

A Color-coded Imaging Model of the Interaction of α_v Integrin-GFP Expressed in Osteosarcoma Cells and RFP Expressing Blood Vessels in Gelfoam[®] Vascularized *In Vivo*

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Abstract. The integrin family of proteins has been shown to be involved in the malignant behavior of cells. We report here development of a color-coded imaging model that can visualize the interaction between α_v integrin linked to green fluorescent protein (GFP) in osteosarcoma cells and blood vessels in Gelfoam[®] vascularized after implantation in red fluorescent protein (RFP) transgenic nude mice. Human 143B osteosarcoma cells expressing α_v integrin-GFP were generated by transfection with an α_v integrin-GFP vector. Gelfoam[®] (5×5 mm) was transplanted subcutaneously in transgenic RFP nude mice. The implanted Gelfoam[®] became highly vascularized with RFP vessels within 14 days. Skin flaps were made at days 7, 14, 21, 28 after transplantation of Gelfoam[®] for observing vascularization of the Gelfoam[®] using fluorescence imaging. Gelfoam[®] is a useful tool to observe angiogenesis *in vivo*. 143B cells (5×10^5) expressing α_v integrin-GFP were injected into the Gelfoam[®] seven days after transplantation of Gelfoam[®]. Seven days after cancer-cell injection, cancer cells and blood vessels were observed in the Gelfoam[®] by color-coded confocal microscopy via the skin flap. The 143B cells expressing α_v integrin-GFP proliferated into the Gelfoam[®], which contained RFP-expressing blood vessels. Strong expression of α_v integrin-GFP in 143B cells was observed near RFP vessels in the

Gelfoam[®]. The observation of the behavior of α_v integrin-GFP and blood vessels will allow further understanding of the role of α_v integrin in cancer cells.

The α_v integrin subfamily consists of at least five members, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ (1). α_v Integrins, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$, have been implicated in angiogenesis and tumor progression (2-5). Up-regulation of α_v integrin has been observed in osteosarcoma (6).

Gelfoam[®] is derived from pigskin and has been used as a substrate for 3-dimensional growth of tumors (7, 8) and other tissues (9). Gelfoam[®] was previously implanted in nestin-driven green fluorescent protein (ND-GFP) transgenic mice (10). The implanted Gelfoam[®] was rapidly vascularized with ND-GFP-expressing blood vessels. At day 7 after transplantation, the ND-GFP-expressing nascent blood vessels were observed, *via* skin flap imaging, forming a network on the surface of the β FGF-treated Gelfoam[®]. The ND-GFP vessels in the Gelfoam[®] stained positively for CD31, demonstrating the presence of endothelial cells (11).

We have previously developed a red fluorescent protein (RFP) transgenic nude mouse, in which the organs all brightly expressed RFP (12).

In the present study, we implanted Gelfoam[®] into the RFP transgenic nude mouse followed by implantation of α_v integrin GFP-expressing 143B osteosarcoma cells in order to visualize the behavior of α_v integrin in cancer cells interacting with blood vessels, using color-coded imaging.

Materials and Methods

Cells. 143B human osteosarcoma cells were maintained with RPMI 1640 medium (Irvine Scientific, Santa Ana, CA, USA) with 10% fetal bovine serum (FBS) (Omega Scientific, San Diego, CA, USA) and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO₂.

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Key Words: α_v Integrin, green fluorescent protein, blood vessels, red fluorescent protein, Gelfoam[®], osteosarcoma, transgenic nude mouse, confocal microscopy.

