

A transgenic Tie2-GFP athymic mouse model; a tool for vascular biology in xenograft tumors

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Abstract

We report the generation of a transgenic Tie2-GFP athymic nude mouse, carrying green fluorescent blood vessels throughout the body. This transgenic mouse is a tool for studies in vascular biology, and is especially of interest for imaging of tumor angiogenesis and the study of anti-angiogenesis strategies in (human) xenografts. Intravital microscopy identified the presence of blood conducting structures that are not lined by endothelial cells. Dedifferentiation of aggressive tumor cells can lead to acquisition of endothelial characteristics. This process of tumor cell plasticity, also referred to as vasculogenic mimicry, has been suggested to contribute to the circulatory system in a tumor. In plastic EW7 Ewing sarcoma tumors in these Tie2-GFP mice, we observed blood flow in both regular blood vessels and non-fluorescent tumor cell-lined channels, visualizing *in vivo* hemodynamics in vasculogenic channels. These results demonstrate that the transgenic Tie2-GFP athymic mouse model is a valuable tool for vascular biology research.

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The importance of the vascular system during pathological conditions such as cancer, atherosclerosis, and wound healing has been well recognized for years [1]. In order to study the biology of vessel formation in development and disease, a transgenic mouse model with green fluorescent protein (GFP) expression in the vasculature was created [2,3]. This model allowed visualization of blood vessels at a single cell resolution. The FVB/N genetic background of these mice precluded the use in many tumor models. In order to develop a more widely applicable model, we backcrossed these mice into the Balb/c nu/nu athymic mice, to allow vascular studies in

(human) xenograft tumor models. In this model, we studied blood circulation in human Ewing sarcoma tumors by intravital microscopy. Plasticity of Ewing sarcoma tumor cells allows the dedifferentiation of these cells into a more stem cell-like phenotype, which can result in the acquisition of endothelial cell characteristics. In aggressive tumors, this process may result in the formation of blood conducting vascular structures [4]. In this context, the term vasculogenic mimicry was introduced [5,6]. Different approaches have suggested that such channels are connected to the blood circulation and also contribute to it [5,7–14]. We previously demonstrated that Ewing sarcoma in patients, as well as models for Ewing sarcoma in mice, abundantly contain vasculogenic structures [4]. The newly developed transgenic Balb/c Tie2-GFP mouse model was used to study this phenomenon.

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Materials and methods

Animals. We used transgenic Balb/c nude Tie2-GFP mice housed under sterile conditions. This strain originated from an FVB/N-Tie2-GFP background, in which the vascular endothelial cells express green fluorescent protein (GFP) under the direction of the endothelial-specific receptor tyrosine kinase (Tie2) promoter (Jackson Laboratory, Bar Harbor, ME, USA), and was backcrossed to Balb/c nude mice for over 10 generations. Phenotyping was executed by cutting a piece of the ear, mounting it in PBS and visualize GFP-positive vessels under a fluorescence microscope (Nikon Eclipse E800, Japan).

GFP expression in organs. For analysis of GFP expression in adult tissues, Balb/c nude Tie2-GFP mice were anesthetized with isoflurane and perfused with cold 4% PFA/PBS. Tissues (heart, lung, kidney, spleen, liver, brain, calf muscle) were further processed for cryosectioning as described in Motoike et al. [3] and 10–20 μ m were cut using a Microm HM500M cryostat (Microm, Walldorf, Germany). GFP expression in blood vessels in the various tissues was visualized using a confocal Laser Scanning Microscope (Zeiss LSM510; Zeiss, Jena, Germany). GFP was excited at 488 nm and detected using a 505–530 bandpass filter.

Cell culture. Human Ewing sarcoma cells (EW7) were cultured in RPMI-1640 (Life technologies, Breda, the Netherlands) containing 10% of inactivated fetal calf serum (Hyclone, Etten-Leur, the Netherlands) and 2 mM L-glutamin (Life technologies). These cells were harvested using 0.125% of trypsin.

