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By Different Cellular Mechanisms, Lymphatic Vessels Sprout by Endothelial Cell Recruitment Whereas Blood Vessels Grow by Vascular Expansion

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Abstract

The development of effective vascular therapies requires the understanding of all modes of vessel formation contributing to vasculogenesis, angiogenesis (here termed hemangiogenesis) and lymphangiogenesis. We show that lymphangiogenesis proceeds by blind-ended vessel sprouting via recruitment of isolated endothelial progenitor cells to the tips of growing vessels, whereas hemangiogenesis occurs by non-sprouting vessel expansion from the capillary network, during mid-development in the quail chorioallantoic membrane (CAM). Blood vessels expanded out of capillaries that displayed transient expression of alpha smooth muscle actin (αSMA), accompanied by mural recruitment of migratory progenitor cells expressing αSMA. Lymphatics and blood vessels were identified by confocal/fluorescence microscopy of vascular endothelial growth factor (VEGF) receptors VEGFR-1 and VEGFR-2, αSMA (expressed on CAM blood vessels but not on lymphatics), homeobox transcription factor Prox-1 (specific to CAM lymphatic endothelium), and the quail hematopoetic/vascular marker, QH-1. Expression of VEGFR-1 was highly restricted to blood vessels (primarily capillaries). VEGFR-2 was expressed intensely in isolated hematopoietic cells, lymphatic vessels and moderately in blood vessels. Prox-1 was absent from endothelial progenitor cells prior to lymphatic recruitment. Although vascular endothelial growth factor-165 ($VEGF_{165}$) is a key regulator of numerous cellular processes in hemangiogenesis and vasculogenesis, the role of $VEGF_{165}$ in lymphangiogenesis is less clear. Exogenous $VEGF_{165}$ increased blood vessel density without changing endogenous modes of vascular/lymphatic vessel formation or marker expression patterns. However,

$VEGF_{165}$ did increase the frequency of blood vascular anastomoses and strongly induced the anti-maturational dissociation of lymphatics from blood vessels, with frequent formation of homogeneous lymphatic networks.

Introduction

The understanding of all modes of vessel formation utilized in pathological vasculogenesis, hemangiogenesis and lymphangiogenesis is required for the development of effective vascular therapies. Developmental models are useful for initial understanding of these complex processes. Vasculogenesis is defined as the formation of a homogeneous, primary vascular network or plexus either within a developing embryo or organ, or during other de novo vascular growth such as wound-healing. The primary vascular network is usually remodeled by sprouting and/or non-sprouting modes of angiogenesis into a mature, nonhomogeneous branching tree (Risau, 1997; Gerhardt et al., 2003; Burri et al., 2004). In general, the term angiogenesis has been used to describe the growth of new blood vessels from pre-existing blood vessels. For increased clarity, we will refer to the angiogenic growth of blood vessels as hemangiogenesis (for example, see Cursiefen et al., 2004). Lymphangiogenesis is the growth of new lymphatic vessels and until recently, has been less studied with the tools of cellular and molecular biology (Oliver and Detmar, 2002; Scavelli et al., 2004). New results suggest that vasculogenesis includes the growth of either blood vessels or lymphatic vessels as homogeneous vascular endothelial networks (Wilting et al., 2004).

Blood vessels first form by vasculogenesis as homogeneous networks in the mouse embryonic allantois (Argraves et al., 2002), myocardial trabeculation and the yolk sac (Risau, 1997; Wang et al., 1999), the primordial quail vascular plexus (Vrancken Peters et al.; Wilting et al., 1997), the extraembryonic quail and chick chorioallantoic membrane (CAM, Caprioli et al., 2001), postnatal developing rat lung (Burri et al., 2004) and postnatal developing mouse retina (Benjamin et al., 1998). In the postnatal mouse retina, the homogeneous blood endothelial network is pruned into a mature branching vascular tree by cells expressing αSMA (Benjamin et al., 1998), and VEGF gradients expand the vascular network at its periphery by inducing new, sprouting angiogenic vessels guided by the filopodia of endothelial "tip cells" (Gerhardt et al., 2003). During hemangiogenesis in the avian CAM, the blood vascular tree grows and fills in by a quantifiable fractal growth process (Kirchner et al., 1996; Parsons-Wingerter et al., 1998; Parsons-Wingerter et al., 2000a).

The mammalian retina and avian CAM are useful for vascular morphogenesis studies because the vascular trees are highly two-dimensional (2D) and optically accessible within transparent tissue. In the bilaminar CAM, a homogeneous capillary network resides in the outer chorionic layer contiguous to the inner allantoic layer containing the blood vascular and lymphatic trees. Early homogenous vascular networks originating at E3-E4 in the quail CAM develop by E6 into branching blood vascular trees, and hemangiogenesis is complete by E9-E10. In embryonic vertebrates, lymphangiogenesis follows earlier development of the blood vascular system in a complex, rather intertwined fashion (Wigle and Oliver, 1999; Karkkainen et al., 2004; Wilting et al., 2004).

Vascular endothelial growth factor-A (VEGF-A) is a major regulator of blood vascular morphogenetic events that include endothelial cell proliferation, adhesion and migration, tubulogenesis, and vessel fusion and pruning (Ferrara et al., 2003; Nagy et al., 2003), but the effects of VEGF-A on lymphangiogenesis are not as well understood. $VEGF_{165}$ is generally the most hemangiogenic isoform of VEGF-A. In the quail CAM (Oh et al., 1997), exogenously applied isomers of VEGF-A, including $VEGF_{165}$, stimulated hemangiogenesis but not lymphangiogenesis. Conversely, exogenously applied VEGF-C, a VEGF family member that is recognized by VEGFR-3 and VEGFR-2 on lymphatic endothelium, stimulated lymphangiogenesis but not hemangiogenesis.

Quail hemangioblasts, which can differentiate into blood or lymphatic angioblasts, co-express VEGF receptor VEGFR-2 (also termed Flk-1, KDR and in quail, Quek-1) and the quail-specific hematopoietic marker QH-1 that is also expressed in blood and lymphatic endothelium. Blood endothelial cells in the quail CAM retain expression of QH-1 and VEGFR-2 (Caprioli et al., 2001). However, VEGFR-1 (also

termed Flt-1), which is widely co-expressed with VEGFR-2 in mammalian blood vessels, has not been identified in avian embryos until recently. VEGFR-1, strongly expressed in the trophoblast layer of mammalian placenta and currently viewed as a probable hemangiogenesis maturation factor, was identified in digests of the chicken embryo and CAM (Yamaguchi et al., 2002). Lymphatic endothelial cells in developing quail co-express QH-1, VEGFR-2, the lymphatic endothelial-specific VEGFR-3 (in quail, Quek-2) and a lymphatic endothelial-specific marker, the nuclear homeobox transcription factor Prox-1 (Papoutsi et al., 2001; Wilting et al., 2001; Scavelli et al., 2004; Wilting et al., 2004).

In this study, we investigated endogenous lymphangiogenesis and hemangiogenesis, and also exogenous stimulation by $VEGF_{165}$, in the rapidly growing quail CAM at E7-E8. By an interesting rescaling process during this 24-hour mid-developmental period, the CAM and its vascular tree double in size while continuing to increase vessel density (Parsons-Wingerter et al., 1998; Parsons-Wingerter et al., 2000a). We report here that vessel formation in the growing lymphatics and blood vessels occur by two simultaneous but contrasting modes of sprouting and non-sprouting vessel morphogenesis.

