Single Cell Analysis of Endothelial Morphogenesis *In Vivo*

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ABSTRACT

Vessel formation has been extensively studied at the tissue level, but difficulty in imaging the endothelium with cellular resolution has hampered study of the morphogenesis and behavior of endothelial cells (EC) in vivo. We are using endothelial-specific transgenes and high-resolution imaging to examine single endothelial cells in the zebrafish. By generating mosaics with transgenes that simultaneously mark endothelial nuclei and membranes we are able to definitively identify and study the morphology and behavior of individual EC during vessel sprouting and lumen formation. Using these methods, we show that developing trunk vessels are composed of EC of varying morphology, and that single cell analysis of EC can be used to quantitate alterations in morphology and dynamics in EC defective in proper guidance and patterning. Finally, we use single cell analysis of intersegmental vessels (ISV) undergoing lumen formation to demonstrate the coexistence of seamless transcellular lumens and single or multicellular enclosed lumens with autocellular or intercellular junctions, suggesting heterogeneous mechanisms contribute to vascular lumen formation in vivo. The tools we have developed for single EC analysis should facilitate further rigorous qualitative and quantitative analysis of EC morphology and behavior in vivo.
INTRODUCTION

Proper formation of functional blood vessel networks is essential for vertebrate organogenesis and physiological homeostasis (Chung and Ferrara, 2011). Insufficient or abnormal vessel growth frequently results in organ malfunction and is associated with diseases such as tumor growth and age-related macular degeneration (AMD) (Chung and Ferrara, 2011). During angiogenesis, new vessels assemble from vascular sprouts emerging from previously formed parental blood vessels. Once neighboring sprouts interconnect, in a process called anastomosis, newly formed vessel segments stabilize, lumenize, and form a functional network with directional blood circulation. At the sub-tissue level, different regions of sprouting vessels exhibit unique characteristics including heterogeneous phenotypes and distinct gene expression signatures (Aird, 2012). Endothelial cell populations are not homogenous in different vascular beds, or between normal physiological and pathological conditions (Aird, 2007a, b; Nolan et al., 2013). Characterization of ECs in different regions of sprouting vessels demonstrates that vascular morphogenesis is a dynamic process with spatial and temporal differentiation of a variety of coordinated cell behaviors, including directional polarization, collective migration, cell division, and lumen formation (Wacker and Gerhardt, 2011). During sprouting, a leading “tip cell” directs migration, while “stalk cells” behind undergo nascent lumen formation. Although these are not defined differential EC cell fates (tip and stalk cells can and do frequently interchange (Jakobsson et al., 2010; Pelton et al., 2014), tip and stalk cells have been shown to display differential gene expression and distinct morphological features (del Toro et al., 2010; Gerhardt et al., 2003; Strasser et al., 2010).

Most of the available data on vascular morphology and dynamics are from in vitro and ex vivo experimental models. Technical challenges in high-resolution optical imaging of deep tissues and lack of suitable genetic tools for imaging EC at single cell resolution have hampered study of the cell biology of endothelial cells in vivo. This is certainly true for the process of vascular tubulogenesis, a multistep interwoven developmental process involving initiation, expansion and stabilization of the nascent lumen. Recent reports have highlighted a number of different mechanisms potentially involved in assembly of vascular tubes (Charpentier and Conlon, 2014). Cord hollowing with cellular
rearrangements or cell hollowing involving fusion of intracellular vacuoles have both been proposed as mechanisms for lumen formation based on observations from in vitro cell culture, tissue sections, and live imaging of animal models (Folkman and Haudenschild, 1980b; Iruela-Arispe and Beitel, 2013; Lubarsky and Krasnow, 2003). Adhesion to surrounding extracellular matrix (ECM), loss of endothelial cell-cell contacts, or repulsive interaction between endothelial cell surfaces have all been suggested as important forces promoting expansion of nascent lumenal spaces (Eilken and Adams, 2010; Lampugnani et al., 2010; Strilic et al., 2010; Strilic et al., 2009; Wang et al., 2010; Zovein et al., 2010). Other work, primarily in vitro, has uncovered molecular regulators critical for lumenogenesis (Bayless and Davis, 2002; Bayless et al., 2000; Egginton and Gerritsen, 2003; Iruela-Arispe and Davis, 2009; Koh et al., 2009; Koh et al., 2008; Popson et al., 2014; Sacharidou et al., 2010; Sacharidou et al., 2012).

The zebrafish has recently emerged as an important model organism for studying growth and morphogenesis of blood vessels during development (Butler et al., 2011; Gore et al., 2012; Isogai et al., 2003; Kamei et al., 2006; Zovein et al., 2010). A variety of recent studies have taken advantage of the optical clarity of zebrafish embryos and larvae to carry out live imaging of tubulogenesis in developing vessels within living animals (Ellertsdottir et al., 2010; Herbert and Stainier, 2011; Herwig et al., 2011; Jin et al., 2005; Jin et al., 2007; Kalen et al., 2009; Kamei et al., 2006; Lenard et al., 2013; Wang et al., 2010). These studies have provided in vivo evidence substantiating both cell hollowing and cord hollowing models for lumenogenesis. However, difficulties in distinguishing closely apposed endothelial cells using available transgenic tools have made it difficult to comprehensively and accurately assess the contributions of individual endothelial cells to lumen assembly and morphogenesis of vessels in general. In order to monitor the morphology and dynamic behaviors of individual ECs, we have developed new transgenic tools that simultaneously label both the nuclei and the plasma membranes, or tight junctions of single endothelial cells, with different fluorescent proteins. Using high-speed confocal and two-photon imaging of these transgenes in injected mosaics, we are able to identify individual endothelial cells and image and parse some of the complex cellular and subcellular dynamics of individual cells that contribute to vessel sprouting and lumen formation.
RESULTS