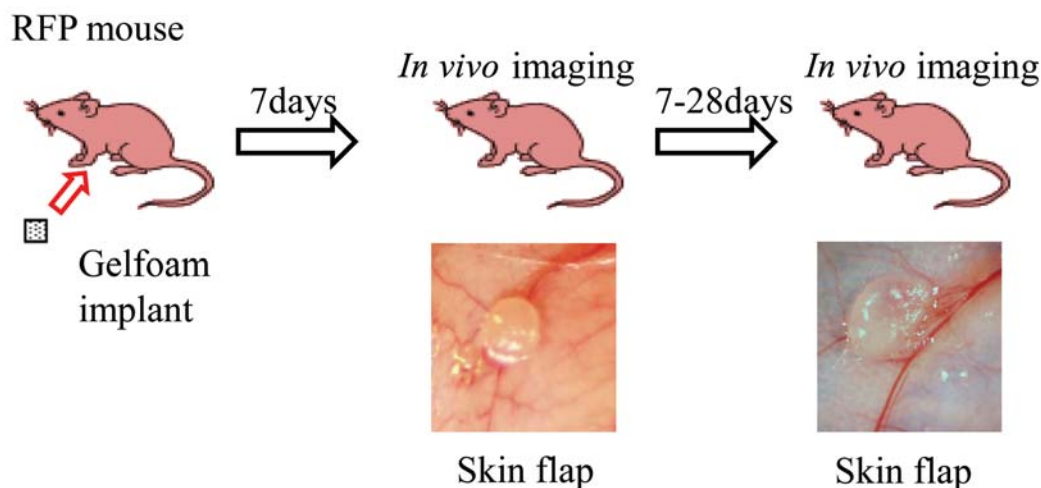


Figure 1. Experimental schema for Gelfoam® angiogenesis. Gelfoam® (5×5 mm), treated with 300 ng β -fibroblast growth factor in 75 μ l RPMI-1640 medium, was transplanted into the subcutis on both flanks of red fluorescent protein (RFP) transgenic nude mice. Skin flaps were made at day 7 after transplantation of Gelfoam® under anesthesia. Angiogenesis was observed in the Gelfoam® in the skin flap using confocal microscopy.

Establishment of human osteosarcoma cells expressing α_v integrin-GFP. The pCMV6-AC-ITGAV-GFP vector, expressing α_v integrin, was obtained from OriGene Technologies (Rockville, MD, USA). 143B cells were seeded at 1×10^6 per 100-mm dish. At 80% confluency, cultures were treated with pCMV6-AC-ITGAV-GFP using Lipofectamine LTX (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. After transfection, stable cells were selected with G418 (800 μ g/ml) (Sigma-Aldrich, St. Louis, MO, USA) starting at 24 h after transfection. Stable colonies were selected and maintained in RPMI 1640 containing 10% FBS and 500 μ g/ml G418.

Mice. Female RFP transgenic nude mice (AntiCancer, Inc., San Diego, CA, 25-30g) were used in this study. Mice were fed with an autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, CA, USA). All animal studies were conducted in accordance with principles and procedures outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals under PHS Assurance Number A3873-01.

Implantation of Gelfoam®. Four-week-old RFP-transgenic nude mice (12) were anesthetized with a ketamine mixture (10 μ l ketamine HCl, 7.6 μ l xylazine, 2.4 μ l acepromazine maleate, and 10 μ l H₂O) *via s.c.* injection. Gelfoam® (Pharmacia & Upjohn, Kalamazoo, MI, USA) (5×5 mm) was treated with 300 ng β fibroblast growth factor (β FGF; Millipore, Billerica, MA, USA) in 75 μ l RPMI-1640 medium (Cellgro, Herndon, VA, USA). The treated Gelfoam® was then transplanted into the subcutis on both flanks of the RFP transgenic nude mouse.

Imaging Gelfoam® vascularization. The RFP transgenic nude mice were anesthetized with the ketamine mixture *via s.c.* injection. An arch-shaped incision was made in the abdominal skin from the axillary to the inguinal region. The subcutaneous connective tissue was separated to free the skin flap without injuring the vessel. Mice were laid flat and the skin flap was spread and fixed on the flat stand (10). Gelfoam® was directly imaged with a FV1000 confocal microscope (Olympus, Tokyo, Japan). The skin was closed with a 6-0 nylon suture.

Immunohistochemical (IHC) staining of vascularized Gelfoam®. Co-localization of RFP and CD31 in frozen sections of the vascularized Gelfoam® was detected with an anti-rat immunoglobulin horseradish peroxidase kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instructions. The primary antibody was a monoclonal antibody specific for CD31 (1:50; Chemicon, Temecula, CA, USA). Substrate chromagen 3,3'-diaminobenzidine staining was used for antigen staining.

