# Hemodynamics regulate spatiotemporal artery muscularization in the developing circle of Willis

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## Abstract

Brain arteries are wrapped by vascular smooth muscle cells (VSMCs). Fully differentiated VSMCs are important for brain artery homeostasis, and they are lost in several cerebrovascular diseases. How healthy VSMCs differentiate on different brain arteries during development is unclear. Such knowledge will help regenerate lost VSMCs in brain arteriopathy. To answer this question, we studied the developmental muscularization of the zebrafish circle of Willis (CW) arteries, the major arterial loop that supplies blood to the brain in all vertebrates. We found that artery specification of CW endothelial cells (ECs) happens after they migrate from primitive veins to form CW arteries. VSMCs differentiate from *pdgfrb*+ common vascular mural cell progenitors at the time when embryo circulation starts and progress temporally and spatially from anterior to posterior CW arteries. Computational fluid dynamic simulation confirms that earlier VSMC differentiation coincide with higher pulsatile flow hemodynamics in anterior CW arteries. Pulsatile blood flow induces the differentiation of human brain *pdgfrb*+ progenitors into VSMCs and reducing pulsatile blood flow by blocking the zebrafish embryo heartbeat after *pdgfrb*+ recruitment but before VSMC differentiation

limits the number of mature VSMCs. Congruently, the flow responsive transcription factor *klf2a* is activated in ECs before VSMC differentiation and knockdown delays VSMC differentiation on CW arteries. Overall, our data place hemodynamic activation of endothelial *klf2a* signaling as key determinant of spatiotemporal VSMC differentiation on CW arteries.

## Introduction

Vascular smooth muscle cells (VSMCs) are contractile mural cells wrapping around endothelial cells (ECs) of large vessels, especially arteries (Ando et al., 2022; Donadon & Santoro, 2021; Stratman et al., 2020). The expression of contractile proteins, such as alpha-smooth muscle actin (encoded by *acta2*), distinguishes VSMC from pericytes, the other type of mural cells primarily associated with small vessels including capillaries (Bahrami & Childs, 2020; Donadon & Santoro, 2021; Stratman et al., 2020). VSMCs are important for arterial homeostasis (Basatemur, Jorgensen, Clarke, Bennett, & Mallat, 2019). In the brain, VSMC constriction and relaxation is essential for functional hyperemia in neurovascular coupling (Hill et al., 2015; Kaplan, Chow, & Gu, 2020). Phenotype switching or dedifferentiation of VSMCs, marked by lower expression of contractile proteins, is prominent in pathological progression of atherosclerosis (Basatemur et al., 2019). Recently, similar VSMC phenotype switching or dedifferentiation have also been described in cell, animal, and human studies of children and adult neurological and cerebrovascular conditions (Aguilar-Pineda et al., 2021; Chou et al., 2022; Milewicz et al., 2010; Oka et al., 2020). These studies suggested that understanding the development of VSMC differentiation on brain arteries might help restore normal VSMC contractility to alleviate various cerebrovascular diseases; such a regenerative strategy requires a deep understanding of how VSMCs acquire a contractile phenotype while differentiating from their progenitors.

VSMCs spatially differentiate from different progenitors from mesoderm and neural crest origin (Ando et al., 2019; Donadon & Santoro, 2021; Whitesell et al., 2019). Previous research described various mechanisms regulating the differentiation of VSMC positive for *acta2* in the trunk vasculature. Arterial Notch signaling activated by blood

flow is necessary for *acta2*+ VSMC appearance on the zebrafish dorsal aorta (X. Chen, Gays, Milia, & Santoro, 2017). Autonomous Notch activation is required for specification of *pdgfrb*+ mural cell progenitors from mesenchyme around arteries, and these progenitors later differentiate into *acta2*+ VSMCs (Ando et al., 2019). Chemokine signaling promotes VSMCs association with the zebrafish dorsal aorta in the trunk, whereas blood flow modulated transcription factor krüppel-like factor 2 (encoded by *klf2a* in zebrafish) prevents their association with the adjacent cardinal vein (Stratman et al., 2020). Differentiation into *acta2*+ VSMCs from *pdgfrb*+ progenitors in the brain requires autonomous expression of an ATP-sensitive potassium channel (Ando et al., 2022). In contrast, *acta2*+ VSMCs in the ventral head of zebrafish are not derived from *pdgfrb*+ progenitors, and their differentiation is regulated by endothelial BMP signaling (Watterston, Zeng, Onabadejo, & Childs, 2019; Whitesell et al., 2019). These studies suggested that VSMC differentiation is highly organotypic and may even be vessel specific.

The circle of Willis (CW) consists of major arteries that supply blood to the vertebrate brain, including the internal carotid arteries and posterior communicating arteries (Campbell et al., 2019; Schröder, Moser, & Huggenberger, 2020). CW arteries are wrapped by VSMCs, and VSMC dedifferentiation and hyperplasia are described in carotid atherosclerosis and the pediatric Moyamoya disease (Chou et al., 2022; Fox, Dorschel, Lawton, & Wanebo, 2021). The internal carotid arteries in the CW are among the earliest circulated arteries in the brain due to their connection to the common carotid arteries (lateral dorsal aorta in zebrafish) (Campbell et al., 2019; Isogai, Horiguchi, & Weinstein, 2001; Schröder et al., 2020). VSMC differentiation on CW arteries, particularly internal carotid arteries, and whether this differentiation is associated with early arterial blood flow in the brain, have not been investigated. The mouse CW resembles that of humans, but its VSMC differentiation is difficult to observe *in vivo*, as the embryos develop in utero and depend on maternal circulation (Isogai et al., 2001; Schröder et al., 2020). The zebrafish CW also resembles that of humans, and the embryos external growth and optical clarity allows for confocal live imaging of developing blood vessels (Isogai et al., 2001). In addition, a few fluorescent transgenic (Tg) lines have been generated to label VSMCs and their progenitors (Ando et al., 2016;

Whitesell et al., 2014). Taking advantage of the zebrafish model, we found a spatiotemporal pattern of VSMC differentiation on CW arteries, and we associated this pattern with hemodynamic signaling pathways in arterial ECs.

| CW vessel nomenclature | |
|---|---|
| CaDI | Caudal division of the internal carotid artery |
| BCA | Basal communicating artery |
| PCS | Posterior communicating segment |

## Results

*Artery muscularization is spatiotemporally regulated in the CW arteries*

Arterial ECs in zebrafish brain emerge from primitive veins (Fujita et al., 2011). Recent single-cell transcriptome profiling of vascular cells in prenatal human brain also supported the fate progression from venous to arterial ECs (Crouch et al., 2022). To establish the stage of CW artery specification in the zebrafish brain, we performed confocal live imaging of Tg(*flt4:yfp*)$^{hu4881}$, which labels venous ECs, and Tg(*kdrl:hras-mcherry*)$^{s896}$, which labels all ECs, at four stages starting at 32 hour post-fertilization (hpf), when CW arteries are assembling (Chi et al., 2008; Hogan et al., 2009; Isogai et al., 2001). As arterial ECs express higher *kdrl*, we used the intensity of *mcherry* as an indicator of artery specification (Figure 1A-1E) (Chi et al., 2008). We found that ECs in CW arteries are primarily venous at 32 hpf (Figure 1A and 1E), and gradually gain *kdrl* expression from 54 hpf to 3-day post fertilization (dpf) (Figure 1B, 1C, and 1E). The *mcherry* intensity at 4 dpf is similar to 3 dpf, suggesting that artery specification of the CW is completed by 3 dpf (Figure 1C-1E).

Then we characterized CW artery muscularization. Previous research on the CW arteries described that VSMCs derive from *pdgfrb*+ mural cell progenitors (Ando et al., 2022; Ando et al., 2019). Thus, to determine VSMC differentiation in real time we

performed confocal live imaging of Tg(*acta2:mcherry*)$^{ca8}$, TgBAC(*pdgfrb:egfp*)$^{ncv22}$, and Tg(*kdrl:cerulean*)$^{sd24}$, to visualize respectively VSMCs (red), *pdgfrb*+ progenitors (green) and the developing arteries (white) at four stages starting from 32 hpf (Ando et al., 2016; Page et al., 2013; Whitesell et al., 2014). We found that overall *pdgfrb*+ progenitors appear on CW arteries after their lumen form around 54 hpf, and most of them do not express *acta2* (Figure 2A-2B, 2E-2F, and S1A-S1B). The number of *pdgfrb*+ progenitors increase temporally from 54 hpf to 3 dpf and spatially on CW arteries from anterior to posterior - from the internal carotid arteries (CaDI), the basal communicating artery (BCA) to the posterior communicating segments (PCS) (Figure 2B-2C). Notably, while many *pdgfrb*+ progenitors on CaDI differentiate into *acta2*+ VSMCs by 3 dpf nearly no *acta2*+ VSMCs were observed in the nearby artery BCA or PCS (Figure 2B-2C, 2E-2F, and S1A-S1B). By 4 dpf, the number of *pdgfrb*+ cells is similar to 3 dpf, but *acta2*+ VSMCs on CaDI continue to differentiate in a greater number over the BCA, and PCS arteries (Figure 2C-2F and S1A-S1B). These results show an anterior to posterior differentiation of *pdgfrb*+ progenitors into *acta2*+ VSMCs with the CaDI being the first CW artery muscularized and having a higher number of *acta2*+ VSMCs.

*CW arteries have spatiotemporal difference in hemodynamics*

Our data suggest that VSMCs in the anterior CaDI differentiate significantly earlier than the anterior BCA and the posterior PCS, even if their progenitors have the same origin and these CW blood vessel formation and arterialization occurs at the same time. We speculate that different hemodynamic distribution may contribute to the spatiotemporal difference in VSMC differentiation on CW arteries. In fact, CaDI is the only vessels in the CW receiving pulsatile arterial blood flow directly from the lateral dorsal aorta and cardiac outflow tract. Furthermore, the CaDI differs from the BCA or PCS in terms of overall vascular geometry, which has been shown to affect local intravascular forces such as the wall shear stress (WSS) (Katritsis et al., 2007). To explore this possibility, we performed microangiography with a high molecular weight fluorescent dextran and used computational fluid dynamics (CFD) to simulate the effect

of pulsatile flow on the CW intravascular forces (ANSYS, 2014; Z. Chen et al., 2019; Fernandes et al., 2022). First, we determined each CW artery diameter, the principal determinant of hemodynamics, and found that there was no significant difference between structures (Figure 3A-3D). Notably, however, the flow velocity across CW arteries was differentially distributed, with the CaDI having the highest blood flow speed starting from 54 hpf to 4 dpf (Figure 3A-3C, and 3E). This is consistent with the CaDI being the inlet vessel for the arterial blood flow within the entire CW circulation. Next, we calculated the WSS across all CW arteries and found an overall increase in average and total WSS from 54 hpf to 4 dpf (Figure 3A-3C, and 3F). Importantly, however, when analyzed individual vessels, we found that the WSS in the CaDI is significantly higher from 54 hpf to 4 dpf, while this increase was not noted in the BCA or PCS (Figure 3A-C, and 3G). Further, WSS in the CaDI is significantly higher than in the BCA and PCS at 3 dpf (Figure 3G), when considerable VSMC differentiation starts on the CaDI but not the BCA or PCS (Figure 2B-2C, 2F, and S1A-S1B). Altogether, the pulsatile blood flow simulation suggests that CW arteries experience different hemodynamic loads that might confer an heterogenous muscularization of CW arteries.