Dynamic and complex collective cell behaviors during trunk angiogenesis

The trunk intersegmental vessels of the developing zebrafish provide a valuable model for investigating sprouting angiogenesis (Isogai et al., 2003; Lawson and Weinstein, 2002). The relatively simple cell composition, repeating pattern, and stereotypical assembly (Supp. Figure 1) of these vessels make them ideal for examining in vivo cellular behaviors during sprouting angiogenesis and lumenization (Figure 1A,B). However, available transgenic tools do not permit reliable identification of single endothelial cells and tracking and imaging of their morphology and behavior, making it difficult to assess how individual endothelial cells contribute to the assembly of the functional vessels. Endothelial cells migrate collectively and are extensively intertwined with one another in vascular sprouts. They also display highly dynamic behaviors in growing vessel segments. We used two photon time-lapse imaging to examine the movements of entire populations of endothelial cells in developing ISVs in Tg(fli1a:nls-egfp)\textsuperscript{y7}; Tg(kdrl:mcherry-caax)\textsuperscript{y171} double transgenic animals with green fluorescent EC nuclei and red fluorescent EC cell membranes (Figure 1C,D). Endothelial cells in growing ISV are highly dynamic, undergoing cell division, passing one another, and exchanging positions within extending vascular sprouts (Figure 1C,D; Supplemental Movie 1). As visualized in the Tg(fli1a:egfp-F)\textsuperscript{y288} transgenic line, ISV sprouts also extend numerous protrusions that appear and disappear, and the overall morphology of the extending ISV sprouts can change dramatically from one time point to the next (Supplemental Figure 2A). Using these and other available transgenic lines it is not possible to accurately distinguish boundaries between endothelial cells in growing vessel segments, or to assess the morphology and dynamics of individual endothelial cells. By creating mosaics by injecting Tg(fli1a:egfp-F)\textsuperscript{y288} endothelial expression constructs into Tg(kdrl:mcherry-caax)\textsuperscript{y171} germline transgenic zebrafish, it is possible to image sub-portions of the developing vasculature in isolation (Supplemental Figure 2B, arrows). However, it is impossible to determine with certainty whether these represent individual endothelial cells or multiple adjacent cells. In order to parse complicated, interwoven endothelial cell behaviors we designed a novel transgenic approach to identify and analyze the morphology of individual endothelial cells.
Visualizing individual endothelial cells within developing zebrafish embryos

To investigate the detailed architecture and dynamic behaviors of individual cells within developing blood vessels, we developed transgene constructs to reliably mark single endothelial cells. We prepared Tol2 transgene constructs in which the *fli1a* endothelial promoter (Lawson and Weinstein, 2002) drives expression of H2B-TagBFP (nuclear-localized blue fluorescence) followed by eGFP-farnesyl (membrane-localized green fluorescence), linked together by the self-cleaving p2A peptide (Kim et al., 2011) permitting stoichiometric co-translation of both fluorescent proteins (Figure 2A). This Tol2(*fli1a::h2b-tagBFP-2A-egfp-f*) transgene is used to generate mosaics by injecting it into Tg(*kdrl::mRFP-F*)y286 germline transgenic animals, allowing simultaneous observation of the nucleus (blue fluorescence) and membranes (green fluorescence) of the same endothelial cell and conclusive determination of the number of endothelial cells being observed and identification of single cells, all in the context of the complete vasculature (red fluorescence) (Figure 2B). Endothelial cells expressing the injected transgene (blue/green/red) do not show any evidence of growth delay or altered morphology or behavior compared to adjacent non-transgene-expressing endothelial cells (red only), and injected animals are otherwise indistinguishable from their uninjected siblings (data not shown). The membrane tethered farnesyl GFP protein permits clear identification of cell membranes and fine membrane-based subcellular structures such as intracellular vesicles and extending processes (Figure 2B). Thus, this approach allows us to investigate morphology and dynamic behaviors of single endothelial cells during vascular development in the living animal.

Endothelial cell morphology in the developing embryonic trunk vascular network

We used single-cell analysis to examine the morphology of endothelial cells contributing to intersegmental vessels during their initial assembly in the zebrafish trunk. The trunk vessel network forms in a stereotypic fashion (Isogai et al., 2003). Angiogenic sprouts emerge from the dorsal aorta, extending dorsally along the intersomitic boundaries until they reach the dorsal-lateral surface of the neural tube (Supplemental Figure 1, Isogai et al., 2003). Here, they branch rostrally and caudally and fuse with similar vascular sprouts from adjacent ISV segments to form continuous dorsal longitudinal anastomotic vessels.
We generated mosaics containing identifiable single endothelial cells by injecting the \textit{Tol2(\textit{fli1a}:h2b-tagBFP-2A-egfp-f)} transgene into \textit{Tg(kdrl:mRFP-F)}\textsuperscript{y286} germline transgenic animals as noted above. By examining a large number of individual endothelial cells within the ISV at stages just post-DLAV interconnection, as assembly of the ISV/DLAV network is completed and lumenization is beginning, we are able to identify a number of different morphologic classes (Figure 3). Half of the endothelial cells are found exclusively within the dorsally extending ISV segments, with a limited number extending the entire length of the ISV (Class I, 4.9%; Figure 3A) but most extending only part way along the ISV (Class II, 45.1%; Figure 3B). Other cells are found exclusively within the DLAV (Class III, 11.7%; Figure 3C) or in an “L shape” bridging the DLAV and ISV (Class IV, 16.7%; Figure 3D) or bridging the ISV and dorsal aorta (Class V, 21.6%; Figure 3E). The five classes and their relative proportions are summarized in Figure 3F,G. Higher-magnification imaging of endothelial cells within newly formed intersegmental vessels shows that they are often extensively interdigitated with one another, frequently extending fine processes along the vessel well beyond where the nucleus and main body of the cell are situated (Figure 3H,I). This shows that cell-cell contacts between endothelial cells in the trunk vascular network are complex, making it difficult to accurately discern endothelial cell boundaries and morphology without methods that reliably permit identification and visualization of single endothelial cells.

