Endothelial cell invasion is controlled by dactylopodia

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Sprouting angiogenesis is fundamental for development and contributes to cancer, diabetic retinopathy, and cardiovascular diseases. Sprouting angiogenesis depends on the invasive properties of endothelial tip cells. However, there is very limited knowledge on how tip cells invade into tissues. Here, we show that endothelial tip cells use dactylopodia as the main cellular protrusion for invasion into nonvascular extracellular matrix. We show that dactylopodia and filopodia protrusions are balanced by myosin IA (NMIIA) and actin-related protein 2/3 (Arp2/3) activity. Endothelial cell-autonomous ablation of NMIIA promotes excessive dactylopodia formation in detriment of filopodia. Conversely, endothelial cell-autonomous ablation of Arp2/3 prevents dactylopodia development and leads to excessive filopodia formation. We further show that NMIIA inhibits Rac1-dependent activation of Arp2/3 by regulating the maturation state of focal adhesions. Our discoveries establish a comprehensive model of how endothelial tip cells regulate its protrusive activity and will pave the way toward strategies to block invasive tip cells during sprouting angiogenesis.

Significance

In this report, we describe how endothelial cells, the cells lining the interior of blood vessels, invade into tissues to form new vessels through sprouting angiogenesis. We found that endothelial cells use a specific lamellipodia-related membrane protrusion for invasion, which we termed dactylopodia. These protrusions have a special morphology, originate from filopodia, and are specialized in invading into avascular extracellular matrix. Our work lays the foundations for drug discovery targeting sprouting angiogenesis.