Methods and Materials

Culture, Assay and Mounting

Embryonic culture has been described previously (Parsons-Wingerter et al., 1998; Parsons-Wingerter et al., 2000a; Parsons-Wingerter et al., 2000b). Fertilized eggs of Japanese quail (*Coturnix coturnix japonica*, Boyd's Bird Co., Pullman, WA) were incubated at 37.6 ± 0.2 °C under ambient atmosphere, cracked at embryonic day three (E3, following incubation of eggs for 56 hours (h)) and cultured further at the same incubator conditions in petri dishes (cross-sectional area = 10 cm^2). Quail egg culture and experimental methods were approved by the Animal Care Committee, University of Washington, and by the Chief Veterinarian Officer of NASA. At E7 (following incubation for an additional 96 h), 0.5 ml prewarmed PBS solution containing 100 μg/ml ovalbumin (Sigma, albumin chicken egg grade VII) and 0, 1.25 and 5 μg human recombinant $VEGF_{165}$ expressed in *E. coli* (G143AB, generous gift of Genentech) was applied dropwise to the surface of each CAM. Because the incubation solution is quickly absorbed into CAM tissue, the total amount of cytokine, rather than cytokine concentration, is the governing parameter and is therefore expressed as μg/CAM. CAM's responded positively to $VEGF_{165}$ from 1.25 to 5 μg/CAM; embryonic lethality increased at doses ≥ 10 μg/CAM. Results were consistent with those produced by human recombinant $VEGF_{165}$ (293-VE, R & D Systems) expressed in *SF 21* insect cells, except that 293-VE $VEGF_{165}$ appeared active at slightly lower concentrations. Following treatment with $VEGF_{165}$ and further incubation for 24 h, the embryos were fixed in 4 percent paraformaldehyde/PBS for fluorescence immunohistochemistry (IHC). Stimulation by $VEGF_{165}$ persisted in specimens treated for 48 h, but was not as strong as stimulation after 24 h.

Fluorescence Immunohistochemistry

Polyclonal, affinity-purified IgG antisera and monoclonal antibodies were applied to CAM specimens by conventional whole-mount fluorescence IHC (Drake and Little, 1995; Parsons-Wingerter et al., 2000b). Staining for nonspecific IgG controls in the IHC was low, and observations were made from numerous experiments and imagings. VEGFR-1 was identified primarily by goat sc-316g and confirmed by rabbit sc-316 (1:200 dilution, proprietary epitope from c-terminus of human VEGFR-1 with established cross-reactivity in human, mouse and rat, Santa Cruz Biotechnology). VEGFR-2 was labeled by RB-1526 (1:200 dilution, epitope from a 1326 to 1345, c-terminus of mouse Flk-1 precursor; Lab Vision). Lymphatics were identified by rabbit antiserum produced by immunization with the highly conserved C-terminus of Prox-1 that is cross-reactive in fish, chick, mouse, rat and human lymphatic endothelial cells (Protein A-purified, RDI-102PA30, Research Diagnostics). Smooth muscle cells were labeled by Cy3-conjugated anti-αSMA mouse monoclonal clone 1A4 (C6198, Sigma). The QH-1

monoclonal antibody (mouse ascites diluted 1:1000, Development Studies Hybridoma Bank, University of Iowa) specifically recognizes quail vasculature and hematopoietic precursor cells (Pardanaud et al., 1987; Coffin and Poole, 1991). Fluorescently labeled secondary antibodies conjugated to Alexa Fluor 488 and 568 were obtained from Molecular Probes, and secondary antibodies conjugated to Cy3 and Cy5, from Jackson Immunoresearch. Nuclei were labeled with Hoechst 33342 (Molecular Probes MP H-3570).

After fixation with 4 percent paraformaldehyde/PBS at 4 °C for 48 h at E6-E7 and 24 h at E8-E9, specimens were blocked in 5 percent normal donkey serum (NDS)/1 percent Triton X-100/PBS, and incubated overnight at 4 °C with primary antibody in 0.2 percent NDS/PBS (except for RB-1526, incubated for 2 hours the following day), followed by incubation with secondary antibodies and mounting with 50 percent polyvinyl alcohol (PVA)/glycerol solution (Parsons-Wingerter et al., 1998), and 50 percent Vectashield™ (Vector Labs). Specimens were imaged with a Leica DM-RXE confocal microscope with TCS-SP2 scanner/Leica confocal software version 2.5 or a Leica DM-R fluorescence microscope with Micromax CCD-1300-V camera, Princeton Instruments and Image-Pro Plus Version 4.1.1.2 software. Volocity™ (sic) software (Improvision) was used for 3D reconstruction of confocal z-series images. Confocal images at 633x total magnification from specimens treated with VEGFR-2 antiserum RB-1526 were post-processed in Adobe Photoshop CS (version 8.0) to remove nonspecific background.

Results

Contrasting modes of vessel formation during endogenous lymphangiogenesis and hemangiogenesis, and the effects of exogenous $VEGF_{165}$ on these processes, were investigated in the quail CAM at E7-E8 with antibodies recognizing VEGFR-1, VEGFR-2, αSMA and Prox-1. The QH-1 monoclonal antibody was also used as a well-established marker of quail embryonic blood vasculature, hematopoietic precursor cells and lymphatic vessels according to QH-1 positivity and morphology described previously (Oh et al., 1997; Papoutsi et al., 2001). Lymphatics and blood vasculature were identified and distinguished from each other by morphology, and by the restricted localization of Prox-1 to lymphatics and αSMA to blood vessels. Smooth muscle cells are not present on lymphatics in the CAM (Papoutsi et al., 2001). Exogenous $VEGF_{165}$ was tested at concentrations that strongly stimulate increased vascular density (1.25 μg/CAM) and increased vascular diameter (5 μg/CAM). The fractal-based quantification of these concentration-dependent morphological changes induced by $VEGF_{165}$ will be described in detail elsewhere. Application of $VEGF_{165}$ stimulated hemangiogenesis and lymphangiogenesis, and induced several striking morphological changes described below. However, VEGF did not change lymphatic and vascular modes of vessel formation, and did not alter the expression patterns of lymphatic/vascular markers.

Staining for VEGFR-2 and QH-1 was highly colocalized, and expression of VEGFR-1 or VEGFR-2 was generally reciprocal (fig. 1). In control specimens at E7 and E8 (beginning and end of treatment by $VEGF_{165}$), VEGFR-2 and the QH-1 epitope were intensely expressed on round or elongate isolated cells, on lymphatic vessels, and at low or moderate levels on blood vessels and capillaries (fig. 1A and B). VEGFR-1 was abundantly expressed in a punctate fashion at the edges of capillaries (fig. 1D), was present at moderate to low levels on blood vessels (appearing more intense on smaller blood vessels), and was very weakly expressed or absent from lymphatic vessels and isolated cells (fig. 1C and D). The rather punctate nature of VEGFR-1 expression on capillaries, and the outlining of small blood vessels, was confirmed by two different polyclonal preparations (goat and rabbit IgG) recognizing the same human VEGFR-1 epitope compared to nonspecific IgG controls (results not shown). Expression of VEGFR-1 (fig. 1C to G) and VEGFR-2/QH-1 (figs. 1, 3, and 5) was not significantly altered by treatment with $VEGF_{165}$ at 1.25 and 5 μg/CAM. Lymphatic vessels were further identified by localization of Prox-1 to lymphatic nuclei (fig. 2), as described previously by others (Papoutsi et al., 2001; Wilting et al., 2001).