**Measurement of protrusive activity and polarity in single endothelial cells \textit{in vivo}**

Vessel sprouting involves the collective migration of a cohesive group of asymmetrically polarized endothelial cells, with more and longer protrusions at their leading ends and fewer, shorter protrusions at their trailing ends. Although a recent study reported that partial inhibition of actin polymerization and endothelial cell protrusion does not limit intersegmental vessel growth (Phng et al., 2013), the correlation between protrusive activity and directional migration of endothelial cells \textit{in vivo} remains largely unexplored and difficult to measure quantitatively. We used our single cell imaging tools to investigate spatial and temporal morphodynamics of endothelial tip and stalk cell protrusions in growing intersegmental vessels of either normal animals or animals defective in trunk ISV guidance and assembly due to loss of \textit{plxnd1}. In a previous study...
we showed that growing intersegmental vessel sprouts in zebrafish lacking the endothelial-specific semaphorin receptor \textit{plxnd1} are unable to respond to somite-derived semaphorin guidance cues that normally restrict their growth to intersomitic boundaries, resulting in loss of proper directionality in their growth and formation of disorganized trunk ISV networks (Torres-Vazquez et al., 2004) (Figure 4A-D). Since \textit{plxnd1} morpholino-injected animals exhibit identical phenotypes to \textit{plxnd1}^{fov01b} mutants, without additional off-target effects (Torres-Vazquez et al., 2004), we used morpholino knockdown to examine and quantitate the morphology and dynamics of verified individual \textit{plxnd1}-deficient \textit{Tol2(fli1a::h2b-tagBFP-2A-egfp-f)} endothelial cells in \textit{Tg(kdrl:mRFP-F)} \textit{y286} germline transgenic animals (Figure 4E,F). Visual inspection suggested that endothelial cells indeed displayed loss of directional growth, but to determine whether this could be validated using objective metrics and to gain insights into the relationship between endothelial protrusiveness and loss of directionality, we performed a rigorous quantitative analysis of single endothelial cell protrusions. For each of six individual randomly chosen trunk endothelial cells found above the level of the dorsal aorta, we measured the length of all of their protrusions (filopodia). The average total length of endothelial protrusions was similar between control and \textit{plxnd1} morpholino injected animals (Figure 4G). To examine the directionality of these protrusions, we grouped the direction of measured protrusions into three categories depending on whether the filopodia were oriented in a dorsal (120° arc), lateral (60° arcs), or ventral (120° arc) direction (Figure 4H). We measured protrusions in tip cells and stalk cells separately in both control and \textit{plxnd1} morphants (Figure 4I,J). There was a small but statistically significant increase in side and ventral protrusiveness along with a less significant decrease in dorsal protrusiveness in the tip cells of \textit{plxnd1} morphants compared to controls (Figure 4I). For stalk cells, \textit{plxnd1} morpholino injected animals showed more substantial increases in protrusiveness compared to controls, particularly dorsal and ventral protrusions (Figure 4J). The lack of change in average protrusion length (Figure 4G), and changes in distribution of protrusions (Figure 4I,J) together suggest that the spatial distribution and not total amount of protrusiveness is altered in \textit{plxnd1}-deficient endothelial tip cells. The more pronounced effect on stalk cells compared to tip cells also hints that reduced EC-EC contact inhibition could be a
contributing factor to the increased sprouting and branching of trunk vessels observed in \textit{plxnd1}\textcopyright-deficient animals.

Endothelial membrane protrusions are not static structures, but transient membranous extensions with highly dynamic behaviors. We use time-lapse two-photon imaging of \textit{Tol2(fli1a::h2b-tagBFP-2A-egfp-f)} injected \textit{Tg(kdrl:mRFP-F)}\textsuperscript{y286} animals to capture and evaluate the temporal dynamics of filopodia in single \textit{plxnd1}\textcopyright-deficient or control trunk endothelial cells (Figure 4K,L). Time-lapse recording revealed that membrane protrusions have a significantly longer average lifetime in \textit{plxnd1}\textcopyright-deficient endothelial cells than in control endothelial cells (Figure 4M,N). Longer protrusive lifetimes were observed in both tip (Figure 4M) and stalk (Figure 4N) cells.

**Single endothelial cell analysis reveals coexistence of both seamless transcellular lumens and multicellular sealed lumens in newly formed intersegmental vessels**

As noted in the introduction, recent studies have suggested a number of possible mechanisms contributing to vascular lumen formation (Charpentier and Conlon, 2014). Lumen formation is thought to occur mainly by either “cell hollowing” (formation of intracellular luminal spaces by fusion of intracellular vacuoles) or “cord hollowing” (generation of intercellular luminal spaces by rearrangement of and creation of spaces between multiple endothelial cells), although the extent to which lumen formation initiates intracellularly vs. between cells is still not clear. As noted above, transgenic and other tools used to date for analysis of lumen formation \textit{in vivo} have not permitted conclusive determination of how many cells were being examined, and therefore of where one cell ends and another cell begins, complicating interpretation. As a first attempt to address this, we began by using our single cell analysis methods to determine what proportion of endothelial cells in intersegmental vessels immediately after initial lumenization contain enclosed lumens (i.e., luminal spaces completely surrounded by a single endothelial cell). We captured images of single \textit{Tol2(fli1a::h2b-tagBFP-2A-egfp-f)} endothelial cells in \textit{Tg(kdrl:mRFP-F)}\textsuperscript{y286} germline transgenic animals and then carried out 3D image reconstruction using active contour-based image segmentation to examine the cross-sectional profiles of the vessels the endothelial cells were incorporated in, to determine what proportion of each identified single endothelial cell was entirely
enclosing the lumen (Figure 5A). Frequently, cross-sectional profiles of identified single endothelial cells revealed that the lumenal spaces adjacent to the “ends” of the cell were surrounded by multiple cells, (Figure 5B; see cross-sections 1 and 4), as might be expected given the extensive interdigitation of endothelial cells in assembling trunk vascular networks noted earlier, while the lumen adjacent to the middle of an endothelial cell was entirely enclosed by that single cell (Figure 5B; see cross-sections 2 and 3). Lumen-enclosing endothelial cells are detected in all positions in the intersegmental vessel (Figure 5C,D). Approximately one-third of all ISV/DLAV endothelial cells are lumen-enclosing (Figure 5D, “Sum”), with substantially more lumen-enclosing endothelial cells in the DLAV (Figure 5D, Classes III and IV) and fewer in the more ventral ISV segments (Figure 5D, Classes II and V). Of the lumen-enclosing cells, on average one-third of the length of each of these cells completely encircles the lumen at the time of initial lumenization (Supplemental Figure 3), with additional cells abutting the lumen at the ends (see sections 1 and 4 in Figure 5B). These observations suggest the coexistence of both unicellular and multicellular lumens in newly formed trunk intersegmental vessels. However, as discussed further below, the presence of unicellular lumens does not necessarily imply that these form “seamlessly.”