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NMIIA, using the Myh9-eGFP transgenic animals (SI Appendix, Fig. S2A) (23). In addition to higher expression, we found that NMIIA was enriched at the base of filopodia protrusions (Fig. 1A and C and SI Appendix, Fig. S2 B and C). NMIIIB followed a similar pattern to NMIIA. It is highly expressed by endothelial tip cells, and it is enriched at the base of filopodia (SI Appendix, Fig. S2C). This spatial distribution of NMII isoforms correlates with high levels of phospho-myosin light chain and filamentous actin (SI Appendix, Fig. S2 B-D). These results suggest that endothelial tip cells have higher levels of actomyosin contractility at their leading edges, in particular at the base of filopodia. To evaluate the function of NMIIA and NMIIIB in ECs, we deleted Myh9 or Myh10 alone, or both simultaneously, using the PDGFB-CreERT2 line (24). Single deletion of Myh10 in ECs (NMIIIB EC knockout [KO]) did not significantly affect retinal vascular morphogenesis, although we noticed a small decrease in the number of filopodia in endothelial tip cells (SI Appendix, Fig. S3 A-C). Efficient depletion of NMIIIB was observed by immunofluorescence (SI Appendix, Fig. S3B). Single deletion of Myh9 (NMIIA EC-KO) showed a very distinct phenotype. NMIIA EC-KO retinas showed a mild decrease in radial expansion, an increase in vessel density, decreased proliferation, and an increase in the number of tip cells (Fig. 1D–F). In accordance with a mild radial expansion phenotype, vascularization of the superficial in NMIIA EC-KO animals reached the retina margin by postnatal day 12 (P12) (SI Appendix, Fig. S4). Yet the most striking phenotype is a dramatic change in tip cell morphology (Fig. 1E and SI Appendix, Fig. S4A). NMIIA-deficient tip cells showed an abnormal branched morphology with extremely long and enlarged membrane protrusions and a severe decrease in filopodia number (Fig. 1E and SI Appendix, Fig. S5A). Remarkably, the absence of filopodia and enlarged membrane protrusions was tip cell specific, as ECs in nonsprouting areas showed numerous bona fide filopodia morphologically similar to wild-type (WT) control littermates, even if NMIIA-deficient ECs in the plexus show a trend to have a decreased number of filopodia (SI Appendix, Fig. S5 B and C). Immunofluorescence analysis confirmed the efficient depletion of NMIIA (Fig. 1G) and that the NMIIA EC-KO phenotype manifested even in the presence of high levels of NMIIB in tip cells (SI Appendix, Fig. S5D). Despite the notable changes in tip cell morphology, filamentous actin did not appear to be severely compromised; however, detectable actin cables present at the leading edge of ECs were reduced in NMIIA EC-KO tip cells, with the exception of the outer segment apical process (OSAP) (SI Appendix, Fig. S5D). This result suggests that membrane ruffling is driven by actin polymerization. Given that NMIIA WT tip cells also showed substantial filopodia membrane ruffling (Movies S4–S6), we reasoned that the formation of long membrane protrusions might be a particular feature of WT tip cells that become overrepresented in NMIIA EC-KO. Indeed, careful analysis of WT tip cells showed similar morphological membrane protrusions, comparable to the prominent ones observed in NMIIA EC-KO tip cells, yet their frequency and size were much reduced (Fig. 2C–E and SI Appendix, Fig. S8A). In WT tip cells, these membrane protrusions were characterized by a mean length of ~20 μm (ranging from ~5 to ~33 μm) and a mean size of ~1.56 μm (ranging from ~0.65 to ~3.3 μm). Filopodia showed substantial variation in actin and NMIIA, and from which numerous filopodia emanated (Fig. 2C and D and SI Appendix, Fig. S8B). Given their morphology (finger-like protrusions), originated from filopodia and linked to membrane-ruffling activity, we named these naturally occurring protrusions as dactylopodia. Interestingly, the presence of dactylopodia correlated with regions where endothelial tip cells contacted with nonvascular extracellular matrices (ECM), highlighted by decreased staining of the vascular basement membrane marker collagen IV (Fig. 2F and SI Appendix, Fig. S8C). Moreover, dactylopodia were associated with increased levels of active ITGB1, a marker for matrix-bound FAs (SI Appendix, Fig. S8C). Dactylopodia in NMIIA-deficient ECs also correlated with sites of contact with nonvascular extracellular matrix and with activated ITGB1; however, its expression pattern appeared to be more diffused along dactylopodia and not enriched at the base of filopodia, as in WT cells (SI Appendix, Fig. S8C). Altogether, these data suggest that dactylopodia are endothelial tip cell–specific protrusions derived from filopodia that might play roles in invasion and migration into nonvascular ECM.

