RESEARCH ARTICLE

Quantification of Angiogenesis and Lymphangiogenesis in the Dual *ex vivo* Aortic and Thoracic Duct Assay

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Abstract: *Background*: Lymphatic vessel formation (lymphangiogenesis) plays important roles in cancer metastasis, organ rejection, and lymphedema, but the underlying molecular events remain unclear. Furthermore, despite significant overlap in the molecular families involved in angiogenesis and lymphangiogenesis, little is known about the crosstalk between these processes. The *ex vivo* aortic ring assay and lymphatic ring assay have enabled detailed studies of vessel sprouting, but harvesting and imaging clear thoracic duct samples remain challenging. Here we present a modified *ex vivo* dual aortic ring and thoracic duct assay using tissues from dual fluorescence reporter Prox1-GFP/Flt1-DsRed (PGFD) mice, which permit simultaneous visualization of blood and lymphatic endothelial cells.

Objective: To characterize the concurrent sprouting of intrinsically fluorescent blood and lymphatic vessels from harvested aorta and thoracic duct samples.

Methods: Dual aorta and thoracic duct specimens were harvested from PGFD mice, grown in six types of endothelial cell growth media (one control, five that each lack a specific growth factor), and visualized by confocal fluorescence microscopy. Linear mixed models were used to compare the extent of vessel growth and sprouting over a 28-day period.

Results: Angiogenesis occurred prior to lymphangiogenesis in our assay. The control medium generally induced superior growth of both vessel types compared with the different modified media formulations. The greatest decrease in lymphangiogenesis was observed in vascular endothelial growth factor-C (VEGF-C)-devoid medium, suggesting the importance of VEGF-C in lymphangiogenesis.

Conclusion: The modified *ex vivo* dual aortic ring and thoracic duct assay represents a powerful tool for studying angiogenesis and lymphangiogenesis in concert.

Keywords: Thoracic duct, aorta, lymphangiogenesis, angiogenesis, VEGF, cancer metastasis.

1. INTRODUCTION

Angiogenesis and lymphangiogenesis, the sprouting of blood and lymphatic vessels, respectively, from pre-existing ones, are heavily regulated processes that occur through tight cell–cell coordination and environmental queues, which signal the differentiation, proliferation, migration, and matrix adhesion of vascular cells [1, 2]. The development of blood and lymphatic vessels allows for efficient transport of nutrients, signaling molecules, circulating cells, and fluid throughout the body. Due to their extensive reach into our physiology, aberrant vascular development leads to increased risks of a vast range of pathologies, including tissue ischemia, lymphedema, and other conditions caused by reduced access to vessels. On the other hand, stimulation of angiogenesis and lymphangiogenesis has been associated with cancer metastasis, as tumor cells are provided access to systemic circulation to travel to distant tissue or organs [3-7]. The growth of blood and lymphatic vessels has also been associated with elevated transplant rejection rates, likely due to increased access of immune cells to the graft [8]. As the blood and lymphatic vessels serve as a conduit for molecules and cells involved in many pathological processes, understanding the regulatory signals of angiogenesis and lymphangiogenesis may open doors for the design of targeted therapies [8-10].

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A large variety of *in vivo* and *in vitro* assays have been used so far to understand these mechanisms and to identify activators and inhibitors of angiogenesis and lymphangiogenesis [11-16]. Analyses involving *in vivo* models have offered an in-depth view of neovascularization, through the use of clinical imaging (*e.g.*, magnetic resonance imaging, computed tomography, angiography) and fluorescent dyes, as well as various transgenic animal models [8, 16, 17]. Although they can provide great insight, *in vivo* assays are relatively expensive and can be prone to interference by background inflammatory reactions. Moreover, while an obvious benefit of *in vivo* studies is the observation of vasculogenesis in its native, biological environment, it can often be difficult to differentiate the responses of blood vessels and the effects of adjacent tissues [11, 12]. *In vitro* or cell-based assays, on the other hand, cannot fully reflect the interactions between developing vessels, extracellular matrix materials, and supporting cells such as pericytes, which have been shown to contribute to the stability of newly formed blood vessels [14, 18, 19]. The contrasting limitations of *in vivo* and *in vitro* studies create a need for a model that is practical and can accurately mimic both physiologic and pathologic conditions involving angiogenesis and lymphangiogenesis.

The *ex vivo* aortic ring and thoracic duct assays bridge the gap between *in vivo* and *in vitro* assays by allowing the study of vascular sprouting from a stabilized, large tissue sample [18, 19]. Several studies have used *ex vivo* methods to characterize pro- or anti-angiogenic and lymphangiogenic factors and screen for potential therapeutic targets. However, the important interactions between angiogenesis and lymphangiogenesis have not been thoroughly studied due to challenges of imaging both endothelial cell types simultaneously [6, 19-21] The aortic ring assay was first devised by Nicosia and Ottinetti [22] in 1990 using a rat model, and it has been applied using mouse tissues [18, 23, 24] in several other studies with some technical modifications. Interestingly, in 1987, Nicosia also published the first lymphatic culture study using the rat thoracic duct [25]. The lymphatic ring assay was introduced much later in 2008 by Bruyère *et al.,* [26] using mouse thoracic duct tissue and was adapted from the aortic ring assay first presented by Nicosia and Ottinetti in 1990. Although the aortic ring can be easily harvested, isolation of the lymph-containing thoracic duct traditionally requires the use of dyes (*e.g.*, Evans blue) due to its lack of color [27]. It is possible to combine these assays to study angiogenesis and lymphangiogenesis in concert, but the outgrowth of fibroblasts from the thoracic duct and aortic ring may overlap with vascular sprouting, making it difficult to quantify vascular growth under brightfield microscopy. Typically, a well-defined view and cell identification are possible only by immunostaining [18, 26].

