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COMMENTARY

BRAF, A gatekeeper controlling endothelial permeability

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The RAF/MEK/ERK signal transduction pathway is activated by growth factors, hormones, and cytokines, and regulates a broad variety of cell responses including proliferation, apoptosis, migration, differentiation, and senescence. Upon external stimuli (i.e., growth factors), the membranous small G protein RAS becomes activated [guanosine triphosphate (GTP)-bound form] and recruits the serine/threonine kinases RAFs at the plasma membrane. Vertebrates express three conserved RAF isoforms, namely ARAF, BRAF, and RAF1, all harboring different affinities for their activator RAS and their downstream targets MEK1/2 [1]. By homology with lower animals, BRAF is considered as the archetypal RAF kinase. BRAF acts as an allosteric activator for ARAF and RAF1 and is crucial to form the required RAF homo- and heterodimers necessary for RAFs to ignite a phosphorylation cascade involving MEK1/2 and ERK1/2 [1]. ERK1/2 will in turn phosphorylate a large panel of membrane, cytosolic, and nuclear targets.

BRAF mutations have been described in about 50–60% of human melanomas [1]. Notably, the constitutively active BRAF^{V600E} mutant has been increasingly associated with cancer development and its oncogenic role extensively studied *in vitro* and *in vivo* with approved clinical applications [1]. However, such treatments face drug resistance with the onset of side effects due to parallel signaling circuits activation, via

yet incompletely understood combinations of RAF paralogs [2]. The article by Dorard *et al.* [3], published in this issue of *The FEBS Journal*, studied the role of wild-type BRAF during tumorigenesis, and particularly the mechanism controlling vascular permeability, a parameter which is deregulated during inflammation and cancer [4]. Increased permeability, with endothelial cells (ECs) as the main players, contributes to pathological features ranging from leukocyte or cancer cell transmigration (metastasis), fluid leakage (edema), poor drug delivery, to tumor angiogenesis [4].

BRAF controls endothelial permeability *in vitro*

A mouse model of BRAF ablation in the endothelial compartment (using a VE-Cadherin-Cre reporter) did not cause any developmental defects and tissue architecture of the major organs tested remained unaffected. Besides, postnatal retinal angiogenesis and adult angiogenesis were normal, as tested via Matrigel plug and sprouting bead assays using BRAF-deficient primary microvessel-derived mouse lung endothelial cells (pMECs) [3]. These results are in agreement with the observation that BRAF is not required for EC survival in embryonic and adult mice [5]. However, general BRAF knockout (KO) mice die during mid-gestation due to vascular defects which include

Abbreviations

GTP, guanosine triphosphate; LIMK, LIM kinase; pMEC, primary microvessel-derived mouse lung endothelial cells; ROK α , Rho-associated protein kinase 2; VEGF, vascular endothelial growth factor.

irregular shaped large blood vessels, incomplete lining with ECs, and hemorrhages into brain ventricles [6,7]. Although the exact underlying mechanism is still unclear, it was shown that vascular endothelial growth factor (VEGF) deficiency due to BRAF ablation in the placenta was causative for this phenotype [5].

By characterizing BRAF-deficient pMECs *in vitro*, the authors brought to light a reduced FITC-dextran leakage and increased transendothelial resistance upon treatment with permeability-inducing stimuli, altogether indicating a higher endothelial barrier function upon BRAF deficiency. This was further supported by the fact that BRAF-deficient confluent endothelial monolayers did not show radial stress fiber formation—normally formed upon permeability-inducing stimuli—nor intracellular gaps even after stimulation with VEGF, a potent permeability inducer frequently upregulated in tumors. BRAF-deficient pMECs were characterized by prominent circumferential actin bundles which correlated with less F-actin content [3]. These results are in accordance with previous findings in the literature reporting that BRAF KO mouse embryonic fibroblasts have reduced actin stress fibers and F-actin content [8].

Mechanistic insights into BRAF-dependent control of permeability

RAF1, a RAF paralog activated by BRAF [1], induces the activation of the Rho kinase (RHOA), a downstream target which triggers the consecutive activations of Rho-associated protein kinase 2 (ROK α , also known as ROCK2) and LIM kinase (LIMK). LIMK is the kinase that phosphorylates COFILIN, thereby inhibiting its actin-depolymerizing activity and contracting actin stress fibers which results in stabilization of the actin filaments (Fig. 1). Consistently with what was found in BRAF KO mouse embryonic fibroblasts [8], Dorard *et al.* [3] found reduced phosphorylation of LIMK and its downstream target COFILIN in BRAF-deficient ECs. However, VEGF signaling upstream of ROK α was unaltered suggesting a roadblock in RHOA signaling at the level of ROK α . ROK α expression was mostly unchanged in BRAF wild-type and KO—although the increase in RHOA-GTP levels in KO pMECs was interpreted as a response to the reduced ROK α activity rather than a primary phenotype—indicating that a distinct mechanism impacts ROK α signaling [3]. It was previously described that BRAF can promote actomyosin formation, cell contractility and endothelial permeability through its effectors MEK/ERK, which activate myosin light chain kinases [9]. This mechanism was

excluded by Dorard *et al.* [3] using a MEK inhibitor which dampened ERK activation in wild-type pMECs but had no impact on BRAF KO pMECs, demonstrating that reduced MEK/ERK activation in BRAF-deficient pMEC is not the cause of decreased permeability.