Next, we investigated the mechanism driving dactylopodia formation in ECs. Actin-based membrane ruffling is known to rely on Arp2/3-dependent, dendritic actin networks (6). To confirm if Arp2/3 is involved in dactylopodia formation, we specifically deleted Arp4, a structurally essential component of the Arp2/3 complex (5, 30), in ECs in vivo. Deletion of Arp4 in ECs, Arp4 EC-KO, led to a very prominent vascular phenotype. ECs without a functional
Fig. 1. (A) Representative images of tip cells from LifeAct-GFP mouse retinas labeled for NMIIA (red), actin (green), and CD31 (blue). Red arrowheads point to sites enriched in NMIIA at the base of filopodia. (Scale bar, 20 μm.) (B) Box plot of Myh9 fluorescence intensity between tip and stalk cells from PDGFB-iCRE::mTmG mouse retinas (n = 14 tip cells and n = 12 stalk cells). P value from unpaired Student’s t test. (C, Upper) Representative image in which red arrowheads point to sites enriched in NMIIA at the base of filopodia. (Scale bar, 20 μm.) (Bottom) Graph of the normalized line-scan fluorescence intensity profile (blue line) for NMIIA and mGFP signals from the endothelial tip cell’s leading edge (n = 6 endothelial tip cells). Lines represent the beginning of the plasma membrane and peak of NMIIA levels. The NMIIA signal shows a peak of intensity subsequent to the beginning of the filopodia burst. (D, Upper) Timeline of tamoxifen injection (tam. inj.) and age killed of mouse pups. The representative images of mouse retinas from Myh9 WT and Myh9 EC-KO are labeled for CD31. (Scale bar, 250 μm.) (E) Representative images of tip cell and filopodia in the angiogenic sprouting front of mouse retinas from Myh9 WT and Myh9 EC-KO labeled for CD31. (Scale bar, 50 μm.) (F) Box plots of vascular outgrowth, vessel density, EC density, EC proliferation, number of tip cells, and number of filopodia per filopodia burst in Myh9 WT (n = 19 retinas) and Myh9 EC-KO (n = 24 retinas) mouse retinas. P value from unpaired Student’s t test. (G, Left) High-magnification images of tip cells labeled for CD31 (blue), F-actin (green), and NMIIA (red) from Myh9 WT and Myh9 EC-KO. (Right) Higher-magnification images from delineated regions in Left for single z-plane of tip cells in two different planes. Red arrowheads point toward leading edges of tip cells; blue arrowheads point toward cortical actin cables in tip cells. (Scale bar, 50 μm.)
Fig. 2. (A) Time-course still images of dactylopodia and filopodia dynamics in \textit{Myh9} fl/wt::PDGFB-iCRE::R26mTmG and \textit{Myh9} fl/fl::PDGFB-iCRE::R26mTmG mouse retinas. Red dashed line contour represents dactylopodia initial state. Blue dashed line contour represents dactylopodia final state. Red arrow indicates filopodia initiation; green arrow indicates filopodia extension; purple arrow indicates filopodia membrane ruffling; and blue arrow indicates filopodia-to-dactylopodia conversion. (Scale bar, 10 μm.) (B) Box plots of filopodia initiation rate per tip cell (filopodia/second) (\textit{Myh9} WT \textit{n} = 10 tip cells and \textit{Myh9} EC-KO \textit{n} = 11 tip cells) and filopodia membrane ruffling (ruffling/second) in \textit{Myh9} WT (\textit{Myh9} WT \textit{n} = 10 filopodia) and \textit{Myh9} EC-KO (\textit{Myh9} EC-KO \textit{n} = 9 filopodia) mouse retinas. \textit{P} value from unpaired Student’s \textit{t} test. (C) Representative images of a tip cell from a PDGFB-iCre::R26mTmG mouse retina labeled for NMIIA (red), cell membrane (green), and CD31 (blue), highlighting dactylopodia in tip cells (red dashed line) with numerous filopodia in their extremity. (Scale bar, 20 μm.) (D) Representative images of \textit{Myh9} WT and \textit{Myh9} EC-KO mouse retinas labeled for NMIIA (green) and CD31 (blue). (Scale bar, 10 μm.) Red dashed line contours dactylopodia. (E) Box plots of number of dactylopodia (<20 μm or >20 μm) in \textit{Myh9} WT (\textit{n} = 4 retinas) and \textit{Myh9} EC-KO mouse retinas (\textit{n} = 5 retinas) and dactylopodium width and length in \textit{Myh9} WT (\textit{n} = 7 retinas) and \textit{Myh9} EC-KO mouse retinas (\textit{n} = 8 retinas). \textit{P} value from unpaired Student’s \textit{t} test. (F) Representative images of dactylopodia in tip cells from a PDGFB-iCre::R26mTmG (green) P6 mouse retina labeled for vascular endothelial–cadherin (blue), Col.IV (red), and nuclei (DAPI, gray). (Scale bar, 20 μm.) Red dashed line contours dactylopodia.
Arp2/3 complex were unable to invade into avascular areas, leading to a dramatic reduction in radial expansion at P6 (Fig. 3 A–C). Higher magnification of tip cells demonstrated that Arpc4-deficient cells had a reduced number of dactylopodia, which correlated with an increase in the number and length of filopodia (Fig. 3 B, D, and E). Strikingly, Arp4 EC-KO phenotype inversely mirrors NMIIA EC-KO phenotype in terms of tip cells' filopodia and dactylopodia number and morphology (compare Figs. 1 E and F and 3 B and C). Yet Arp2/3 deficiency did not alter NMIIA distribution in endothelial tip cells (Fig. 3D). Analysis of P12 retinas showed that radial expansion was completely abrogated, as radial outgrowth was mostly at the same distance from the optic nerve as in P6 retinas (SI Appendix, Fig. S9A). Altogether, we conclude that dactylopodia are formed by Arp2/3-dependent actin polymerization and that endothelial tip cell invasiveness is entirely determined by Arp2/3 activity. Moreover, it proves that the presence of filopodia is insufficient for tip cell invasion.