To enable simultaneous observation of angiogenesis and lymphangiogenesis in the same specimen, we bred transgenic Prox1-GFP/Flt1-DsRed (PGFD) mice, in which the blood and lymphatic endothelial cells inherently express distinct fluorescently labeled proteins [8]. This transgenic model provides a unique advantage for identifying and harvesting the thoracic duct based on the fluorescence of lymphatic endothelial cells. Moreover, a dual aortic ring and thoracic duct assay can be performed using tissues from PGFD mice without the need to perform immunostaining for two distinct types of sprouting vessels. This transgenic model also allows for the continuous temporospatial observation of angiogenesis and lymphangiogenesis, reducing the labor and animal sacrifices typically needed to measure data over time.

2. METHODS

2.1. Prox1-GFP/Flt1-DsRed (PGFD) Mice

All animal experiments were done in accordance with guidelines and approved by Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Chicago.

PGFD mice were bred as previously described [8] and housed under pathogen-free conditions in ventilated individual cages. Mice were kept in a 12-h light–dark cycle and provided free access to food and water. Postnatal day 2 (male or female) PGFD mice were used for the present study, as they possess less fat tissue around the aorta and thoracic duct compared to adult mice, which minimizes damage to the aorta and thoracic duct during the removal of the fibroadipose tissue. Polymerase chain reaction (PCR) analysis was performed to confirm the PGFD mouse genotype. Furthermore, mouse skin was harvested for confocal microscopic imaging to verify that the blood and lymphatic vessels fluoresced at their respective wavelengths (Figure **1A-C**).

2.2. Preparation of the Three-dimensional Aortic Ring and Thoracic Duct Co-Cultures

Mice were sacrificed and surface-sterilized with 70% ($^{v}/_{v}$) ethanol prior to aorta and thoracic duct isolation. Under an Axio Zoom fluorescent microscope (Zeiss, Germany), the aorta and thoracic duct were harvested together between the level of the thymus and diaphragm, along the vertebral column by microsurgery using very thin micro-dissecting forceps and Vannas scissors. Compared with the aorta, the thoracic duct is colorless and has a thinner wall with a diameter less than half that of the aortic wall. Under the fluorescent microscope, the thoracic duct is identified by green fluorescence (Figure **1D** & **E**). The periaortic fibroadipose tissue as well as the fibroadipose tissue surrounding the thoracic duct were removed using fine micro-dissecting forceps and iridectomy scissors, taking extra care to avoid any damage to the aortic and thoracic duct wall, respectively. During the dissection, the thoracic cavity bounded by the rib cage and intact diaphragm was continuously filled with sterile phosphate-buffered saline (PBS, pH 7.4). The harvested dual aorta and thoracic duct specimen were then transferred to a sterile dish containing endothelial cell growth medium (EGM-2, Lonza, Switzerland).