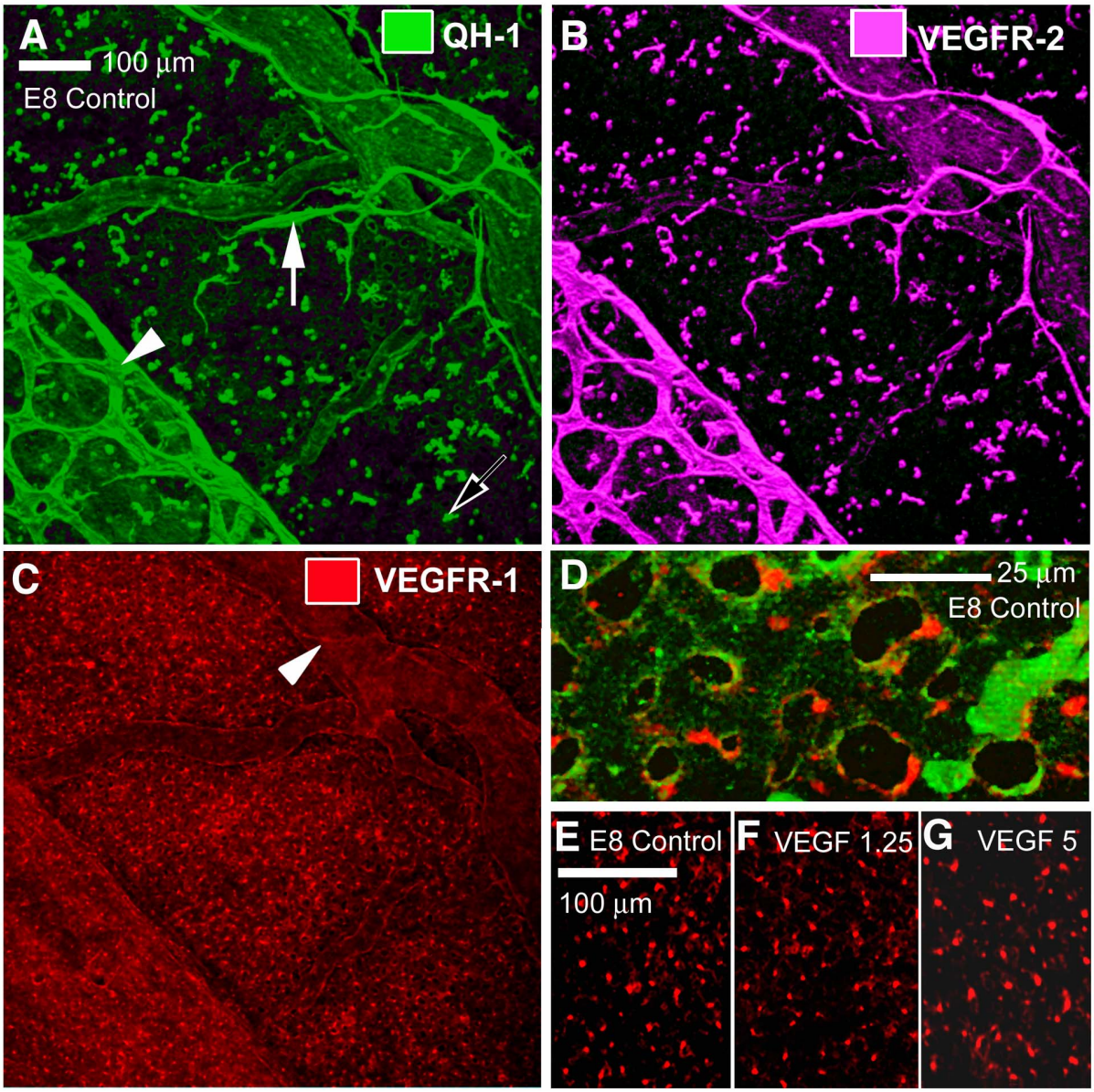

Figure 1.—VEGFR-1 and VEGFR-2 are reciprocally expressed in lymphatics and blood vessels. (A, B) By IHC of control specimens at E8, the vascular/hematopoietic marker QH-1 (A) and VEGFR-2 (B) were essentially co-localized, although QH-1 stained smaller blood vessels and the capillaries with somewhat greater intensity. Expression of QH-1 and VEGFR-2 was most intense in single isolated cells (hollow arrow, A) and in lymphatic vessels (large lymphatic plexus surrounding large blood vessel, arrowhead; smaller lymphatic vessel adjacent to smaller blood vessels, arrow), with moderate to low expression on blood vessels (hollow arrowhead) and capillaries (red arrowhead, see also C). The lymphatics were further distinguished from the blood vasculature by their distinctive morphology and by the lymphatic-specific marker Prox-1 (see fig. 2). VEGFR-1 expression (red, C to G) was generally reciprocal to VEGFR-2 (B) and QH-1 (A and D). VEGFR-1 was localized primarily to the capillary lumen in a punctate fashion (C to G), was present in moderate abundance on some smaller blood vessels (arrowhead, C), and was essentially absent from lymphatic vessels, larger blood vessels and most isolated cells. Relative to untreated controls (D and E), VEGFR-1 expression was not altered significantly in response to application of $VEGF_{165}$ at 1.25 µg/CAM (F) and 5 µg/CAM (G). A to G: All confocal images.

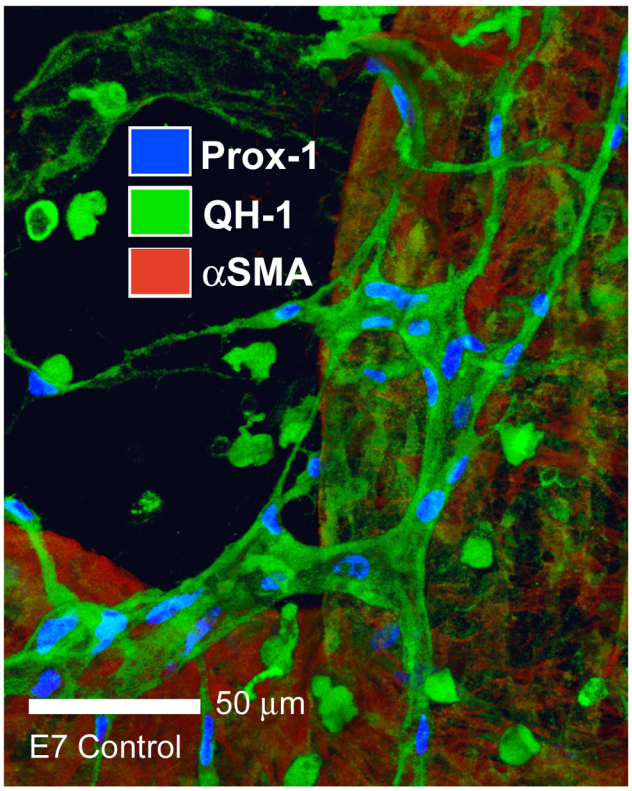

Figure 2.—Expression of Prox-1 in lymphatic vessels. (A) Expression of the lymphatic endothelial-specific marker Prox-1, a homeobox transcription factor localized to the nucleus, was restricted to lymphatic endothelium. Prox-1 further confirmed identification of the lymphatic vessels enclosing the blood vessels.

