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Profilin phosphorylation as a VEGFR effector in angiogenesis

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Abstract

Vascular endothelial growth factor (VEGF) signalling induces embryonic vascular development and angiogenesis in adult tissues. Direct phosphorylation of the actin-binding protein profilin by VEGF receptors is now shown to increase its affinity for actin, and to be essential for adult but not embryonic arteriogenesis.

Twenty years after cloning VEGF-A and its receptors, the complexity of the intracellular signalling downstream of VEGF still challenges our ability to develop a complete picture of this pathway. VEGF-A binds to three principal cell-surface receptors on endothelial cells: the tyrosine kinase receptors VEGFR1 (also known as FLT1) and VEGFR2 (FLK1), and the non-kinase receptor neuropilin 1 (NRP1)¹. Endothelial VEGFR1, which is the receptor with the highest affinity for VEGF-A, is generally thought to be a decoy receptor whose function is to soak up excess VEGF, thereby limiting the amount that reaches VEGFR2, the principal signalling receptor¹. VEGF-A is also subject to alternative splicing, which gives rise to distinct isoforms with different extracellular-matrix-and NRP1-binding abilities (VEGF-A 204, 189 and 165 have NRP1-binding domains and also bind heparan sulphates, whereas VEGF-A 145 and 121 do not). Thus, depending on the form of VEGF-A present, the extracellular matrix and NRP1 can facilitate VEGF-A binding to VEGFR2 and modulate its signalling. Although each VEGF receptor functions as a homodimer, receptor heterodimers (VEGFR1–VEGFR2) have also been described; however, their functional significance remains unknown¹.

VEGF-A is critical for early vascular development; so much so that deletion of a single allele leads to embryonic lethality². In adult tissues, VEGF stimulates angiogenesis, a process that involves the migration and proliferation of endothelial cells, followed by formation of patent endothelial tubes, recruitment of mural cells and stabilization of the new vessels². VEGF also plays an important part in regulating vascular permeability². Given the sensitivity of these processes to VEGF input, it is not surprising that the entire cascade is tightly regulated at multiple levels, including by the concentration of VEGF available for VEGFR2 binding, the extent and duration of phosphorylation of various VEGFR2 tyrosine

residues, and the rate of receptor cytoplasmic trafficking and degradation³. At the cellular level, similarly to other receptor tyrosine kinases, VEGF-A binding to VEGFR2 initiates a series of receptor phosphorylation events that induce binding of adaptor molecules, which in turn mediate activation of various 'second messenger' pathways. Examples of these adaptors and their downstream pathways include TSAd (also known as SH2D2A) and Src, Shb and FAK, NCK and p38 MAPK, and PLC- γ and p42/44 MAPK1. On page 1046 of this issue, Fan *et al.*⁴ introduce a previously uncharacterized facet of VEGFR2 signalling that departs from the usual adaptor/second-messenger/receptor-tyrosine-kinase signalling paradigm, by showing that VEGFR2 stimulates actin remodelling by direct phosphorylation of profilin-1 (Pfn-1). Profilin is a small, ubiquitously expressed actin-binding protein that also binds phosphoinositides and a large number of proline-rich sequences, such as those on actin-regulatory proteins including formins, NWASP (also known as WASL) and VASP^{5,6}. Although originally identified as a G-actin-sequestering protein that inhibited actin polymerization, subsequent work demonstrated that under most conditions profilin promotes assembly of actin filaments through several activities. For example, profilin catalyses the exchange of ADP for ATP on G-actin, which facilitates actin polymerization^{5,6}. The profilin-actin complex also associates with formins by binding to their FH1 domain, which accelerates processive formin-mediated actin assembly onto the barbed ends of actin filaments⁷. Additionally, profilin binds thymosin- β 4, ENA/VASP family proteins and NWASP, in all cases enhancing actin polymerization, and also interacts with phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5) P₂), which induces the release of polymerization-competent G-actin from its complex with profilin^{5,6}. Consistent with the localization of actin polymerization to the leading edge of motile cells⁸, profilin is concentrated at this site and has been found to control cell migration⁹.

Endothelial cell migration is fundamental to various angiogenic steps regulated by VEGF. The key element in new vessel development is thought to be the formation of tip cells that lead invasive endothelial sprouts¹⁰. Tip cells are highly polarized, extending long, actin-rich filopodia in the direction of the VEGF concentration gradient. In the present study, Fan *et al.* showed that VEGFR2 directly phosphorylates Tyr 129 of Pfn-1 and that this phosphorylation enhances actin polymerization at the leading edge of the cell⁴. Moreover, by generating an endothelial-cell-specific knock-in mouse expressing a phosphorylation-deficient Pfn-1 mutant (Pfn-1^{Y129F}), they demonstrated that absence of VEGFR2-dependent Pfn-1 phosphorylation impairs endothelial cell migration and adult, but not developmental, angiogenesis and arteriogenesis. The authors also showed that Tyr 129 phosphorylation increases the affinity of Pfn-1 for actin, suggesting that phospho-Tyr 129 Pfn-1 promotes angiogenesis through enhanced actin polymerization. Thus, from the perspective of VEGF biology, this pathway provides a direct link between the polarized localization and activation of VEGFR2 in tip cells and cytoskeletal remodelling.