Analysis of endothelial junctions reveals coexistence of seamless, autocellular, and multicellular sealed lumens

Intracellular lumen formation would be expected to not only result in unicellular lumenal spaces, but to form them transcellularly or “seamlessly” without an autocellular junction (Figure 6Ai). Autocellular junctions could result from lumen formation by single endothelial cells “wrapping” on themselves (Figure 6Aii). The membrane-tethered fluorescent proteins used in the transgenes described thus far (egfp-f, mRFP-F) outline boundaries between cells but do not permit direct conclusive visualization of cell-cell or autocellular junctions. To address this issue, we developed a genetic approach to label endothelial cell junctions in vivo using fluorescent proteins. A fusion protein construct was generated (Figure 6B) using the coding sequences for the endothelial-specific tight junction protein claudin5b and EGFP. This construct was placed under the control of the endothelial fli1a promoter in a Tol2 vector, and stable double germline transgenic Tg(fli1a:egfp-claudin5b)y287; Tg(kdrl:mRFP-F)y286 animals were generated. The
Tg(fli1a:egfp-claudin5b)y287; Tg(kdrl:mRFP-F)y286 double transgenic animals, and the vessels within them, develop indistinguishably from control Tg(kdrl:mRFP-F)y286 animals. The egfp-claudin5b fusion protein co-localizes with endogenous ZO-1, as shown by co-immunostaining with anti-egfp and anti-ZO-1 antibodies (Supplemental Figure 4), validating that this transgenic line permits detailed visualization of endothelial cell tight junction complexes in living animals (Figure 6C,D). We carried out extensive confocal and two-photon microscopy using this line to examine the junctional morphology of the newly assembled ISV and DLAV in the trunk of zebrafish embryos. We were able to identify a variety of junctional types suggestive of different types of endothelial cell-cell contacts in these vessels (Figure 6D-G, Supplemental Movies 2-4). Interestingly, we found that approximately one quarter of the length of all vessel segments lacked any obvious junctions at all (Figure 6E,H,I, Supplemental Movie 2). The proportion of vessel length lacking GFP-claudin5b junctional localization was greater in the DLAV (34.9±7.7%) than in the ascending ISV (17.9±5.6%). These “gaps” in junctional localization along the vessels were not a result of lack of transgene expression in selected cells, as the gaps were also observed in mosaics generated by injecting a Tol2(kdrl:mCherry-p2A-egfp-claudin5b) transgene simultaneously labeling endothelial cell bodies and tight junctions. In these animals approximately one-quarter of the length of transgene-labeled endothelial cells showed “dark” gaps in egfp-claudin5b fusion protein localization despite expressing mCherry throughout the cell (Figure 6J-M, Supplemental Movie 5). 3-D examination of egfp-claudin5b patterns in these mosaics also reveals the complexity of endothelial junctional morphology and the difficulty in interpreting morphology from static single-view images (compare Supplemental Movie 5 and Figure 6M). Tight junction-free vessel segments are also detected by endogenous ZO-1 staining in the perfused ISV and DLAV (Supplemental Figure 4). Tight junction-free vessel segments in Tg(fli1a:egfp-claudin5b)y287; Tg(kdrl:mRFP-F)y286 double transgenic animals persist for at least a day after intersegmental vessel lumenization, (Supplemental Figure 5A-C) showing that these are not simply transient intermediates during vessels, although the overall length of these segments does decrease over time (Supplemental Figure 5D,E). Together, our results suggest that, at least for small-caliber early trunk vessels (ISV, DLAV), there are multiple types of tubular lumens and
cell junction distributions, with lumens either hollowed inside the cell, enclosed by single EC, or sealed by multiple ECs (Figure 6A). These observations, in concert with the results from single EC 3D configuration analysis, suggest that multiple mechanisms of lumen formation coexist in the early developing vasculature.

**Intracellular vacuoles within individual endothelial cells likely contribute to initial lumenization**

Our laboratory and others have previously reported intracellular vacuoles contributing to lumen formation in vitro and in vivo (Bayless and Davis, 2002; Davis et al., 2011; Folkman and Haudenschild, 1980a; Kamei et al., 2006; Koh et al., 2009; Sacharidou et al., 2012). However, other recent work has highlighted the importance of cellular rearrangements and multicellular/extracellular lumenization (Wacker and Gerhardt, 2011), and the existence of intracellular vacuoles or nascent lumenal compartments has been questioned. This is in part because of the challenges in imaging these compartments in vivo and the difficulty of discerning whether these spaces are found within single endothelial cells or between multiple endothelial cells. In order to examine whether intracellular vacuoles are present during ISV/DLAV assembly, we used two-photon imaging to visualize subcellular compartments in single Tol2(fli1a::h2b-tagBFP-2A-egfp-f) endothelial cells in Tg(kdrl:mRFP-F) y286 animals. Using the h2b-tagBFP tag to verify that we were indeed examining single endothelial cells, we were able to observe EGFP-farnesyl-labeled intracellular membrane structures of varying sizes that appeared to be entirely enclosed within the cytoplasm (Figure 7A,B, Supplemental Movies 6,7). These were observed frequently within individual ECs, especially at the early stages before lumen formation. Using time-lapse imaging we were also able to observe fusion of multiple smaller vesicles to generate larger vacuolar compartments within single endothelial cells (Figure 7C,D, Supplemental Movie 8). We were also able to image emergence of a vacuolar compartment that appeared to be enclosed within a single endothelial cell and its subsequent enlargement and eventual fusion with the nascent adjacent extracellular space (Supplemental Figure 6, Supplemental Movie 9). Intracellular vesicular/vacuolar compartments were observed more frequently in the un-lumenized tip cells than in lumen-forming stalk cells. Taken together with our results showing an increased proportion of seamless vessel segments in the newly formed
DLAV, these findings suggest that intracellular membrane-bound compartments may contribute to the initial lumenization of angiogenic trunk vessels.