The diameters of lymphatic vessels, and perhaps lymphatic density, increased in response to $VEGF_{165}$ at 1.25 and 5 μg/CAM (fig. 3), which is consistent with the established effect of $VEGF_{165}$ as a vascular permeability factor resulting in increased serum leakage from blood vessels. Notably, the lymphatics were highly detached from medium-sized and smaller blood vessels, relative to control specimens (fig. 3A, and C to E). The frequent network-like organization of lymphatics resembled early lymphatic morphology as a homogeneous vascular network observed at E5-E6 in untreated specimens (fig. 3B), prior to the later, more mature, close association of lymphatic vessels with blood vessels. Treatment with $VEGF_{165}$ at 1.25 μg/CAM resulted in increased blood vascular density accompanied by the increased frequency of blood vascular anastomoses (fig. 4A, C, and D). These anastomoses were relatively frequent in normal specimens at E6-E7 and became infrequent by E8-E10. Increases in the diameter of blood vessels were observed most frequently in specimens treated with 5 μg $VEGF_{165}$/CAM (results not shown, because increases in vessel diameter are dependent on branching location in the vascular tree; see figs. 1 and 3).

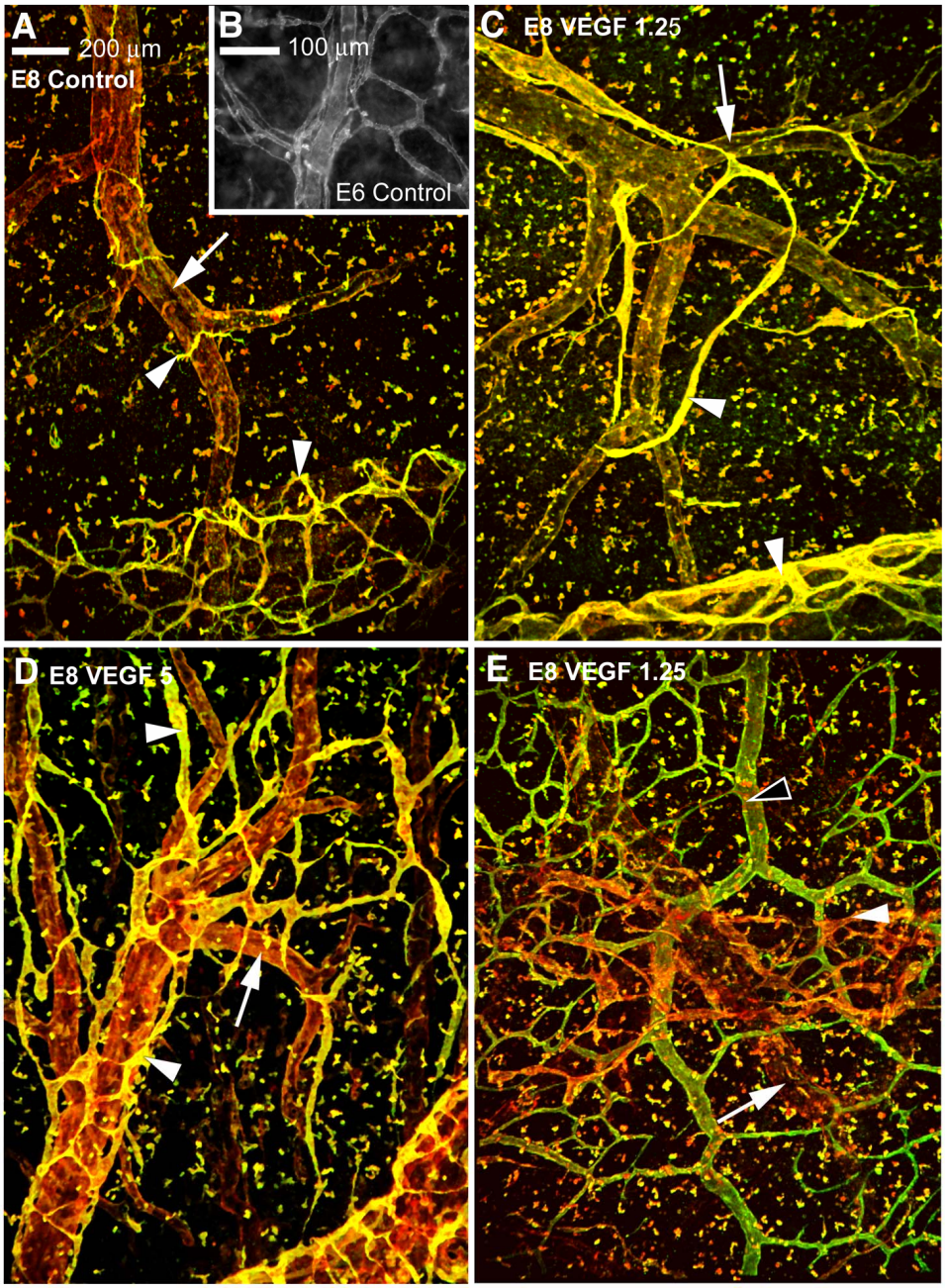

Figure 3.—$VEGF_{165}$ increases the diameter and density of lymphatic vessels, and induces lymphatic dissociation from blood vessels. (A and C to E) By IHC of VEGFR-2 (green) and QH-1 (red) relative to control specimens (A), lymphatic vessels (arrowheads) enlarged and dissociated from blood vessels (arrows) in response to the application of $VEGF_{165}$ at 1.25 µg/CAM (C) and 5 µg/CAM (D). (E) The lymphatics frequently reassociated as a network (hollow arrowhead) when treated with 1.25 or 5 µg VEGF/CAM, which is a regression of lymphatic morphology to homogeneous vascular networks observed in the normal CAM at E5-E6 (B, visualized by QH-1). (A and C to E) Note large numbers of single, isolated cells co-expressing VEGFR-2 and QH-1. A and C to E: Confocal images. B: Fluorescence image.