Next, we investigated how the balance between filopodia and dactylopodia in endothelial tip cells is established. Remarkably, NMIIA KO phenotype was characterized by a loss of filopodia and a gain in dactylopodia, while Arp4c EC-KO displayed excessive filopodia and a reduced number of dactylopodia. Moreover, we showed that dactylopodia originated from filopodia. Thus, we hypothesized that NMIIA could balance the ability of tip cells to form filopodia over dactylopodia by regulating Arp2/3 complex activity. A prediction from this model is that inhibition of the Arp2/3 complex in NMIIA-deficient cells should rescue the ability of ECs to produce filopodia. To test this hypothesis, we turned to in vitro human umbilical vein EC (HUVEC) cultures. ECs’ growth on glass or plastic did not show the ability to form long filopodia. However, when seeded on top of fibroblast monolayers, HUVECs acquired this capacity (31). However, in both in vitro conditions, HUVECs did not display protrusions similar to dactylopodia but rather standard lamellipodia. DMSO-treated or CK666-treated (a specific inhibitor of Arp2/3) cells have a similar number of filopodia in normal conditions. In contrast, blebbistatin (BBS), an inhibitor of NMII activity, abrogated filopodia formation. Remarkably, filopodia formation in BBS-treated cells was partially rescued by cotreatment with CK666 (SI Appendix, Fig. S10A and B). These results were further confirmed by small interfering RNA-mediated knockdown of NMIIA and Arp2/3 complex (Fig. S12A and B). To these observations, we used an optogenetic tool that allows timed and local activation of Cdc42 (32), the main regulator of EC protrusions in vivo and in vitro (13). We observed that, in DMSO-treated conditions, Cdc42 activation led to high lamellipodia activity and few filopodia, which was reverted by an inhibitor of Arp2/3 (CK666) (Fig. 4 A and B and Movies S14 and S15). In contrast, BBS treatment abrogated filopodia formation (Fig. 4 A and B and Movie S16). The capacity of forming filopodia in BBS-treated cells was restored by cotreatment with CK666 (Fig. 4 A and B and Movie S17), supporting the hypothesis that NMII activity promotes filopodia formation by limiting the activation of Arp2/3. To verify if NMIIA EC-KO leads to a lack of filopodia and an excess of dactylopodia due to unrestrained Arp2/3 activity in vivo, we generated a double loss of function of both Arp2/3 and NMIIA by crossing NMIIA EC-KO and Arp4c EC-KO mice. Overall, Arp4c/NMIIA EC double KO mice showed a phenotype very similar to Arp4c EC-KO, with a strong reduction in radial expansion and compaction at the vascular front (Fig. 4 C and D). Yet abrogation of Arp2/3 rescued filopodia formation in NMIIA-deficient ECs in vivo (Fig. 4 E and F and SI Appendix, Fig. S9B). Altogether, these results demonstrate that NMIIA enables filopodia stability by restricting Arp2/3 activation in endothelial tip cells in vivo.