Imaging of 143B cells expressing α_v integrin-GFP interacting with RFP vessels in Gelfoam®. 143B cells (5×10^5), expressing α_v integrin-GFP, were injected into the Gelfoam® previously implanted in RFP mice with a 0.5 ml 28 G latex-free insulin syringe (TYCO Health Group LP, Mansfield, MA, USA). Skin flaps were made on days 7, 14, 21, 28 days after injection and the inside surface of the skin flap and Gelfoam® was directly imaged. The skin was closed with a 6-0 suture.

Imaging. A Fluoview FV1000 laser scanning confocal microscope with a XLUMPLFL $\times 20$ (0.95 numerical aperture [NA]) water-immersion objective was used for imaging. GFP was excited at 488 nm, and RFP was excited at 559 nm with an Argon laser. Images were produced with FV10-ASW Fluoview software (Olympus) and ImageJ (NIH, Bethesda, MD, USA) and were not modified beyond the standard adjustment of intensity levels.

Results

Visualization of Gelfoam® in RFP transgenic nude mice. To obtain Gelfoam® angiogenesis Gelfoam® (5×5 mm) was treated with 300 ng β FGF and then implanted into the subcutis on both flanks of RFP nude mice (Figure 1). Seven days after transplantation, skin flaps were made and observed with an Olympus FV1000 confocal laser scanning microscope. Vessels had not yet appeared in the Gelfoam®