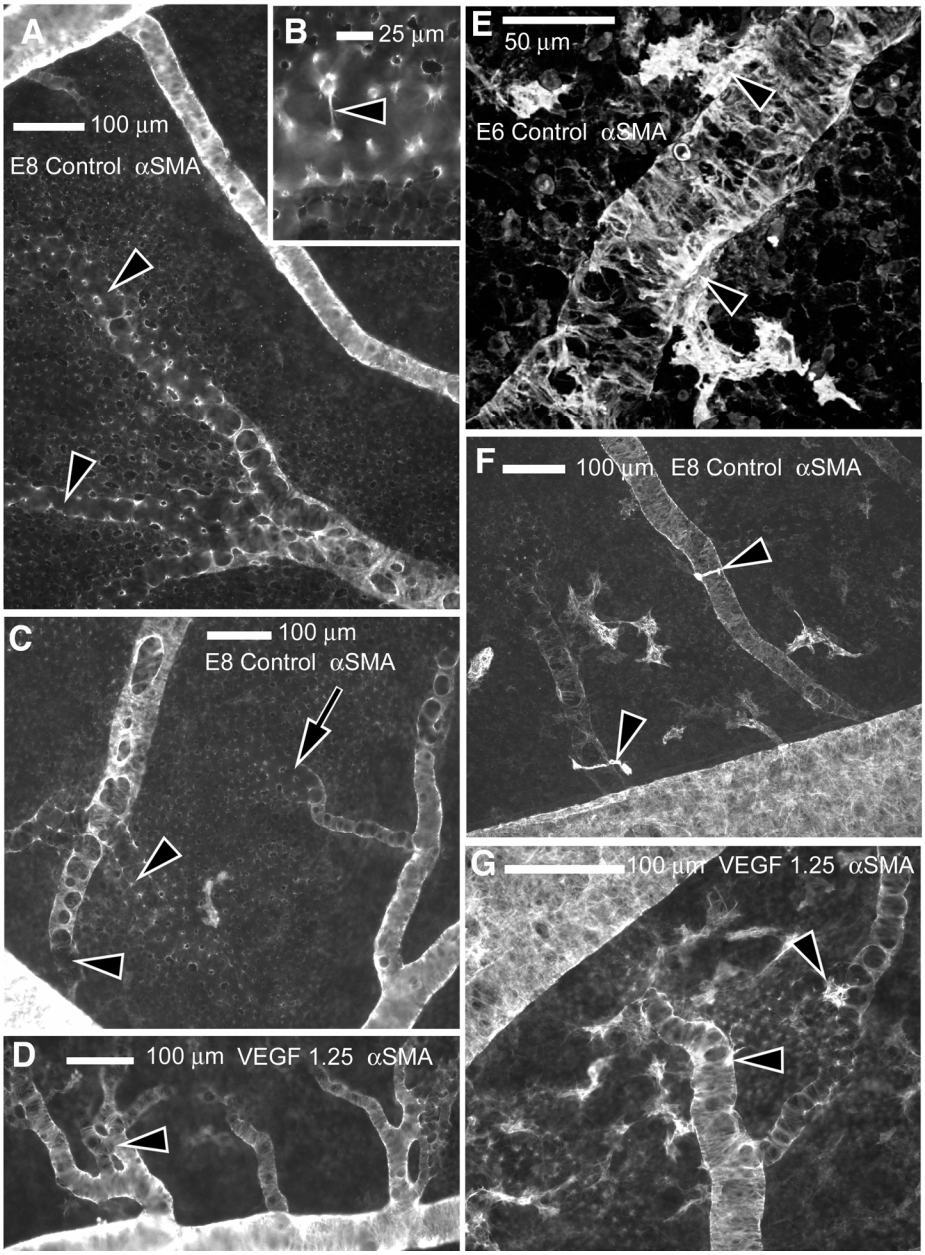

Figure 4.—$VEGF_{165}$ increases blood vessel density during hemangiogenesis by vascular expansion from the capillary network. (A and C) In control specimens expressing αSMA at E8, post-capillary venules (PCV, arrowheads) and an arteriole (arrow) emerge from the capillary network, displaying larger round gaps arranged in a parallel fashion, and brighter staining, compared to smaller, less intense, non-parallel round gaps (holes) of the intercapillary spaces. Staining of the round capillary gaps is clear but transitory, and is no longer present in mature capillaries at E9-E10 or on immunological control specimens untreated with antibody recognizing αSMA (results not shown). (B) Fine processes (arrowhead) of αSMA, an enlarged detail from PCV (A, arrowhead), span the distance between two neighboring gaps of the PCV emerging from the capillary bed. (C) Arrowheads indicate post-capillary venules (PCVs), and the arrow points to an arteriole, according to the more discontinuous pericyte and smooth muscle coverage of the PCVs, as well as venular and arteriolar connections to larger parent veins and arteries (not shown). (D) Following application of $VEGF_{165}$ at 1.25 µg/CAM, blood vessels displayed more frequent, abnormal anastomoses (arrowhead) relative to controls (A and C). (E) At E6 in untreated controls, many single cells expressing αSMA are merging with blood vessels (arrowheads). Compared to decreased frequencies in untreated controls at E8 (A, C, and F), αSMA-positive cells merge with blood vessels at increased frequencies in control specimens at E6 (E) and VEGF-treated specimens at E8 (G) although these frequencies vary throughout CAMs of all specimens. A to D and G: Fluorescence images. E and F: Confocal images.