Finally, the dual specimen was cut into sections of about 0.5–1 mm in width with a microscissors and immediately transferred to a glass-bottom culture dish (14-mm microwell diameter, MatTek, Ashland, MA) containing 150–200 µL Matrigel (Corning Life Sciences, Tewksbury, MA). Each
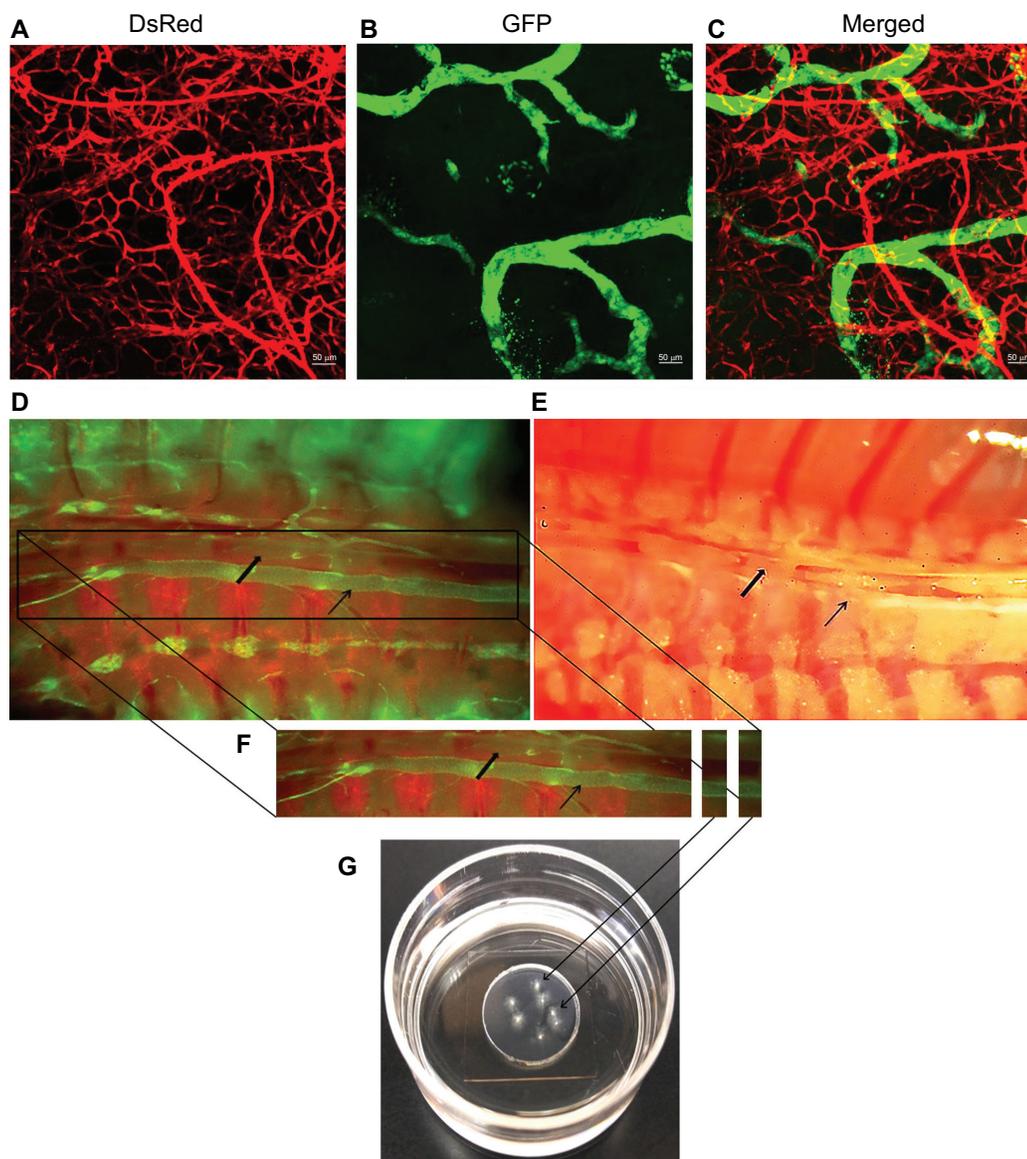
Figure 1. (**A-C**) Confirmation of the PGFD genotype by confocal microscopic imaging displaying expression of DsRed and GFP in blood and lymphatic vessels, respectively, in skin explants from PGFD mice. (**D-E**) Images of the aorta (thick arrow) and thoracic duct (thin arrow) under Axio Zoom fluorescence imaging *versus* brightfield. (**F**) Dual aortic ring and thoracic duct samples were transferred to (**G**) a glass-bottom dish containing Matrigel. *Images A-F were taken from different batch of PGFD mice, demonstrating the fluorescence of the PGFD mice.*

culture dish contained multiple sections of each dual specimen of aorta and thoracic duct. This step must be done promptly before the Matrigel solidifies (Figure **1F-G**). The dish was placed in a culture incubator at 37°C for 30 min, and 2 mL EGM-2 (containing 10% Fetal Bovine Serum [FBS], Sigma-Aldrich, St. Louis, MO) and 100 ng/mL vascular endothelial cell growth factor C (VEGF-C, R&D Systems, Minneapolis, MN) was added for culture at 37°C with 5% CO_2.

2.3. Immunofluorescence Imaging of Sprouting Blood and Lymphatic Vessels

The glass-bottom culture dishes containing the dual aortic ring and thoracic duct samples were first washed with 1x PBS, and the tissues were fixed by addition of 2 mL 4% ($^v/_v$) paraformaldehyde and incubated for 2 hours at room temperature. Nonspecific antigens were blocked using 10% FBS at 37°C for 30 minutes. Samples were then incubated in solutions of primary antibodies for CD31 (rat anti-mouse, 1:100, BD Bioscience, San Jose, CA) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1; rabbit anti-mouse, 1:100, AbCam, UK) at 37°C for 48 hours. Next, samples were incubated in solutions of secondary antibodies, goat anti-rat and goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), at 37°C for 12 hours. After washing with 1x PBS, the samples were ready for imaging *via* confocal microscopy.

2.4. Quantitation of Blood and Lymphatic Vessel Sprouting

Confocal microscopy was used to observe the sprouting, spread, and regression of new lymphatic and blood vessels at days 1, 7, 15, 21, and 28. Samples were imaged at room temperature. Z-stack confocal images obtained using the Zen program (Zeiss, Germany) provided detailed views of vessels growing out in different layers and were used for measurements. Adobe Photoshop (Adobe, San Jose, CA) was used to quantitate branch numbers and measure branch lengths. In addition, outgrowths of fibroblasts were easily excluded because they lacked fluorescence.

To determine the effects of individual growth factors on angiogenesis and lymphangiogenesis, the aortic ring and thoracic duct assays were conducted by culturing tissues in six different endothelial cell growth media formulations (EGM-2 based). The control endothelial cell growth medium included the following: Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Insulin-Like Growth Factor (IGF), VEGF-A, and VEGF-C. The remaining growth media were each deprived of one of the growth factors (growth medium including all growth factors except FGF, *etc.*). The number of branches and branch length were evaluated on days 1, 7, 15, 21, and 28 and compared with the outgrowth of both vessel types in control endothelial cell

growth medium to assess the effect of the absence of a specific growth factor on vessel growth.

2.5. Statistical Analysis

Statistical analysis to compare the number and length of sprouting aortic and thoracic branches was performed using linear mixed models for repeated measures. The trend in growth within each medium was compared versus that in the control group. The analysis controlled for time and sample, addressing any effects from correlations between measurements. The plot of residuals was performed for each model to test for normality. All analyses were performed with SAS (SAS Institute Inc., Cary, NC) and R (The R Foundation, Vienna, Austria).