*Blood flow is required for differential CW artery muscularization*

Our data suggest that ECs under pulsatile arterial flow might favor the differentiation of *acta2*+ VSMCs from *pdgfrb*+ progenitors. To test this hypothesis, we set up an *in vitro* cell co-culture experiment where GFP-PDGFRB+ human brain pericytes (GFP-HBVPs) were cultured in flow amenable outlet slides, then covered by a thin layer of collagen type 1. Confluent human endothelial cells (HUVECs) were seeded on top of that and then exposed to steady-state laminar flow or pulsatile flow conditions (Figure 4A) (Abello, Raghavan, Yien, & Stratman, 2022). Maturation of PDGFRB+ human brain pericytes into ACTA2+ VSMCs was then quantified after 24 hours from the introduction of flow addition. We found that brain pericytes under pulsatile flow were larger and showed higher expression of VSMC differentiation markers including PDGFRB, ACTA2, and TRANSGELIN (Robin et al., 2013) (Figure 4B-4D), suggesting that pulsatile blood flow favored PDGFRB+ progenitor differentiation.

Next, we tested the effect of pulsatile flow in the CW on VSMC differentiation *in vivo*. Previous research suggested that blood flow hemodynamics is not necessary for *pdgfrb*+ progenitor recruitment at CW arteries, as shown by zebrafish embryos lacking blood flow such as the *tnnt2a* morphants (MO) (Ando et al., 2016), however their differentiation to *acta2*+ cells has not been documented. Thus, to determine whether blood flow affects *acta2*+ VSMC differentiation on CW arteries, we injected 0.35 ng of *tnnt2a* MO into Tg(*acta2:mcherry*, *kdrl:gfp*)$^{ca8/zn1}$ at the one to two-cell stage, to abrogate cardiac contractility and thus pulsatile flow (Sehnert et al., 2002; Whitesell et al., 2014; Zhong et al., 2006). Notably, we found no *acta2*+ VSMCs on CW arteries of *tnnt2a* MO at 3 to 4 dpf (Figure 4F, 4H-4I, and 4K), suggesting that blood flow is dispensable for *pdgfrb*+ progenitor recruitment but is required for *acta2*+ VSMC differentiation on the CW arteries.

To further explore the temporal requirement of blood flow, we treated embryos with 25 μM nifedipine, a drug shown to reduce heart rate in zebrafish embryos (Gierten et al., 2020), from 54 hpf, right before the onset of *pdgfrb*+ progenitors' expression of *acta2* (Figure. 2A and B). We found that after 18 hours at 3 dpf the number of *acta2*+ VSMCs is greatly reduced on the CaDI of treated embryos compared to untreated controls (Figure 4G and 4M); *acta2*+ VSMCs on the BCA and the PCS at 4 dpf are also reduced significantly (Figure 4L and 4N), suggesting that blood flow reduction after 54 hpf delays VSMC differentiation on CW arteries with the earliest and strongest effect on the CaDI muscularization (Figure 4G and 4M).

To determine whether blood flow is differentially required for the maintenance of VSMCs on CW arteries, we treated embryos with nifedipine from 4 dpf, after VSMC differentiation on CW arteries, and imaged at 5 dpf. The number of *acta2*+ VSMCs on the CaDI and PCS in treated embryos is similar to untreated controls (Figure S2A-S2C), suggesting that blood flow is required for differentiation but not for short-term maintenance of VSMCs.

*Blood flow-regulated transcription factor klf2a is required for spatiotemporal CW artery muscularization*

Our results suggest that ECs might express a flow pattern-dependent transcriptional program that favors spatiotemporal VSMC muscularization in the CW arteries. For example, previous studies showed that the site and level of the transcription factor *Klf2* (Parmar et al., 2006; Sweet, Fan, Hsieh, & Jain, 2018; Warboys, Amini, de Luca, & Evans, 2011) in artery ECs closely follow the predicted pattern of elevated intravascular forces (Lee et al., 2006). Consistently, higher *Klf2* expression can be induced by unidirectional pulsatile flow mimicking arterial flow (Dekker et al., 2002). Furthermore, *klf2* is implicated in VSMC migration on arterial blood vessels during mouse development: loss of *klf2* leads to aorta VSMC deficiency (Wu, Bohanan, Neumann, & Lingrel, 2008) while endothelial *klf2a* expression prevents VSMC association with primitive veins in zebrafish trunk (Stratman et al., 2020). Hence, we tested the hypothesis that *klf2a* might be the signaling spatially regulated in the CW artery to control VSMC maturation. We first imaged Tg(*klf2a:h2b-egfp*, *kdrl:hras-mcherry*)$^{ig11/s896}$, which labels nuclei with active *klf2a* expression, and quantified the number of *klf2a*+ ECs in the CW arteries (Chi et al., 2008; Steed et al., 2016). To account for the overall changes in EC number during development (Ulrich, Ma, Baker, & Torres-Vazquez, 2011), as well as the length of each vessels, we also imaged Tg(*fli1:nls-gfp*, *kdrl:hras-mcherry*)$^{y7/s896}$, which labels all EC nuclei, at four stages from 32 hpf (Roman et al., 2002). Notably, we found a slight increase in ECs number per 100 μm vessel in the CaDI from 32 to 54 hpf (Figure S3A-S3C), but a much more significant increase in *klf2a*+ EC nuclei in the CaDI (Figure 5A and 5B), before *acta2*+ VSMCs appear (Figure 2B-2C, 2F, and Figure S1A-S1C). The number of ECs in the CaDI stayed the same from 54 hpf through 4 dpf (Figure S3A-S3C), while *klf2a*+ ECs in the CaDI increased until 3 dpf (Figure 5A and 5B). In tandem, *acta2*+ VSMCs on the CaDI increase through 4 dpf (Figure 2C-2D, 2F, and Figure S1A-S1C). EC number per 100 μm vessel in the BCA and PCS are similar from 54 hpf to 4 dpf (Figure S3A-S3C), whereas the number of *klf2a*+ ECs increase significantly over time (Figure 5A and 5B). This increase predates the presence of *acta2*+ VSMCs on the BCA and PCS (Figure 2B-2D, 2F, and Figure S1A-S1C). These results suggest that the increase of *klf2a*+ ECs in the CW arteries is not due to an increase in EC number and precedes the spatiotemporal VSMC differentiation observed on the CW arteries.

To further define the role of *klf2a* in CW artery muscularization, we knocked down *klf2a* in Tg(*acta2:mcherry*, *kdrl:gfp*)$^{ca8/zn1}$ with 11 ng MO injection at the one to two-cell stage and imaged at 3 to 4 dpf (Nicoli et al., 2010; Whitesell et al., 2014; Zhong et al., 2006). We validated the effect of *klf2a* MO knockdown using the Tg(*klf2a:h2b-egfp*, *kdrl:hras-mcherry*)$^{ig11/s896}$ transgenic line (Figure S3D-S3F) (Chi et al., 2008; Steed et al., 2016). Compared to uninjected control, *klf2a* morphants have significantly less *acta2*+ VSMCs on the CaDI at 3 dpf but a normal number by 4 dpf (Figure 6A-6D), suggesting that *klf2a* promotes timely initiation of CaDI muscularization. Together these data suggest that CW muscularization is associated with endothelial *klf2a* activation and necessary for spatiotemporal VSMC differentiation under pulsatile blood flow conditions.

## Discussion

Here, we used confocal live imaging of fluorescence transgenic zebrafish embryos to characterize the spatiotemporal dynamics of VSMC differentiation on the CW, which consists of major arteries that supply blood to the vertebrate brain. We found that CW morphogenesis preceded arterial specification. *pdgfrb*+ mural cell progenitors start to express the VSMC marker *acta2* after these mural cell progenitors were recruited to CW arteries. VSMCs differentiated earlier on anterior CW arteries, which are under higher WSS than their posterior counterparts, due to the high velocity of incoming pulsatile blood flow. We used an *in vitro* co-culture assay, genetic manipulation, and drug treatments to provide evidence that pulsatile blood flow can contribute to spatiotemporal VSMC differentiation. We found that the flow responsive transcription factor *klf2a* is activated from anterior to posterior in the CW arteries, preceding VSMC differentiation. *klf2a* knockdown delayed VSMC differentiation on anterior CW arteries. Together, these data support the conclusion that pulsatile flow activation of endothelial *klf2a* promotes spatiotemporal VSMC differentiation on CW arteries in the brain (Figure 7).

Our work suggests *klf2a*-mediated blood flow regulation of VSMC differentiation on zebrafish brain arteries, and thus raises the question of how Klf2 transduces endothelial signals to mural cell progenitors and VSMCs. *In vitro*, endothelial Klf2 upregulates miR-143/145, which are transported into co-cultured VSMCs within extracellular vesicles

(EV) to promote a contractile phenotype in VSMCs (Hergenreider et al., 2012). How EV transport of miR-143/145 works *in vivo* during development remains unknown.

Notch signaling appears a plausible downstream effector of Klf2 activation. Like Klf2, Notch also responds to flow in heart valve development (Fontana et al., 2020), and Klf2 appears to upregulate Notch (Duchemin, Vignes, & Vermot, 2019). Non-canonical Notch signaling is induced by shear stress to mediate endothelial barrier formation (Polacheck et al., 2017). Notch1 is a proposed mechanosensor in adult arteries (J. J. Mack et al., 2017). In addition, Notch regulation of VSMC development is well established. Additionally, flow activation of Notch in the dorsal aorta (DA) is required for VSMC recruitment (X. Chen et al., 2017). Notch signaling is also activated in DA VSMC progenitors during development, when DA ECs express Jag1 (jagged canonical Notch ligand 1) (Chang et al., 2012; High et al., 2008). Notch2 and Notch3 are compensatory for VSMC development on the DA (Q. Wang, Zhao, Kennard, & Lilly, 2012). In VSMC differentiation on coronary arteries, ECs express Jag1 and pericyte progenitors express Notch3 (Volz et al., 2015). Thus, reciprocal Notch signaling between ECs and VSMCs may regulate VSMC differentiation on brain arteries, although how Notch signaling activates Myocd (myocardin) (Huang et al., 2008; Li, Wang, Wang, Richardson, & Olson, 2003), Srf (serum response factor) (C. P. Mack & Owens, 1999), and miR-143/145 (Boettger et al., 2009) to enable expression of Acta2 and other contractile proteins remain incompletely understood.

It is important to recognize the versatile roles of Notch signaling in vascular development for process both prior to and in tandem with VSMC differentiation. Notch signaling represses proliferation of arterial ECs during angiogenesis (Hasan et al., 2017; Pitulescu et al., 2017), and maintains arterial identity after morphogenesis (Lawson et al., 2001; Shutter et al., 2000; Villa et al., 2001). Notch signaling is required for recruitment of mural cells to brain arteries, in which Notch2 and Notch3 are redundant for upregulation of Pdgfrb in mesenchymal progenitors (Ando et al., 2019). Notch3, in which mutations cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), is broadly required for mural cell development in the brain (Domenga et al., 2004; Joutel et al., 1996; Y. Wang, Pan,

Moens, & Appel, 2014). Thus, thorough dissection of how Notch signaling regulates VSMC differentiation on brain arteries would require development of novel methods that enable spatiotemporal resolution of Notch ligands and receptors in different vascular cell types.

Wnt signaling is another possible downstream effector of Klf2. In heart valve development, endocardial Klf2 upregulates Wnt9 (wingless-type MMTV integration type family member 9), which activates Wnt signaling in neighboring mesenchymal cells and thereby regulates their proliferation and condensation (Goddard et al., 2017). The pathway interacts with Notch signaling in heart valve morphogenesis (Paolini, Fontana, Pham, Rodel, & Abdelilah-Seyfried, 2021). Non-canonical Wnt signaling attenuates EC sensitivity to shear stress and stabilizes blood vessels (Franco et al., 2016). In brain vascular development, Wnt signaling controls brain specific developmental angiogenesis and endothelial barrier formation (Daneman et al., 2009; Liebner et al., 2008; Zhou et al., 2014). Wnt signaling in mural cells activates expression of Lama2 (laminin subunit alpha 2), a major component of the brain vascular basement membrane, to promote the neurovascular unit and blood-brain barrier maturation (Biswas et al., 2022). Wnt signaling regulates VSMC proliferation, migration, and survival in cardiovascular diseases (Mill & George, 2012), but relatively little is known about the role of Wnt signaling in VSMC differentiation, especially organotypic VSMC differentiation on brain arteries.