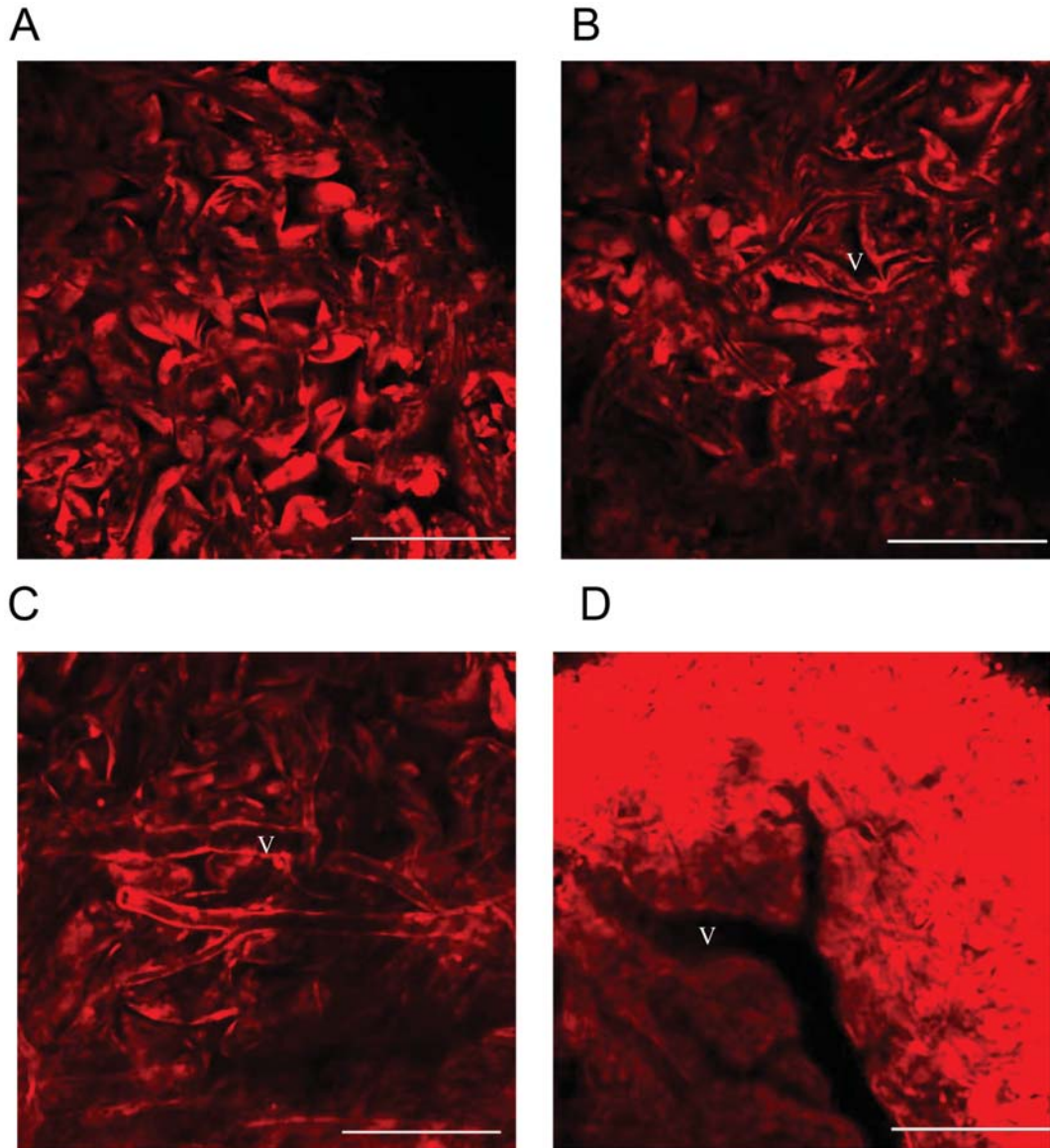


Figure 2. Vascularization of implanted Gelfoam[®] with RFP-expressing vessels. Seven days after Gelfoam[®] transplantation, skin flaps were made and observed with the FV1000 laser scanning confocal microscope. A: Seven days after Gelfoam[®] transplantation, RFP-expressing cells infiltrated the Gelfoam[®], but no vessel structures were observed. Bar=200 μ m. B: Fourteen days after transplantation, RFP-expressing vessel structures were observed on the surface of Gelfoam[®]. Bar=200 μ m. C: Twenty-one days after transplantation, RFP-expressing vessels were observed invading the Gelfoam[®]. Bar=200 μ m. D: Twenty-eight days after transplantation, RFP-expressing vessels were thick and long and invaded the Gelfoam[®]. “V” in the figure indicates blood vessels. Bar=200 μ m.

(Figure 2A). Fourteen days after transplantation, skin flaps were made, and RFP-expressing vessel structures were observed on the surface of the Gelfoam[®] (Figure 2B). Twenty-one days after transplantation, RFP-expressing vessels were observed to be penetrating deeply in the Gelfoam[®] (Figure 2C). Twenty-eight days after transplantation, RFP-expressing vessels were thick and long and had invaded deeply in into the Gelfoam[®] (Figure 2D).

Co-localization of RFP and CD 31 in blood vessels in Gelfoam[®]. Frozen sections of Gelfoam[®] were made at day 28 after transplantation of Gelfoam[®] into the subcutis of RFP transgenic nude mice. At that time, RFP-expressing blood vessels were growing into the Gelfoam[®] (Figure 3A). IHC staining showed that CD31 (Figure 3B) and RFP fluorescence co-localized in the RFP-expressing blood vessels in the Gelfoam[®].