We next investigated the mechanism by which NMIIA limits Arp2/3 activity. Arp2/3 is activated by WASP/WAVE complexes downstream of the Rho GTPases, Rac1 and Cdc42 (33). A common trigger for Rac1/Cdc42 activation and migration is signaling from immature FAs at the leading edge of cells (33, 34). Our observations showed that Arp2/3-dependent dactylopodia correlated with local invasion into extravascular matrices (Fig. 2F and SI Appendix, Fig. S7), suggesting that integrin-mediated signaling could activate Arp2/3 when engaging with extravascular matrices. In accordance, ITGB1 EC-KO shows striking similarities with Arp4c EC-KO, including excessive and longer filopodia formation, blunted sprouting front (reminiscent of a lack of dactylopodia), and severely reduced invasion to deeper layers (20). Moreover, it is well established that NMIIA-dependent activity promotes maturation of integrin adhesions (35). Thus, we hypothesized that Fas could be a platform promoting the cross-talk between NMIIA and Arp2/3 complex at the leading edge of tip cells. To evaluate the state of Fas, we analyzed integrins (ITGAI5 and activated ITGB1) and phosphorylated paxillin (pPAX, a marker for more mature FAs). WT tip cells showed high levels of pPAX and activated ITGB1 at the base of filopodia and in dactylopodia but also an enrichment of both ITGAI5 and ITGB1 (Fig. 5A and SI Appendix, Fig. S11 A and B). NMIIA-deficient tip cells showed a significant reduction of pPAX, and signals for both ITGAI5 and activated ITGB1 were diffused all over dactylopodia when compared to WT tip cells (Fig. 5 A and B and SI Appendix, Fig. S11 A and B). These observations suggest that the inhibition of NMIIA leads to significant differences in integrin location and activation state. Similar results were observed in ECs in vitro. BBS-mediated inhibition of NMII in HUVEC fibroblasts cocultures led to a strong decrease in the number of mature fibrillar Fas and a strong decrease in pPAX, both at immunofluorescence and Western blot level, while CK666 treatment did not modify levels of pPAX (Fig. 5 C and D and SI Appendix, Fig. S12 A–C), which correlates with a decrease in the number of filopodia (SI Appendix, Fig. S10). Our results are suggestive of a mechanism in which immature/nascent adhesions would activate Arp2/3, and NMIIA inhibits this signaling axis by promoting maturation of Fas, as previously reported (35, 36). We next assessed if BBS treatment would promote activation of Rac1, a major positive regulator of Arp2/3 in ECs downstream of integrin signaling. We observed that BBS treatment increased the levels of Rac1-GTP, the active form of Rac1, when compared to DMSO treatment (Fig. 5E). Accordingly, inhibition of Rac1 activation with NSC23766 led to a rescue of filopodia formation in BBS-treated cells (Fig. 5G and SI Appendix, Fig. S13). Tiam1, DOCK180, and Arhgef7/P-PIX were previously implicated in Rac1 activation downstream of integrins (37). In addition, previous work showed that NMIIA inhibits P-PIX recruitment to and activation by FAs (36). Thus, we tested if knockdown of P-PIX (SI Appendix, Fig. S14A) led to a partial rescue in filopodia number in BBS-treated ECs (Fig. 5 H and I and SI Appendix, Fig. S14B). Taken together, these results suggest that NMIIA balances dactylopodia and filopodia formation by promoting FA maturation and thereby limiting Arp2/3 activation through inhibition of Rac1 activation downstream of integrin P-PIX in nascent adhesions.

Discussion

Deciphering the mechanisms employed by ECs to migrate and invade is essential to understand sprouting angiogenesis and to develop novel anti-angiogenic therapies. Altogether, these results demonstrate 1) that Arp2/3 activity is necessary for endothelial tip cell invasiveness and formation of proinvasive dactylopodia; 2) that dactylopodia derive from filopodia; 3) that filopodia are not required for tip cell migration; and 4) that NMIIA enables filopodia stability by restricting Arp2/3 activation in endothelial tip cells in vivo. We found that invasiveness of endothelial tip cells during angiogenesis in vivo depends on the formation of specific Arp2/3-dependent protrusions, the dactylopodia. We found that dactylopodia are derived from filopodia at sites in contact.
with nonvascular extracellular matrix. Our findings also support the idea that filopodia are not per se sufficient for EC invasion and migration, as previously reported (21). Yet filopodia play a regulatory role as they serve as dactylopodia precursors. Interestingly, our live-imaging analysis showed that only a very small number of filopodia showed membrane-ruffling activity, and WT tip cells present an excess number of filopodia (∼30) over dactylopodia (∼4). Thus, the mechanisms allowing dactylopodia formation must be tightly regulated.