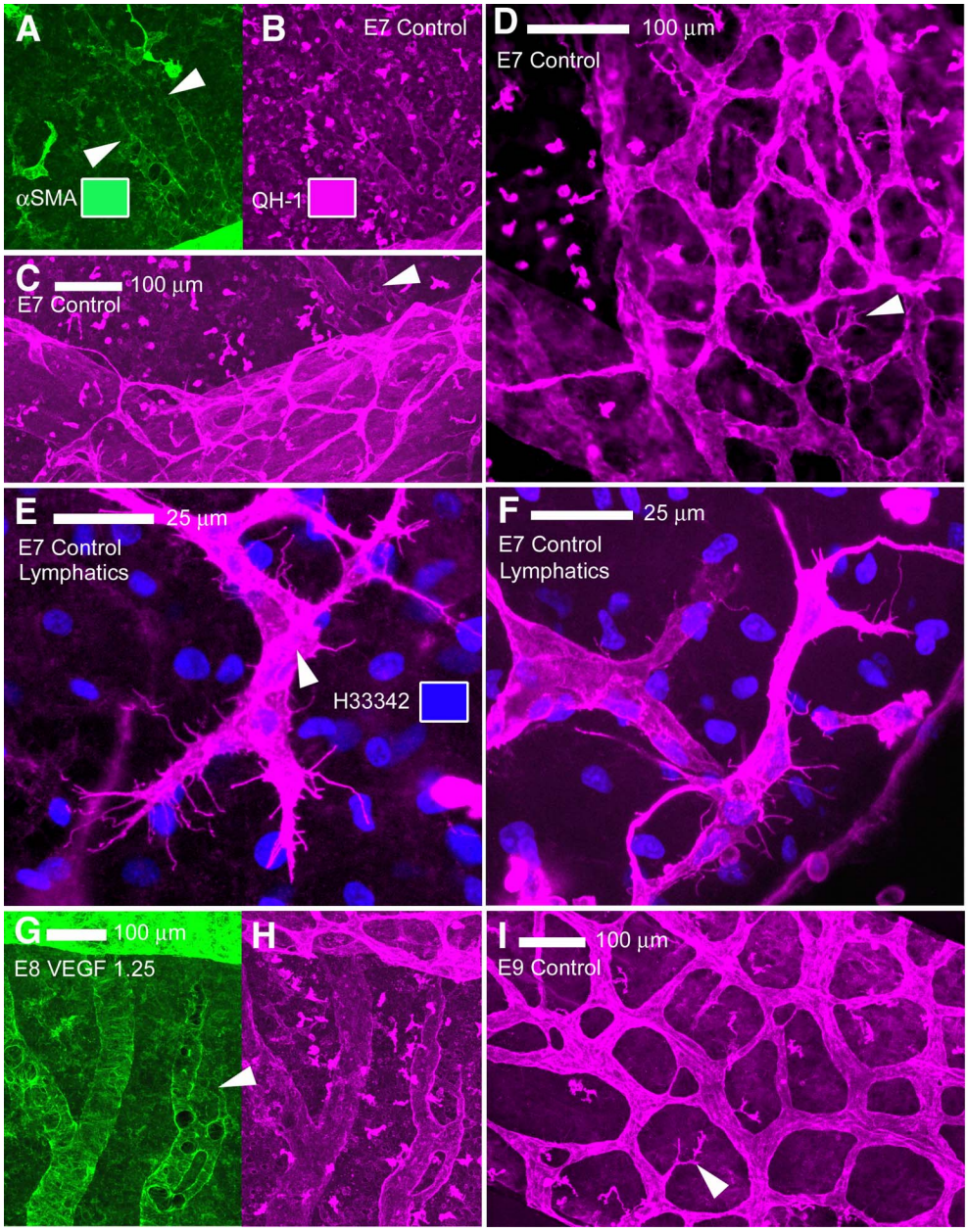

Figure 5.—$VEGF_{165}$ stimulates lymphangiogenesis, but not hemangiogenesis, by progenitor cell recruitment to the tips of sprouting vessels. By IHC of control specimens at E7 expressing (A) αSMA and (B) QH-1 (as co-localized with VEGFR-2), small blood vessels (arrowheads) emerge directly from the capillary network. This field (A and B) is a small region (C, arrowhead) adjacent to the larger blood vessel enclosed by a lymphatic network containing numerous blind-ended lymphatic sprouts. (D) At higher magnification, lymphatic sprouts led by tip cells with filopodia (arrowhead) are growing by recruitment of isolated endothelial cells to the lymphatics. (E and F) Filopodial processes of lymphatic tip cells (arrowhead) are clearly shown. The lymphatic tip cells are details from a well-developed lymphatic network enclosing a large blood vessel, as shown at lower magnification in C and D. (G and H) In specimens treated with $VEGF_{165}$ at 1.25 µg/CAM, the small, anastomotic blood vessels emerge directly from the capillary network (arrowhead). (I) In the thickened lymphatic network enclosing a large blood vessel from a more mature control specimen at E9, lymphatic sprouts with tip cells displaying filopodia are still present.

Single cells expressing αSMA and displaying a pericyte/smooth muscle migratory phenotype were prominent in control and VEGF-treated samples (figs. 3 and 4). These cells were often imaged as merging with blood vessels (fig. 4E to G). The frequency of αSMA-positive cells was variable throughout a specimen. Treatment with $VEGF_{165}$ appeared to increase the numbers of isolated cells expressing αSMA (figs. 3 and 4). In untreated specimens, isolated cells expressing either VEGFR-2/QH-1 or αSMA were very numerous at E6-E7, declined in number by E9, and were infrequent at E10. Capillaries consistently expressed αSMA at low levels as cortical staining around the intercapillary gaps (fig. 4). This pattern of αSMA expression, the size of the circular intercapillary gaps, and the linear realignment of the gaps into a parallel arrangement increased significantly in capillary regions where either pre-existing arterioles and venules merged into the capillary network or new arterioles and venules were beginning to form. This transient capillary expression of αSMA, which appears to support the process of blood vessel expansion out of the capillaries, stopped by E9-E10.

Non-Sprouting Hemangiogenesis by Vessel Expansion From the Capillary Network

At E7-E8, endogenous and VEGF-stimulated hemangiogenesis in the CAM resulted by a non-sprouting vascular expansion (i.e., emergence) of arterioles and post-capillary venules from the capillary network (figs. 4A to C; 5A and B, and G to H). No vascular tip cell displaying extended filopodia or positive expression of VEGFR-2/QH-1 was observed on blood vessels in control or VEGF-treated specimens during this relatively mature phase of hemangiogenesis in the CAM. Discontinuous walls of smooth muscle cells appeared to emerge from the capillary network in part by stronger expression of αSMA on the side of the blood vessel proximal to capillaries. These changes in the expression of αSMA colocalized with the expression patterns of QH-1 in endothelial cells that continued in part to resemble the plexus-like morphology of capillaries (fig. 5A and B). New, small blood vessels subsequently were invested with smooth muscle coverage on the distal side of growing vessels by extending and connecting the αSMA-positive processes, and then broadening these processes.

Lymphangiogenic Sprouting via Filopodial Guidance of Tip Cells

At E7-E8, lymphangiogenesis in both control and VEGF-treated specimens (fig. 3) proceeded by the sprouting of lymphatic vessels guided by the filopodia of tip cells, as identified by positive expression of Prox-1, VEGFR-2 and QH-1 (figs. 1, 3, and 5). The tip cells appeared to be recruited to the blind-ended vessel sprouts from a large population of isolated, polarized endothelial progenitor cells located on: (1) large, well-developed lymphatic networks tightly enclosing larger blood vessels, (2) the ends of smaller lymphatics loosely associated with smaller blood vessels, and (3) isolated lymphatics or lymphatic networks either at the periphery of the CAM or throughout the CAM following stimulation by $VEGF_{165}$. In control specimens, lymphatic tip cells displaying filopodia were very frequently visible at E6-E7 (fig. 3B) and decreasing in number by E8. Tip cells were still present at E9, although overall lymphatic architecture was smoother, thicker and accompanied by fewer numbers of VEGFR-2+ isolated cells (fig. 5I). Lymphatic tip cells thus appear to play a critical role in the development, and perhaps maintenance and function, of well-connected CAM lymphatic networks. Although clearly recruited from isolated endothelial progenitor cells, it is not known whether tip cells were also produced by cell division within blind-ended lymphatic sprouts. We saw little evidence for recruitment of isolated endothelial precursors to the growing trunks of lymphatic vessels, but do not rule this out as a possible additional contribution to the overall lymphangiogenic process.

Summary

During endogenous lymphangiogenesis, isolated endothelial progenitor cells were recruited to the tips of sprouting, blind-ended lymphatic vessels. During endogenous hemangiogenesis, new blood vessels

connected to the rapidly expanding vascular tree grew by non-sprouting vessel expansion out of the pre-existing, homogeneous capillary network. Specimens treated exogenously with human $VEGF_{165}$ displayed increased vascular density, more frequent vascular anastomoses, the anti-maturational, regressive dissociation of lymphatic vessels from blood vessels, and the frequent reassociation of lymphatics into a homogenous vascular network. However, no major differences in the sprouting and non-sprouting modes of lymphatic and blood vessel formation were found between control and VEGF-treated specimens.