The biochemical pathway by which Klf2 activates Notch or Wnt signaling remains incompletely understood. Recent chromatin occupancy and transcription studies showed that Klf2 has context specific binding patterns and transcriptional targets in the heart and lung endothelium (Sweet et al., 2023). Thus, Klf2 may have multiple direct or indirect paths to activate Notch or Wnt signaling. The context specificity may also explain different effects of *klf2a* on VSMC development in the zebrafish brain and trunk: previous research suggested that *klf2a* expression in the cardinal vein prevents VSMC association (Stratman et al., 2020), whereas this study suggests that *klf2a* activation in brain arteries promotes VSMC differentiation. It is plausible that *klf2a* has different

expression levels, binding patterns, transcriptional targets, and downstream effects in brain arterial ECs compared with trunk venous ECs.

Our findings raise interesting new questions on whether stable *klf2a* expression in CW arterial ECs supports further maturation of VSMCs. Previous research found that *acta2*+ VSMCs on the BCA and PCS express pericyte enriched *abcc9* (ATP binding cassette subfamily C member 9) at 4 dpf (Ando et al., 2022), which is gradually lost from 5 dpf to 6 dpf (Ando et al., 2022) as these cells become *acta2*+. Interestingly, *acta2*+ VSMCs on CaDI, BCA, and PCS still retain expression of *pdgfrb* at 6 dpf (Ando et al., 2021). Thus, it is possible that stable expression of *klf2a* in CW arterial ECs will support later on complete VSMC maturation, for example in Transgelin positive cells (Colijn, Nambara, & Stratman, 2023). Alternatively, *acta2*+ VSMCs with *pdgfrb* expression are maintained as such in the CW arteries (Ando et al., 2021).

It is important to note the differences between the trunk and our data focused on the CW brain arterial VSMC differentiation. On the dorsal aorta of the trunk, as soon as VSMCs are recruited, they begin expressing Acta2 and Tagln, suggesting simultaneous recruitment and differentiation from sclerotome progenitors (Ando et al., 2016; Stratman et al., 2017), compared with the spatiotemporal dynamics we see on brain arteries. It is also important to note the difference between arterial and venous VSMCs. Brain arterial VSMCs strongly express Acta2, whose expression is weak in venous VSMCs (Crouch, Joseph, Marsan, & Huang, 2023; Hill et al., 2015). Venous VSMCs share more common gene expression signature with pericytes, as they both express Abcc9 and Kcnj8 (Ando et al., 2019; Bondjers et al., 2006; Chasseigneaux et al., 2018; He et al., 2016; Vanlandewijck et al., 2018). Based on our data, it is possible to speculate that pulsatile flow reinforces brain arterial specification before VSMC differentiation. Pulsatile flow contributes to both arterial specification and VSMC differentiation, although the two processes are difficult to dissect *in vivo*, as both require Notch signaling and overlap temporally. In addition, pulsations dampen from dorsal aorta to internal carotid arteries, and flow eventually becomes stable in capillaries and veins (Humphrey & Schwartz, 2021). Current *in vivo* methods involving genetic manipulations and drug treatments are only capable of qualitative reduction in all types of flow, including pulsatile. *In vitro* three-

dimensional (3D) vascular culture models, which combine ECs and mural cells (Mirabella et al., 2017; Vila Cuenca et al., 2021), could be further optimized to simulate complex geometry of brain arteries. Combining these models with microfluidics, which allows precise calibration of flow velocity and pulse, would enable a more thorough analysis of endothelial mechanotransduction and its contribution to VSMC differentiation on different arterial beds (Abello, Raghavan, Yien, & Stratman, 2022; Gray & Stroka, 2017; Griffith et al., 2020).

In conclusion, our work highlights organotypic differences in VSMC differentiation in the CW brain arteries and points to hemodynamics as a main driver of spatiotemporal dynamics of VSMC maturation on the brain arteries. Impaired VSMC differentiation in development could be implicated in cerebrovascular diseases, such as Moyamoya disease, which is linked to mutations in the human *ACTA2* gene that results in VSMC hyperplasia specifically on the internal carotid arteries (Fox et al., 2021; Guo et al., 2007; Guo et al., 2009; Lin et al., 2012). Our study points to endothelial cell mechanotransduction of pulsatile flow as a key signaling network that could render specific VSMC populations on brain arteries more susceptible compared to VSMCs associated with other vessels in the same tissue. Our data will therefore inform the screening of new genes that in combination to known genetic variants, such as *ACTA2*, could contribute to our understanding of the susceptibilities in cerebrovascular diseases.

## Material and Methods

### Zebrafish husbandry and transgenic lines

Zebrafish were raised and maintained at 28.5 °C using standard methods. Protocols are approved by the Yale Institutional Animal Care and Use Committee (2020-11473). Transgenic lines in Table 1 were established previously.

Table 1. List of zebrafish fluorescent transgenic lines used in the study

| Transgenic Line | ID | Lab of Origin | Reference |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Tg(*flt4:yfp*)$^{hu4881}$ | ZDB-ALT-100208-1 | S. Schulte-Merker | Hogan et al., 2009 |
| Tg(*kdrl:hras-mcherry*)$^{s896}$ [Tg(*kdrl:ras-mcherry*)$^{s896}$] | ZDB-ALT-081212-4 | D.Y.R. Stainier | Chi et al., 2008 |
| Tg(*acta2:mcherry*)$^{ca8}$ | ZDB-ALT-120508-2 | S.J. Childs | Whitesell et al., 2014 |
| TgBAC(*pdgfrb:egfp*)$^{ncv22}$ | ZDB-ALT-160609-1 | N. Mochizuki | Ando et al., 2016 |
| Tg(*kdrl:cerulean*)$^{sd24}$ | ZDB-ALT-131024-2 | D. Traver | Page et al., 2013 |
| Tg(*kdrl:grcfp*)$^{zn1}$ [Tg(*kdrl:gfp*)$^{zn1}$] | ZDB-ALT-051114-10 | Zygogen Research Department | Cross et al., 2003 |
| Tg(*klf2a:h2b-egfp*)$^{ig11}$ | ZDB-ALT-161017-10 | J. Vermot | Steed et al., 2016 |
| Tg(*fli1:negfp*)$^{y7}$ [Tg(*fli1:nls-gfp*)$^{y7}$] | ZDB-ALT-060821-4 | B.M. Weinstein | Roman et al., 2002 |

Confocal fluorescence microscopy

Zebrafish embryos were raised in 0.003% 1-phenyl-2-thiourea (PTU, phenylthiocarbamide, or n-phenylthiourea, Sigma P7629) from gastrulation stage to prevent pigmentation. Embryos imaged live by confocal fluorescence microscopy were anesthetized in 0.1% tricaine methanesulfonate (TMS, MS-222, or Syncaine, Western Chemical, NC0872873) and mounted in 1% low melt agarose within glass bottom microwell dishes. Fluorescence images were captured with an upright Zeiss LSM 980 confocal microscope using a 20X objective.

Image analysis

Confocal fluorescence images were analyzed with Imaris microscopy image analysis software (Bitplane, Oxford Instruments). Average fluorescence intensity of mcherry in Tg(*flt4:yfp, kdrl:hras-mcherry*)$^{hu4881/s896}$ was estimated by generating volume objects covering the artery of interest with Surfaces module. Vessel lengths of the arteries were manually traced with Filament module. Numbers of *pdgfrb*+ mural cell progenitors, *acta2*+ vascular smooth muscle cells, and *klf2a*+ and *fli1*+ endothelial nucleus were counted with Spots module.

Morpholino injections

Morpholino antisense oligonucleotides were synthesized by Gene Tools. Morpholinos in Table 2 were validated previously. Optimized dose injected into each embryo are listed. Uninjected siblings were used as controls.

Table 2. List of morpholino antisense oligonucleotides used in the study

| Morpholino | Sequence | ID | Reference | Dose (ng/embryo) |
|---|---|---|---|---|
| MO1-tnnt2a | 5' - CATGTTTGCTCTGATCTGACACGCA - 3' | ZDB-MRPHLNO-060317-4 | Sehnert et al., 2002 | 0.35 |
| MO1-klf2a | 5' - GGACCTGTCCAGTTCATCCTTCCAC - 3' | ZDB-MRPHLNO-100610-8 | Nicoli et al., 2010 | 11 |

Nifedipine treatment

Nifedipine was dissolved into 20 mM in dimethyl sulfoxide (DMSO). The stock solution was diluted into 25 μM in egg water with 0.003% PTU to treat zebrafish embryos from 54 hpf to 3 dpf or 4 dpf. The stock solution was diluted into 20 μM in egg water with 0.003% PTU to treat zebrafish embryos from 4 dpf to 5 dpf. The same

volume of DMSO was added into egg water with 0.003% PTU for sibling control embryos.

Microangiography

Embryos were anesthetized in 0.1% tricaine methanesulfonate, placed on an agarose mold, and injected pericardially with 4 nL Tetramethylrhodamine Dextran (2,000,000 molecular weight, Thermo Fisher) at a concentration of 10 mg/mL. Subsequently, the vasculature of embryos was checked for Dextran fluorescence signal under a stereomicroscope (Olympus, MVX10). Embryos with Dextran fluorescence were mounted in 1% low melt agarose within glass bottom microwell dishes and imaged with confocal microscope.

Computational fluid dynamic simulation

Computational fluid dynamic simulation was performed as previously described with modification (ANSYS, 2014; Barak et al., 2021). Three-dimensional (3D) geometry models of the circle of Willis (CW) arteries were reconstructed from confocal microangiography images of Dextran injected Tg(*kdrl:gfp*) embryos with the Filament module in Imaris (Bitplane, Oxford instruments). The CW 3D geometry models were pre-processed in ANSYS SpaceClaim 2022 R1 software. Pre-processed models were then meshed in ANSYS Fluent 2022 R1 software using computational fluid dynamics application settings. Inlets and outlets were specified. Meshing orthogonal quality was calculated, and objects less than 0.01 were excluded. Blood density and gauge pressure were considered as 1,060 kg/m$^3$ and 13,332 pascals, respectively. Each analysis consisted of 200 iterations.

Cell Culture

Pooled primary Human Umbilical Vein Endothelial Cells (HUVEC) (PCS-100-010$^{TM}$, ATCC) were seeded at an initial concertation of 5,000 cells per cm$^2$, in 1x M119 media (110343-023, Gibco) supplemented with 16% FBS (Gibco), 84 µg/mL of heparin sodium salt (H3393, Sigma-Aldrich), 25 µg/mL of endothelial cell growth supplement (02-102, EMD Millipore Corp.) and 1x Antibiotic-antimycotic solution (15240-062, Gibco). Cell

cultures were maintained at 37$^{o}$C, 5% $CO_2$ and 95% humidity, until the cell reached 80% confluence.