Experimental protocol and intravital microscopy. EW7 cells (2×10^6) were inoculated s.c. on both flanks and on both legs of two 10 weeks old Balb/c nude Tie2-GFP male transgenic mice. On day 28, mice were

anesthetized by s.c. administration of a mixture of ketamine and xylazine (0.1 and 0.05 mg/g, respectively; Nimatek, Sedamun, Ad Usem Veterinarium, Cuijk, the Netherlands). Intravital microscopy was done as described before [15]. Vessels were visualized using a Leitz intravital microscope adapted for telescopic imaging equipped with an SW25 objective lens (numerical aperture 0.60). Microscopic images were recorded using a digital camera (Hamamatsu, EM-CCD, Phototronics, Germany) with the imaging program Wasabi (version 1.5, Hamamatsu Photonics). Body temperature was kept at 37 °C by an infrared heating lamp. In both mice, we screened for fluorescent vessels in the ear, muscle and tumor. In the tumor, non-fluorescent vessels with an irregular shape, were considered as vasculogenic mimicry channels. Before sacrificing, mice were injected with 10–20 μ l rhodamine 6G solution in the tail vein, to enable the observation of leukocytes. For GFP recordings we used an I2 filter (Leitz, BP 450–490, RKP 510, LP 515) and for rhodamine recordings we used a N2.1 filter (Leitz, BP 515–560, RKP 580, LP 580). Diameters of blood vessels and vasculogenic mimicry channels were analysed with a calibration curve.

Immunohistochemistry. Paraffin sections (6 μ m thickness) were deparaffinized in ethanol, incubated in 0.3% H₂O₂ in methanol for 20' to quench endogenous peroxidase activity, after which antigen retrieval was carried out by heating the sections in a Tris–EDTA buffer (10 mM Tris–1 mM EDTA, pH 8) for 15' in a microwave. Subsequently, the slides were incubated for 30' in 20% FCS–1% BSA–0.1% Tween-20 in PBS for blocking of non-specific antibody binding. Sections were incubated for 1 hour with an endothelial-specific antibody 9F1 [16], followed by a biotin-labelled donkey anti-rat IgG (Jackson Immunosearch Laboratories, West