3. RESULTS

3.1. Assay Establishment and Confirmation of Blood and Lymphatic Vessel Sprouting

Samples of dual aortic ring and thoracic duct specimens from PGFD mice were immersed in Matrigel for three-dimensional culture to quantitate the progression of blood and lymphatic vessel sprouting (Figure **1**). Immuno-fluorescence characterization of vascular or lymphatic endothelial cell sprouting from aortic ring and thoracic duct
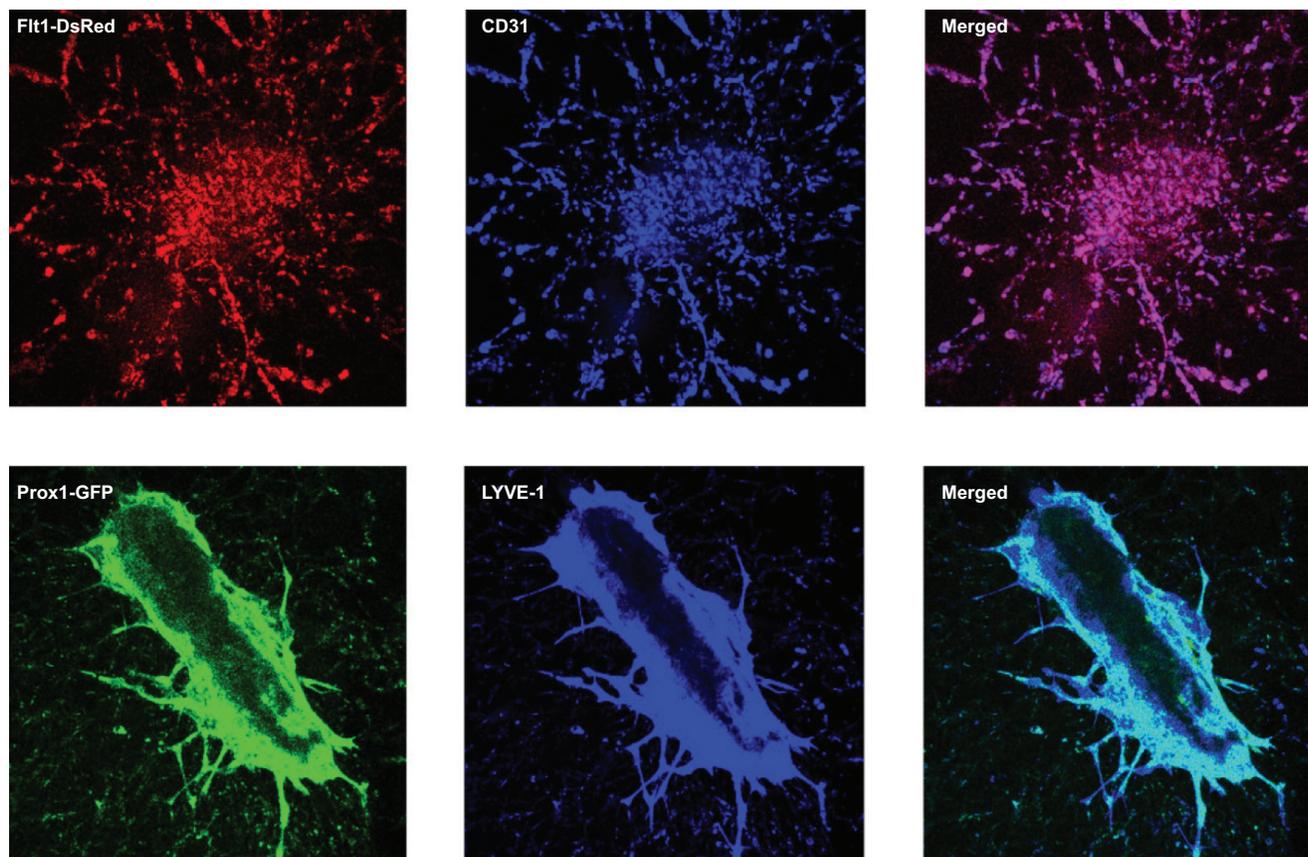
Figure 2. Immunofluorescence confirmation of blood and lymphatic vessel sprouting in the dual aortic ring and thoracic duct assay under confocal microscopy. Immunoreactivity for CD31 expression on blood vessels and LYVE-1 expression on lymphatic cells was co-localized with Ds-Red and GFP fluorescence, respectively.
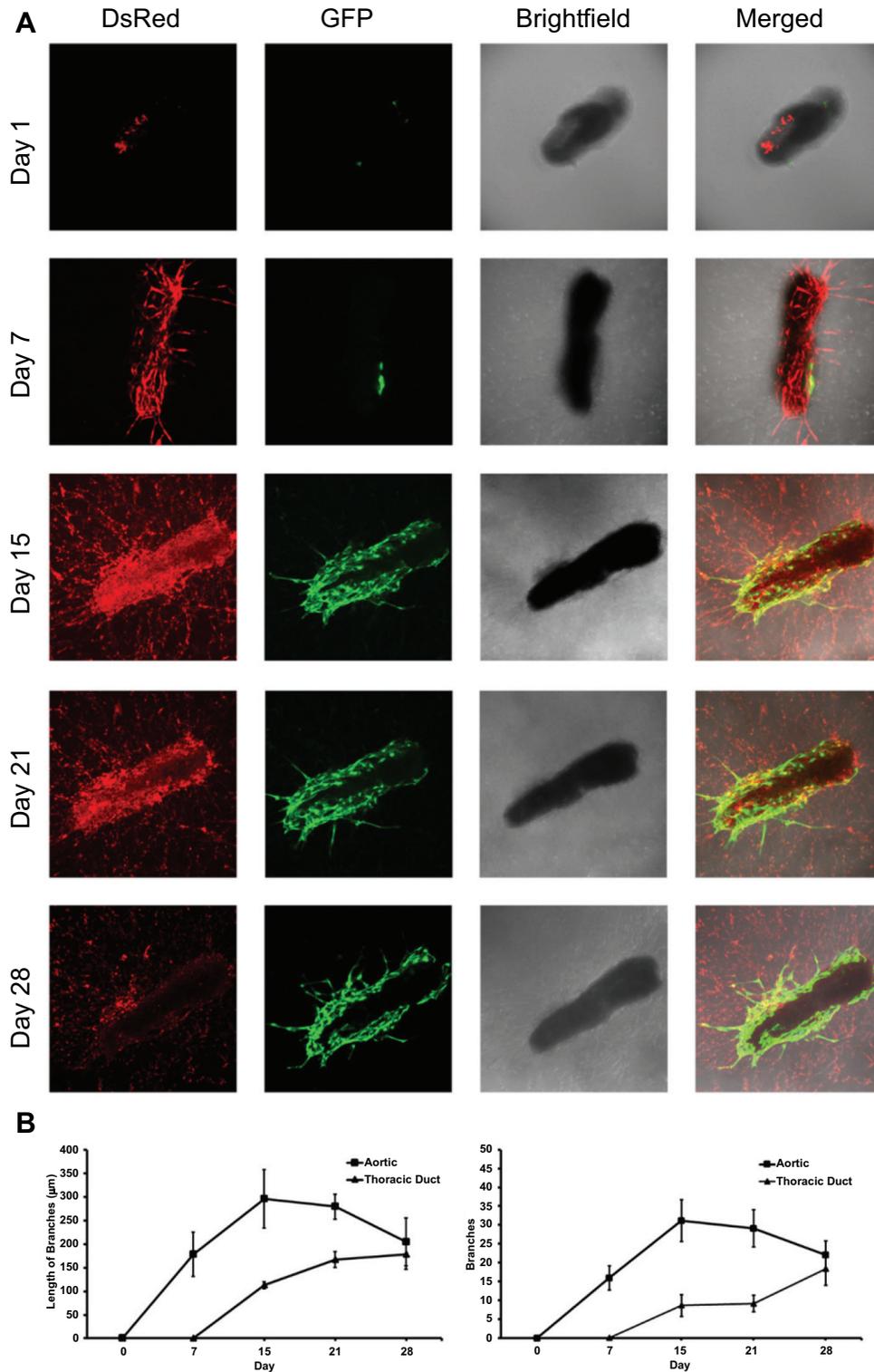
Figure 3. Temporospatial observation with confocal microscopy of blood and lymphatic vessel sprouting in the dual aortic ring and thoracic duct assay. (**A**) The dual aortic ring and thoracic duct assay revealed growth of newly sprouted blood (red) and lymphatic (green) vessels from out of the sample border. (**B**) Numbers and lengths of newly sprouted blood and lymphatic vessels from day 1 to day 28.