To facilitate cell visualization in cell co-cultures, Human Brain Vascular Pericytes (HBVP) (1200, ScienCell) were transfected using lentiviral particles (LVP310, GenTarget, Inc.) to induce GFP expression under EF1a promotor. Cells cultures were initiated by seeding 5,000 cells per cm$^2$ in 175 cm$^2$ plastic flasks pre-coated with gelatin and 1x DMEM (11995-065, Gibco) supplemented with 10% FBS (Gibco), and 1x Antibiotic-antimycotic solution under the same cell culture conditions described above. When cells cultures were at 80% confluence, 200 µL containing 2 x 10$^6$ GFP-lentiviral particles were added to each 175 flasks. After 72 hours, fresh cell culture media supplemented with 10µg/mL of Blasticidin (15205, Sigma-Aldrich) were added to each flask to select the positive transfected cells.

Flow assays and immunostaining

HBVP cells were harvested and seeded at a concentration of 1.3 x 10$^5$ cells in 0.4 optical plastic flow microslides (80176, Ibidi) precoated with 1 mg/mL gelatin and incubated for 24 hours under standard culture conditions. After the initial incubation, 100 µg/mL of collagen I (354249, Corning) diluted in DMEM cell culture media was added to the slides to create a thin layer on top of the HBVP cells. After 2 hours, the media was removed and 2.5 x 10$^5$ HUVECs were seeded on top of the collagen I layer and incubated for additional 24 hours. After cell co-cultures were established, the slides were exposed to laminar (15 dyn/cm$^2$) or pulsatile (12-15 dyn/cm$^2$) flow for 24 hours, implementing a peristaltic pump adapted to produce different types of flow (Abello, Raghavan, Yien, & Stratman, 2022). After 24 hours, cultures were rinsed with 1x PBS and fixed for 30 minutes in 4% paraformaldehyde at room temperature. Cell cultures were immunostained with α-Smooth muscle actin D4K9N XP® rabbit monoclonal antibody (19245, Cell Signaling), followed by Alexa Fluor 633 goat anti-rabbit IgG (A21071, Invitrogen). Confocal images were obtained using a 40x objective with a W1 Spinning Disk confocal microscope, a Fusion camera, and the Nikon Eclipse Ti2-E base. Fiji image processing software was used for image analysis and fluorescence intensity quantification.

Statistics

All statistical analyses were performed with GraphPad Prism (version 10.0.3). Mann–Whitney test was used to compare two groups to test mean differences (protein level, morpholino and nifedipine treatment). Two-way analyses of variance followed by Tukey's multiple comparisons was used to compare more than two groups to test mean differences (average fluorescence intensity, cell number, vascular diameter, flow velocity, and WSS).

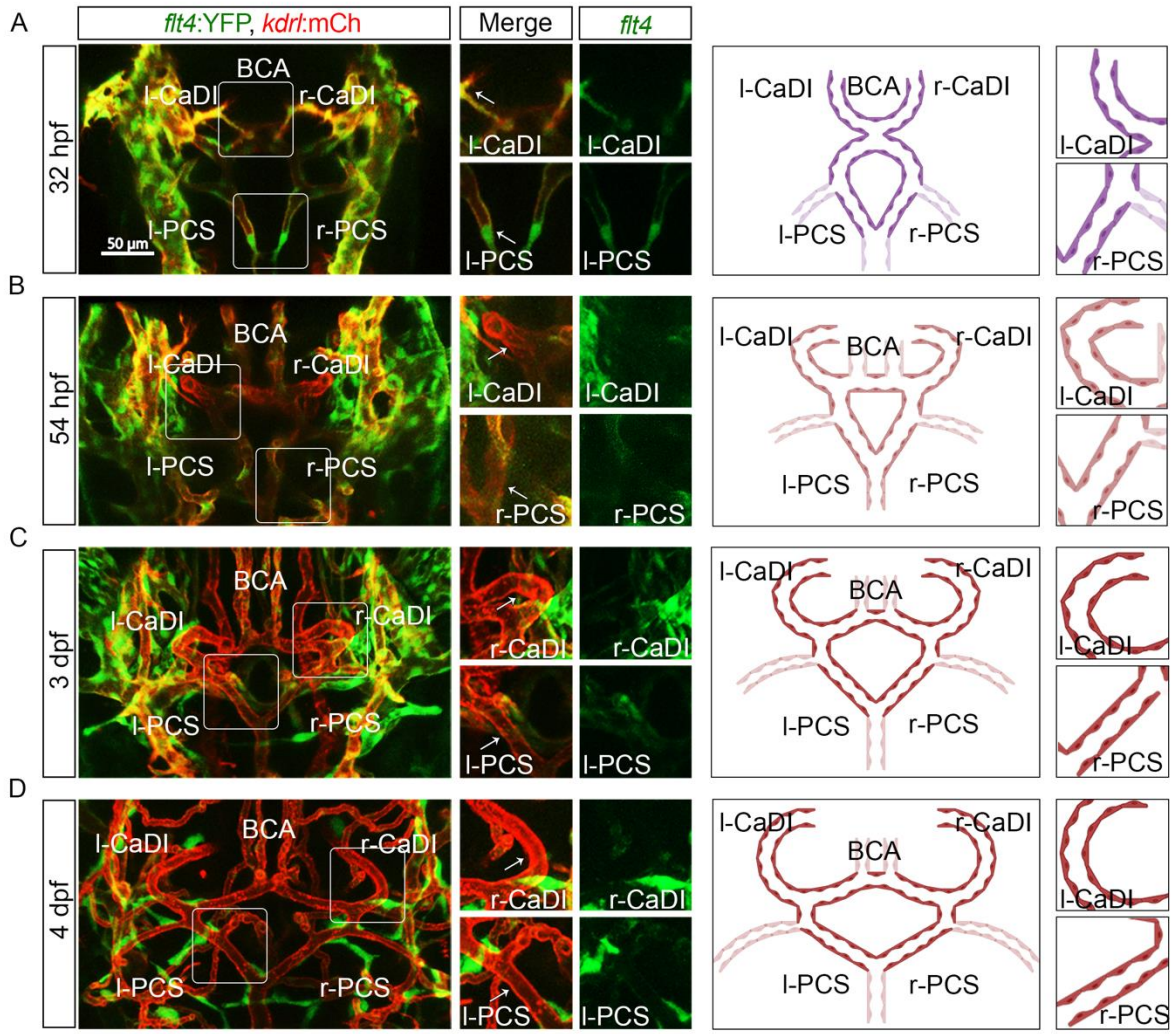
**Acknowledgements**

We thank the labs of S. Schulte-Merker, D.Y.R. Stainier, S.J. Childs, N. Mochizuki, D. Traver, J. Vermot, and B.M Weinstein for sharing zebrafish transgenic lines. We thank N. Semanchik for all the assistance with zebrafish adult colonies and husbandry. Experiments in the manuscript were supported by R01NS109160 and R01DK118728 awarded to S.N. and AHA23POST1025829 post-doctoral fellowship awarded to I.F.X. The paper is based on a dissertation submitted by S.C. to fulfill in part the requirements for the degree of Doctor of Philosophy, Yale University. S.C. was supported by a Gruber Science Fellowship from Yale Graduate School of Arts and Sciences.

**Author Contributions**

S.C. and I.F.X contributed to the experiments, the data analysis, and composed the figures. R.W. performed dextran microangiography experiments. J.A. and A.N.S. performed the *in vitro* experiments. S.C., I.F.X and S.N. wrote the manuscript and prepared figures. J.A. and A.N.S. edited the paper.

**Figures and Figure Legends**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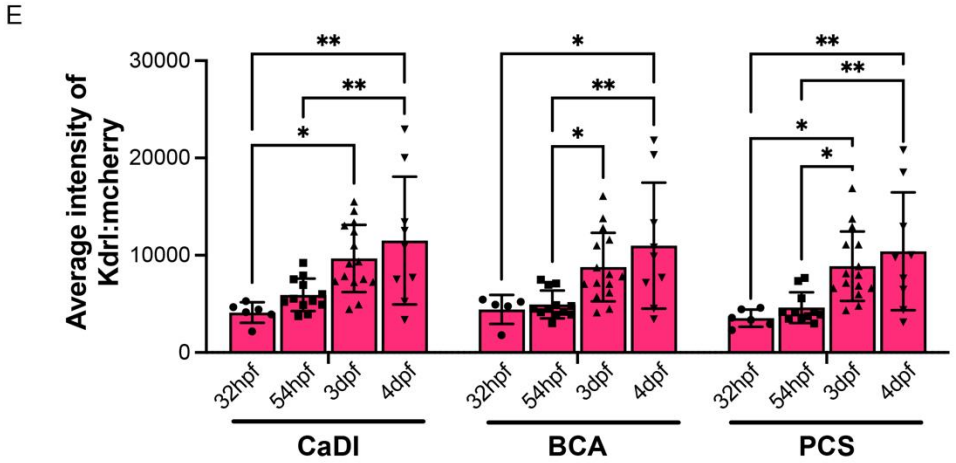
flt4:YFP, kdrl:mCh
Merge
flt4
32 hpf
BCA
l-CaDI
r-CaDI
l-PCS
r-PCS
50 µm
B
54 hpf
C
3 dpf
D
4 dpf
Primitive venous ECs (flt4 high, and kdrl low)
Transitioning ECs (flt4 low to kdrl high)
Arterial ECs (flt4 negative, and kdrl high)


E
Average intensity of Kdrl:mcherry
30000
20000
10000
0
32hpf
54hpf
3dpf
4dpf
CaDI
BCA
PCS

