

Werner Risau Preis: Boris Strilic

The molecular mechanism of vascular lumen formation

The cardiovascular circulatory system is pivotal for embryonic development, homeostatic regulation in the adult and is involved in various pathological situations such as atherosclerosis and cancer. In mammals, the circulatory system is composed of endothelial cells (ECs) that form multicellular tubes with a central vascular lumen. The process of vascular lumen formation in the developing aorta is called vasculogenesis, since this blood vessel forms *de novo* from ECs. In contrast, the formation of secondary blood vessels, for example the intersomitic vessels, is called angiogenesis, because these blood vessels form from pre-existing vessels (i.e. the aorta). Interestingly, lumen formation during both vasculogenesis and angiogenesis involves three distinct steps: (i) formation of a multicellular EC cord (Fig. 1A), (ii) formation of a central vascular lumen in this cord (Fig. 1B), and (iii) initiation of blood flow through the vascular lumen (Fig. 1C). In larger blood vessels, the latter step is subsequently followed by further expansion and remodeling of the blood vessel (Fig. 1D).

Several proteins have been identified as important for blood vessel lumen formation. In particular, deletion of one allele of the vascular endothelial growth factor VEGF-A results in formation of blood vessels with no vascular lumen (Carmeliet et al., 1996; Ferrara et al., 1996). Besides VEGF-A, the vascular endothelial-cadherin (VE-cadherin) is

also required for blood vessel lumen formation (Carmeliet et al., 1999). This cadherin is exclusively expressed on ECs and deletion, similar to the deletion of VEGF-A, results in embryonic death.

The mechanism by which EC cords form a vascular lumen has been debated (Blum et al., 2008; Kamei et al., 2006; Nelson and Beitel, 2009). For many decades, lumen formation was believed to result from intracellular *vacuole coalescence* (Folkman and Haudenschild, 1980; Kamei et al., 2006; Sabin, 1920). According to this model, each EC within an EC cord forms a large intracellular vacuole through fluid up-take, called pinocytosis, and the vacuoles from adjacent ECs subsequently coalesce to form a continuous vascular lumen (Kamei et al., 2006). This model received some support from *in vivo* imaging of intersomitic vessels in zebrafish embryos (Kamei et al., 2006), since vacuole-like structures were observed at the onset of lumen formation in these vessels. However, two years later, Blum and colleagues re-investigated lumen formation in these blood vessels and found that the vascular lumen does not develop from intracellular vacuoles, but instead forms extracellularly between adjacent ECs (Blum et al., 2008). Taken together, so far neither model (*vacuole coalescence* versus *extracellular vascular lumen formation*) explained the molecular mechanism underlying blood vessel lumen forma-

tion and thus did not explain the roles of VEGF-A and VE-cadherin in this important process.

In the present study, we used the developing mouse (dorsal) aorta as a model system to investigate the molecular mechanism of vascular lumen formation. The aorta is the first and largest arterial blood vessel to develop in all mammals and allows precise temporal and spatial staging of the transition from a non-lumenized EC cord to a vascular lumen (see Fig. 1) (Strilic et al., 2009). In addition, embryos isolated prior to lumen formation of the aorta and those injected with intervening substances, can be used to study *in vivo* the process of vascular lumen formation (Strilic et al., 2009). These whole embryo cultured (WEC) embryos develop a functional circulatory system, fully lumenized aortae and a beating heart under control conditions. The aorta thus offers several advantages over *in vitro* systems, which are known to be prone to artifacts and often do not represent the mammalian *in vivo* situation (Kucera et al., 2009).

Using this model system, we found that at embryonic day (E) 8.0 at the 1-2 somite (S) stage of mouse development, the aortic EC cords have not yet formed a vascular lumen, whereas at the 6-8S stage all cords have formed a vascular lumen larger than 5 μm in diameter (Strilic et al., 2009). This shows that in the aorta the transition from a non-lumenized cord to a lumenized blood vessel only takes several hours and can be staged, depending on the number of somites (Strilic et al., 2009). More importantly, by careful analyses using single plane illumination microscopy (SPIM), confocal light microscopy and electron microscopy we found that lumen formation in the aorta occurs in the absence of intracellular vacuoles. Instead, our data suggested that the lumen develops extracellularly at the cell-cell contact between adjacent ECs (Strilic et al., 2009).