Previous studies have highlighted that Arp2/3 and formins compete with each other to regulate the organization of filamentous actin and to dictate the type of cellular protrusions (38, 39). Remarkably, we show that NMIIA balances the relative proportion of two types of cellular protrusions, favoring filopodia over dactylopodia, a lamellipodia-like structure. This is rather surprising as increased contractility is generally inversely correlated with protrusive activity, as shown in several different models (9, 10). Moreover, we discovered that NMIIA balances the protrusive activity of tip cells

Fig. 3. (A) Representative images of mouse retinas from Arpc4 WT and Arpc4 EC-KO labeled for CD31. (Scale bar, 250 μm.) Please note that images were generated by tiling multiple fields of view, and that imperfect tiling generated misalignments between fields of view, which can be seen in the images. (B) Representative images of tip cells and filopodia in the angiogenic sprouting front of mouse retinas from Arpc4 WT and Arpc4 EC-KO labeled for CD31. (Scale bar, 50 μm.) (C) Box plots of vascular outgrowth, vessel density, EC density, EC proliferation, and number of tip cells per sprouting front length (micrometer) in Arpc4 WT (n = 7 retinas) and Arpc4 EC-KO (n = 9 retinas) mouse retinas. P values from unpaired Student’s t test. (D) Representative images of tip cells from Arpc4 WT and Arpc4 EC-KO mouse retinas labeled for CD31 and NMIIA. (Scale bar, 20 μm.) (E) Box plots of number of filopodia per 100 μm (<20 μm or >20 μm) in Arpc4 WT (n = 4 retinas) and Arpc4 EC-KO (n = 7 retinas) mouse retinas. P values from unpaired Student’s t test.
Fig. 4. (A) Representative images of HUVECs expressing an optogenetic activator of Cdc42 (ILID) in DMSO, BBS, CK666, and BBS + CK666 conditions. (Scale bar, 10 μm.) (B) Box plots of rate of new filopodia and total number of filopodia in different conditions (DMSO n = 7 cells; BBS n = 6 cells; CK666 n = 8 cells; and BBS + CK666 n = 4 cells). P values from unpaired ANOVA test. (C) Representative images of mouse retinas from Arpc4;Myh9 WT and Arpc4;Myh9 dEC-KO mouse retinas labeled for CD31. (Scale bar, 250 μm.) (D) Box plot of vascular outgrowth and vessel density in Arpc4;Myh9 WT (n = 8 retinas) and Arpc4;Myh9 dEC-KO (n = 10 retinas) mouse retinas. P values from unpaired Student’s t test. (E) Representative images of tip cells and filopodia/dactylopodia from Arpc4;Myh9 WT, Arpc4 EC-KO, Myh9 WT, and Arpc4;Myh9 dEC-KO mouse retinas labeled for CD31 (gray). (Scale bar, 20 μm.) (F) Box plots of number of filopodia per filopodia burst and number of dactylopodia (<20 μm or >20 μm) in Arpc4;Myh9 WT (n = 5 retinas) and Arpc4;Myh9 dEC-KO mouse retinas (n = 5 retinas). P value from unpaired Student’s t test.