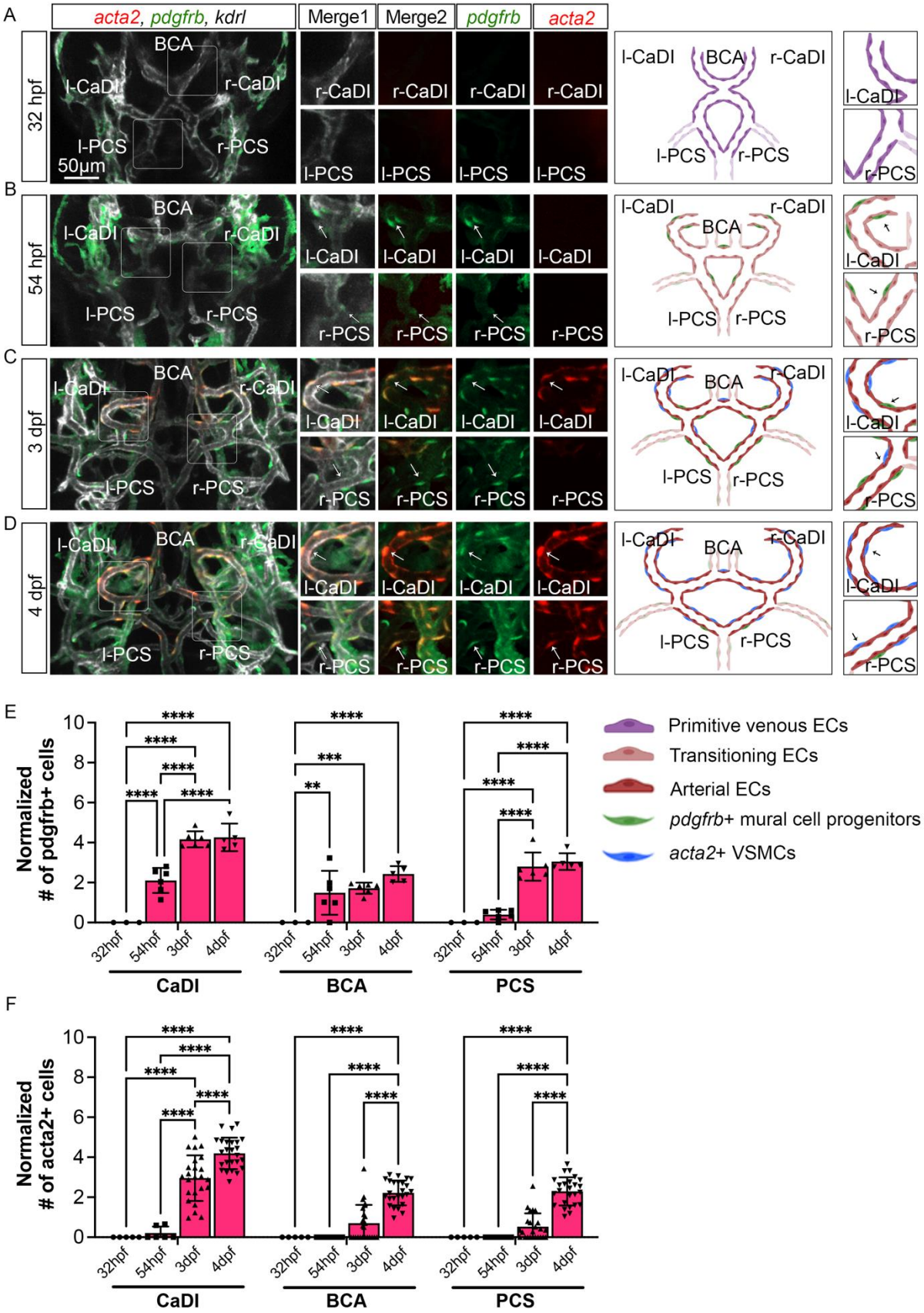
492

**Figure 1.** Artery specification of endothelial cells (ECs) in circle of Willis (CW) arteries

(A-D) Confocal live images of Tg(*flt4:yfp*, *kdrl:hras-mcherry*)$^{hu4881/s896}$ and scheme representation of CW arteries in zebrafish brain at 32 hour post fertilization (hpf) (A), 54 hpf (B), 3 day post fertilization (dpf) (C), and 4 dpf (D). Green channel represents *flt4:yfp* fluorescence, Red channel represents *kdrl:hras-mcherry* fluorescence, and Merge panel combines both channels. Arrows point to the CW arteries with *kdrl:hras-mcherry* signal. Scale bar = 50 μm

(E) Average intensity of *kdrl:hras-mcherry* in caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 32 hpf (n=6, 2 independent experiments), 54 hpf (n=12, 4 independent experiments), 3 dpf (n=15, 4 independent experiments), and 4 dpf (n=9, 2 independent experiments), two-way analyses of variance followed by Tukey's multiple comparisons, represented with mean ± SD, * $p \leq 0.05$, ** $p \leq 0.01$

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, EC: endothelial cell, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment


A
acta2, pdgfrb, kdrl
Merge1
Merge2
pdgfrb
acta2
32 hpf
BCA
l-CaDI
r-CaDI
l-PCS
r-PCS
50µm
B
54 hpf
C
3 dpf
D
4 dpf
E
Normalized # of pdgfrb+ cells
32hpf
54hpf
3dpf
4dpf
CaDI
BCA
PCS
Primitive venous ECs
Transitioning ECs
Arterial ECs
pdgfrb+ mural cell progenitors
acta2+ VSMCs
F
Normalized # of acta2+ cells

511

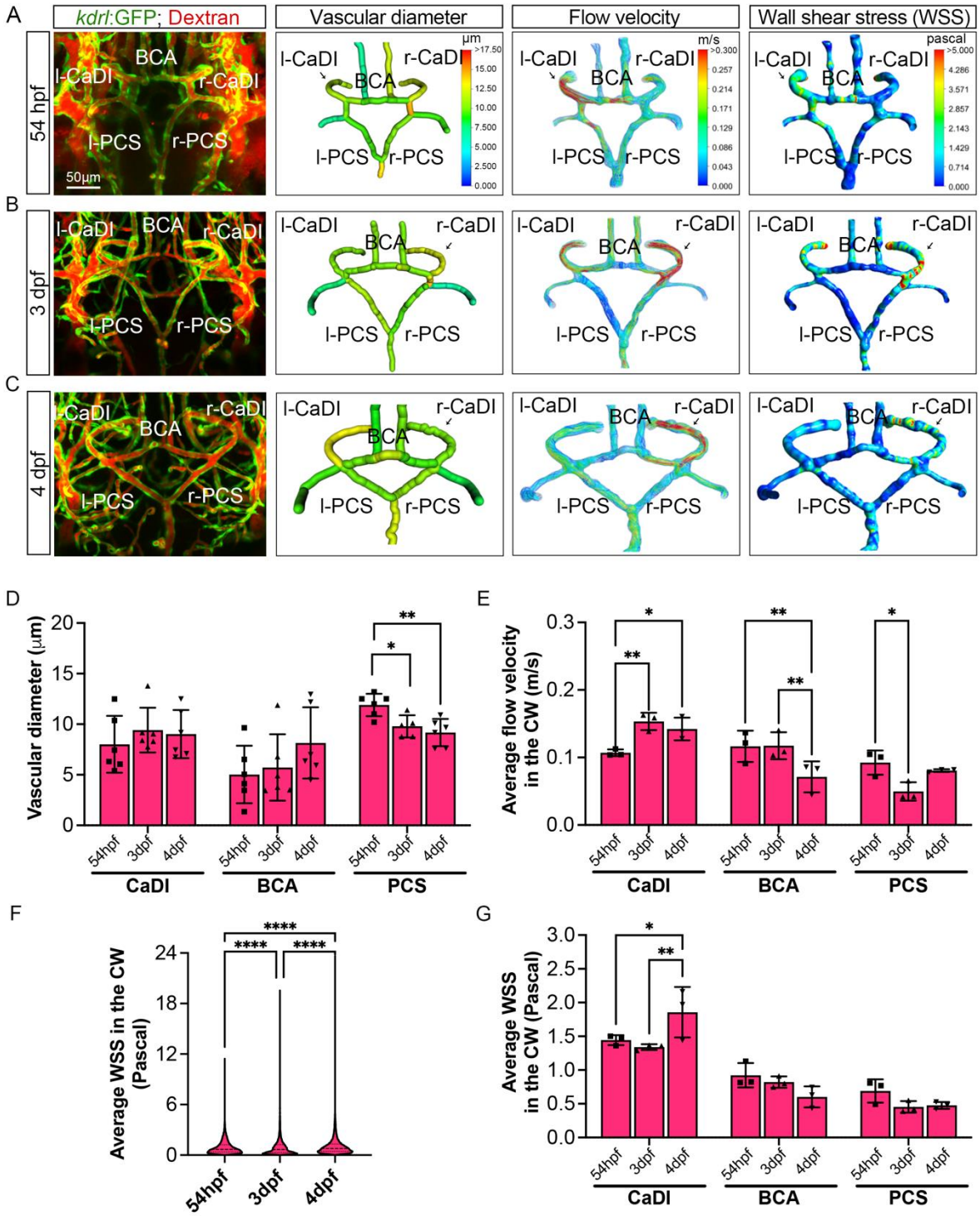
**Figure 2.** Vascular smooth muscle cell (VSMC) differentiation on circle of Willis (CW) arteries

(A-D) Confocal live images of Tg(*acta2:mcherry*, *kdrl:cerulean*)$^{ca8/sd24}$ TgBAC(*pdgfrb:egfp*)$^{ncv22}$ and scheme representation of vascular endothelium and mural cells on CW arteries in zebrafish brain at 32 hour post fertilization (hpf) (A), 54 hpf (B), 3 day post fertilization (dpf) (C), and 4 dpf (D). White channel represents *kdrl:cerulean* fluorescence, Red channel represents *acta2:mcherry*, Green channel represents *pdgfrb:egfp,* Merge 1 panel combines all three channels, Merge 2 combines *acta2:mcherry* in red and *pdgfrb:egfp* in green. Arrows point to the CW arteries with *pdgfrb:egfp* and *acta2:mcherry* signal. Scale bar = 50 µm

(E) Number of *pdgfrb*+ vascular mural cell progenitors per 100 µm vessel length on caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 32 hpf (n=3, 1 independent experiment), 54 hpf (n=6, 1 independent experiment), 3 dpf (n=6, 1 independent experiment), and 4 dpf (n=5, 1 independent experiment), two-way analyses of variance followed by Tukey's multiple comparisons, represented with mean ± SD, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

(F) Number of *acta2*+ VSMCs per 100 µm vessel length on CaDI, BCA, and PCS at 32 hpf (n=5, 1 independent experiment), 54 hpf (n=6, 2 independent experiments), 3 dpf (n=24, 6 independent experiments), and 4 dpf (n=25, 6 independent experiments), two-way analyses of variance followed by Tukey's multiple comparisons, represented with mean ± SD, **** $p \leq 0.0001$

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, EC: endothelial cell, VSMC: vascular smooth muscle cell, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment


A
kdrl:GFP; Dextran
Vascular diameter
Flow velocity
Wall shear stress (WSS)
54 hpf
3 dpf
4 dpf
BCA
l-CaDI
r-CaDI
l-PCS
r-PCS
50µm
µm
m/s
pascal
B
C
D
Vascular diameter (µm)
54hpf
3dpf
4dpf
CaDI
BCA
PCS
E
Average flow velocity in the CW (m/s)
F
Average WSS in the CW (Pascal)
G
Average WSS in the CW (Pascal)

539

**Figure 3.** Computational fluid dynamic (CFD) simulation of circle of Willis (CW) arteries

(A-C) Confocal live images of Tg(*kdrl:gfp*)$^{zn1}$ injected with dextran and representation of vascular diameter, CFD simulated wall shear stress (WSS), and flow velocities in CW arteries at 54 hour post fertilization (hpf) (A), 3 day post fertilization (dpf) (B), and 4 dpf (C). Arrows point to the CW arteries with high flow velocity and WSS. Scale bar = 50 μm

(D) Average vascular diameter in caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 54 hpf (n=6, 1 independent experiment), 3 dpf (n=6, 1 independent experiment), and 4 dpf (n=6, 1 independent experiment), two-way analyses of variance followed by Tukey's multiple comparisons, * $p \leq 0.05$, ** $p \leq 0.01$

(E) Average flow velocity in CaDI, BCA, and PCS at 54 hpf (n=3, 1 independent experiment), 3 dpf (n=3, 1 independent experiment), and 4 dpf (n=3, 1 independent experiment), two-way analyses of variance followed by Tukey's multiple comparisons, * $p \leq 0.05$, ** $p \leq 0.01$

(F) Average wall shear stress (WSS) throughout the CW arteries at 54 hpf (n=3, 1 independent experiment), 3 dpf (n=3, 1 independent experiment), and 4 dpf (n=3, 1 independent experiment), two-way analyses of variance followed by Tukey's multiple comparisons, **** $p \leq 0.0001$

(G) Average wall shear stress (WSS) in CaDI, BCA, and PCS at 54 hpf (n=3, 1 independent experiment), 3 dpf (n=3, 1 independent experiment), and 4 dpf (n=3, 1 independent experiment), two-way analyses of variance followed by Tukey's multiple comparisons, * $p \leq 0.05$, ** $p \leq 0.01$

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, WSS; wall shear stress, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment

