Color-coded Imaging of Spontaneous Vessel Anastomosis *In Vivo*

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Abstract. *Vessel anastomosis is important in tumor angiogenesis as well as for vascularization therapy for ischemia and other diseases. We report here the development of a color-coded imaging model that can visualize the anastomosis between blood vessels of red fluorescent protein (RFP)-expressing vessels in vascularized Gelfoam® previously transplanted into RFP transgenic mice and then re-transplanted into nestin-driven green fluorescent protein (ND-GFP) mice where nascent blood vessels express GFP. Gelfoam® was initially transplanted subcutaneously in the flank of transgenic RFP nude mice. Skin flaps were made at 14 days after transplantation of Gelfoam® to allow observation of vascularization of the Gelfoam® using confocal fluorescence imaging. The implanted Gelfoam® became highly vascularized with RFP vessels. Fourteen days after transplantation into RFP transgenic nude mice, the Gelfoam® was removed and re-transplanted into the subcutis on the flank of ND-GFP transgenic nude mice in which nascent blood vessels express GFP. Skin flaps were made and anastomosis between the GFP-expressing nascent blood vessels of ND-GFP transgenic nude mice and RFP blood vessels in the Gelfoam® was imaged 14 and 21 days after re-transplantation. The results presented in this report indicate a possible mechanism for tumor angiogenesis and suggest a new paradigm of therapeutic revascularization of ischemic organs requiring new blood vessels and in other diseases.*

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Angiogenesis occurs in embryogenesis development, regeneration, the menstrual cycle, and pathologically in cancer, macular degeneration, and rheumatoid arthritis (1, 2). The process of blood vessel formation is essential for tumor development and metastasis.

We previously developed a transgenic nude mouse for the imaging of human tumor angiogenesis. In this mouse model, the nestin promoter drives green fluorescent protein (GFP) [nestin-driven GFP (ND-GFP)] which is expressed in nascent blood vessels (3).

In another previous study, Gelfoam® (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) was transplanted into ND-GFP mice and was vascularized with GFP-expressing vessels in the presence of an angiogenesis stimulator. Antiangiogenesis agents inhibited the vascularization (4).

Angiogenesis was quantified in the Gelfoam® by measuring the total length of ND-GFP-expressing nascent blood vessels imaged in a skin flap by *in vivo* fluorescence microscopy. At day 7 after transplantation, the ND-GFP-expressing nascent blood vessels were observed *via* a skin flap to form a network on the surface of the basic fibroblast growth factor (bFGF)-treated Gelfoam®. Implanted Gelfoam® that was not treated with bFGF was not vascularized. The ND-GFP vessels in the Gelfoam® stained positively for CD31 antigen demonstrating the presence of endothelial cells (4).

We previously developed a color-coded imaging model that can visualize the interaction between cancer cells expressing GFP and blood vessels in Gelfoam® vascularized after implantation in red fluorescent protein (RFP) transgenic nude mice. The implanted Gelfoam® became highly vascularized with RFP-expressing vessels. Cancer cells expressing α_v integrin-GFP were injected into the Gelfoam® seven days after transplantation of Gelfoam®. Seven days after cancer cell injection, the GFP-expressing cancer cells interacting with RFP-expressing blood vessels were observed by color-coded confocal microscopy (5).