Discussion

The major finding of our investigative study of simultaneous, coordinate hemangiogenesis and lymphangiogenesis in the quail CAM at E7-E8 is that vessel formation occurred by the two contrasting morphogenetic modes of non-sprouting vessel expansion versus vessel sprouting, respectively (fig. 6). Exogenous application of $VEGF_{165}$ accelerated, but did not change, endogenous lymphatic and vascular modes of vessel formation. However, $VEGF_{165}$ did induce frequent anastomoses in smaller blood vessels and the anti-maturational dissociation of lymphatics from their close, highly organized association with blood vessels, as well as frequent lymphatic reassociation into more homogeneous networks. Exogenous $VEGF_{165}$ did not alter the expression patterns of various vascular and lymphatic markers.

During hemangiogenesis (fig. 6A and B), new blood vessels emerged from out of the chorionic capillaries to enlarge the rapidly growing allantoic vascular tree. As identified by QH-1, VEGFR-1, VEGFR-2 and αSMA, the blood vessels did not grow by vascular sprouting. Instead, a growing vessel remained connected to the capillary bed at one end and to a larger parent artery or vein at the other. The parent arteries and veins vary considerably in size because the most frequent branching event in the growing CAM (as in the heart and lung of the adult dog and pig), is the offshoot of a small child or daughter vessel from a larger parent vessel, not the dichotomous branching of a parent vessel into two equivalent "child" or daughter vessels (Gan et al., 1993; Kassab et al., 1993; Kassab et al., 1994; Parsons-Wingerter et al., 2000a; Parsons-Wingerter et al., 2000b). Cortical expression of αSMA occurred transiently within capillaries, presumably to facilitate the process of vessel expansion. Capillary expression of αSMA intensified in the regions that were either merging or transforming into new vessels. Bundled cables of αSMA were extended around the growing new vessels; isolated, αSMA-positive cells of polarized, migratory phenotype were also recruited to the walls of smooth muscle or pericyte cells discontinuously covering new blood vessels.

The non-sprouting expansion of the blood vessels reported by us shares important characteristics with the non-sprouting mode of hemangiogenesis termed intussusception, described for the hemangiogenic transformation of homogeneous capillary networks formed in developing rat lung and the chicken CAM by vascular casting and electron microscopy (Patan et al., 1996; Burri et al., 2004). For example, according to both non-sprouting modes of hemangiogenesis, parallel holes in the capillary plexus define emerging blood vessels. These intercapillary holes, described by Burri and co-workers as the sites of non-vascular tissue pillars, become increasingly prominent as new vessels grow and separate from the capillary bed. In the hemangiogenic vessel expansion observed by us, new, emergent vessels often appear as incomplete, transitional tubes, and not as the closed tubes described by intussusceptive hemangiogenesis (see especially fig. 5A and B). We recognize that this could be due to our incomplete labeling of truly extant tubular blood vessels by the vascular markers QH-1 and VEGR-2. Coverage of post-capillary venules by pericytes and smooth muscle cells is generally discontinuous. On the other hand, the anastomotic blood vessels stimulated by our treatment with $VEGF_{165}$ appear as closed tubes, and strongly resemble the remodeling blood vessels created by enlarging tissue pillars during intussusception (see fig. 5G and H).

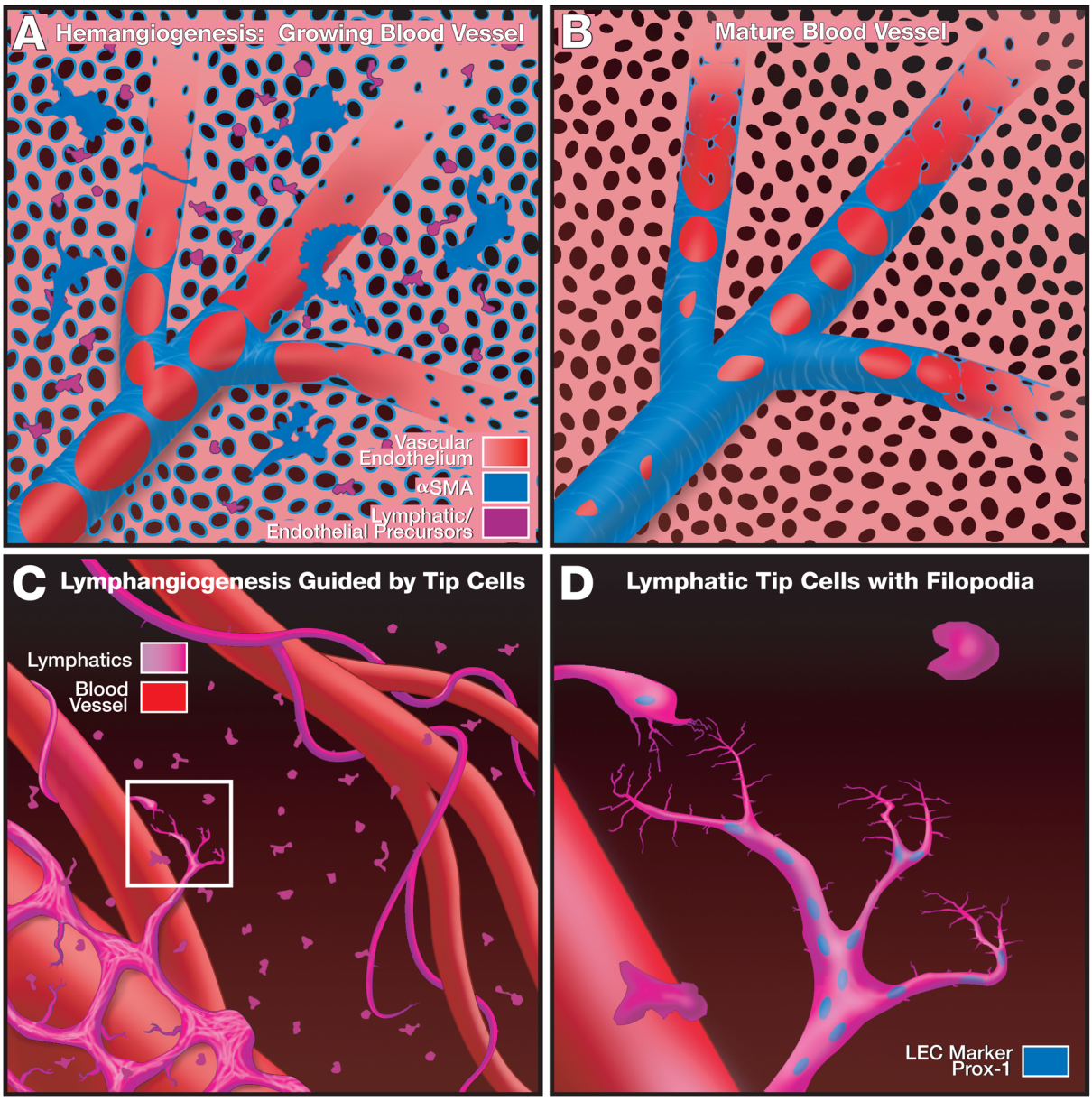

Figure 6.—Model of hemangiogenic expansion and lymphatic sprouting as stimulated by $VEGF_{165}$ in the CAM. (A) ***Hemangiogenic Expansion***: At E6-E8, the capillary network and vascular tree are actively developing in response to stimulation by $VEGF_{165}$ and ongoing hemangiogenesis. Migrating cells positive for αSMA are frequently recruited to growing blood vessels, and the highly plastic capillaries are temporarily positive for αSMA. Small new arterioles and post-capillary venules (PCV) arise out of the capillary network. New, small regions of these emerging vessels display a larger gap size in the smooth muscle layer relative to more mature regions that are proximal to larger blood vessels. (B) Round gaps of smooth muscle become less prominent as blood vessels enlarge and mature, and isolated cells positive for αSMA or VEGFR-2 disappear. It is not clear to us whether the precursor cells expressing VEGFR-2 are recruited to growing blood vessels. (C) ***Lymphatic Sprouting***: The growing lymphatic network, which is relatively homogeneous at E6, differentiates by attaching and growing as prominent networks around large blood vessels, and as loosely associated vessels around smaller blood vessels. The lymphatics, which are strongly positive for VEGFR-2 and negative for αSMA, recruit VEGFR-2 expressing lymphatic progenitor cells to the tips of blind-ended sprouts on vessels and in networks. (D) As a detail of (C), tip cells display numerous, prominent filopodia, most of which later fuse with the growing lymphatic vessel. Newly recruited tip cells continue to express VEGFR-2 and begin to express Prox-1.