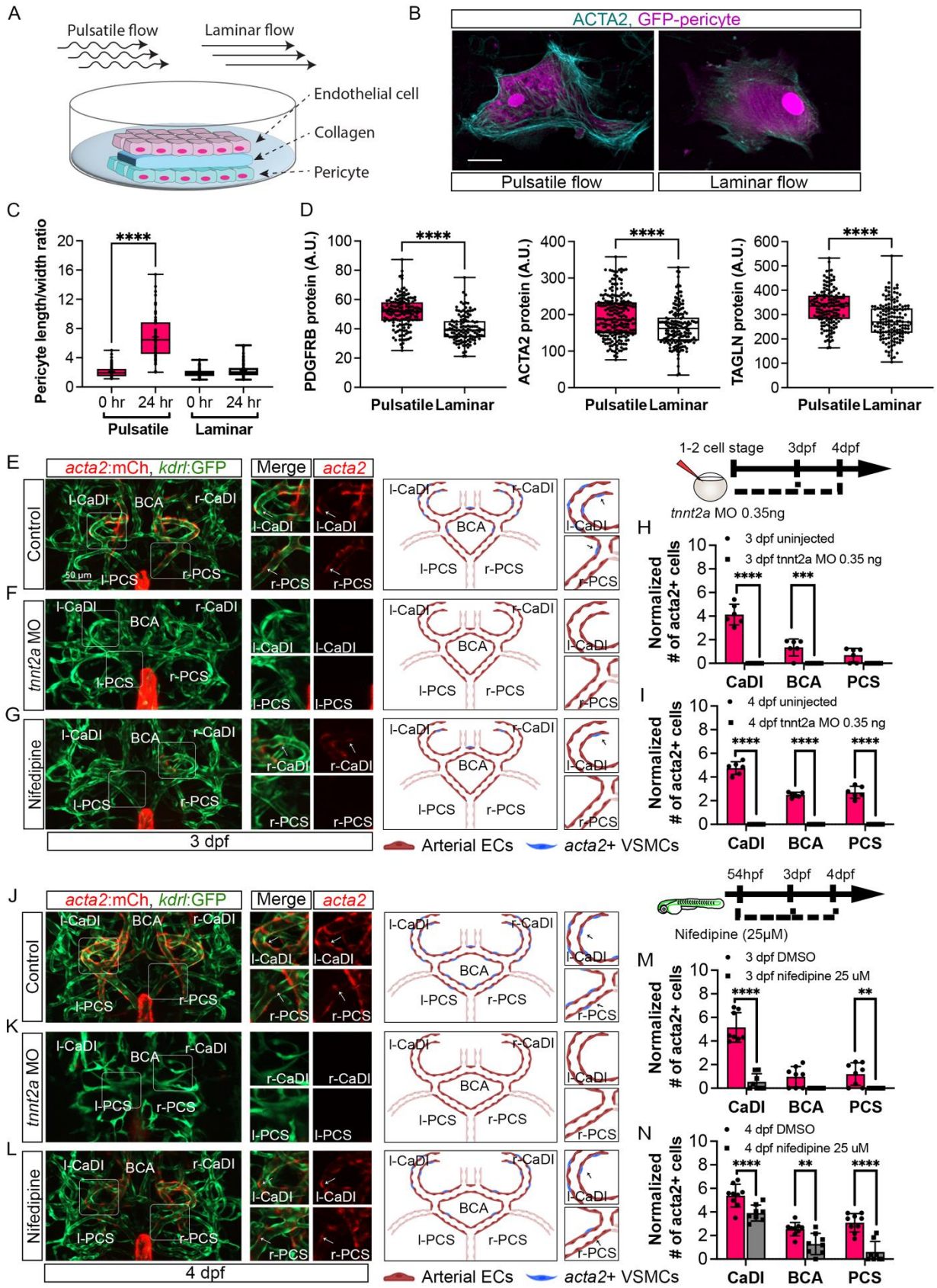

A
Pulsatile flow
Laminar flow
Endothelial cell
Collagen
Pericyte
B
ACTA2, GFP-pericyte
Pulsatile flow
Laminar flow
C
Pericyte length/width ratio
****
0 hr
24 hr
0 hr
24 hr
Pulsatile
Laminar
D
PDGFRB protein (A.U.)
ACTA2 protein (A.U.)
TAGLN protein (A.U.)
****
Pulsatile
Laminar
E
F
G
acta2:mCh, kdrl:GFP
Merge
acta2
Control
tnnt2a MO
Nifedipine
l-CaDI
r-CaDI
BCA
l-PCS
r-PCS
50 µm
3 dpf
Arterial ECs
acta2+ VSMCs
1-2 cell stage
3dpf
4dpf
tnnt2a MO 0.35ng
H
3 dpf uninjected
3 dpf tnnt2a MO 0.35 ng
Normalized # of acta2+ cells
CaDI
BCA
PCS
I
4 dpf uninjected
4 dpf tnnt2a MO 0.35 ng
J
K
L
4 dpf
54hpf
3dpf
4dpf
Nifedipine (25µM)
M
3 dpf DMSO
3 dpf nifedipine 25 uM
N
4 dpf DMSO
4 dpf nifedipine 25 uM

**Figure 4.** Blood flow is required for vascular smooth muscle cell (VSMC) differentiation on circle of Willis (CW) arteries

(A) Scheme representation of *in vitro* cell co-culture experiment

(B) Representative immunofluorescence images of brain pericytes after exposure of pulsative flow and laminar flow. Cells were stained for ACTA2 (cyan), and cytosolic GFP label (magenta). Scale bar = 10 μm

(C) Morphological measurement of brain pericyte length/width ratio before and after exposure of pulsative flow and laminar flow, 3 independent experiments, two-tailed Mann–Whitney test, represented with mean ± SD, **** p≤0.0001

(D) Protein level of PDGFRB (3 independent experiment), ACTA2 (5 independent experiment), and TAGLN (TRANSGELIN, 3 independent experiment) in arbitrary unit (A.U.) after exposure of pulsative flow and laminar flow, two-tailed Mann–Whitney test, represented with mean ± SD, **** p≤0.0001

(E-G) Confocal live images of Tg(*acta2:mcherry*; *kdrl:gfp*)$^{ca8/zn1}$ and scheme representation of vascular endothelium and VSMCs on CW arteries at 3 day post fertilization (dpf) in control embryos (E), embryos injected with 0.35 ng *tnnt2a* morpholino (MO) (F), embryos treated with 25 μM nifedipine from 54 hour post fertilization (hpf) (G). Red channel represents *acta2:mcherry*, Green channel represents *kdrl:gfp*, and Merge panel combines both channels. Arrows point to the CW arteries with *acta2:mcherry* signal. Scale bar = 50 μm

(H) Number of *acta2*+ VSMCs per 100 μm vessel length on caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 3 dpf in uninjected control (n=6, 1 independent experiment) and embryos injected with 0.35 ng *tnnt2a* MO at one to two cell stage (n=6, 1 independent experiment), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, *** p≤0.001, **** p≤0.0001

(I) Number of *acta2*+ VSMCs per 100 μm vessel length on CaDI, BCA, and PCS at 4 dpf in uninjected control (n=6, 1 independent experiment) and embryos injected with

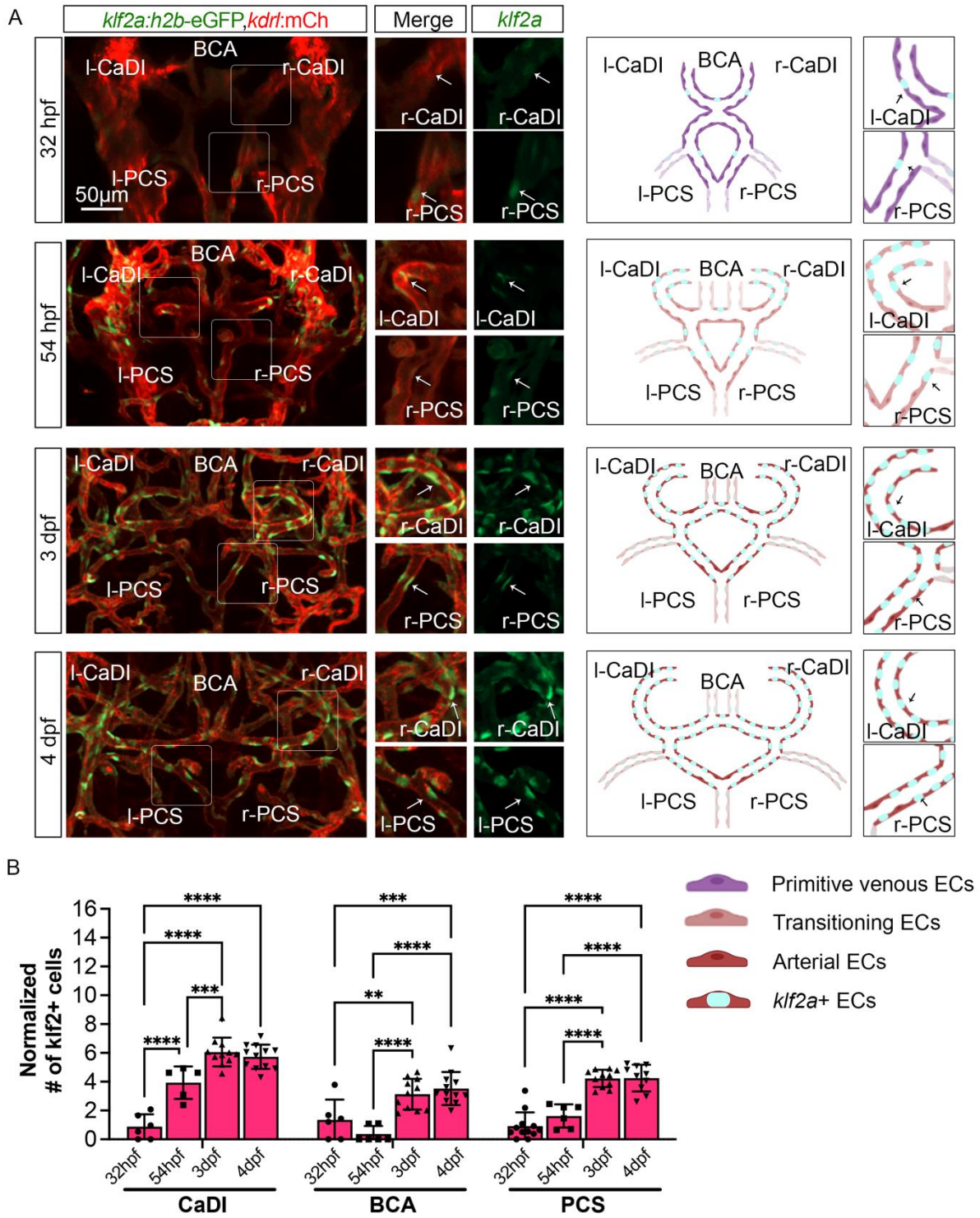
0.35 ng *tnnt2a* MO at one to two cell stage (n=6, 1 independent experiment), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, **** p≤0.0001

(J-L) Confocal live images of Tg(*acta2:mcherry*; *kdrl:gfp*)$^{ca8/zn1}$ and scheme representation of vascular endothelium and VSMCs on CW arteries at 4 dpf in control embryos (J), embryos injected with 0.35 ng *tnnt2a* MO (K), embryos treated with 25 μM nifedipine from 54 hpf (L). Red channel represents *acta2:mcherry*, Green channel represents *kdrl:gfp*, and Merge panel combines both channels. Arrows point to the CW arteries with *acta2:mcherry* signal. Scale bar = 50 μm

(M) Number of *acta2*+ VSMCs per 100 μm vessel length on CaDI, BCA, and PCS at 3 dpf in DMSO control (n=8, 2 independent experiments) and embryos treated with 25 μM nifedipine from 54 hpf (n=8, 2 independent experiments), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, ** p≤0.01, **** p≤0.0001

(N) Number of *acta2*+ VSMCs per 100 μm vessel length on CaDI, BCA, and PCS at 4 dpf in DMSO control (n=9, 2 independent experiments) and embryos treated with 25 μM nifedipine from 54 hpf (n=8, 2 independent experiments), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, ** p≤0.01, **** p≤0.0001

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, EC: endothelial cell, VSMC: vascular smooth muscle cell, MO: morpholino, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment