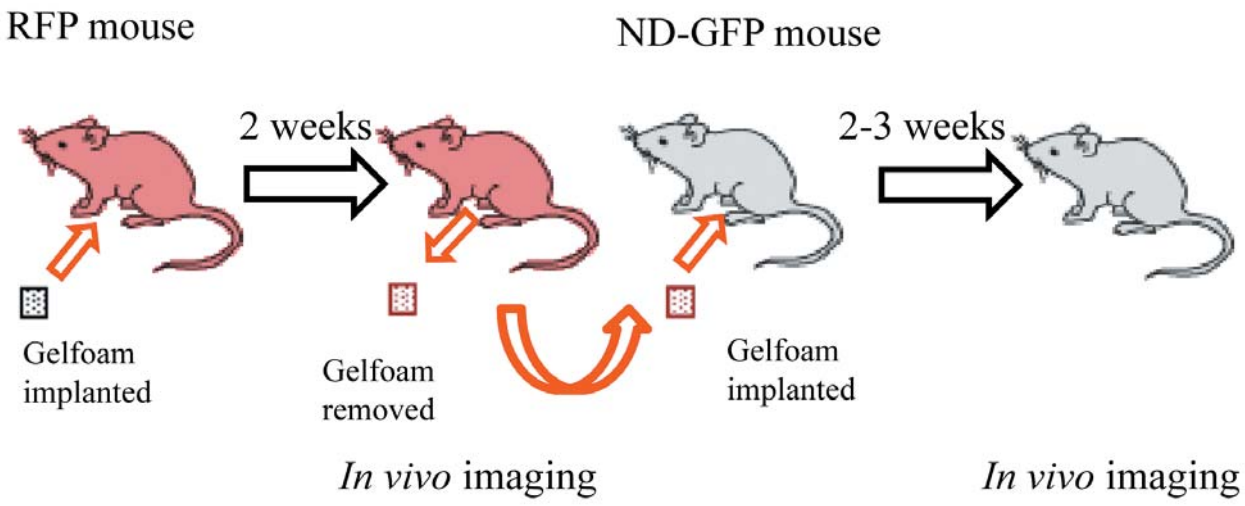

Figure 1. *Experimental schema for Gelfoam® angiogenesis. Gelfoam® (5×5 mm), pretreated with 300 ng basic fibroblast growth factor in 75 μl RPMI-1640 medium, was transplanted into the subcutis of both flanks of red fluorescent protein (RFP) transgenic nude mice. Skin flaps were made at day 14 after transplantation of Gelfoam® under anesthesia. Angiogenesis was imaged in the Gelfoam® via the skin flap using confocal microscopy. The transplanted Gelfoam® was then removed and transplanted into the subcutis of the flank of nestin-driven green fluorescent protein (ND-GFP) transgenic nude mice for color-coded imaging of blood vessel anastomosis.*

In the present study, we generated RFP-expressing vascularized Gelfoam® in RFP transgenic mice and then transplanted the vascularized Gelfoam® to ND-FP transgenic nude mice in order to observe vessel anastomosis using color-coded imaging.

Materials and Methods

Mice. Female RFP transgenic nude mice and ND-GFP transgenic nude mice (AntiCancer Inc., San Diego, CA, USA) 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 (5, 6) were anesthetized with a ketamine mixture (Butler-Schein, Dublin, OH, USA) (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H_2O) *via s.c.* injection. Gelfoam® (Pharmacia & Upjohn Company) (5×5 mm) was pretreated with 300 ng bFGF (Millipore, Billerica, MA, USA) in 75 μl RPMI-1640 medium (Cellgro, Herndon, VA, USA). The treated Gelfoam® was then transplanted into the subcutis on both flanks of RFP transgenic nude mice.

Skin-flap window. Skin flap imaging windows were made as previously described (7). The RFP transgenic nude mice were anesthetized with the ketamine mixture *via s.c.* injection. An arc-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 vessels. Mice were laid flat, and the skin flap was spread and fixed on the flat stand. The implanted Gelfoam® was directly imaged with confocal microscopy. The skin was subsequently closed with a 6-0 nylon suture.

Re-implantation of Gelfoam®. Four-week-old ND-GFP transgenic nude mice (4) were anesthetized with the ketamine mixture as described above. Fourteen days after implantation, the Gelfoam® previously implanted in the RFP transgenic nude mice, was removed and re-implanted into the subcutis on the flank of ND-GFP transgenic nude mice. The Gelfoam® was observed *via* a skin flap with a confocal microscope 14 and 21 days after re-implantation.

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

Imaging. An Fluoview FV 1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan) with an XLUMPLFL ×20 (0.95 numerical aperture) 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 (8).