However, as always with VEGF, the situation proved to be more complex. First, as well as direct VEGFR2-mediated phosphorylation of Pfn-1, VEGFR2-activated Src kinase also phosphorylates this site, with the two kinases making roughly equal contributions. Second, VEGFR1 seems to contribute as well, as its depletion inhibited VEGF-dependent phosphorylation of Pfn-1. Although VEGFR1 was shown to have kinase activity towards

Pfn-1 *in vitro*, it differed from VEGFR2 in being unable to phosphorylate it when expressed in fibroblasts. Given that VEGF binding by VEGFR1 is normally thought to inhibit VEGF signalling, these results may imply that Pfn-1 phosphorylation is accomplished by VEGFR1–VEGFR2 heterodimers rather than by VEGFR2 alone. Alternatively, VEGFR1 may promote the ability of VEGFR2 to phosphorylate Pfn-1 in some as yet undefined manner.

An interesting finding in the work by Fan *et al.* is that VEGF-induced Pfn-1 phosphorylation is important for adult but not developmental angiogenesis and arteriogenesis⁴. The authors observed normal vascular development in the Pfn-1^{Y129F} knock-in mice, including retinal angiogenesis, whereas angiogenesis in adult mice in the setting of tissue injury and subsequent wound healing was impaired, as was arteriogenesis in the hindlimb ischaemia model. The dissociation between the developmental and adult angiogenesis and arteriogenesis processes has been previously reported in the case of CD31 (refs 11,12). This suggests either that VEGF utilizes different signalling pathways in developing versus adult endothelial cells, or that different cellular processes are involved in adult versus developmental angiogenesis. Another explanation for these differences is the involvement in developmental and adult angiogenesis of distinct immune cells^{13,14} that are essential components of the angiogenic process, but can undergo substantial maturation and changes in function during development.

Although the results obtained by Fan *et al.*⁴ are consistent with existing data on the role of profilin in cell motility, a number of points remain to be elucidated. First, it is not obvious that increasing the affinity of profilin for actin will enhance rates of polymerization. Profilin must also release G-actin to permit polymerization and, indeed, this release step is thought to be crucial¹⁵. The notion that the vast majority of unphosphorylated Pfn-1 is suboptimal is somewhat surprising and has not been investigated. Second, it is unclear whether increasing the rate of leading-edge actin polymerization is the only mechanism by which Pfn-1 affects angiogenesis. In fact, it is uncertain whether the 50% inhibition of migration speed fully accounts for the potent blockade of angiogenesis. Third, profilin has also been implicated in the formation and stability of both cell–cell and cell–extracellular-matrix adhesions, which are critical for new blood vessel formation and stabilization. Effects on adhesions could therefore contribute to the effects observed *in vivo*. Fourth, the role of actin in directionality of movement, which is distinct from migration speed, could be important. Cell polarization towards chemotactic factors is critical in angiogenesis, and is a complex process that involves both positive and negative feedback loops that maintain the distinct front and rear domains of migrating cells. The cell front acquires higher sensitivity to chemotactic factors through a positive feedback loop involving actin, the small GTPase Rac and phosphoinositides⁸. Profilin binds phosphoinositides and regulates small GTPases⁵, and is thus positioned to affect these pathways. It would be very interesting to investigate the potential contribution of phosphorylated Pfn-1 to the polarization of migrating cells.

Overall, the study by Fan *et al.*⁴ adds another layer to the already complex picture of VEGF signalling (Fig. 1). These complexities may highlight the difference between ‘growth factors’ that merely induce proliferation and tissue expansion, and angiogenic factors that initiate formation of a blood vessel network with a defined architecture. The latter require

precise modulation by multiple environmental cues to generate a well-organized vasculature that efficiently perfuses its target tissues. Elucidating how the ever-increasing number of VEGFR adaptors, targets and regulatory checkpoints function within this context is likely to be a fascinating direction for future work.

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Figure 1.

Profilin in VEGF signalling. Binding of VEGF to VEGF receptors on endothelial cells induces phosphorylation of Pfn-1 at Tyr 129 (indicated by PO_4) through both VEGFR and Src tyrosine kinases. Phosphorylation increases the affinity of Pfn-1 for actin. Changes in actin polymerization are required for protrusion formation (filopodia and lamellipodia), cell migration and angiogenesis. Other known profilin-interacting proteins that influence actin polymerization and organization are also depicted, although their contributions to the observed effects remain unexplored.