In the past, the lab of Prof. Baccarini already reported that ROK α interaction with VE-Cadherin is RAF1-dependent and is abolished in BRAF- and RAF1-ablated pMECs (BRAF Δ/Δ /RAF1 Δ/Δ) [10]. The association between VE-Cadherin, the cytoskeleton, and ROK α is increased in BRAF-deficient pMECs while ERK phosphorylation was comparable between BRAF Δ/Δ /RAF1 Δ/Δ and BRAF Δ/Δ pMECs. A similar phenotype has already been described in primary keratinocytes by the same group suggesting redundancy across cell types [11]. Strikingly, the cytoskeletal phenotype, accompanied by restored VEGF-induced permeability, was rescued when RAF1 was ablated in BRAF mutants, thus mimicking a similar phenotype earlier described in RAF KO ECs [10]. To gain insight into the mechanism by which BRAF impacts the binding of RAF1 to ROK α , Dorard *et al.* employed elegant models including the use of (a) kinase-dead BRAF, (b) BRAF mutant which cannot bind to RAS or RAPI (GTPase required for BRAF activation), and (c) BRAF mutants with higher or lower affinity for RAF1. Altogether, their findings revealed that the BRAF kinase activity and its ability to bind the complex RAS/RAPI and dimerize with RAF1 are crucial for controlling endothelial permeability.

BRAF controls endothelial permeability *in vivo*

As a proof of concept that BRAF is required for the control of endothelial permeability, Dorard *et al.* used the Miles assay with intradermal injection of permeability-inducing stimuli (VEGF, histamine, and thrombin) in BRAF Δ/Δ and control littermates (Fig. 1). BRAF-deficient vessels were more resistant to the permeability-inducing stimuli, while these stimuli induced similar levels of permeability in RAF1 Δ/Δ , BRAF Δ/Δ /RAF1 Δ/Δ , and F/F mice confirming their earlier *ex vivo* findings [3]. To assess if the permeability impacts tumor growth, the authors took advantage of a pathological model of melanoma using ectopic B16F10 xenograft. Tumor cell extravasation in the lung vasculature was less efficient in BRAF Δ/Δ than in control littermates. Consistent with these results, permeability stimuli promoted the migration of B16F10 melanoma cells through a monolayer of F/F and BRAF Δ/Δ /RAF1 Δ/Δ but not BRAF Δ/Δ ECs. These