Results

Imaging of Gelfoam® vascularization in RFP transgenic mice. To obtain Gelfoam® vascularization in RFP nude mice, Gelfoam® (5×5 mm) was pre-treated with 300 ng

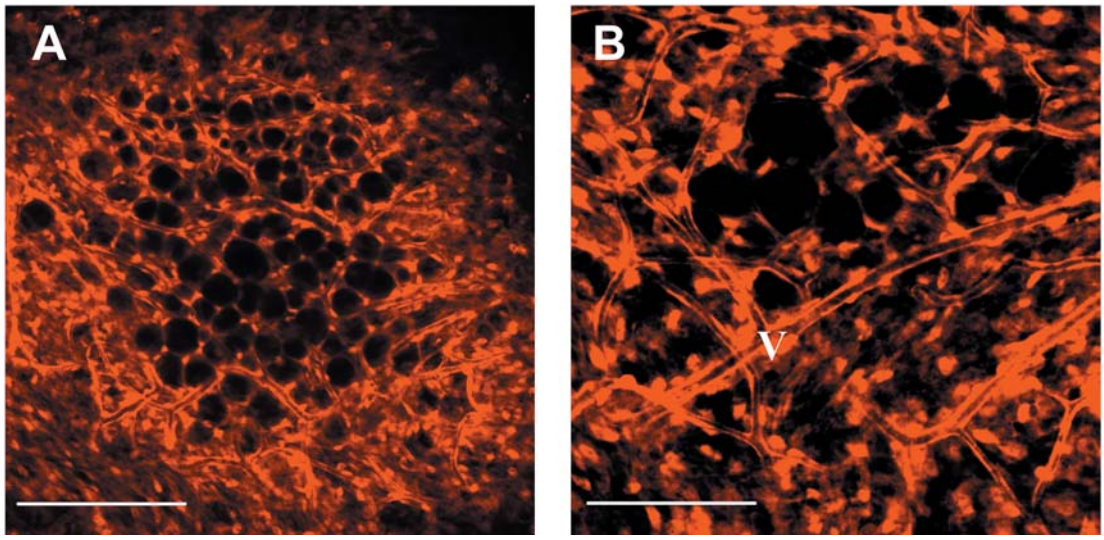

Figure 2. *Vascularization of implanted Gelfoam® with red fluorescent protein (RFP)-expressing vessels. Fourteen days after Gelfoam® transplantation in RFP transgenic mice, skin-flap windows were made for observation with an FV1000 confocal microscope. A: Fourteen days after transplantation, RFP-expressing vessels were observed in the Gelfoam®. Bar=200 μm. B: At higher magnification (×40), RFP-expressing vessel structures (V) were observed in the Gelfoam®. Bar=100 μm.*