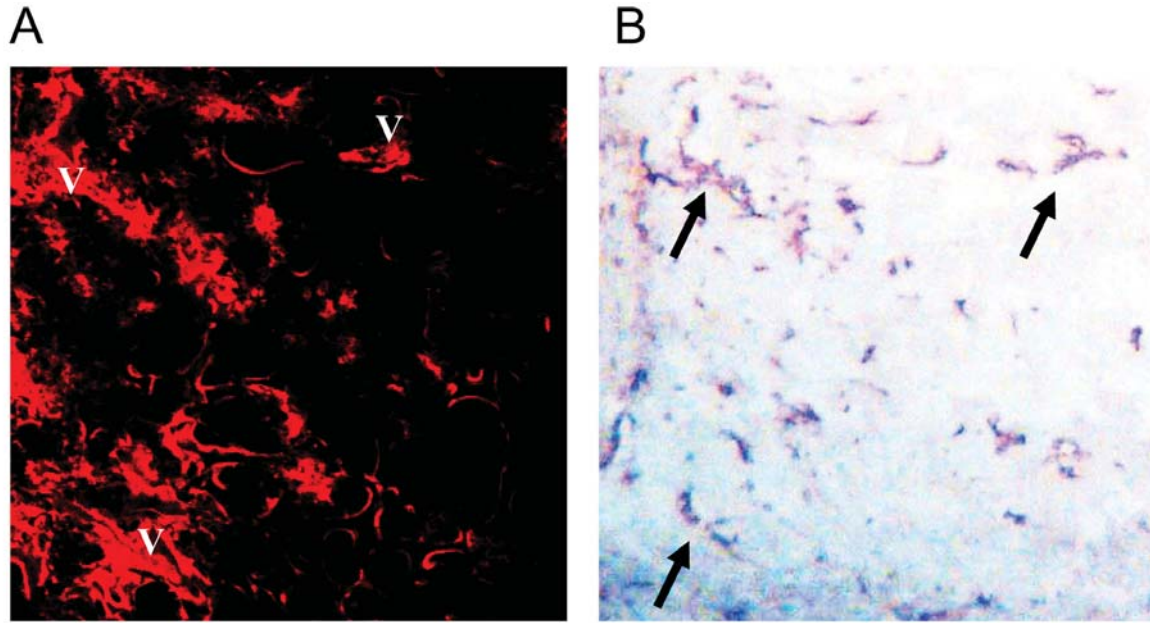


Figure 3. Co-localization of RFP and CD31 in blood vessels in Gelfoam[®]. Frozen sections were made at day 28 after transplantation of Gelfoam[®], treated with β -fibroblast growth factor, into the subcutis of RFP transgenic nude mice. A: RFP-expressing blood vessels were growing into the Gelfoam[®]. "V" indicates blood vessels. B: Immunohistochemical staining showed that CD31 and RFP fluorescence co-localized in the RFP-expressing blood vessels. Arrows indicate where blood vessels are located.