**DISCUSSION**

Developmental angiogenesis is a finely controlled process that involves complex and diverse endothelial cellular behaviors. Over the past decade, a great deal of effort has been focused on understanding the molecular mechanisms regulating angiogenesis, but most of this work has been carried out using *in vitro* endothelial cell culture methods or by studying vessels *in vivo* at the tissue level. Our understanding of the cellular mechanisms regulating angiogenesis *in vivo* remains incomplete, at least in part due to the limitations of live imaging of endothelial cells *in vivo* and lack of suitable genetic tools for analyzing the morphology and behavior of individual endothelial cells in the context of the entire vasculature. Although studies of vessel formation at the organ and tissue levels have provided important insights into the underlying genetic basis for embryonic vascular development and organogenesis, endothelial morphology and/or dynamics have mostly been analyzed in groups of endothelial cells or whole growing vessels rather than in individual endothelial cells, which generally cannot be definitively distinguished from their neighbors.

In this study, we describe an approach for labeling and definitively identifying single endothelial cells *in vivo*. We prepared transgene constructs driving simultaneous expression of a blue fluorescent “tag” in endothelial cell nuclei (H2B-TagBFP), to definitively identify individual cells by their nuclei, and a green fluorescent “tag” in endothelial cell membranes (EGFP-F), to visualize cellular and subcellular morphology. The H2B-TagBFP and EGFP-F proteins are stoichiometrically co-expressed from the same construct by linking them together using a viral 2A peptide sequence (Kim et al., 2011) permitting co-translational expression of both proteins (Provost et al., 2007; Wang et al., 2011). Injection of the *Tol2(fli1a::h2b-tagBFP-2A-egfp-f)* transgene into *Tg(kdrl:mRFP-F)* germline transgenic zebrafish results in mosaic co-expression of H2B-TagBFP and EGFP-F in a subset of endothelial cells within globally mRFP-F positive vasculature. By examining the nuclear H2B-TagBFP and cellular EGFP-F expression in the endothelial cells of mosaic injected animals, it is straightforward to
identify individually “tagged” endothelial cells for morphological and behavioral characterization that have no immediately adjacent tagged neighbors. This makes it possible to examine the morphology and behavior of individual endothelial cells within the context of multicellular vascular cords or tubes.

As a proof-of-principle to demonstrate the usefulness of this method for qualitative and quantitative analysis of differences in endothelial cell morphology and behavior between normal and “defective” vasculature, we carried out single endothelial cell analysis in plexinD1-deficient embryos. In previous studies we and others have shown that growing intersegmental vessels lacking the endothelial-specific semaphorin receptor *plxnd1* due to genetic mutation or knock-down are unable to respond to somite-derived semaphorin guidance cues that normally restrict their growth to intersomitic boundaries, resulting in loss of proper directionality in their growth and formation of disorganized trunk vascular networks (Gitler et al., 2004; Gu et al., 2005; Torres-Vazquez et al., 2004). By measuring the amount and dynamics of membrane protrusions in individual trunk endothelial cells, we were able to show that while the total length of endothelial protrusions is similar in control and plexinD1 morpholino-injected animals, the directionality of protrusions is altered. Our results also revealed a prolonged persistence of endothelial membrane protrusions in plexinD1 morphants compared to controls, as might be expected given the inability of these endothelial cells to respond to repulsive semaphorin cues produced by the adjacent somites. Similar prolongation of protrusions has been noted in axonal growth cones in the absence of semaphorin-plexin repulsion (Ayoob et al., 2004; Chauvet et al., 2007).

Vascular lumen formation is a complicated morphogenetic process involving the structural transition from a cord of endothelial cells to a hollow tube. As discussed in detail in the introduction, recent reports have highlighted a number of different mechanisms potentially involved in endothelial tubulogenesis (Charpentier and Conlon, 2014). It has been proposed that vascular tubes form via either “cord hollowing,” involving cellular rearrangements, or “cell hollowing,” involving formation and fusion of intracellular vacuoles (Charpentier and Conlon, 2014; Ellertsdottir et al., 2010; Kamei et al., 2006; Lampugnani et al., 2010; Lubarsky and Krasnow, 2003; Strilic et al., 2009; Wang et al., 2010). Mechanisms such as adhesion to surrounding extracellular matrix
(ECM), loss of endothelial cell-cell contacts, repulsive interaction between endothelial cell surfaces, and formation of intracellular vacuole and their intra-or inter-cellular fusion have all been suggested as important forces promoting expansion of nascent luminal spaces. The cellular basis for endothelial tube formation in vivo has been difficult to study due to the challenge of observing individual endothelial cell behaviors within the vasculature. Our single-cell analysis in intact, live animals suggests that endothelial tube formation occurs in a heterogeneous manner, using a combination of different cellular mechanisms (Figure 8). We observe both single cell-enclosed lumens and lumens lined by multiple endothelial cells within newly formed tubes in the trunk vascular network. Live imaging of endothelial cell junctions reveals that many lumenal segments enclosed by single endothelial cells lack apparent autocellular junctions, suggesting that these are “seamless” vascular tubes formed at least in part by a cell hollowing mechanism. The existence of “seamless” vascular tubes in mammals has also been suggested from EM analysis of capillary vessels from various vascular beds (Bar et al., 1984), and by VE-Cadherin staining of the mouse retinal vasculature (Ehling et al., 2013; Fan et al., 2014). We also document the existence of small vesicles and larger vacuoles within individual endothelial cells of angiogenic ISVs, although additional work will be needed to definitively conclude whether these vacuole-like structures fuse and enlarge intracellularly, directly form extracellular spaces between adjacent endothelial cells, or a combination of both. Taken together our observations and those of many other groups, using a variety of different approaches, strongly suggest that angiogenic sprouts, especially those forming small caliber vessels such as capillaries, become lumenized through multiple distinct cellular mechanisms including both cord hollowing and cell hollowing. The coexistence of multiple cellular mechanisms for lumen formation may be advantageous for endothelial adaptation to the local challenges of distinct microenvironments.