A
klf2a:h2b-eGFP,kdrl:mCh
Merge
klf2a
32 hpf
54 hpf
3 dpf
4 dpf
BCA
l-CaDl
r-CaDl
l-PCS
r-PCS
50µm
B
Normalized # of klf2+ cells
16
14
12
10
8
6
4
2
0
****
***
**
32hpf
54hpf
3dpf
4dpf
CaDl
BCA
PCS
Primitive venous ECs
Transitioning ECs
Arterial ECs
klf2a+ ECs

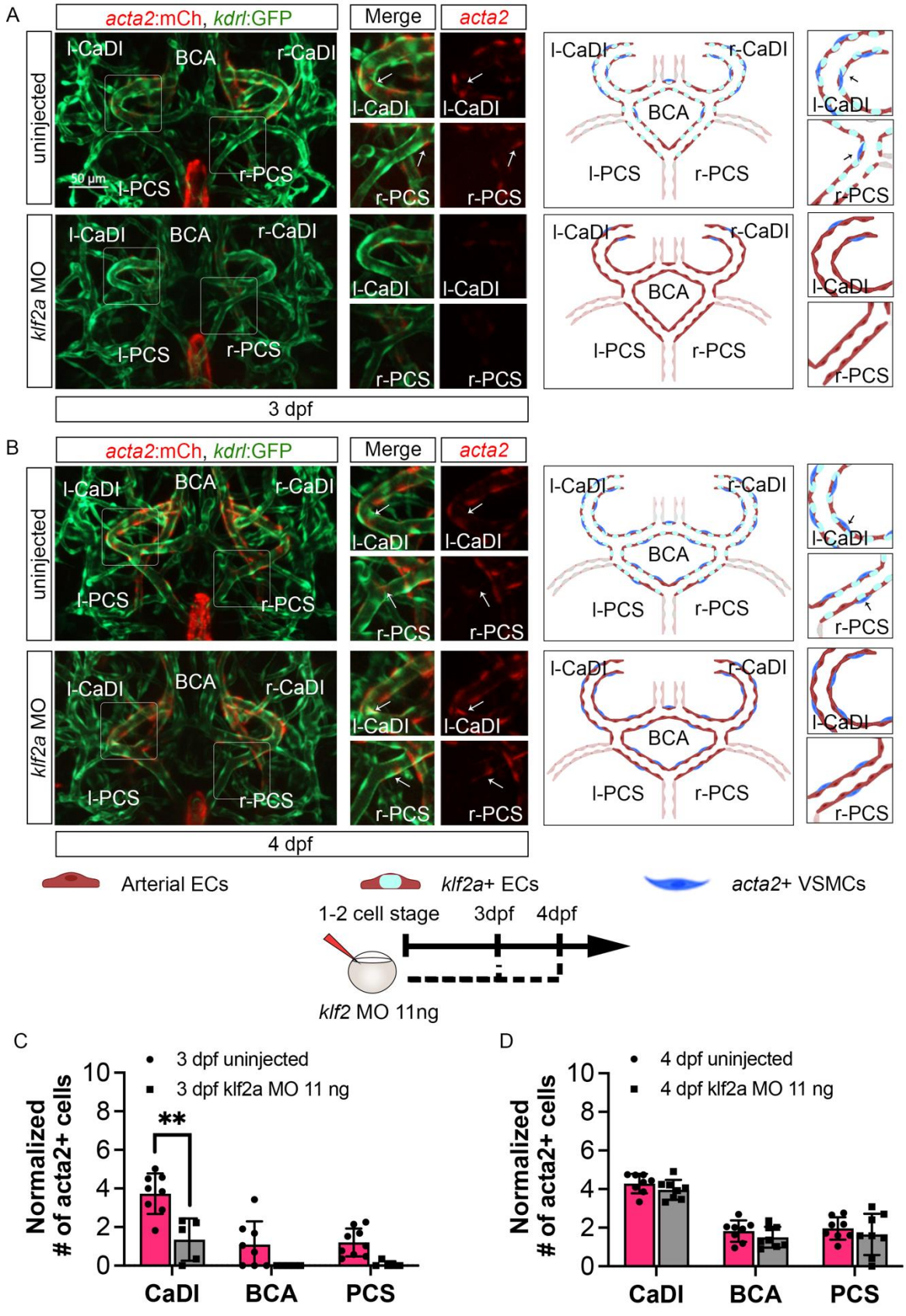
617

**Figure 5.** Blood flow regulated transcription factor *klf2a* is expressed in circle of Willis (CW) arteries

(A) Confocal live images of Tg(*klf2a:h2b-gfp*, *kdrl:ras-mcherry*)$^{ig11/s896}$ and scheme representation of endothelial cells (ECs) in CW arteries in zebrafish brain at 32 hour post fertilization (hpf), 54 hpf, 3 day post fertilization (dpf), and 4 dpf. Green channel represents *klf2a:h2b-gfp*, Red channel represents *kdrl:ras-mcherry*, and Merge panel combines both channels. Arrows point to the CW arteries with *klf2a:h2b-gfp* signal. Scale bar = 50 μm

(B) Number of *klf2a*+ ECs per 100 μm vessel length on caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 32 hpf (n=6, 3 independent experiments), 54 hpf (n=6, 2 independent experiments), 3 dpf (n=11, 3 independent experiments), and 4 dpf (n=12, 3 independent experiments), two-way analyses of variance followed by Tukey's multiple comparisons, represented with mean ± SD, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, EC: endothelial cell, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment


A
acta2:mCh, kdrl:GFP
Merge
acta2
uninjected
klf2a MO
l-CaDI
BCA
r-CaDI
l-PCS
r-PCS
50 µm
3 dpf
B
4 dpf
Arterial ECs
klf2a+ ECs
acta2+ VSMCs
1-2 cell stage
3dpf
4dpf
klf2 MO 11ng
C
3 dpf uninjected
3 dpf klf2a MO 11 ng
Normalized # of acta2+ cells
**
CaDI
BCA
PCS
D
4 dpf uninjected
4 dpf klf2a MO 11 ng

638

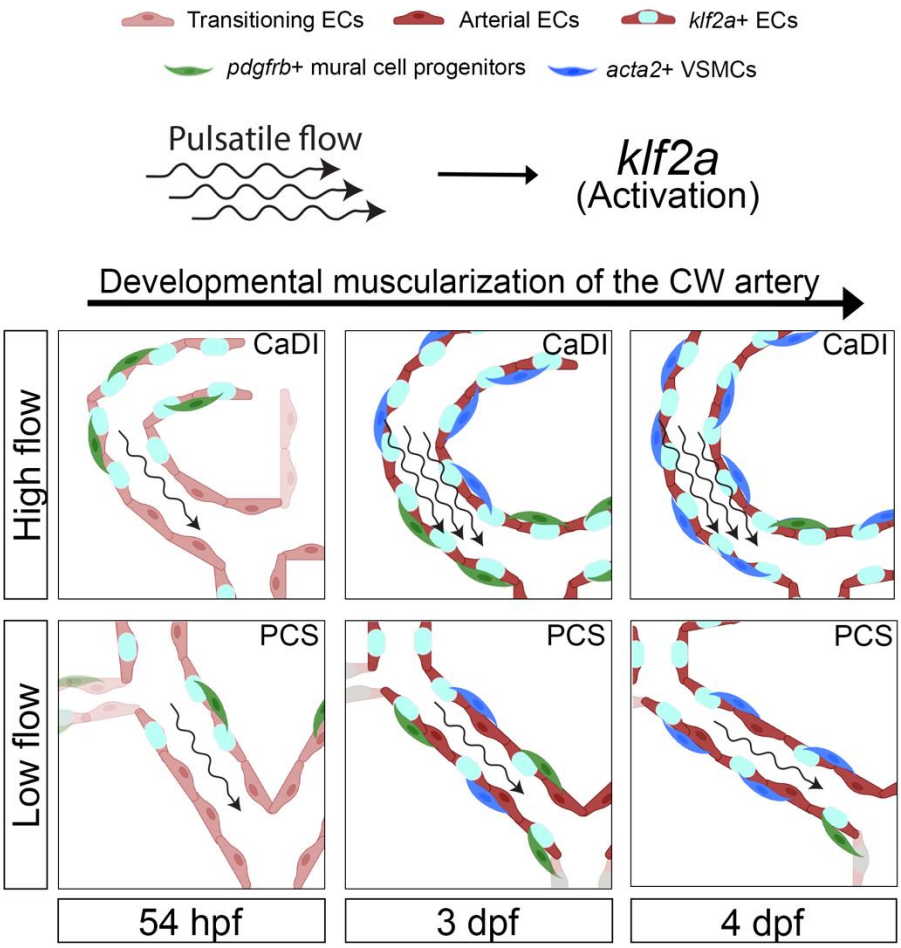
**Figure 6.** *klf2a* promotes vascular smooth muscle cell (VSMC) differentiation on anterior circle of Willis (CW) arteries

(A-B) Confocal live images of Tg(*acta2:mcherry*; *kdrl:gfp*)$^{ca8/zn1}$ and scheme representation of vascular endothelium and VSMCs on CW arteries at 3 dpf (A) and 4 dpf (B) in uninjected control embryos, embryos injected with 11 ng *klf2a* morpholino (MO). Red channel represents *acta2:mcherry*, Green channel represents *kdrl:gfp*, and Merge panel combines both channels. Arrows point to the CW arteries with *acta2:mcherry* signal. Scale bar = 50 μm

(C) Number of *acta2*+ VSMCs per 100 μm vessel length on caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 3 dpf in uninjected control (n=8, 2 independent experiments) and embryos injected with 11 ng *klf2a* MO at one to two cell stage (n=8, 2 independent experiments), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, ** $p \leq 0.01$

(D) Number of *acta2*+ VSMCs per 100 μm vessel length on CaDI, BCA, and PCS at 4 dpf in uninjected control (n=8, 3 independent experiments) and embryos injected with 11 ng *klf2a* MO at one to two cell stage (n=8, 3 independent experiments), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, EC: endothelial cell, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment


Transitioning ECs
Arterial ECs
klf2a+ ECs
pdgfrb+ mural cell progenitors
acta2+ VSMCs
Pulsatile flow
klf2a
(Activation)
Developmental muscularization of the CW artery
High flow
Low flow
CaDI
PCS
54 hpf
3 dpf
4 dpf

661

**Figure 7.** Schematic model of the developmental muscularization of the CW arteries

The model shows how pulsatile flow generate higher hemodynamics in anterior CW arteries like the caudal division of internal carotid artery (CaDI) via the activation of endothelial *klf2a* signaling. Other posterior CW arteries with straight shape like the posterior communicating segment (PCS) experience less hemodynamic force and showed moderate *klf2a* activation and VSMC differentiation.