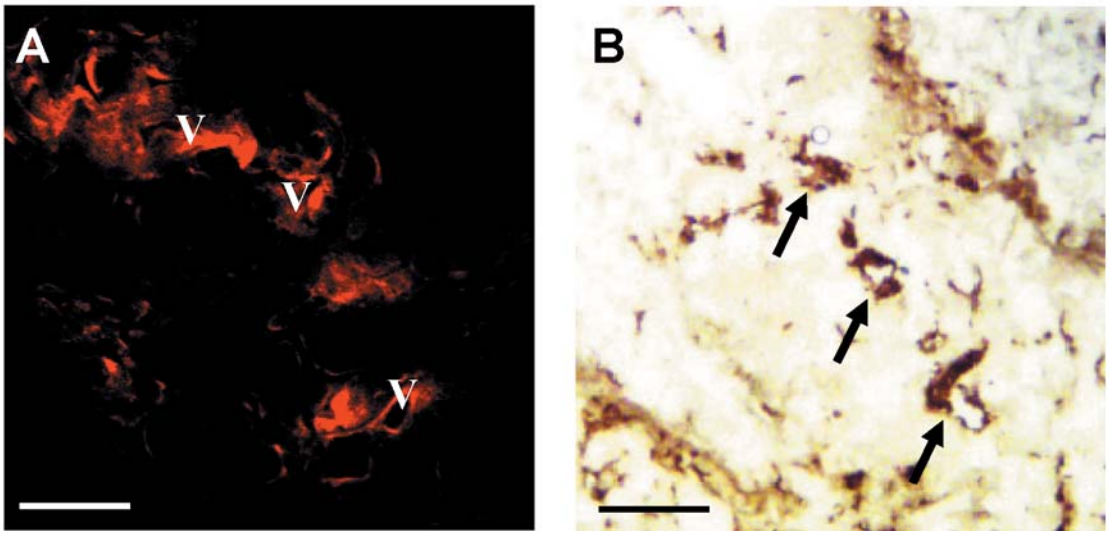

Figure 3. *Co-localization of red fluorescent protein (RFP) and CD31 in blood vessels in Gelfoam®. Frozen sections were made at day 14 after transplantation of Gelfoam®, pretreated with basic fibroblast growth factor (bFGF), into the subcutis of RFP transgenic mice. A: RFP-expressing blood vessels (V) were growing in the Gelfoam®. B: Immunohistochemical staining showed that CD31 and RFP fluorescence co-localized in the RFP-expressing blood vessels (arrow). Bar=50μm*

bFGF, and transplanted into the subcutis on both flanks of RFP nude mice (Figure 1). Fourteen days after transplantation, skin flaps were made and the Gelfoam® was observed with an Olympus FV1000 confocal laser-scanning microscope. RFP-expressing vessels structures were observed in the Gelfoam® (Figure 2).

Co-localization of RFP and CD31 in blood vessels in Gelfoam®. Gelfoam® was harvested and frozen sections were made at day 14 after transplantation of the Gelfoam® into the subcutis of RFP transgenic nude mice. At that time, RFP-expressing blood vessels were observed growing in the Gelfoam® (Figure 3A). IHC staining

