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Polo-like kinase 2 regulates angiogenic sprouting and blood vessel development

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ABSTRACT

Angiogenesis relies on specialized endothelial tip cells to extend toward guidance cues in order to direct growing blood vessels. Although many of the signaling pathways that control this directional endothelial sprouting are well known, the specific cellular mechanisms that mediate this process remain to be fully elucidated. Here, we show that Polo-like kinase 2 (PLK2) regulates Rap1 activity to guide endothelial tip cell lamellipodia formation and subsequent angiogenic sprouting. Using a combination of high-resolution *in vivo* imaging of zebrafish vascular development and a human umbilical vein endothelial cell (HUVEC) *in vitro* cell culture system, we observed that loss of *PLK2* function resulted in a reduction in endothelial cell sprouting and migration, whereas overexpression of *PLK2* promoted angiogenesis. Furthermore, we discovered that PLK2 may control angiogenic sprouting by binding to PDZ-GEF to regulate RAP1 activity during endothelial cell lamellipodia formation and extracellular matrix attachment. Consistent with these findings, constitutively active RAP1 could rescue the endothelial cell sprouting defects observed in zebrafish and HUVEC *PLK2* knockdowns. Overall, these findings reveal a conserved PLK2-RAP1 pathway that is crucial to regulate endothelial tip cell behavior in order to ensure proper vascular development and patterning in vertebrates.

Introduction

Angiogenesis is a highly integrative and reiterative cellular process involving the migration and proliferation of endothelial cells (EC) to form new blood vessels from pre-existing ones. It is dependent on the specification of specialized EC subtypes (Adams and Alitalo, 2007; De Smet et al., 2009; Lamalice et al., 2007) that play distinct roles in angiogenic sprouting. To this end, endothelial tip cells can extend filopodia- and lamellipodia-like processes to sense growth factors, extracellular matrix (ECM) components and attractive/repulsive cues that direct EC sprouting and migration (Adams and Alitalo, 2007; De Smet et al., 2009; Lamalice et al., 2007). In contrast, endothelial stalk cells, which reside behind the leading endothelial tip cells, proliferate to elongate the growing blood vessel. Recent studies have shown that Vascular endothelial growth factor (VEGF) and Notch signaling tightly control the differentiation of these EC subtypes (Gerhardt et al., 2003; Hellstrom et al., 2007; Roca and Adams, 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). In particular, VEGF can induce tip cell specification and the expression of the Notch ligand Delta-like 4, which in turn activates Notch signaling in neighboring stalk cells to suppress VEGF receptor 2 expression and tip cell behavior. Furthermore, VEGF and other developmental signaling cues (Bedell et al., 2005; Herbert et al., 2009; Larrivee et al., 2009; Lee et al., 2002; Lu et al., 2004; Park et al., 2003; Torres-Vazquez et al., 2004; Weitzman et al., 2008) can also guide endothelial tip cell membrane extensions to control the proper development and patterning of the vascular network (Carmeliet and Tessier-Lavigne, 2005; Mukouyama et al., 2002; Zacchigna et al., 2008).

In order for ECs to directionally sprout and migrate to form the vasculature, a series of organized cellular events are required for endothelial tip cells to extend their membranes and migrate