Since the formation of specialized luminal plasma membranes is thought to be a key step during lumen formation in epithelial cells (reviewed by (Bryant and Mostov, 2008; Lubarsky and Krasnow, 2003)), we checked

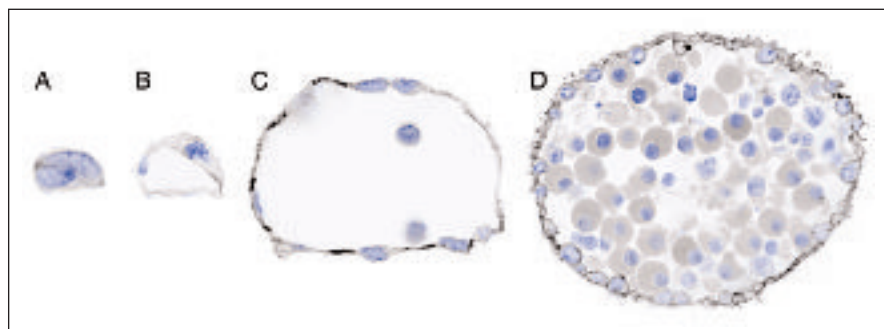


Figure 1: Transition from non-lumenized vascular cord to lumenized blood vessel
Confocal images of a series of sections through the developing mouse aorta are shown. From the left to the right, the aorta starts its development as an endothelial cell cord (A) that develops a central vascular lumen (B), which is slowly filled with red blood cells (C) and expands further (D). Cell nuclei are shown in blue, and endothelial cells are shown in black.

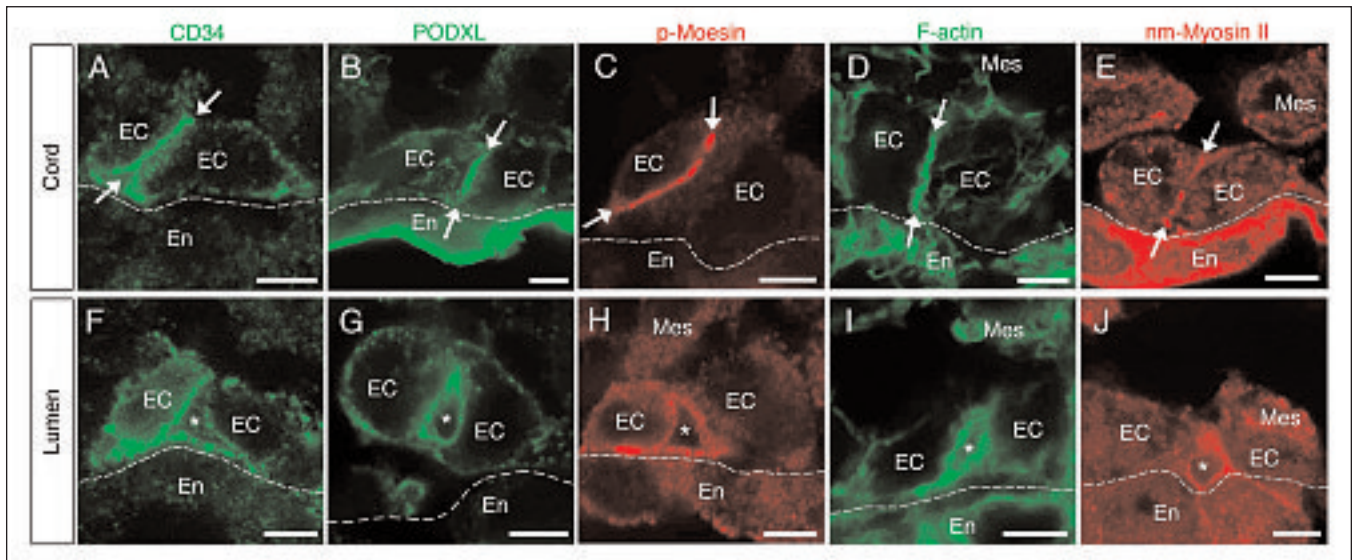


Figure 2: Localization of CD34-sialomucins, phospho-moesin, F-actin and nm-myosin II at the endothelial cell-cell contact at the onset of vascular lumen formation

Confocal images of transverse sections through the developing aortae are shown. Arrows point to the endothelial cell-cell contact. Asterisks mark the developing lumen. En, endoderm; Mes, mesenchyme.