tissues confirmed co-localization of the lymphatic-specific hyaluronan receptor LYVE-1 with GFP fluorescence and vascular endothelial cell-specific CD31 expression with DsRed fluorescence (Figure **2**).

The sprouting of lymphatic vessels from the thoracic duct began at day 15 (D15), and these vessels progressively branched to form a microvascular network at the edges of the explants, which achieved maximal complexity at D28. In

contrast, the sprouting of blood vessels from the aorta initially occurred on D7, and then these vessels achieved peak growth by D15 and started to regress at D21 in terms of both branch length and number (Figure **3A & B**).

3.2. Influence of Growth Factors on Blood and Lymphatic Vessel Sprouting in the Dual Aortic Ring and Thoracic Duct Assay

The effect of molecular growth factors on vascular development were studied by measuring the number of branches and the branch length of sprouting vessels from aortic ring or thoracic duct tissue in the absence of a specific growth factor in comparison to those observed during culture in control medium (Figure **4**). Compared to culture in the control medium, an EGM-2 based medium with all growth factors included in this study (*i.e.*, FGF, EGF, IGF, VEGF-A, and VEGF-C), culture in media devoid of each individual growth factor resulted reduced development of vessels. The experimental results are graphically represented in Figure **5A-D**. With a few exceptions, the control medium generally induced superior growth, measured in number of branches and length of branches, compared to variable media. The branching rates of the aorta were reduced in IGF-devoid (p=0.0310), VEGF-A–devoid (p=0.0282), and VEGF-C–devoid (p=0.0117) media compared to that in control media (Figure **5A**). The growth rates of vessels sprouting from the aorta, measured in terms of the length of branches, in all variable media were reduced when compared to that in the control media. A linear mixed model estimated growth rates were less than that of control by 33 μm/day in FGF-devoid medium (p=0.0082), 61 μm/day in IGF-devoid medium (p=0.0043), 61 μm/day in VEGF-A–devoid medium (p<0.0001), 80.4 μm/day in EGF-devoid medium (p<0.0001), and 119 μm/day in VEGF-C–devoid medium (p<0.0001; Figure **5B**). Furthermore, the branching rates of lymphatics from the thoracic duct were reduced in IGF-devoid (p=0.0166), VEGF-A–devoid (p<0.0001), EGF-devoid (p<0.0001), and VEGF-C–devoid (p<0.0001) media when compared to that in the control media (Figure **5C**). The growth rates of lymphatic vessels sprouting from the thoracic duct, measured in terms of the length of branches, in all variable media were reduced when compared to that in the control media. A linear mixed model estimated that the growth rates were less than that in control media by 54 μm/day in FGF-devoid medium (p=0.0012), 65 μm/day in IGF-devoid medium (p=0.0001), 78 μm/day in VEGF-A–devoid medium (p<0.0001), 124 μm/day in EGF-devoid medium (p<0.0001), and 155 μm/day in VEGF-C–devoid medium (p<0.0001; Figure **5B**). Notably, the VEGF-C–devoid medium produces the greatest decrease in lymphatic vessel sprouting and growth. This finding emphasizes the importance of VEGF-C as a mediator of lymphangiogenesis [4, 28-31].