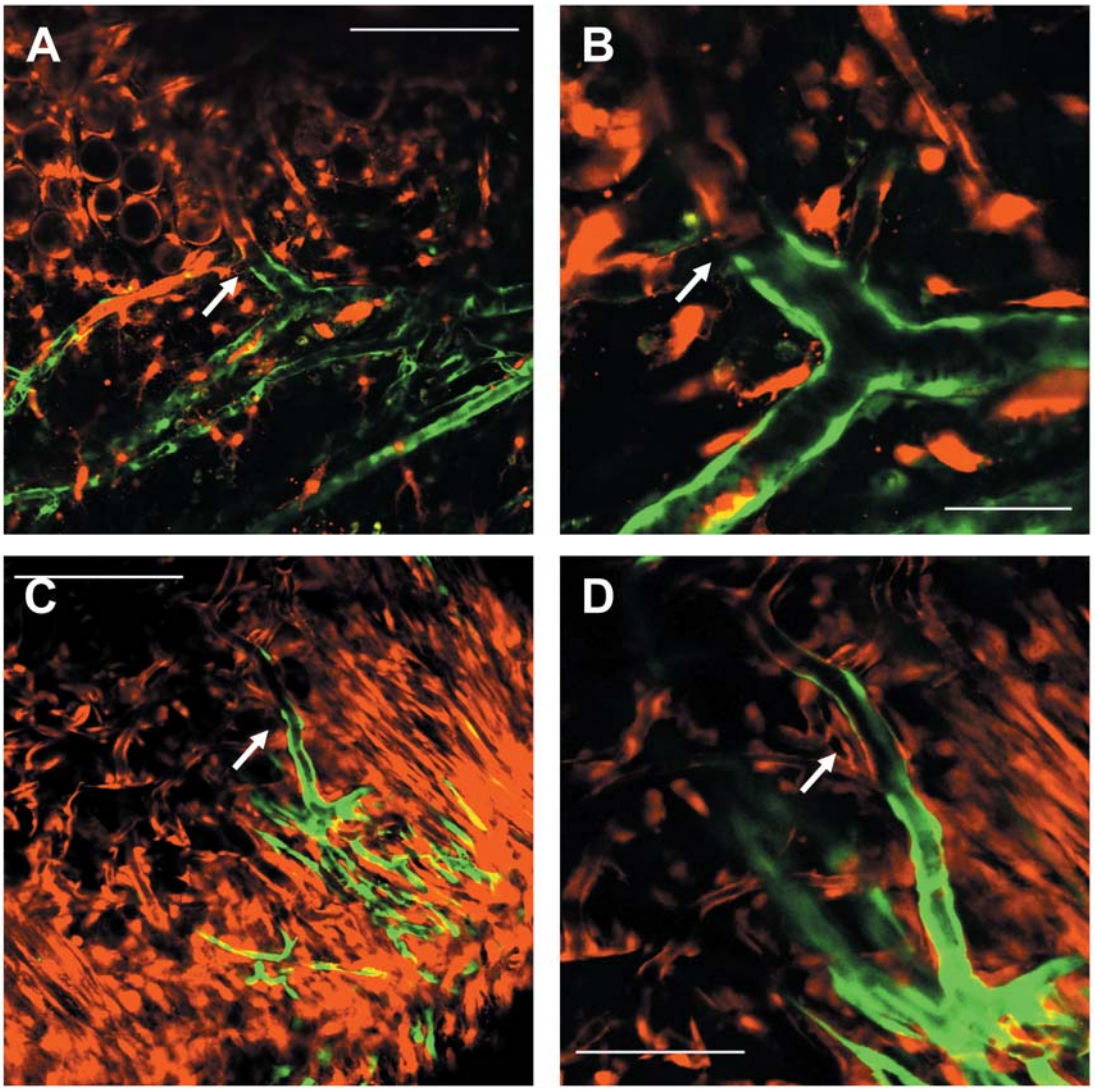

Figure 4. *In vivo visualization of anastomosis between red fluorescent protein (RFP) vessels in Gelfoam® and host vessels in nestin-driven green fluorescent protein (ND-GFP) transgenic nude mice. Skin flaps were made after re-transplantation of vascularized Gelfoam®, previously transplanted in RFP nude mice. A: Fourteen days after re-transplantation, RFP-expressing vessels were observed anastomosed to ND-GFP-expressing blood vessels (arrow). Bar=200 μm. B: At higher magnification (×60), the vessel anastomosis was imaged (arrow). Bar=30 μm. C: Twenty-one days after re-transplantation, RFP-expressing vessels and ND-GFP-expressing blood vessels were anastomosed (arrow). Bar=200 μm. D: At higher magnification (×40), a hybrid vessel with mixed expression of RFP and ND-GFP was imaged (arrow). Bar=100 μm.*

showed that CD31 (Figure 3B) and RFP fluorescence co-localized in the RFP-expressing blood vessels in the Gelfoam®.

In vivo imaging of the anastomosis between blood vessels in ND-GFP transgenic nude mice and RFP blood vessels in Gelfoam®. Fourteen and 21 days after re-implantation of the vascularized Gelfoam® in ND-GFP mice, the ND-GFP- and RFP-expressing blood vessels were observed in the RFP vascularized Gelfoam® (Figure 4). RFP blood vessels in the Gelfoam® had anastomosed with the ND-GFP blood vessels of the host mice thereby forming hybrid blood vessels.

Discussion

Gelfoam®, impregnated with proangiogenic factors, is readily vascularized in vivo (9). In this study, Gelfoam® was implanted into RFP transgenic nude mice and became vascularized with

RFP blood vessels. The vascularized Gelfoam® was then transplanted into ND-GFP transgenic nude mice in order to visualize the interaction between transplanted vessels and host vessels. RFP-expressing blood vessels anastomosed to host ND-GFP-expressing blood vessels in the Gelfoam® by 14 days after re-transplantation. By 21 days after re-transplantion, RFP vessels and ND-GFP vessels were further fused. These results suggest that an angiogenesis system using Gelfoam® could be used to connect transplanted blood vessels to host vessels and supplement or even replace host blood vessels. The color-coded model described in this report should be a powerful tool for investigating the behavior of new vessels, such as those arising in tumors as well as transplanted vessels, for example in ishchemic organs. Gelfoam® is a useful tool for observing angiogenesis *in vivo* and the interactions between vessels, which will allow further understanding of the mechanism of tumor and normal angiogenesis using high-resolution imaging methods of fluorescent protein expression (10-12).

Conflicts of Interest

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

Acknowledgements

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