Endothelial cell abnormalities in form and function are common features of many conditions involving pathological angiogenesis, notably cancer (Baluk et al., 2005). Although there has been enormous recent progress in identifying genetic and epigenetic factors contributing to changes in blood vessel form and function at the tissue level, thorough investigation of the underlying in vivo cellular mechanisms at high resolution is
needed to understand how molecular alterations in endothelial cells translate into changes in the form and function of the vasculature at the tissue level. The new tools we describe here should be useful in helping to bridge this knowledge gap.
EXPERIMENTAL PROCEDURES

Zebrafish

Zebrafish were maintained according to standard protocols (Westerfield, 2000) and the Guide for the Care and Use of Laboratory Animals of National Institutes of Health in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited facility. Wild type EK strain was obtained through natural spawning from Ekkwill Breeders in Florida. All transgenic lines were maintained in the EK strain. Transgenic lines Tg(fli1a:nls-egfp)y7 and Tg(kdrl:mcherry-caax)y171 were referred to previous publications (Fujita et al., 2011; Siekmann and Lawson, 2007).

Plasmids Constructs and Morpholinos

Tol2fli1a:H2B-TagBFP-p2A-eGFP-F construct: TagBFP tagged human histone H2B was purchased from Evrogen (pTagBFP-H2B, Cat. #FP176). Porcine 2A viral peptide (DNA sequence: GGAAGCGAGCTACTAACCTCAGCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCTGGACCTGGAGAG) was fused between TagBFP-H2B and eGFP-farnesy sequences. H2B-TagBFP-p2A-eGFP-F was subcloned into miniTol2 containing vector harboring endothelial promoter fli1a. Zebrafish claudin-5b gene (NCBI accession NM_001006044) was amplified from a cDNA library from 48hpf zebrafish embryos. Cladun-5b was subcloned into pEGFP-C1 to generate eGFP-claudin5b. EGFP-claudin5b was digested by NheI and MluI and subcloned into miniTol2 containing vector harboring endothelial promoter fli1a. For construct (I-SceI)kdrl:mCherry-p2A-eGFP-Claudin5b, p5’E:mCherry-p2A, pME:eGFP-Claudin5b and p3’E:polyA were cloned into vector pDEST:kdrl containing meganuclease I-SceI sequences by using MultiSite Gateway Construction Kit (Invitrogen). Morpholino oligos targeting plexinD1 (TGAGGGTATT-TACAGTCGCTCCGC) were injected at one to four-cell stage at the dose of 5ng (Torres-Vazquez et al., 2004).

Mosaic Analysis and Germline Transgenesis

For mosaic single cell analysis, 1 nl solution with 25 ng/μl DNA plasmid Tol2fli1a:H2B-TagBFP-p2A-eGFP-F and 30 ng/μl miniTol2 RNA were coinjected into transgenic embryos Tg(kdrl:mRFP-F)y286 at one-cell stage. Injected embryos with
membrane GFP fluorescence in developing vasculature were selected for single EC morphology analysis under 2-photon confocal microscope. For stable transgenic germline generation, DNA plasmids Tol2fli1a:eGFP-Claudin5b or ISceIkdr1:mRFP-F were coinjected with miniTol2 RNA or I-SceI meganuclease protein into wild type EK embryos at one-cell stage. Embryos with fluorescent blood vessels were selected as F0 founders and maintained for descendant generations.

**Microscopy and Imaging Processing**

Live embryos were mounted in 0.7% low-melting agarose gel and culture in embryo water for confocal imaging by using a long working distance objective (20X, water-immersion, 507701, Leica). Confocal images were acquired from FluoView 1000 Confocal microscope (Olympus, Center Valley, PA, USA) or Leica TCS SP5II Confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an imaging chamber mounted by MatTek 60mm glass bottom dishes. Z-series stacks were captured with 0.25 μm spacing between image planes. The active contour-based image segmentation and three-dimensional reconstruction are performed by using the software Volocity 6.0 (Perkin Elmer, Waltham, MA) and Imaris 7.4 (Bitplane, South Windsor, CT). 3D rotation movies were made using image processing software ImageJ (National Institutes of Health) and MAYA. Lasers used in this study: 850nm 2P for TagBFP, 488nm for eGFP, Argon laser for mRFP and mCherry.

**Statistical Analysis**

Statistical analysis was performed using Prism 6.0 (GraphPad). Statistical graphs of Box-and-Whisker with minimum and maximum, and geometric plots with mean ± standard deviation (S.D.) were generated from Prism Graphs. The difference among indicated groups was evaluated by unpaired two-tailed Student’s t-test. A P-value of less than 0.05 was considered as statistical significance between test groups. Polar graphs for protrusiveness (filopodia) spatial distribution were generated by open-source software Gnumeric 1.12.16 (www.gnumeric.org).
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FIGURES

Figure 1

Complex cellular architecture and behavior of endothelial cells in the developing zebrafish trunk. (A) Schematic diagram of a zebrafish with the position of trunk vessels imaged in panel B noted with a red box. (B) Confocal image of trunk ISV and DLAV vessels in a 48 hpf $Tg(fli1:EGFP)^{y1}$ embryo. (C) Confocal image time series of a single growing trunk ISV sprout in a $Tg(fli1a:nls-eGFP)^{y7}; Tg(kdrl:mCherry-caax)^{y171}$ double-transgenic embryo at 31 hpf (i), 34 hpf (ii), 37 hpf (iii) and 40 hpf (iv). The mCherry-positive endothelial cells are in grey, while nEGFP-positive endothelial cell nuclei are highlighted in red, blue, and green. (D) Quantitative measurement of the dorsal-ventral position of each of the endothelial nuclei in panel C (distance from the dorsal aorta in microns), measured every 10 minutes over a 10-hour time course. The open arrowhead shows where two EC nuclei exchanged positions in the vessel segment and the arrow shows where a nuclear division occurred. Scale bar = 20 µm.
Figure 2