At the cord stage, during onset of vascular lumen formation, the molecules CD34 (A), PODXL (B), phospho (p)-moesin (C), F-actin (D) and some nm-myosin II (E) localize at the endothelial cell-cell contact. All molecules remain enriched at the plasma membrane facing the developing lumen (F-J). Scale bars, 5 μ m. Image adopted from (Strilic et al., 2009).

for CD34-sialomucins at the endothelial cell-cell contact at the onset of vascular lumen formation. CD34-sialomucins are transmembrane proteins with a highly glycosylated and negatively charged extracellular domain, and are commonly used as an apical/luminal cell surface marker in epithelial cells (Meder et al., 2005). In line with the notion that the vascular lumen develops at the endothelial cell-cell contact, we observed that the CD34-sialomucins CD34 and podocalyxin (PODXL) localized at the endothelial cell-cell contact at the cord stage (Fig. 2A and B) and remained localized at the luminal plasma membrane thereafter (Fig. 2F and G). CD34-sialomucins can furthermore link the luminal plasma membrane to the underlying F-actin cytoskeleton via adapter molecules (for review see (Bretscher et al., 2002)), and these adapter molecules were shown to be required for epithelial tubulogenesis (Gobel et al., 2004; Van Furden et al., 2004). In addition, we found the activated adapter molecule phospho (p)-moesin together with F-actin localized at the apical pole interface between adjacent ECs (Fig. 2C and D) and at the luminal plasma membrane (Fig. 2H and I). Furthermore, F-actin is known to interact with non-muscle (nm) myosin II to induce cell shape changes in different cell types, including ECs (Furman et al., 2007). Similarly, nm-myosin II was enriched at the endothelial cell-cell contact before (Fig. 2E) and during initial vascular lumen formation (Fig. 2J). Together, our data suggested that

negatively charged CD34-sialomucins initially separate the ECs from each other and subsequently, an apical actomyosin complex consisting of F-actin and nm-myosin II exerts the physical force to further separate the ECs from each other, giving rise to a vascular lumen.

Despite the detailed morphological data on vascular lumen formation in the aorta, we still did not understand the molecular mechanism and in particular we did not know when and how VE-cadherin and VEGFA are involved in this process. To address these questions, we used several genetic and pharmacologic approaches. We first investigated the role of VE-cadherin, since cadherins are known to induce cell polarity in epithelial cells (Nejsum and Nelson, 2007), and establishment of polarity was important in vascular lumen formation (see Fig. 2). Interestingly, mice deficient for VE-cadherin failed to form a proper lumen in the aorta, and this defect was not due to a decreased number of ECs or an increased rate of apoptosis (Strilic et al., 2009). Instead, these embryos failed to localize the above-mentioned molecules (CD34-sialomucins, p-moesin, F-actin and nm-myosin II) at the endothelial cell-cell contact (Strilic et al., 2009). Therefore, we could show that VE-cadherin, possibly via the phosphatase and tensin homolog (PTEN), is required for establishing endothelial cell polarity and vascular lumen formation. This data is also in line with other data

showing that cadherins associate with PTEN (Vogelmann et al., 2005) and that PTEN is necessary for establishing apical cell polarity and lumen formation in epithelial cells (Martin-Belmonte et al., 2007).