Fig. 5. (A) Representative images of tip cells from Myh9 WT and Myh9 EC-KO mouse retinas labeled for pPax Y118 (blue), activated ITGB1 (aITGB1, green), and CD31 (red). Red arrowheads point to sites enriched in aITGB1 and pPAX at the base of filopodia in Myh9 WT tip cells, which is absent or more homogenously distributed along dactylopodia of Myh9 EC-KO tip cells. (Scale bar, 10 μm.) (B) Box plot of pPax fluorescence in tip cells from Myh9 WT (n = 9 retinas) and Myh9 EC-KO (n = 8 retinas) mouse retinas. P value from unpaired Student’s t test. (C) Representative images of HUVECs expressing CAAX-Venus at the cell membrane in DMSO, CK666, and BBS + CK666 conditions. HUVECs are labeled for aITGB1 (red), cell membrane (CAAX-Venus, green), and nuclei (DAPI, gray). (Scale bar, 10 μm.) (D) Box plots of pPax fluorescence intensity and aITGB1 fluorescence intensity in HUVECs expressing CAAX-Venus at the cell membrane in DMSO (n = 20 cells), BBS (n = 15 cells), CK666 (n = 24 cells), and BBS + CK666 (n = 13 cells) conditions. P values from one-way ANOVA. (E) Western blot analysis of levels of active GTP-bound Rac1 and total Rac1 in DMSO- or BBS-treated HUVECs. (F) Representative images of HUVECs expressing CAAX-Venus at the cell membrane in NSC23766 and NSC23766 + BBS conditions. HUVECs are labeled for aITGB1 (red), cell membrane (CAAX-Venus, green), and nuclei (DAPI, gray). (Scale bar, 10 μm.) (G) Box plots of filopodia number in DMSO (n = 21 cells), BBS (n = 20 cells), NSC23766 (n = 18 cells), and NSC23766 + BBS (n = 20 cells). P values from one-way ANOVA. (H) Representative images of HUVECs expressing CAAX-Venus at the cell membrane in siArhgef7 HUVECs treated with DMSO or BBS. HUVECs are labeled for aITGB1 (red), cell membrane (CAAX-Venus, green), and nuclei (DAPI, gray). (Scale bar, 10 μm.) (I) Box plot of filopodia number in siControl and siArhgef7 HUVECs treated with DMSO (n = 6 cells in siControl and 10 in siArhgef7) or BBS (n = 10 cells in siControl and 11 in siArhgef7). P values from one-way ANOVA.
by limiting Arp2/3 activation through actomyosin contractility-induced maturation of FAs. Notably, this effect was restricted to NMIIA and not NMIIB, in agreement with reports demonstrating that NMIIA isoforms have unique roles (23–25). Recently, Ma et al. have also revealed the importance of NMIIA and NMIIB in sprouting angiogenesis during embryonic development (40). While the relative importance of each NMII isoform for a correct development of the vascular network is consistent with our observations, the effects of NMIIA deletion on cortical stability models was not consistent with our in vivo live-imaging results. Instead of an excess of cortical protrusions (filopodia and dactylopodia), we observed a net decrease in the overall number of cortical protrusions. Moreover, no blebbing behavior was observed, in contrast with observations in Ma et al. (40). These differences may be explained by the different models used for live imaging in the two studies, in vitro embryoid body spraying assays in Ma et al. and our ex vivo assays. This highlights the specific differences and adaptive mechanisms in cell behavior from in vitro and in vivo conditions.