Design and validation of an expression cassette for analyzing the morphology and dynamics of individual endothelial cells within developing vessels. (A) Diagram of a Tol2(fli1a:H2B-TagBFP-p2A-eGFP-Farnesyl) transgene construct for simultaneously marking endothelial nuclei (with H2B-TagBFP) and endothelial cellular internal and plasma membranes (with EGFP-farnesyl). (B) Confocal micrograph of a growing trunk ISV/DLAV segment in a 32 hpf Tg(kdrl:mRFP-F) embryo injected with a Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene, showing blue (upper left), green (upper right), red (lower left) and all three (merged, lower right) fluorescent channels. This segment contains two individual transgene-labeled endothelial cells (as indicated by the presence of a single H2B-TagBFP nucleus in each). EGFP-F fluorescence is concentrated on the cell surface as well as internal membranes, although this is not readily evident in this low-magnification reconstructed image. Scale bars = 20 µm.
**Figure 3**

**Heterogeneous morphology of individual endothelial cells in newly forming ISV/DLAV vessel segments.** (A-E) Representative confocal micrographs of verified single *Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F)* transgene-expressing endothelial cells in trunk ISV/DLAV segments within a 42 hpf *Tg(kdrl:mRFP-F) y286* germline transgenic embryo, showing green (left), red (middle left) and red/green merged, (middle right), and blue (right) fluorescent channels. (F) Morphological classification of endothelial cells contributing to the ISV/DLAV. Class I, extending along an entire ascending ISV segment (see panel A); Class II, extending partially along an entire ascending ISV (see panel B); Class III, exclusively in the DLAV; Class IV, in both DLAV and ISV (see panel C);
Class V, in both DA and ISV (see panel D). (G) Quantification of the proportion of endothelial cells found in each of the five morphological classes. (H,I) High-magnification GFP fluorescence (H) and merged GFP/BFP/RFP fluorescence (I) images of a representative verified single Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene-expressing endothelial cell in a trunk ISV segment in a 42 hpf Tg(kdrl:mRFP-F) y286 germline transgenic embryo, showing the morphological complexity of the cell, with fine processes extending up and down the vessel segment past the main part of the endothelial cell body. Scale bars = 20 µm (A-E), 10 µm (H,I).
Figure 4

Measuring endothelial protrusiveness, polarity, and dynamics using single endothelial cell imaging in vivo. Spatial distribution of filopodia in individual ECs is polarized during normal ISV network patterning. (A-D) Confocal images of mid-trunk vessels in 30 hpf (A,B) or 42 hpf (C,D) Tg(fli1a:EGFP)y1 control (A,C) or pldnd1 (B,D) morpholino injected embryos, showing disorganization of the the intersegmental vessel
network in plxnd1-deficient animals. (E,F) Higher magnification confocal micrographs of verified single Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene-expressing endothelial cells in the trunks of approximately 30 hpf Tg(kdrl:mRFP-F)\textsuperscript{y286} control (E) or plxnd1 (F) morpholino injected embryos. GFP-F fluorescence is in grey, H2B-TagBFP-positive nuclei are in blue. (G) Quantification of the average total length of endothelial cell protrusions in endothelial cells from control (left) or plxnd1 (right) morpholino injected embryos. (H) Schematic diagram showing the quadrants used for assessing the directionality of endothelial protrusions – dorsal, ventral, and side. (I) Quantification of the average total length (in microns) of protrusions from endothelial tip cells (cells at the leading front) in control or plxnd1 morpholino injected embryos grouped by whether they protrude dorsally (left), to the side (middle), or ventrally (right). (J) Quantification of the average total length (in microns) of protrusions from endothelial stalk cells (cells not at the leading front) in control or plxnd1 morpholino injected embryos grouped by whether they protrude dorsally (left), to the side (middle), or ventrally (right). The data in panels G, I, and J are collected from six independent experiments. (K,L) Representative confocal kymographs of endothelial protrusions on verified single Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene-expressing endothelial cells in the trunks of approximately 30 hpf Tg(kdrl:mRFP-F)\textsuperscript{y286} control (K) or plxnd1 (L) morpholino injected embryos. GFP-F fluorescence is in grey, H2B-TagBFP-positive nuclei are in blue. (M) Quantification of the average lifetime (in minutes) of protrusions emerging from endothelial tip cells (cells at the leading front) in control or plxnd1 morpholino injected embryos. (N) Quantification of the average lifetime (in minutes) of protrusions emerging from endothelial stalk cells (cells not at the leading front) in control or plxnd1 morpholino injected embryos. The data in panels M and N were collected from four independent experiments. Box-and-Whisker graphs in panels G, I, J, M, and N represent interquartile range (IQR, spanning from first to third quartile with median segment inside) in the box, minimum and maximum values (whiskers) at both ends. P-value was derived from unpaired two-tailed Student’s t-test. (n.s., not significant). All images are lateral views with rostral to the left. Scale bars = 25 \mu m (A-D), 10 \mu m (E,F,K,L).
Figure 5