Since PODXL and moesin were the first molecules to localize at the endothelial cell-cell contact, we asked whether these molecules were required for vascular lumen formation. To this end, we analyzed mice that were deficient for either PODXL or moesin. Interestingly, analysis of each individual knockout mouse revealed that lumen formation in the aorta was delayed and that lumens were significantly smaller (Strilic et al., 2009). Importantly, in both cases, F-actin was not enriched at the endothelial cell-cell contact, providing genetic evidence that the CD34-sialomucin PODXL and the adapter molecule moesin are both required for linking the luminal plasma membrane to the F-actin cytoskeleton and for vascular lumen formation.

Next, we asked how the activation of moesin is regulated, since only phosphorylated moesin links CD34-sialomucins to the F-actin cytoskeleton (for review see (Bretscher et al., 2002)). Using several inhibitors against pan-PKC- or ROCK I/II-mediated signaling, we found *in vitro* that inhibition of PKC prevented phosphorylation of moesin, but did not affect phosphorylation of myosin light chain (MLC), another known target downstream of

PKC signaling. In contrast, inhibition of ROCK I/II prevented phosphorylation of MLC, but did not affect phosphorylation of moesin. This data was confirmed *in vivo* using WEC. ECs of developing aortae from mice injected with either PKC- or ROCK-inhibitors were normally polarized (i.e. PODXL was localized at the endothelial cell-cell contact). However, in mouse embryos injected with the PKC inhibitor, p-moesin, F-actin and nm-myosin were not enriched at the endothelial cell-cell contact (Strilic et al., 2009). In contrast, embryos injected with the inhibitor against ROCK, showed normal phosphorylation of moesin and F-actin localization at the endothelial cell-cell contact, but failed to position nm-myosin II to F-actin (Strilic et al., 2009). In both cases, vascular lumens formed to a lesser extent. This data showed that PKC-signaling is required for phosphorylation of moesin, recruitment of F-actin to the cell-cell contact and vascular lumen formation, while ROCK-mediated signaling is required for phosphorylation of MLC, nm-myosin II recruitment to the F-actin cytoskeleton at the cell-cell contact and vascular lumen formation.

VEGF-A is known to regulate ROCK activity and is required for embryonic blood vessel formation (Carmeliet et al., 1996; Ferrara et

al., 1996). Similar to the results that we obtained after injection of ROCK-inhibitors into mouse embryos, mice that were haplo-insufficient for VEGF-A showed correct polarization, phosphorylation of moesin and localization of F-actin at the endothelial cell-cell contact but failed to position nm-myosin II to the latter complex (Strilic et al., 2009). Importantly, this defect was not due to decreased endothelial cell proliferation or an increased rate of apoptosis. Since the analyzed mouse embryos still harbored half of the endogenous VEGF-A, it was likely that this reduced amount of VEGF-A was sufficient for moesin phosphorylation and F-actin recruitment. For this reason, we tested two differentially acting inhibitors against VEGF-A-mediated signaling both *in vitro* and *in vivo* in WEC (Flt1-Fc and SU5416). The data confirmed that in the absence of VEGF-mediated signaling polarity establishment and moesin phosphorylation were normal, but MLC phosphorylation was reduced, nm-myosin II was absent from the endothelial cell-cell contact and lumen formation was impaired.

Conclusively, this study for the first time proposes a model that explains how ECs form a vascular lumen on a molecular level and integrates key players such as VE-cadherin

and VEGF-A. We could show that the aortic lumen develops extracellularly at the endothelial cell-cell contacts between ECs, rather than through coalescence of intracellular vacuoles. Furthermore, we identified a molecular pathway comprising CD34-sialomucins, (p)-moesin, F-actin and nm-myosin II. These proteins are localized to the endothelial cell-cell contacts to define the luminal cell surface and to trigger cell shape changes required for aortic lumen formation. More precisely, CD34-sialomucins localize to EC contacts in a VE-cadherin dependent-manner, and the repellent activity of these CD34-sialomucins initiates lumen formation (Fig. 3A to B). The CD34-sialomucins are also required for the recruitment of moesin, a step that is driven by the activation and phosphorylation by PKC (Fig. 3B to C). Moesin helps to stabilize the F-actin network at the cell-cell contact. Importantly, VEGF-A and ROCK are not involved in the early steps of vascular lumen formation (i.e. polarization and moesin phosphorylation), but recruit nm-myosin II to the cortical F-actin network by phosphorylating MLC, and furthermore regulate cell-shape changes required for vascular lumen formation (Fig. 3C to D). Finally, components of this pathway are also present during blood vessel formation in other organ systems and tumors (Strilic et