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toward guidance cues. They initially extend multiple filopodia at their distal tips to sense guidance cues in their environment and subsequently form lamellipodia to create an EC leading edge that protrudes toward the chemotactic signal. These EC protrusions then attach to the ECM at focal adhesions to stabilize the sprouting EC membrane and prepare the endothelial tip cell for subsequent migration. A central cellular component in regulating many of these specific cellular events is the activation of small GTPases (Kiosses et al., 2001; Spindler et al., 2010; Wojciak-Stothard et al., 1998). In response to VEGF, Cdc42 promotes the growth of filopodia and mediates cell polarization through microtubule organization (Petrovic et al., 2007), whereas Rac1, together with PAK, controls lamellipodia generation (Kiosses et al., 1999, 2001; Somanath and Byzova, 2009). Moreover, RhoA/ROCK and Ras-associated protein 1 (Rap1) have also been discovered to regulate angiogenesis through the phosphorylation of focal adhesion kinase (FAK) as well as the activation of β1 and αVβ3 integrins (Carmona et al., 2009; Lakshmikanthan et al., 2011; van Nieuw Amerongen et al., 2003; Zeng et al., 2002). Consistent with these findings, both loss of function of Rap1 and PDZ-GEF, a guanine nucleotide exchange factor (GEF) for Rap1, results in vascular developmental defects in both mouse and zebrafish (Carmona et al., 2009; Chrzanowska-Wodnicka et al., 2008; Lakshmikanthan et al., 2011; Wei et al., 2007). Thus, specific small GTPases control distinct cellular events during EC sprouting and discovering the factors that regulate these small GTPases may provide insight as to how the activity of these small GTPases are coordinated to promote directional EC angiogenic sprouting and migration.

The polo-like kinase (PLK) family proteins have been previously shown to play pivotal roles in regulating the cell cycle (Simmons et al., 1992), including entry into mitosis, centrosome maturation, and exit from mitosis with the initiation of cytokinesis (Liu and Erikson, 2003). They contain two conserved domains – the canonical serine/threonine kinase domain and the non-catalytic polo box domain (PBD), which binds to substrates and targets the kinase to specific subcellular zones (Lowery et al., 2005; Strebhardt, 2010). Upon ablation of the kinase domain, the PBD domain alone becomes a dominant-negative form of PLKs (Seeburg et al., 2008). Despite their roles in cell cycle regulation, recent reports have suggested PLKs may possess additional cellular functions (Strebhardt, 2010). For example, Plk3 has been shown to suppress tumor angiogenesis, and *Plk3*$^{-/-}$ mice developed tumors in various organs at advanced age, with enhanced angiogenesis (Xu et al., 2012, 2010a, 2010b; Yang et al., 2008). Additionally, PLK2 has been observed to control dendritic spine sprouting and the density of synapses in neurons by governing RAP1 activity *via* the regulation of PDZ-GEF (Lee et al., 2011a, 2011b). Interestingly, previous studies have also revealed that human *PLK2* transcripts can be observed in human fetal lung, kidney, spleen and heart (Simmons et al., 1992), and additional expression analysis showed that *PLK2* may also be found more specifically expressed in the vascular system in early developmental stages (Duncan et al., 2001; Zhong et al., 2010), suggesting that PLK2 may also regulate vascular development. Thus, given its role in controlling RAP1 activity, we speculate that during vascular development, PLK2 may also serve to mediate lamellipodia formation and attachment to the ECM in ECs through regulating EC RAP1 function.

Here, we report that PLK2 is expressed in the vascular system and can control angiogenesis during vascular development by specifically regulating the EC lamellipodia but not filopodia formation. Using a combination of high-resolution *in vivo* imaging of zebrafish vascular development and a human umbilical vein endothelial cell (HUVEC) *in vitro* cell culture system, we observed that loss of *PLK2* function resulted in a reduction in EC sprouting and migration, whereas overexpression of *PLK2* promoted angiogenesis. Moreover, PLK2 appears to impart its function through the regulation of RAP1 to mediate focal adhesion and lamellipodia formation in migrating ECs. Overall, our data reveal a conserved PLK2-RAP1 pathway that is crucial to regulate endothelial tip cell behavior in order to ensure proper vascular development and patterning in vertebrates.

Material and methods

Zebrafish strains

Embryos and adult fish were maintained under standard laboratory conditions as described previously (Zhang et al., 2013). The following lines were used: *Tg(kdrl:mcherry-ras)*^{s896} (Chi et al., 2008), *Tg(kdrl:GFP)*^{s843} (Jin et al., 2005), *Tg(fli1a:EGFP)*^{y1} (Lawson and Weinstein, 2002), and *Tg(hsp70:dn-MAML-GFP)*^{fb10} (Zhao et al., 2014).