Endothelial lumen heterogeneity revealed by single-cell 3D reconstruction. (A) Schematic diagrams showing potential configurations of verified single Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene-expressing endothelial cells adjoining lumenal spaces, in cross sections of vessels containing these cells from transgene-injected Tg(kdrl:mRFP-F) y286 embryos. **Unenclosed lumen**: if multiple cells enclose a lumen segment (top diagram), both GFP/RFP double-positive (expressing the injected transgene) and RFP single-positive (not expressing the injected transgene) sectors of the vessel will be detected (see Panel B, 1 and 4). **Enclosed lumen**: if only a single cell encloses a lumen segment (bottom diagram), the vessel wall will be entirely GFP/RFP double-positive (expressing the injected transgene; see Panel B, 2 and 3). (B) Confocal images of a verified single Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene-expressing endothelial cell (yellow) in the dorsal part of a Tg(kdrl:mRFP-F) y286 vessel (red). The left panel shows a lateral view reconstruction of the entire cell, with the single blue fluorescent...
marked nucleus visible. The right panels show red/green merge (left) green (center), and red (right) fluorescence images of four image “slices” noted in the image in the left panel. Slices 1 and 4 show lumens bounded by both the injected transgenic endothelial cell and other endothelial cell(s). Slices 2 and 3 show lumens bounded by only the single injected transgene-positive endothelial cell. (C) Morphological classification of endothelial cells contributing to the ISV/DLAV; see Figure 3 for additional details. (D) Quantification of the proportion of endothelial cells with unenclosed (grey portions of columns) or enclosed (solid colored portions of columns) found in each of the five morphological classes and in total combined trunk endothelial cells. Scale bar = 20 µm.
Figure 6

*In vivo imaging of endothelial cell junctions.* (A) Schematic diagram depicting representative junctional morphologies observed in a unicellular endothelial “seamless” tube lacking junctions along its length (i), a unicellular endothelial tube forming an autocellular junction (ii), and a multicellular endothelial tube with multiple intercellular junctions (iii). (B) Diagram of the *Tg(fli1a:egfp-claudin5b) Tol2* transgene construct used for generating a stable transgenic line marking endothelial junctions. (C,D) Confocal
images of mid-trunk vessels in 42 hpf \(Tg(fli1a:egfp-claudin5b)^{y287}; Tg(kdrl:mRFP-F)^{y286}\) double-transgenic embryos, showing egfp-labeled endothelial tight junctions (Panel D, green) in mRFP-positive endothelial cells (Panel C, red). (E-G) Higher magnification confocal micrographs of mid-trunk vessels in 48 hpf \(Tg(fli1a:egfp-claudin5b)^{y287}; Tg(kdrl:mRFP-F)^{y286}\) double-transgenic embryos, showing vessel segments with a “gap” in egfp-claudin5b-expression (E), with a single line of egfp-claudin5b (F), and with multiple lines of egfp-claudin5b (G). 3-D rotations of the merged images in panels E-G are shown in Supplemental Movies 2-4. (H) Schematic diagram of trunk vascular network, showing ascending ISV (boxed in black) and dorsal DLAV (boxed in green) vascular segments that are quantified for the presence or absence of junctions (localized egfp-claudin5b) in panel I. Arterial vessels (dorsal aorta, intersegmental arteries) are shown in red, venous vessels (posterior cardinal vein, intersegmental veins) are show in blue, and arterial-venous transition zones in the DLAV are denoted with a red-blue color gradient. (I) Quantification of the percentage of vessel segments lacking localized egfp-claudin5b expression in the ascending ISV segments (left column) or dorsal DLAV segments (right column). (J) Diagram of the \(Tg(kdrl1:mCherry-p2A-egfp-claudin5b)\) I-SceI kdrl transgene construct used for mosaic co-labeling of endothelial cell membranes (mCherry) and cell-cell junctions (eGFP-claudin5b). (K-M) Higher magnification confocal micrographs of a 48 hpf embryo injected with a \(Tol2(kdrl:mCherry-p2A-egfp-claudin5b)\) transgene, showing mCherry (K), EGFP (L), and merged (M) fluorescence images. The mCherry-positive segment (K) has egfp-claudin5b (L) localized to the ends but an egfp-claudin5b-negative “gap” in the center of the segment (arrow). A 3-D rotation of the merged image in panel M is shown in Supplemental Movie 5. All images are lateral views with rostral to the left. Scale bar = 20 µm in C,D; scale bar = 10 µm in K-M.
Figure 7

Single cell analysis of intracellular vacuole fusion during vascular lumenization. (A) Schematic diagram of a zebrafish with the position of trunk vessels chosen in panel B noted with a red box. (B) Confocal micrograph of a verified single Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene-expressing endothelial cell in the trunk of an approximately 28 hpf Tg(kdrl:mRFP-F)y286 embryo. (C) A single cell (boxed in B) was chosen for time-lapse analysis. (D) Surface-rendered 3-D reconstruction of an image stack collected of the cell in panel C, with intracellular compartments highlighted in green (see Supp. Movies 7 and 8 for a 3-D rotation of the reconstruction and the deconvolved image stack, respectively). (E) Confocal micrograph time series of a verified single Tol2(fli1a:H2B-TagBFP-p2A-eGFP-F) transgene-expressing endothelial cell in the trunk of an approximately 30 hpf Tg(kdrl:mRFP-F)y286 embryo. The red box in the final time point shows the region magnified in panels F and G. (F,G) Magnified images of the boxed region in the time series shown in panel F, with intracellular vesicular/vacuolar compartments highlighted in red in panel G. Scale bar = 10 µm.
Figure 8

Schematic diagram illustrating proposed mechanisms contributing to lumen formation during trunk vessel formation. Vascular lumens and nascent extracellular luminal spaces are shown in solid black, endothelial cells are shown in semi-transparent colors. (A) Small intracellular vacuoles emerge in endothelial cells of growing vascular segments, most prominently in leading or “tip” cells. (B) Smaller vacuolar compartments merge and/or grow to form larger intracellular compartments. (C) Intracellular vacuoles eventually fuse with the plasma membrane and merge with nascent extracellular luminal spaces. (D) Extracellular nascent luminal spaces can also form via cellular rearrangement (either single cell self-lumenization or multicellular lumenization), most probably in conjunction with luminal enlargement via extracellular deposition of intracellular vacuoles as in (C). (E) Further cell rearrangement occurs to enlarge the lumen and stabilize the mature vascular tube. Lumenization via cell rearrangement and vacuoles likely occurs together throughout this process.