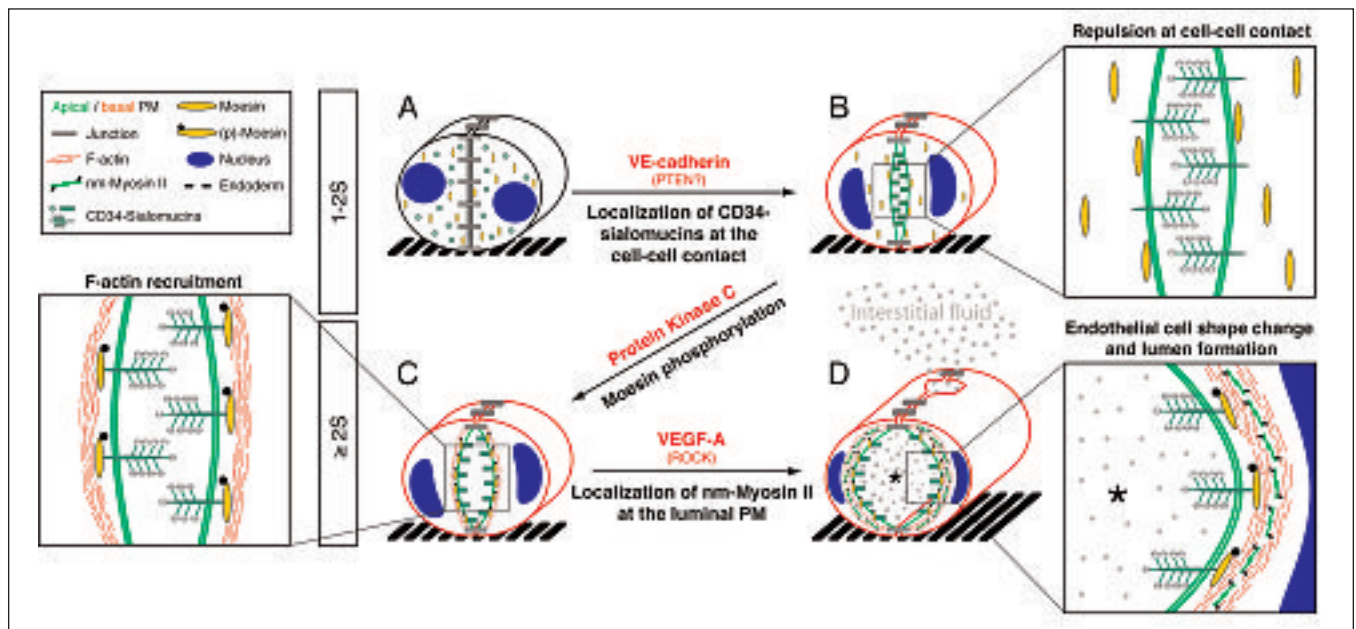


Figure 3: Molecular mechanism of *in vivo* vascular lumen formation in the developing aorta
 (A) Adjacent endothelial cells (ECs) adhere to each other via junctions at multiple positions along the endothelial cell-cell contact.
 (B) VE-cadherin is required for localizing of CD34-sialomucins to the endothelial cell-cell contact, possibly via its interaction with PTEN. The anti-adhesive CD34-sialomucins are involved in separating apical endothelial cell surfaces from each other.
 (C) PKC activity is required for phosphorylating moesin, which is involved in recruiting F-actin to the endothelial cell-cell contact.
 (D) VEGF-A activates ROCK, which is required for myosin light chain (MLC) phosphorylation and recruitment of nm-myosin II to the apically enriched F-actin. VEGF-A and ROCK are required for fully separating apical endothelial cell surfaces from each other, for EC shape changes and for vascular lumen formation. Since the developing lumen (asterisk) is leaky, extravascular interstitial fluid (Fig. 3D, grey dots) passively enters via paracellular openings. Image from (Strilic et al., 2009).

al., 2009), suggesting that this model may represent a general mechanism for vascular lumen formation.