During lymphangiogenesis (fig. 6C and D), isolated endothelial precursor cells intensely co-expressing VEGFR-2 and QH-1 were recruited to the tips of blind-ended lymphatic sprouts. Upon recruitment, the tip cells quickly displayed numerous, long, filopodial processes and positively expressed the lymphatic endothelial marker Prox-1, in addition to continuing expression of VEGFR-2 and QH-1. Tip cells of different lymphatic vessels frequently contacted each other by their filopodia, prior to subsequent joining of the lymphatic vessels into a growing lymphatic network tightly enclosing a large blood vessel, or a more extensive lymphatic branching tree around a smaller blood vessel.

As revealed by fluorescence IHC, expression of the receptors VEGFR-1 and VEGFR-2 was highly reciprocal. Expression of VEGFR-2 in the quail CAM was intense on isolated endothelial progenitor cells, strong on lymphatic endothelial cells and the filopodial processes of the lymphatic tip cells, and weak on blood vessels and capillaries. Strong co-expression of VEGFR-2 and lymphatic-specific VEGFGR-3 was reported for developing lymphatics of the quail and chicken CAM (Oh et al., 1997). The expression of VEGFR-2 on lymphatics has not been widely reported. However, molecular profiling demonstrated that lymphatic endothelial cells can express VEGR-2 (Podgrabinska et al., 2002). The expression of VEGFR-1 was prominent in the capillaries and a few isolated cells, and appeared to weakly line the surface of smaller blood vessels. Perhaps due to its low abundance relative to VEGFR-2, VEGFR-1 has not been identified previously in the blood vessels of the avian CAM. The pronounced punctate expression of VEGFR-1, perhaps related to the regulation of growth cessation in CAM capillaries, is intriguing. However, it is not clear from our histological study how VEGFR-1 expression in the CAM is consistent with the maturational role proposed for VEGFR-1 in the formation of blood vessels, in which VEGFR-1 helps to halt vascular growth (Eriksson and Alitalo, 2002; Luttun et al., 2002). Embryonic mice deficient in VEGR-1 die prenatally with excessive numbers of endothelial cells in the blood vasculature (Fong et al., 1999). In CAM capillaries, VEGFR-1 may also assist in regulation of hematopoietic cell recruitment and mobilization (Hattori et al., 2002).

Spatial gradients of VEGF concentration were reported to guide the filopodia of endothelial tip cells expressing VEGFR-2 during sprouting hemangiogenesis in the postnatal mouse retina (Gerhardt et al., 2003). Precise concentration gradients of VEGF-A support chemotactic cell migration and are required for correct blood vascular development *in vivo* (Carmeliet et al., 1996; Ferrara et al., 1996; Yoshida et al., 1996; Ozawa et al., 2004). In addition to the well-established evidence for induction of lymphangiogenesis by VEGF-C (for example, Jeltsch et al., 1997), there is some evidence that VEGF-A can also induce lymphatic tube formation (Nagy et al., 2002; Cursiefen et al., 2004). Recognized by VEGFR-2 and VEGFR-3 but not VEGFR-1, VEGF-C is expressed throughout the allantoic bud and during later CAM development, and is restricted primarily to the tunica media of the mural wall in larger blood vessels and the allantoic epithelium (Wilting et al., 2001). The localized expression of VEGF-C on larger blood vessels could account for the close attachment of large lymphatic networks to these vessels observed by us and others. The $VEGF_{165}$–induced dissociation of the lymphatics from the blood vessels reported here, and their frequent reassociation into homogeneous lymphatic networks, may be due to disruption of VEGF-C chemotactic gradients, and/or concentration-dependent signaling to its receptor VEGFR-2.

We have described in this report a sprouting mode of lymphangiogenesis simultaneous to a non-sprouting mode of hemangiogenesis in the quail CAM during mid-development at E7-E8. As summarized in the Introduction, sprouting and non-sprouting modes of VEGF-regulated hemangiogenesis following primordial vasculogenesis have been reported previously, particularly in developing mouse, quail and chicken. The complex regulation of hemangiogenesis and lymphangiogenesis by the VEGF family is tissue and program-specific (for example, Byzova et al., 2002), and much has been learned about the cellular and molecular biology of the VEGFs. Fundamental, useful principles of VEGF-regulated blood and lymphatic vessel morphogenesis are also becoming more clear.

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13. ABSTRACT ***(Maximum 200 words)***

The development of effective vascular therapies requires the understanding of all modes of vessel formation contributing to vasculogenesis, angiogenesis (here termed hemangiogenesis) and lymphangiogenesis. We show that lymphangiogenesis proceeds by blind-ended vessel sprouting via recruitment of isolated endothelial progenitor cells to the tips of growing vessels, whereas hemangiogenesis occurs by non-sprouting vessel expansion from the capillary network, during mid-development in the quail chorioallantoic membrane (CAM). Blood vessels expanded out of capillaries that displayed transient expression of alpha smooth muscle actin (αSMA), accompanied by mural recruitment of migratory progenitor cells expressing αSMA. Lymphatics and blood vessels were identified by confocal/fluorescence microscopy of VEGF receptors VEGFR-1 and VEGFR-2, αSMA (expressed on CAM blood vessels but not on lymphatics), homeobox transcription factor Prox-1 (specific to CAM lymphatic endothelium), and the quail hematopoetic/vascular marker, QH-1. Expression of VEGFR-1 was highly restricted to blood vessels (primarily capillaries). VEGFR-2 was expressed intensely in isolated hematopoietic cells, lymphatic vessels and moderately in blood vessels. Prox-1 was absent from endothelial progenitor cells prior to lymphatic recruitment. Although vascular endothelial growth factor-165 ($VEGF_{165}$) is a key regulator of numerous cellular processes in hemangiogenesis and vasculogenesis, the role of $VEGF_{165}$ in lymphangiogenesis is less clear. Exogenous $VEGF_{165}$ increased blood vessel density without changing endogenous modes of vascular/lymphatic vessel formation or marker expression patterns. However, $VEGF_{165}$ did increase the frequency of blood vascular anastomoses and strongly induced the anti-maturational dissociation of lymphatics from blood vessels, with frequent formation of homogeneous lymphatic networks.

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