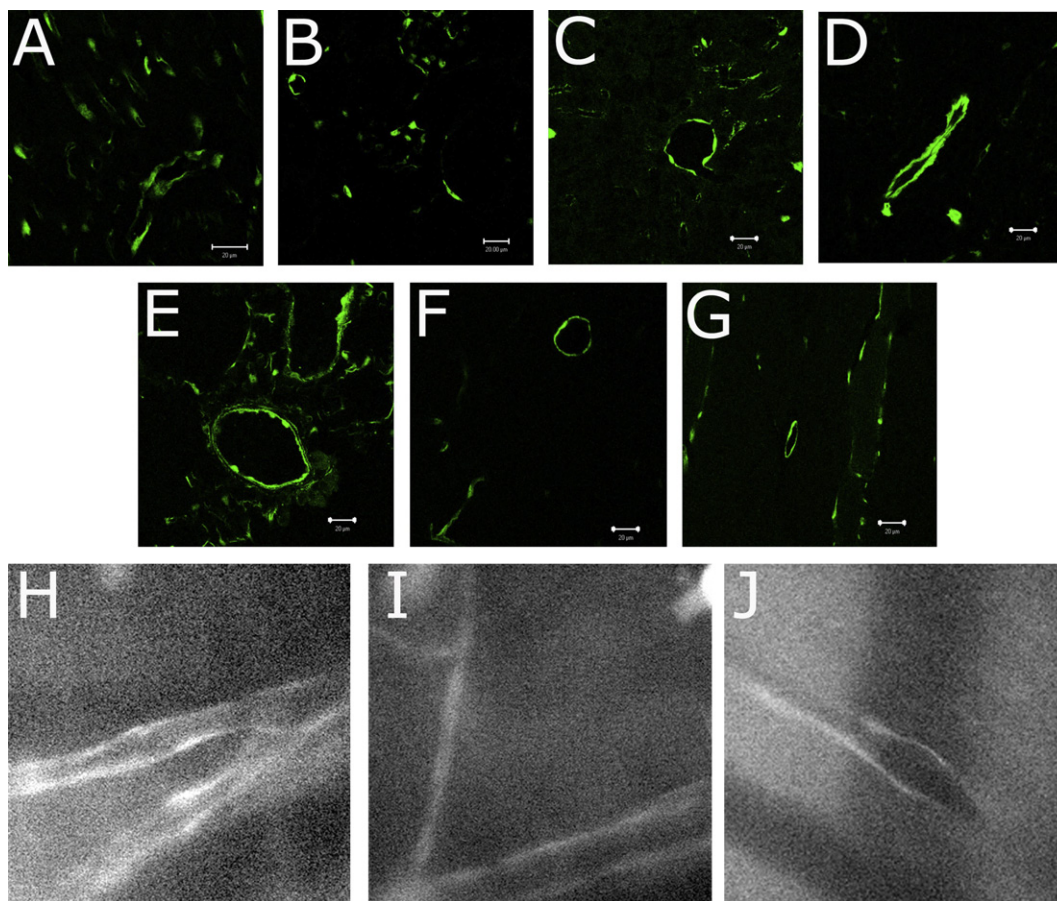


Fig. 1. GFP vessels in the athymic Tie2-GFP transgenic adult mouse. (A–G) Images from heart, kidney, liver, spleen, lung, brain, and calf muscle, respectively. Scale bar represents 20 μ m. (H–J) Stills of intravital fluorescence microscopy images of GFP vessels in the muscle (H–I) and in the ear skin (J). Video movies of these experiments can be viewed at www.angiogenesis.nl.

Grove, PA, USA; 1/100) diluted in 1% BSA-5% mouse serum in PBS for 30' and avidin-biotin complex HRP (Dakocytomation, Glostrup, Denmark; 1/500) for 30'. Diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) was used as a brown chromogen. Finally, after dehydration, slides were mounted with entellan (Merck, Darmstadt, Germany).

Statistical analysis. Statistical calculations were performed with SPSS software using the Mann–Witney *U* test. The statistical significance level was set at $p < 0.05$.

Results and discussion

Balb/c nude Tie2-GFP transgenic mice express GFP in the endothelium of all blood vessels

In order to allow the study of vascular biology and angiogenesis in xenograft tumor models, an immunocompromised (athymic) Tie2-GFP transgenic mouse was generated. These mice were backcrossed from the original FVB/N-Tie2-GFP mice [3]. This resulted in transgenic mice with a specific and uniform expression of GFP in the vascular endothelium. Fluorescence microscopy demonstrated that all blood vessels in heart, kidney, liver, spleen, lung, brain, and muscle (shown in Fig. 1A–G) were GFP-positive. There was no distinction in expression between large blood vessels, smaller vessels, and capillaries, although arterioles were more fluorescent than venules. Using intravital microscopy, vessels in the ear skin and

muscle were observed to express GFP (Fig. 1H–J), allowing the efficient identification of endothelium in vital tissue. Real-time video images of intravital microscopy experiments can be viewed at www.angiogenesis.nl.

Vasculogenic channels are non-fluorescent, contribute to circulation and transport leukocytes

The Tie2-GFP mice were used to study the blood circulation in human Ewing sarcoma xenografts. We described Ewing sarcoma as an aggressive tumor with an unexpected low level of ongoing angiogenesis and the presence of irregularly lined vasculogenic structures. We suggested earlier that these structures, which are called blood lakes and are a hallmark of Ewing sarcoma tissues, contribute to blood circulation by demonstrating that i.v. injected Indian ink is present in regular blood vessels as well as in these blood lakes [4]. In the athymic Tie2-GFP transgenic mice carrying EW7 Ewing sarcoma tumors, we studied the vasculogenic structures, which appeared as non-fluorescent, irregularly lined, tortuous vessel-like structures (Fig. 2A). We found that these structures have a statistically significant larger diameter as compared to the regular endothelium lined blood vessels (ear vs. VM: $p < 0.001$, muscle vs. VM: $p < 0.003$, tumor vs. VM: $p < 0.001$). While regular blood vessels in these xenografts were observed throughout the