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References

Blum, Y., Belting, H.G., Ellertsdottir, E., Herwig, L., Luders, F., and Affolter, M. (2008). Complex cell rearrangements during intersegmental vessel sprouting and vessel fusion in the zebrafish embryo. *Dev Biol* 316, 312-322.

Bretscher, A., Edwards, K., and Fehon, R.G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* 3, 586-599.

Bryant, D.M., and Mostov, K.E. (2008). From cells to organs: building polarized tissue. *Nat Rev Mol Cell Biol* 9, 887-901.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoek, A., Harpal, K., Eberhardt, C., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380, 435-439.

Carmeliet, P., Lampugnani, M.G., Moons, L., Breviaro, F., Compernelle, V., Bono, F., Balconi, G., Spagnuolo,

R., Oostuyse, B., Dewerchin, M., et al. (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 98, 147-157.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380, 439-442.

Folkman, J., and Haudenschild, C. (1980). Angiogenesis in vitro. *Nature* 288, 551-556.

Furman, C., Sieminski, A.L., Kwiatkowski, A.V., Rubinson, D.A., Vasile, E., Bronson, R.T., Fassler, R., and Gertler, F.B. (2007). Ena/VASP is required for endothelial barrier function in vivo. *J Cell Biol* 179, 761-775.

Gobel, V., Barrett, P.L., Hall, D.H., and Fleming, J.T. (2004). Lumen morphogenesis in *C. elegans* requires the membrane-cytoskeleton linker erm-1. *Dev Cell* 6, 865-873.

Kamei, M., Saunders, W.B., Bayless, K.J., Dye, L., Davis, G.E., and Weinstein, B.M. (2006). Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* 442, 453-456.

Kucera, T., Strilic, B., Regener, K., Schubert, M., Laudet, V., and Lammert, E. (2009). Ancestral vascular lumen formation via basal cell surfaces. *PLoS ONE* 4, e4132.

Lubarsky, B., and Krasnow, M.A. (2003). Tube morphogenesis: making and shaping biological tubes. *Cell* 112, 19-28.

Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V., and Mostov, K. (2007). PTEN-mediated apical segregation of phosphoinositides

controls epithelial morphogenesis through Cdc42. *Cell* 128, 383-397.

Meder, D., Shevchenko, A., Simons, K., and Fullekrug, J. (2005). Gp135/podocalyxin and NHERF-2 participate in the formation of a preapical domain during polarization of MDCK cells. *J Cell Biol* 168, 303-313.

Nejsum, L.N., and Nelson, W.J. (2007). A molecular mechanism directly linking E-cadherin adhesion to initiation of epithelial cell surface polarity. *J Cell Biol* 178, 323-335.

Nelson, K.S., and Beitel, G.J. (2009). More than a pipe dream: uncovering mechanisms of vascular lumen formation. *Dev Cell* 17, 435-437.

Sabin, F.R. (1920). Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contr Embryol* 9, 215.

Strilic, B., Kucera, T., Eglinger, J., Hughes, M.R., McNagny, K.M., Tsukita, S., Dejana, E., Ferrara, N., and Lammert, E. (2009). The molecular basis of vascular lumen formation in the developing mouse aorta. *Dev Cell* 17, 505-515.

Van Furden, D., Johnson, K., Segbert, C., and Bossinger, O. (2004). The *C. elegans* ezrin-radixin-moesin protein ERM-1 is necessary for apical junction remodelling and tubulogenesis in the intestine. *Dev Biol* 272, 262-276.

Vogelmann, R., Nguyen-Tat, M.D., Giehl, K., Adler, G., Wedlich, D., and Menke, A. (2005). TGFbeta-induced downregulation of E-cadherin-based cell-cell adhesion depends on PI3-kinase and PTEN. *J Cell Sci* 118, 4901-4912.

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