4. DISCUSSION

Angiogenesis plays a vital role in the evolution of several types of pathological conditions, such as cancer, ischemia, inflammation, infection and immune disorders [3], and the aortic ring assay has been commonly used to investigate the molecular basis of angiogenesis, with multiple key mechanisms being identified in recent decades [32]. By contrast, the transparency of lymphatic vessels, as well as the lack of a lymphatic-specific endothelial cell marker until recent decades, long precluded advancements in similar research on lymphangiogenesis [15]. The discoveries of lymphatic endothelial cell markers, such as VEGFR-3 (FLT4) [33], podoplanin [34], LYVE-1 [35] and Prox1 [36] have advanced research on the lymphatic system, leading to important discoveries of its complex role in pathological and physiological processes [4, 5, 9, 37]. The requirement of immunostaining to view lymphatic vessels though has remaining a limitation though, particularly in studies of the interdependence of angiogenesis and lymphangiogenesis.

5. ADVANTAGES OF DUAL *EX VIVO* AORTIC AND THORACIC DUCT ASSAY COMBINED WITH PGFD MICE

The dual *ex vivo* aortic ring and thoracic duct assay presented herein revealed a pattern of blood and lymphatic vessel growth consistent with findings from other studies [6, 38]. Axio Zoom imaging demonstrated expected reductions in angiogenesis and lymphangiogenesis upon exclusion of individual growth factors known to mediate vascular development (*e.g.*, FGF, EGF, IGF, VEGF-A, and VEGF-C) from the media. Further research is warranted to elucidate the complex interplay between various growth factors and both vascular and lymphatic endothelial lineages. Studying both processes in concert, as enabled by the dual aortic ring and thoracic duct assay, can also greatly benefit efforts to understand the mechanisms of their interdependence.