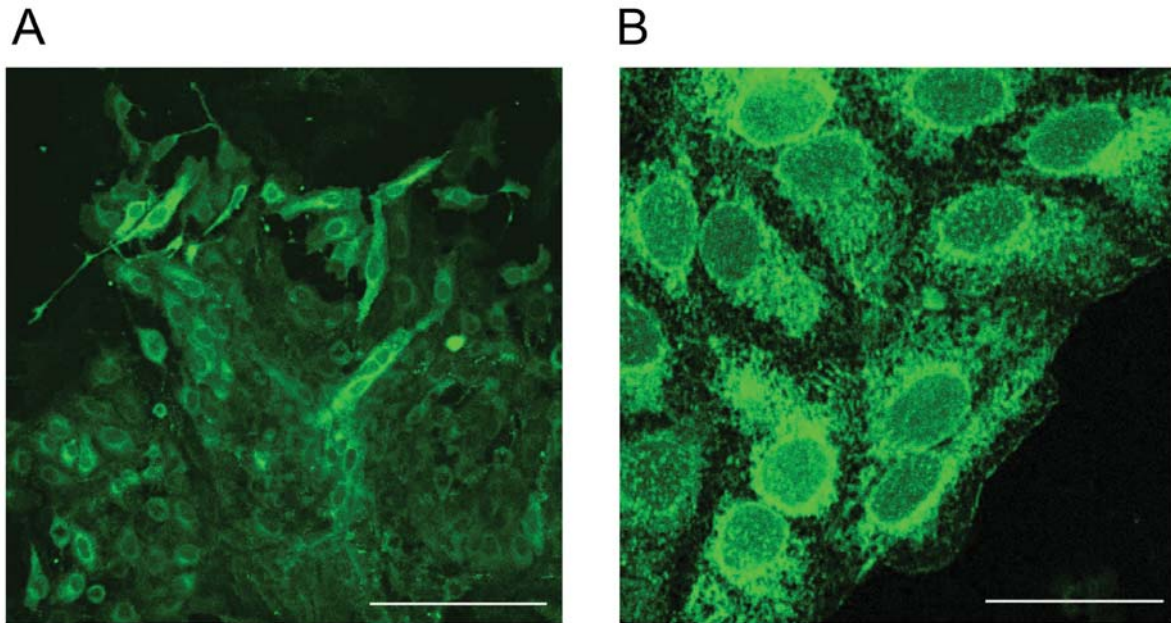


Figure 4. Behavior of α_v integrin GFP-expressing 143B cells in vitro. A: 143B cells stably expressing α_v integrin-GFP in vitro. Bar=200 μ m. B: Punctate expression of α_v integrin-GFP was observed at the bottom of the dish when 143B cells were seeded on a fibronectin-coated dish. Bar=50 μ m.

Expression of α_v integrin-GFP in 143B osteosarcoma cells in vitro. The selected α_v integrin-GFP transduced 143B cells had a strikingly bright GFP fluorescence in their cytoplasm, in vitro. When 143B α_v integrin-GFP cells

were seeded in fibronectin-coated dishes (BD Pharmingen), punctate expression of α_v integrin-GFP was observed under laser scanning confocal microscopy (Figure 4).