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, EC: endothelial cell, VSMC: vascular smooth muscle cell, CaDI: caudal division of internal carotid artery, PCS: posterior communicating segment

**Supplementary Figures and Supplementary Figure Legends**

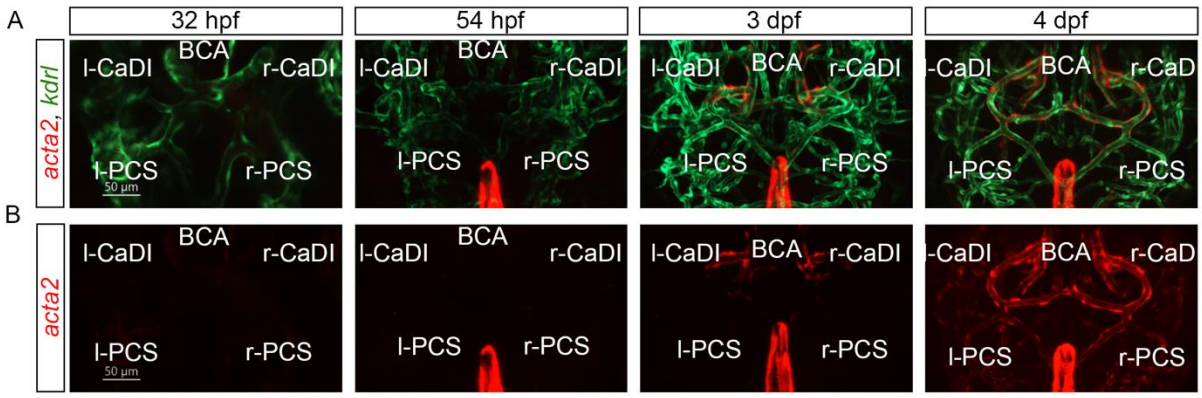
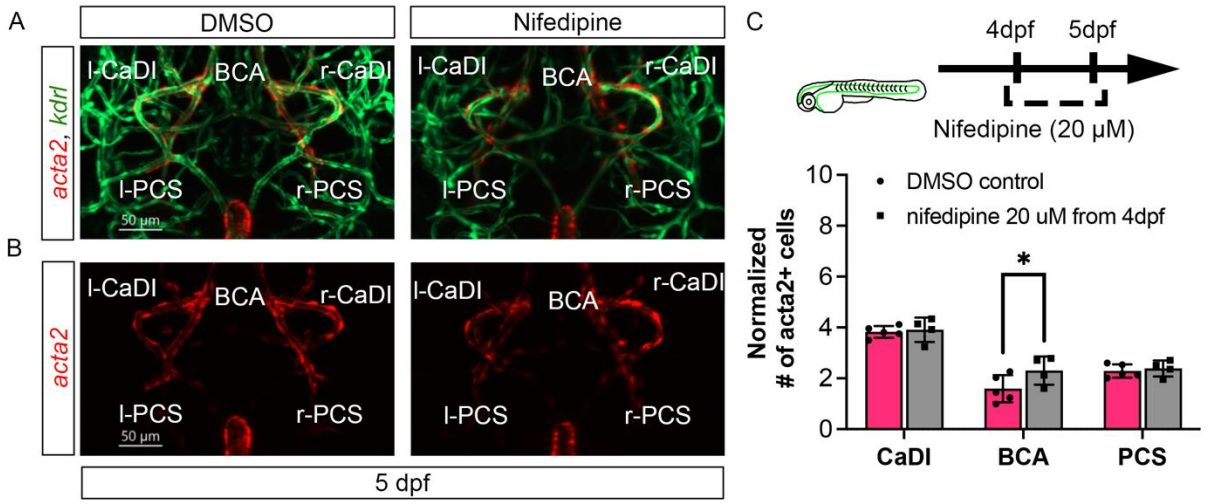

A
32 hpf
54 hpf
3 dpf
4 dpf
acta2, kdrl
BCA
l-CaDI
r-CaDI
l-PCS
r-PCS
50 µm
B
acta2

**Figure S1.** Vascular smooth muscle cell (VSMC) differentiation on circle of Willis (CW) arteries

(A-B) Confocal live images of Tg(*acta2:mcherry*, *kdrl:gfp*)$^{ca8/zn1}$ in CW arteries of zebrafish brain at 32 hour post fertilization (hpf), 54 hpf, 3 day post fertilization (dpf), and 4 dpf. Red channel represents *acta2:mcherry*, Green channel represents *kdrl:gfp*, and Merge panel combines both channels. Scale bar = 50 μm

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, VSMC: vascular smooth muscle cell, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment


A
DMSO
Nifedipine
acta2, kdrl
l-CaDI
BCA
r-CaDI
l-PCS
r-PCS
50 µm
B
acta2
5 dpf
C
4dpf
5dpf
Nifedipine (20 µM)
DMSO control
nifedipine 20 uM from 4dpf
Normalized # of acta2+ cells
CaDI
BCA
PCS

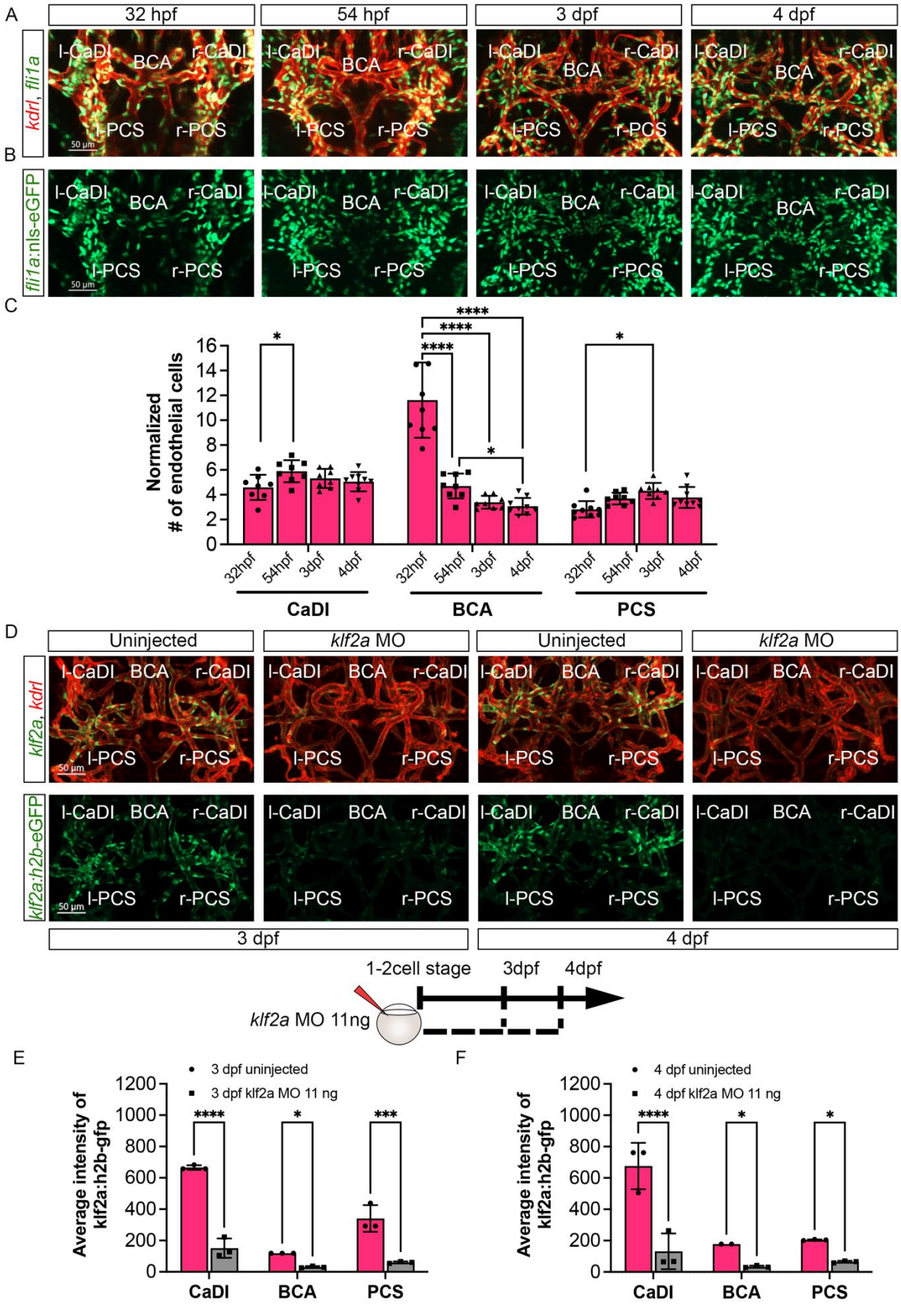
685

**Figure S2.** Blood flow is not required for vascular smooth muscle cell (VSMC) maintenance on circle of Willis (CW) arteries

(A-B) Confocal live images of Tg(*acta2:mcherry*; *kdrl:gfp*)$^{ca8/zn1}$ in CW arteries of zebrafish brain at 5 day post fertilization (dpf) in DMSO control embryos and embryos treated with 20 μM nifedipine from 4 dpf. Red channel represents *acta2:mcherry*, Green channel represents *kdrl:gfp*, and Merge panel combines both channels. Scale bar = 50 μm

(C) Number of *acta2*+ VSMCs per 100 μm vessel length on caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 5 dpf in DMSO control (n=5, 1 independent experiment) and embryos treated with 20 μM nifedipine from 4 dpf (n=4, 1 independent experiment), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, * $p \leq 0.05$

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, VSMC: vascular smooth muscle cell, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment


A
32 hpf
54 hpf
3 dpf
4 dpf
kdrl, fli1a
l-CaDI
BCA
r-CaDI
l-PCS
r-PCS
50 µm
B
fli1a:nls-eGFP
C
Normalized # of endothelial cells
32hpf
54hpf
3dpf
4dpf
CaDI
BCA
PCS
D
Uninjected
klf2a MO
klf2a, kdrl
klf2a:h2b-eGFP
1-2cell stage
3dpf
4dpf
klf2a MO 11ng
E
3 dpf uninjected
3 dpf klf2a MO 11 ng
Average intensity of klf2a:h2b-gfp
F
4 dpf uninjected
4 dpf klf2a MO 11 ng

704

**Figure S3**. Number of endothelial cells (ECs) in circle of Willis (CW) arteries does not increase during *klf2a* activation

(A-B) Confocal live images of Tg(*fli1:nls-gfp*, *kdrl:ras-mcherry*)$^{y7/s896}$ in CW arteries of zebrafish brain at 32 hour post fertilization (hpf), 54 hpf, 3 day post fertilization (dpf), and 4 dpf. Red channel represents *kdrl:ras-mcherry* and Green channel represents *fli1:nls-gfp*. Scale bar = 50 μm

(C) Number of ECs per 100 μm vessel length on caudal division of internal carotid arteries (CaDI), basal communicating artery (BCA), and posterior communicating segments (PCS) at 32 hpf (n=9, 1 independent experiment), 54 hpf (n=8, 1 independent experiment), 3 dpf (n=8, 1 independent experiment), and 4 dpf (n=9, 1 independent experiment), two-way analyses of variance followed by Tukey's multiple comparisons, represented with mean ± SD, * p≤0.05, **** p≤0.0001

(D) Confocal live images of Tg(*klf2a:h2b-gfp*, *kdrl:ras-mcherry*)$^{ig11/s896}$ in CW arteries of zebrafish brain at 3 and 4 dpf in uninjected control and embryos injected with 11 ng of *klf2a* morpholino (MO). Red channel represents *kdrl:ras-mcherry* and Green channel represents *klf2a:h2b-gfp*. Scale bar = 50 μm

(E) Average intensity of *klf2a:h2b-gfp* in CaDI, BCA, and PCS at 3 dpf in uninjected control (n=3, 1 independent experiment) and embryos injected with 11 ng of *klf2a* MO (n=3, 1 independent experiment), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, * p≤0.05, *** p≤0.001, **** p≤0.0001

(F) Average intensity of *klf2a:h2b-gfp* in CaDI, BCA, and PCS at 4 dpf in uninjected control (n=3, 1 independent experiment) and embryos injected with 11 ng of *klf2a* MO (n=3, 1 independent experiment), two-tailed Mann–Whitney test on each vessel's comparison, represented with mean ± SD, * p≤0.05, **** p≤0.0001

Abbreviations: hpf: hour post fertilization, dpf: day post fertilization, l-CaDI: left caudal division of internal carotid artery, r-CaDI: right caudal division of internal carotid artery, BCA: basal communicating artery, l-PCS: left posterior communicating segment, r-PCS: right posterior communicating segment

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