Our results demonstrate a main advantage of using tissues from the PGFD mice rather than those from traditional mice in the dual aortic ring and thoracic duct assay, particularly for the detection of the thoracic duct, which is made feasible by the expression of GFP fluorescence by lymphatic endothelial cells. The overlapping fluorescence of GFP and immunofluorescent staining of LYVE-1 (a lymphatic specific marker) in the fragments of mouse thoracic ducts embedded in a Matrigel confirmed that Prox1-GFP–expressing cells are mainly lymphatic endothelial cells. Similarly, the sprouting of vessels from aortic endothelial cells was identified by DsRed fluorescence and confirmed by overlapping immunofluorescence staining for CD31 (Figure **2**), demonstrating the practical use of PGFD mouse tissues to unambiguously identify and distinguish between blood and lymphatic cell types. Furthermore, the same sample can be repeatedly imaged throughout the experimental period without the need for fixation for staining purposes, allowing for temporospatial observations. Thus, not only are we able to observe the initial development, peak growth, and ultimately, regression of blood and lymphatic vessels, but we can also evaluate the distinct processes within angiogenesis and lymphangiogenesis from endothelial cell migration to the formation of the lumen tube and sprouting from an existing vessel. Additionally, through *ex vivo* culture of these PGFD mouse tissues, we can gain better insight into the interactions of the endothelial cells with their surrounding matrices and other cells. Finally, the ability to analyze the same samples over the experimental time course can greatly reduce the number of animals required for an experiment.
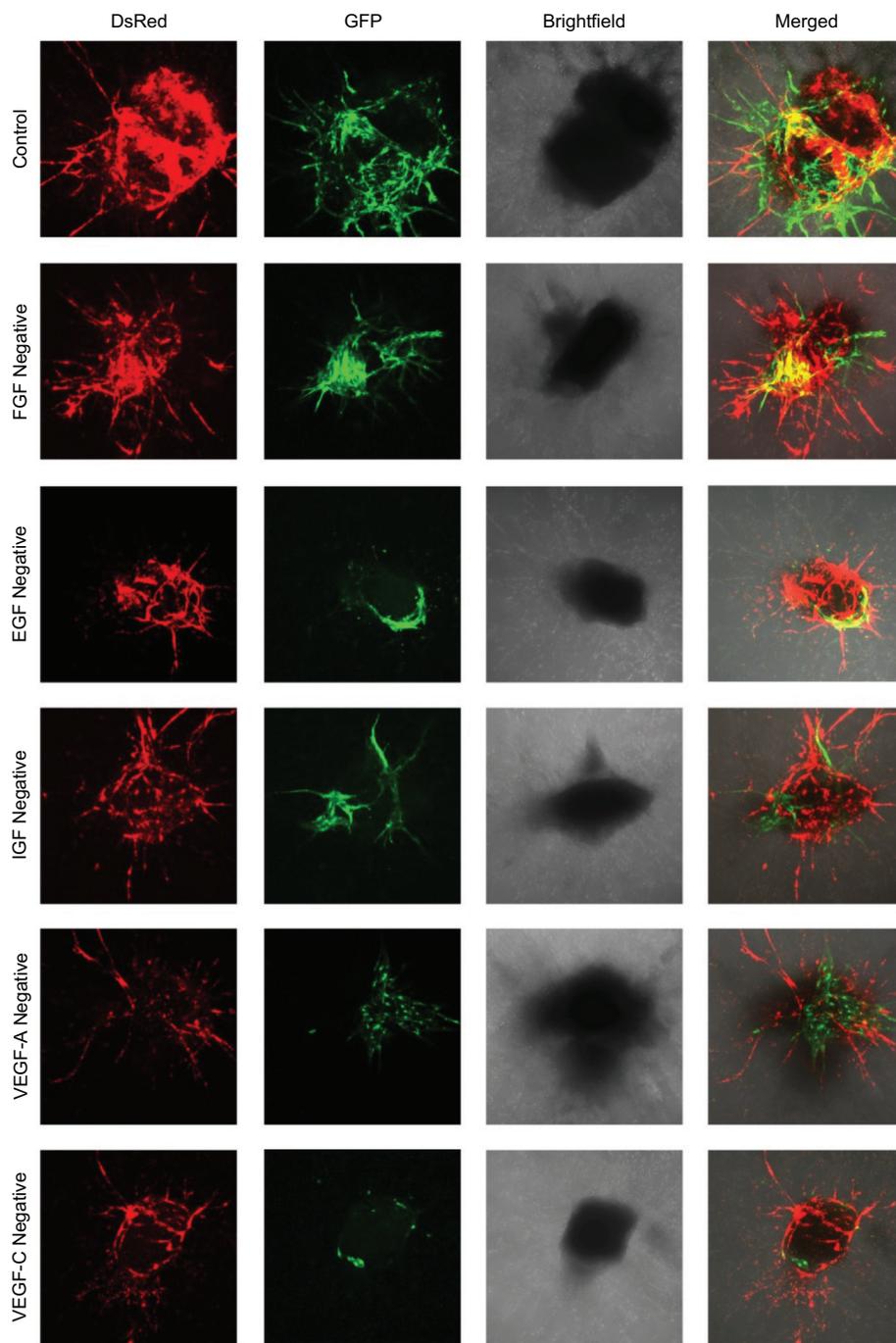
Figure 4. Confocal microscopy images of blood and lymphatic vessel sprouting in dual aortic ring and thoracic duct assays following culture in EGM-2–based media with various specific growth factors individually excluded.

The versatility of the PGFD mouse is also demonstrated in our present study by its use in the investigation of the pro-(lymph)angiogenic roles of various growth factors. We cultured aortic ring or thoracic duct tissue in various EGM-2–based media excluding individual growth factors well known to be angiogenic or lymphangiogenic factors, such as FGF, EGF, IGF, VEGF-A, and VEGF-C [1, 39-42]. By using tissues from PGFD mice in this dual aortic ring and thoracic duct assay, we were able to observe and confirm the angiogenic and lymphangiogenic activities of these factors.

In theory, this method can be extended to investigations of other potential mediators of angiogenesis and lymphangiogenesis, aiding the development of targeted therapies against these processes.