PLK2 sequence alignment

PLK2 sequence alignment was performed by ClustalW multisequence alignment as described (Hegarty et al., 2013).

Morpholino (MO) knockdown and rescue experiments

To knockdown plk2b function, we used an ATG-MO (MO1) against the 5′UTR adjacent to the translation start site of *plk2b* and a splicing MO (MO2) against the 3′ splice site of exon 2. The MO sequences are: MO1 (ATG-*plk2b*-MO): 5′-GCTGTGTGTTACTGTGCTTTCTGTC-3′ and MO2 (splicing-*plk2b*-MO): 5′-TATGCAGTGTTTATCCTACCTTCTC-3′. Five base pairs (bp) of the ATG MO was altered to create a control 5 bp mismatched MO, which did not cause any discernible phenotypes: (ATG-*plk2b*-control-MO): 5′-GCTcTcTGTTAgTGTcCTTTCTcTC-3′. One-cell stage *Tg(kdrl:mcherry-ras)*, *Tg(fli1a:eGFP)* embryos were injected with 10 ng of MO1, MO2 or control MO. To confirm that MO1 blocked the translation of *plk2b*, the following primers were used to PCR amplify a construct that fuses the *plk2b* 5′UTR region to GFP: *plk2b*-GFP-F: 5′-actgtgacagaaagcacagtaacacacagccATGGTGAGCAAGGGCGAGGA-3′ and GFP-R: 5′-TTACTTGTACAGCTCGTCCA-3′. This *5′UTR plk2b-GFP* construct was subcloned into the pCS2 vector, confirmed by sequencing and then transcribed into capped mRNA using a mMessage mMachine SP6 Transcription kit (Cat. no. AM1340, Life Technologies). 50 pg of the *5′UTR plk2b-GFP* RNA with or without 10 ng of *plk2b* MO1 was injected into zebrafish embryos at the one-cell stage. To evaluate the MO2 function, the following primers were used to detect *plk2b* exon 1-4 mRNA from 50 24 hpf (hour post-fertilization) control or MO2 injected embryos: *plk2b*-sMO-rtF: 5′-GGAAATGTTACTGCCGGGGA-3′, *plk2b*-sMO-rtR: 5′-CGTTTTGCGTCTGTTGCTGA-3′. For the dominant negative *plk2b* (*dn-plk2b*) experiments, the *plk2b* PBD domain fragment was cloned into the pCS2 vector and then *in vitro* transcribed using the mMessage mMachine SP6 Transcription kit (Cat. no. AM1340, Life Technologies). The following primers were used for PCR of the PBD domain: *plk2b*-PBDF 5′-ACTCAGGGCTTCATGCCAGAAACGC-3′, *plk2b*-PBDR: 5′-GTTGCATCGCTGCAGCAGCATGTTGA-3′. One-cell stage *Tg(kdrl:mcherry-ras)* or *Tg(fli1a:eGFP)* embryos were injected with 180 pg of *dn-plk2b* mRNA. For mRNA rescue experiments, the *plk2b* coding sequence (CDS), which does not include the *plk2b* ATG MO1 binding site, and the human *PLK2* CDS were cloned into the pCS2 vector and then *in vitro* transcribed using the mMessage mMachine SP6 Transcription kit (Cat. no. AM1340, Life Technologies). The following primers were used for PCR: *plk2b*-cdsF 5′-ATGGAGACACTAAGGAATAC-3′, *plk2b*-cdsR: 5′-TCAGTTGCATCGCTGCAGCAGCATG-3′, *hPLK2*-cdsF 5′-ATGGAGCTTTTGCGGACTAT-3′, *hPLK2*-cdsR: 5′-TCAGTTACATCTTTGTAAGA-3′. One cell stage *Tg(kdrl:mcherry-ras)* embryos were co-injected with 10 ng of *plk2b* MO1 or MO2 and 80 pg of zebrafish *plk2b* RNA, 80 pg of human *PLK2* RNA, or 100 pg, 160 pg of