Based on this study and on previous reports, we propose an integrative view on the mechanisms regulating endothelial tip cell’s invasive behavior: VEGFA controls formins, by directly regulating Cdc42 activity (13, 14) and NMIIA, via SRF transcriptional activity (16). This fosters actomyosin contractility and proliferative activity in endothelial tip cells. Cdc42/formin-dependent filopodia activation engages with extravascular matrices, activating integrin signaling at the base and within filopodia, which promotes Arp2/3 activity downstream of a βPIX/Rac1 pathway. NMIIA positively regulates the maturation state of FAs, which negatively regulates the βPIX/Rac1 pathway and thus prevents excessive Arp2/3 activation. Loss of NMIIA activity leads to overactivation of Arp2/3 and unrestrained conversion of filopodia into dactylopodia. Loss of Arp2/3 activity abrogates dactylopodia formation and thus inhibits cell migration and invasion (SI Appendix, Fig. S15). Excessive filopodia formation in Arp2/3-deficient endothelium may be explained by the previously documented competition between actin nucleators (41). Within this model, an outstanding question relates to the mechanism of local control of NMIIA activity that licenses dactylopodium formation. We can speculate that filopodia-dependent signaling (engagement with local guidance cues and extravascular matrix components or cells) would trigger inhibition of NMIIA at the base of filopodia, leading to Arp2/3 activation and dactylopodia formation. Filopodia have been shown to produce pulling forces on the extravascular matrix, a propriety related to NMIIA activity (11), which could be related to dactylopodia formation. Exploration of this molecular cross-talk might provide therapeutic opportunities to sprouting angiogenesis, based on the ability to block invasion of endothelial tip cells.

Methods

Mice and Treatments. In this study, we used the following mouse strains: Myh9 floxed (42); Myh10 floxed (43); ArpoC3 floxed (30); LifeAct-GFP (44); R26mTmG (45); Myh9-GFP (46); and Pgyb-fCreERT2 (24). Inter-crosses between the different mouse strains generated new double and triple transgenic mouse strains. As controls, Cre-negative littermates were used in all experiments. Both males and females were used without distinction. Tamoxifen (Sigma) was injected intraperitoneally (20 μL of 1 mg/mL solution) at P1 and P3 before mice were collected either at P6 and P12 or injected at P4 and P5 and collected at P12, as described previously (47). Additional details about mouse models, as well as all other materials and methods, are described in detail in SI Appendix.

Data Availability. All study data are included in the article and/or supporting information.

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36. J.-C. Kuo, X. Han, C.-T. Hsiao, J. R. Yates III, C. M. Waterman, Analysis of the myosin-II-responsive focal adhesion proteome reveals a role for β-Pix in negative regulation of focal adhesion maturation. Nat. Cell Biol. 13, 383–393 (2011).

37. S. Huveneers, E. H. J. Danen, Adhesion signaling–Crosstalk between integrins, Src and Rho. J. Cell Sci. 122, 1059–1069 (2009).

38. J. D. Rotty et al., Profilin-1 serves as a gatekeeper for actin assembly by Arp2/3-dependent and -independent pathways. Dev. Cell 32, 54–67 (2015).

39. C. Suarez et al., Profilin regulates F-actin network homeostasis by favoring formin over Arp2/3 complex. Dev. Cell 32, 43–53 (2015).

40. X. Ma et al., Nonmuscle myosin 2 regulates cortical stability during sprouting angiogenesis. Mol. Biol. Cell 31, 1974–1987 (2020).

41. M.-F. Carlier, S. Shekhar, Global treadmilling coordinates actin turnover and controls the size of actin networks. Nat. Rev. Mol. Cell Biol. 18, 389–401 (2017).

42. C. Léon et al., Megakaryocyte-restricted MYH9 inactivation dramatically affects hemostasis while preserving platelet aggregation and secretion. Blood 110, 3183–3191 (2007).

43. A. N. Tullio et al., Nonmuscle myosin II-B is required for normal development of the mouse heart. Proc. Natl. Acad. Sci. U.S.A. 94, 12407–12412 (1997).

44. J. Riedl et al., Lifeact mice for studying F-actin dynamics. Nat. Methods 7, 168–169 (2010).

45. M. D. Muzumdar, B. Tasic, K. Miyamichi, L. Li, L. Luo, A global double-fluorescent Cre reporter mouse. Genesis 45, 593–605 (2007).

46. Y. Zhang et al., Mouse models of MYH9-related disease: Mutations in nonmuscle myosin II-A. Blood 119, 238–250 (2012).

47. C. A. Franco et al., Non-canonical Wnt signalling modulates the endothelial shear stress flow sensor in vascular remodelling. eLife 5, e07727 (2016).