6. PITFALLS AND DISADVANTAGES

Although the presented *ex vivo* assay offers many advantages for observing angiogenesis and lymphangiogenesis, it also has disadvantages and limitations. In our study, this assay required extra equipment and more technical training
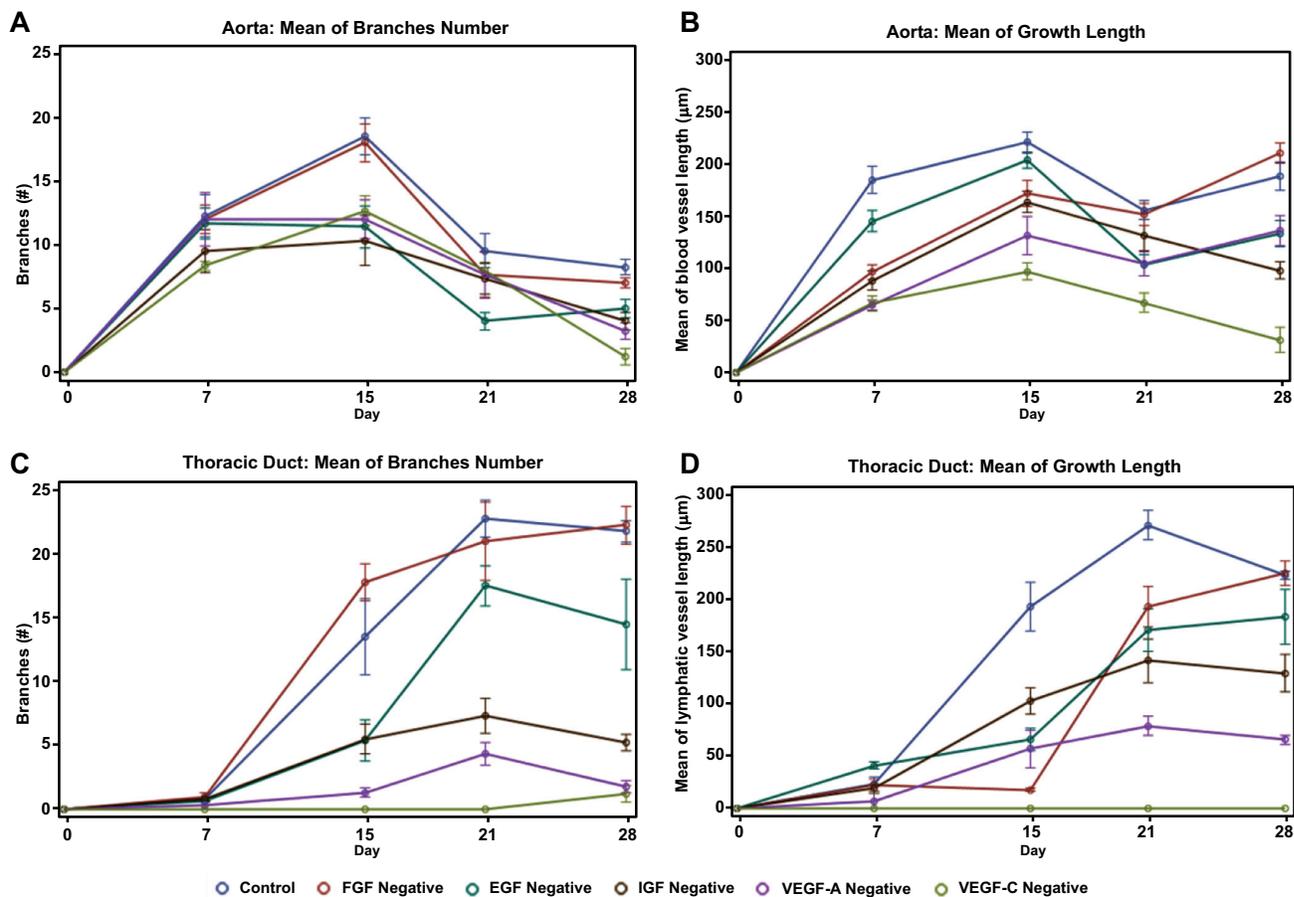
Figure 5. Quantified results of the absence of specific growth factors on blood and lymphatic vessel sprouting in the dual aortic ring and thoracic duct assay. (**A & B**) The branch number and length of blood vessels sprouting from aortic ring samples cultured in endothelial cell growth media with specific growth factors excluded. (**C & D**) The branch number and length of lymphatic vessels sprouting from thoracic duct samples cultured in endothelial cell growth medium lacking specific growth factors.

as the thin and colorless thoracic duct is prone to be damaged during harvesting and isolation. Thus, at present, the assay requires the use of fluorescent transgenic reporter mice (PGFD mice) and fluorescence microscopy for identification. In addition, without the presence of immunostaining or fluorescent markers, the outgrowth of the blood vessel endothelium, lymphatic vessel endothelium, and fibroblasts are virtually undistinguishable.

Another disadvantage according to Kazenwadel *et al*., is that an *ex vivo* assay of the collecting lymph vessels, as opposed to the lymphatic capillaries, generates smaller numbers of primary cells, as the heterogeneity of the endothelial cells in the large collecting vessels is greater than that in the lymphatic capillaries. Immortalization and extended culture will further shift the molecular properties and identity of these endothelial cells [43].

Despite mimicking *in vivo* conditions, the *ex vivo* assay does not completely duplicate the same environment. The normal environmental requirements needed in both angiogenesis and lymphangiogenesis are much more substantial than can be provided in the assay. Multiple factors contributing to lymphangiogenesis and angiogenesis may not be represented in this assay, such as the role of fluid flow

within the lumen and interstitium and neuroendocrine factors, which are lost in this assay. Angiogenesis may be disrupted, as this process requires blood flow that carries rich nutrients, oxygen, and numerous angiogenic factors as its driving force. Additionally, lymphangiogenesis may also be influenced by the interstitial fluid flow, as proposed by Boardman and Swartz [44]. The neuroendocrine system may also play a role in regulating both angiogenesis and lymphangiogenesis, as shown in the development and progression of many tumors. Mukouyama *et al*., reported that cutaneous neurons guide developing arteries in embryonic skin [45]. Although the nervous system has not been found to directly affect lymphangiogenesis, some experiments suggest cross-talk occurs between the nervous system and lymphangiogenesis [46-48].

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In general, *ex vivo* assays provide a middle ground between *in vitro* and *in vivo* assays, combining the selectivity of an *in vitro* assay and the natural environment of an *in vivo* assay. An *ex vivo* assay simulates *in vivo* conditions but also facilitates easier environmental manipulation compared to

the whole living organism. However, with the disadvantages mentioned before, an *ex vivo* assay may not be suitable for long-term culture or observation of angiogenesis and lymphangiogenesis.

The three-dimensional cultivation of dual aortic ring and thoracic duct tissue from PGFD mice provides a method for simultaneous observation of the temporospatial progression and regression of blood and lymphatic vessels. This *ex vivo* assay can be used to identify activators or inhibitors of angiogenesis and lymphangiogenesis within a controlled environment, which will likely support screenings for potential therapeutic agents for the prevention or treatment of a variety of pathological conditions that involve blood and/or lymphatic vessels.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study is approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Chicago, USA.

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. All experiments on animals were performed in accordance with the Institutional guidelines of University of Illinois at Chicago.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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