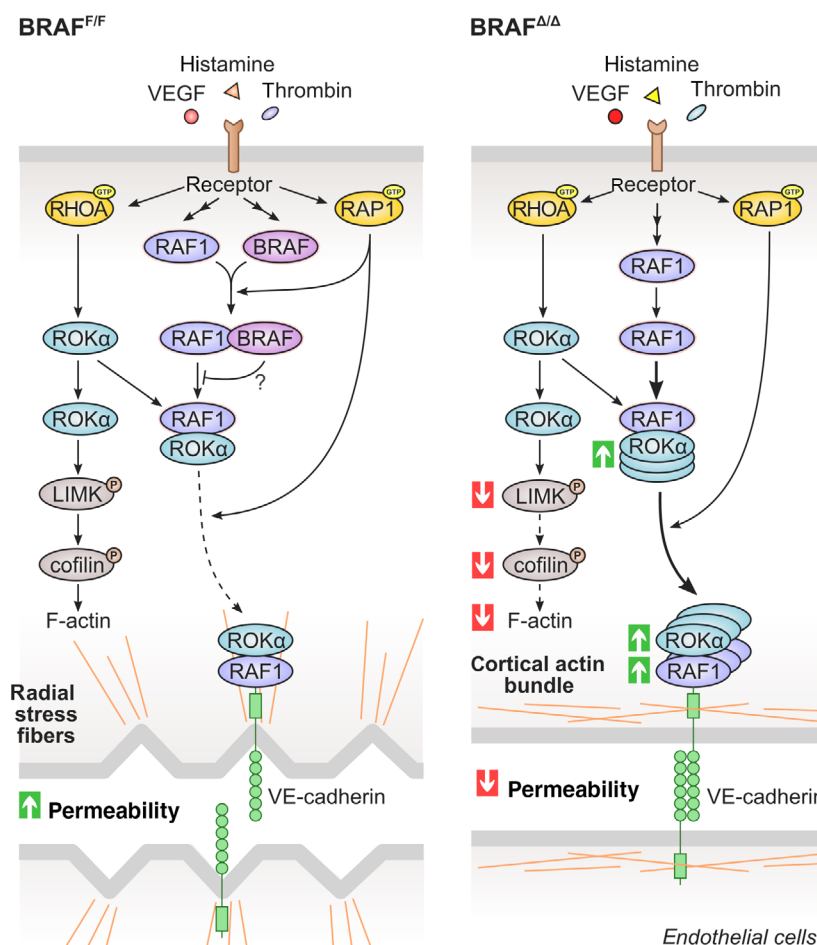


Fig. 1. BRAF, an important player regulating paracellular permeability in ECs. In wild-type cells, upon permeability stimuli, RHOA, RAF1, BRAF, and RAP1 become activated. RHOA-GTP activates the ROK α /LIMK/cofilin pathway, inducing F-actin remodeling, the formation of radial stress fibers (associated with junctional plasticity), and intercellular gap formation, altogether resulting in an increase in cell permeability. The active form of RAP1 increases the dimerization of RAF1 with BRAF and is needed for the relocalization of RAF1/ROK α dimers to the VE-cadherin containing adherens junction. In BRAF ablated cells, more RAF1/ROK α dimerization and relocalization to VE-cadherin occurs, while LIMK and cofilin phosphorylation decreases, leading to a decreased F-actin content, cortical actin bundle formation, and a reduction in vessel permeability.

findings are in accordance with *in vitro* data from Zhang *et al.* who showed that co culture of hyperactive B-RAF^{V600E} EC monolayer with metastatic melanoma cells increase the endothelial permeability and tumor extravasation [12].

Taken together, Dorard *et al.* showed that BRAF is required for the regulation of endothelial permeability *in vitro* and *in vivo* by inducing cytoskeleton rearrangements which affect VE-cadherin, and that this phenotype depends on BRAF kinase activity, the binding of BRAF with RAS/RAP1 and the ability to form BRAF/RAF1 dimers (Fig. 1).

Perspectives

As previously mentioned, BRAF is frequently hyperactivated in cancer. Blocking its binding, dimerization or kinase activity is of therapeutic interest to prevent tumor proliferation but also to improve tumor vessel normalization, prevent metastasis and tumor growth, and increase the efficacy of immunotherapy. In line with their prominent roles in cell proliferation and

tumorigenesis, the components of the RAS/RAF/MEK/ERK network are attractive targets for therapy but more research studies are needed to unravel their precise mechanism of action and interacting signaling pathways. Although redundancy has been observed between tissues, certainly *in vitro*, much less is known *in vivo*. For instance, upon RAF1 ablation, brain ECs angiogenic properties get impaired [13] and it has already been shown in murine models that targeting RAF1 in brain ECs reduces glioma proliferation and migration by targeting the vasculature [14]. Remarkably, Raf-1 ablation did not have any effect on the proliferation and migration of brain tumor cells themselves [13], so it seems that RAF1 response is tissue dependent. It would be worth studying if RAF1 and/or BRAF ablation has the same phenotype in brain ECs where permeability control is tightly orchestrated for an adequate function of the blood brain barrier similarly to what Dorard *et al.* observed in lung ECs. Further evidence of tissue specificity for RAF1 comes from the liver, where RAF1 ablation in hepatocellular carcinoma promoted proliferation and carcinogenesis,

while ECs together with keratinocytes, mouse embryonic fibroblasts or macrophages were unaffected by deletion of RAF1 [15]. Whether BRAF ablation in hepatocellular carcinoma has similar effects is currently unknown and warrants further investigation. In a colon cancer-derived cell line, BRAF ablation induced proliferation and disrupted cell polarity and tight junctions [16]. Endothelial adherens junctions (of which VE-cadherin is a key player) are intimately linked to tight junctions [4]. However, the influence of BRAF ablation in ECs on members of the tight junctions remains to be studied. Selective BRAF ablation in the β -cells of the Langerhans islets had no impact on proliferation, instead it impaired the production of VEGF and TGF- β as well as that of extracellular matrix components. The resulting reduced blood vessel density and stromal responses restrain tumor proliferation and delay tumor progression [17]. It is known that BRAF plays an essential role in regulating angiogenesis during embryogenesis [5,6] and from more recent research it could also be suggested that targeting BRAF has potential to be employed in combinatory approaches to treat cancer. However, as aforementioned, these approaches require caution and further investigations, as different outcomes upon inhibition are observed depending on the cell types targeted.

Conflicts of interest

The authors declare no conflict of interest.

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