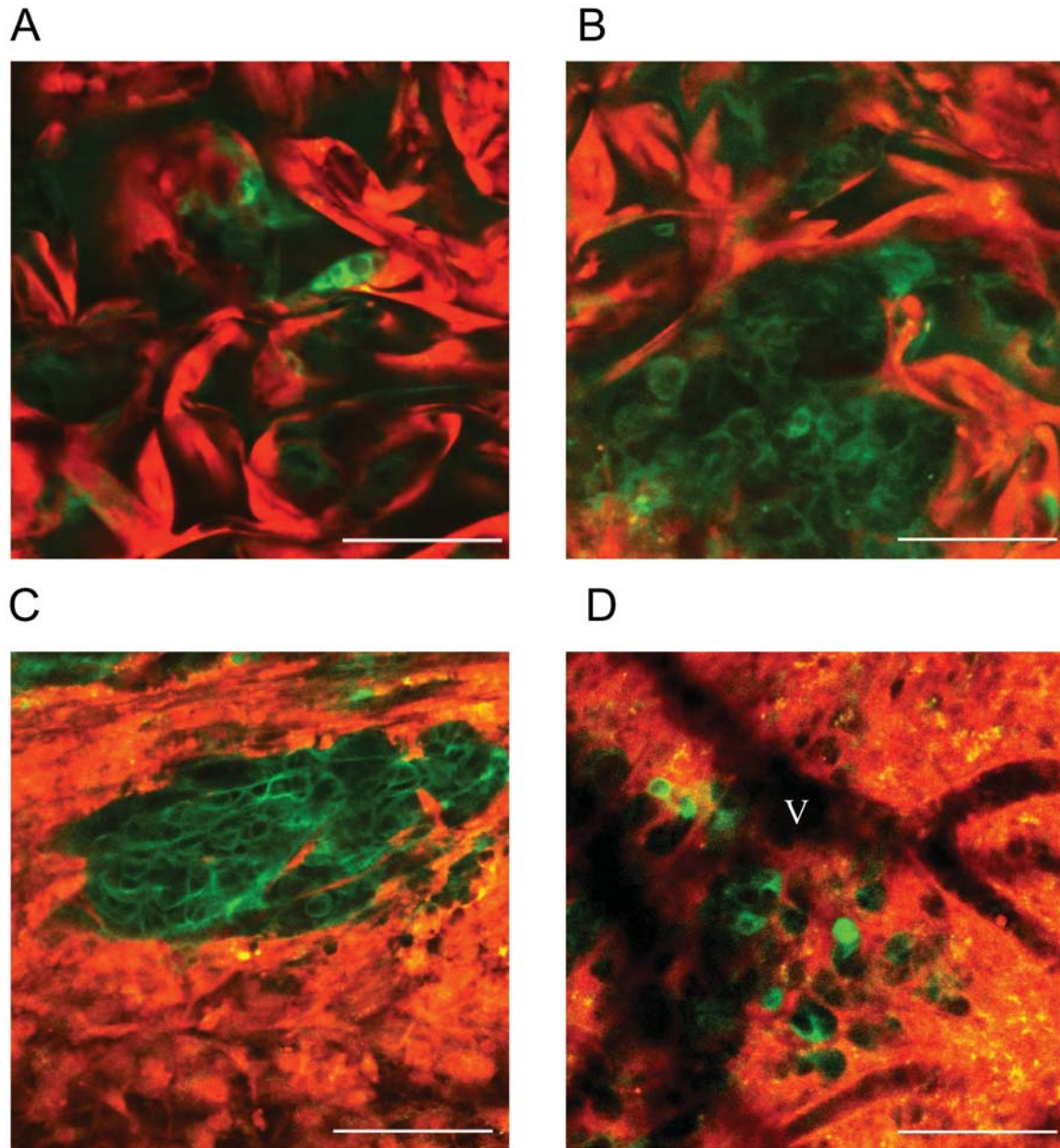


Figure 5. *In vivo* visualization of the interaction between 143B cells expressing α_v integrin GFP and RFP vessels in Gelfoam[®]. Skin flaps were made seven days after transplantation of 143B cancer cells (5×10^5), expressing α_v integrin-GFP, into the Gelfoam[®], previously transplanted in RFP transgenic nude mice. The skin flaps were closed with a 6-0 nylon suture. A: Seven days after cell injection, GFP-expressing cancer cells were observed in the Gelfoam[®]. Bar=100 μ m. B: Fourteen days after injection, GFP-expressing cancer cells were found proliferating in the Gelfoam[®]. Bar=100 μ m. C: Twenty-one days after injection, α_v integrin-GFP-expressing cancer cells were found to be proliferating in the Gelfoam[®]. α_v Integrin-GFP was strongly expressed around the surface of the cells. Bar=100 μ m. D: Twenty-eight days after injection, cancer cells strongly expressed α_v integrin-GFP around a small RFP-expressing vessel (V). Bar=100 μ m.

In vivo visualization of the interaction between 143B cells expressing α_v integrin-GFP and RFP blood vessels in Gelfoam[®]. Seven days after 143B cell injection, the GFP-expressing cancer cells were observed in the Gelfoam[®] in

the RFP nude mice (Figure 5A). Fourteen days after injection, GFP-expressing cells were proliferating in the Gelfoam[®] (Figure 5B). Twenty-one days after injection, GFP-expressing cells were proliferating in the Gelfoam[®]

cavities, and α_v GFP integrin was strongly expressed around the surface of the cells (Figure 5C). Twenty-eight days after injection, cancer cells strongly expressing α_v integrin-GFP were observed around a small vessel (Figure 5D). These data suggests that the pattern of expression of α_v integrin-GFP is associated with the proliferation and invasion of cancer cells.

Discussion

The expression of α_v integrins is associated with tumor progression in various types of human cancer. α_v integrin has been implicated in angiogenesis and tumor progression (2-5). Up-regulation of α_v integrin has been observed in osteosarcoma (6). However, little is known about molecular dynamics of interaction between α_v integrin cancer cells and blood vessels.

In this report, injected 143B cells expressing α_v integrin-GFP proliferated in Gelfoam[®] *in vivo*, and GFP was expressed around the surface of the cells. Cancer cells strongly expressing α_v integrin-GFP were observed in 143B cells interacting with blood vessels in Gelfoam[®]. This suggests that α_v integrin-GFP influenced the proliferation and the interaction between the cancer cells and blood vessels. The molecular color-coded model described in this report should be a powerful tool for investigating the role of integrin expression in host-cancer cell interaction. Gelfoam[®] is a useful tool for observing angiogenesis *in vivo* and the interactions between cancer cells and blood vessels, which will allow further understanding of the role of α_v integrin in malignancy.

Conflicts of Interest

None of the Authors have a conflict of interest in regard to this study.

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