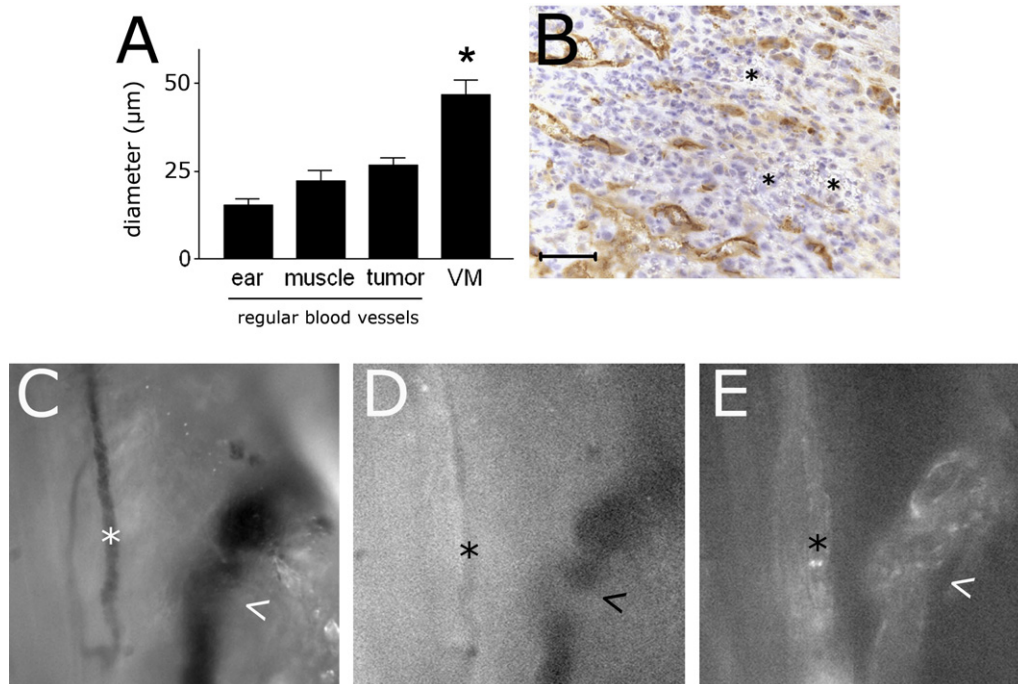


Fig. 2. Non-fluorescent vasculogenic mimicry channels contribute to *in vivo* hemodynamics. (A) Quantification of vessel diameter of different vascular structures in normal tissues and tumor. (B) Blood vessel staining in a Ewing sarcoma xenograft. Non-endothelial cell-lined structures containing red blood cells, also referred to as blood lakes, are indicated by *. Scale bar represents 20 µm. (C–E) Intravital microscopy images of regular GFP expressing blood vessels and non-fluorescent vasculogenic mimicry channels. The tumor blood vessel is located on the right and indicated with an asterisk, while the vasculogenic mimicry channel is indicated with an arrow. The different images are recorded from the same vessels without filter (C), with I2 filter for GFP (D), and with N2.1 filter after injection of rhodamine (E). In Fig. 2D the tumor blood vessel is clearly fluorescent while the VM channel is not. In Fig. 2E, hemodynamics of labelled rhodamine leukocytes are visible in both structures. Video movies of these experiments can be viewed at www.angiogenesis.nl.

tumor, vasculogenic structures (Fig. 2B) were mainly observed in the outer rim of the tumor. Using intravital microscopy, both fluorescent regular blood vessels and non-fluorescent channels were observed (Fig. 2C–D). Under bright field we observed blood flow in the blood capillaries but slower blood flow in the vasculogenic structures. This was expected and in line with earlier findings that blood flow in these structures is sluggish leading to hypoxia in the surrounding tissue [4]. After injection of rhodamine in the tail vein, we observed the circulation of labelled leukocytes in both the regular blood vessels as well as in the vasculogenic structures (Fig. 2E).

In conclusion, we describe the generation of an athymic Tie2-GFP transgenic mouse model expressing GFP in the endothelium of all blood vessels and allowing the study of vascular developmental biology as well as angiogenesis research in xenograft (including human) tumor models. Using intravital microscopy this model allowed the visualization of both fluorescent blood vessels and non-fluorescent tumor cell-lined channels, as well as *in vivo* hemodynamics in these vasculogenic mimicry channels. We believe this tumor model has clearly shown to be a useful tool for the study of vascular biology in a wide variety of tumor models.

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