180, 2008.
- Chishima T, Miyagi Y, Wang X, Yamaoka H, Shimada H, Moossa AR and Hoffman RM: Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression. *Cancer Res* 57: 2042-2047, 1997.
- Yang M, Baranov E, Jiang P, Sun F-X, Li X-M, Li L, Hasegawa S, Bouvet M, Al-Tuwaijri M, Chishima T, Shimada H, Moossa AR, Penman S and Hoffman RM: Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. *Proc Natl Acad Sci USA* 97: 1206-1211, 2000.
- Hoffman RM: The multiple uses of fluorescent proteins to visualize cancer *in vivo*. *Nat Reviews Cancer* 5: 796-806, 2005.
- Yang M, Jiang P, An Z, Baranov E, Li L, Hasegawa S, Al-Tuwaijri M, Chishima T, Shimada H, Moossa AR and Hoffman RM: Genetically fluorescent melanoma bone and organ metastasis models. *Clin Cancer Res* 5: 3549-3559, 1999.
- Yang M, Reynoso J, Bouvet M and Hoffman RM: A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. *J Cell Biochem* 106: 279-284, 2009.
- Hoffman RM and Yang M: Color-coded fluorescence imaging of tumor-host interactions. *Nat Protoc* 1: 928-935, 2006.
- Yamamoto N, Jiang P, Yang M, Xu M, Yamauchi K, Tsuchiya H, Tomita K, Wahl GM, Moossa AR and Hoffman RM: Cellular dynamics visualized in live cells *in vitro* and *in vivo* by differential dual-color nuclear-cytoplasmic fluorescent-protein expression. *Cancer Res* 64: 4251-4256, 2004.
- Kimura H, Lee C, Hayashi K, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Bouvet M and Hoffman RM: UV light killing efficacy of fluorescent protein-expressing cancer cells *in vitro* and *in vivo*. *J Cell Biochem* 110: 1439-1446, 2010.
- Kishimoto H, Zhao M, Hayashi K, Urata Y, Tanaka N, Fujiwara T, Penman S and Hoffman RM: *in vivo* internal tumor illumination by telomerase-dependent adenoviral GFP for precise surgical navigation. *Proc Natl Acad Sci USA* 106: 14514-14517, 2009.
- Kishimoto H, Urata Y, Tanaka N, Fujiwara T and Hoffman RM: Selective metastatic tumor labeling with green fluorescent protein and killing by systemic administration of telomerase-dependent adenoviruses. *Mol Cancer Therap* 8: 3001-3008, 2009.
- Yang M, Jiang P, Sun FX, Hasegawa S, Baranov E, Chishima T, Shimada H, Moossa AR and Hoffman RM: A fluorescent orthotopic bone metastasis model of human prostate cancer. *Cancer Res* 59: 781-786, 1999.
- Yang M, Baranov E, Li X-M, Wang J-W, Jiang P, Li L, Moossa AR, Penman S and Hoffman RM: Whole-body and intravital optical imaging of angiogenesis in orthotopically implanted tumors. *Proc Natl Acad Sci USA* 98: 2616-2621, 2001.
- Yang M, Li L, Jiang P, Moossa AR, Penman S and Hoffman RM: Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. *Proc Natl Acad Sci USA* 100: 14259-14262, 2003.
- Amoh Y, Li L, Yang M, Jiang P, Moossa AR, Katsuoka K and Hoffman RM: Hair follicle-derived blood vessels vascularize tumors in skin and are inhibited by doxorubicin. *Cancer Res* 65: 2337-2343, 2005.
- Vintersten K, Monetti C, Gertsenstein M, Zhang P, Laszlo L, Biechele S and Nagy A: Mouse in red: Red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 40: 241-246, 2004.

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