

Essential role of B-Raf in ERK activation during extraembryonic development

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The kinases of the Raf family have been intensively studied as activators of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) module in regulated and deregulated proliferation. Genetic evidence that Raf is required for ERK activation *in vivo* has been obtained in lower organisms, which express only one Raf kinase, but was hitherto lacking in mammals, which express more than one Raf kinase. Ablation of the two best studied Raf kinases, B-Raf and Raf-1, is lethal at midgestation in mice, hampering the detailed study of the essential functions of these proteins. Here, we have combined conventional and conditional gene ablation to show that B-Raf is essential for ERK activation and for vascular development in the placenta. B-Raf-deficient placentae show complete absence of phosphorylated ERK and strongly reduced HIF-1 α and VEGF levels, whereas all these parameters are normal in Raf-1-deficient placentae. In addition, neither ERK phosphorylation nor development are affected in B-raf-deficient embryos that are born alive obtained by epiblast-restricted gene inactivation. The data demonstrate that B-Raf plays a nonredundant role in ERK activation during extraembryonic mammalian development *in vivo*.

extracellular signal-regulated kinase | placenta | Raf | vascular development | VEGF

The placenta is the first organ to develop during embryogenesis, and it supports the growth of the developing embryo by mediating the exchange of nutrients and wastes between the fetal and maternal circulatory systems. Placentation includes extensive angiogenesis, and reduced placental vascular development is associated with early embryonic mortality. Genetic studies have demonstrated a crucial role of VEGF, FGF, and their receptors in placental angiogenesis. In addition, the ablation of several signaling molecules operating downstream of receptor tyrosine kinases results in defects in placentation, often at the stage of labyrinth formation (1).

The Raf kinases (A-Raf, B-Raf, and Raf-1) relay signals from tyrosine kinase receptors to the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling module. Although most of the early work on the activation of the MEK/ERK module was focused on Raf-1, evidence has accumulated that B-Raf is the main MEK kinase. Raf kinases from lower organisms (*Caenorhabditis elegans lin-45* and *Drosophila D-Raf*) are more similar to B-Raf than to the other two mammalian Raf kinases. Biochemical studies have indicated that B-Raf is the main MEK kinase found in fibroblast and brain lysates (2–5). Consistently, among the three Raf kinases, B-Raf binds best to MEK (6) and has the highest basal MEK kinase activity, both *in vitro* (7) and in fibroblasts, when expressed as a conditionally oncogenic form (8). Finally, B-Raf mutations resulting in increased MEK/ERK activation have been discovered in a broad range of human tumors (9). All these observations hint at B-Raf as the archetypal mammalian MEK kinase, whereas Raf-1 and A-Raf have probably diverged to perform other functions. Growth-factor-stimulated ERK activation is reduced in cells lacking B-Raf but not in A-Raf- or

Raf-1-deficient cells (10–14). However, none of the kinases that activate MEK *in vitro* or in cultured cells has been shown to be essential for the activation of the ERK module *in vivo*.

Ablation of B-Raf, Raf-1, MEK-1, and ERK2 results in embryonic death between embryonic day (E)8.5 (ERK-2) and E12.5 (Raf-1) (13, 15–19). Defects at various stages of placental development have been observed in embryos lacking Raf-1 (13, 14), MEK-1 (15), and, depending on the targeting strategy used, ERK-2 (16, 19). In contrast, B-Raf ablation has been reported to compromise the survival of mature endothelial cells in the embryo proper (18). Although the availability of a conditional knockout has helped establish a MEK-independent role of Raf-1 in apoptosis and migration *in vivo* (20, 21), follow-up work on the effects of B-Raf and MEK-1 ablation has been difficult because of early embryonic lethality. Here, we use conditional mutagenesis to show that the essential role of B-Raf in intrauterine life is restricted to extraembryonic development and that the anomalies observed in the B-Raf knockout (KO) embryos are secondary to placental defects. In addition, we show that B-Raf ablation abrogates ERK phosphorylation in the trophoblast but not in the epiblast. Lack of phosphorylated ERK (pERK) is accompanied by a dramatic reduction in HIF-1 α and VEGF levels. In contrast, ERK activation is unperturbed in Raf-1 KO placentae, and epiblast-restricted ablation fails to rescue embryonic lethality. These data show a nonredundant role of B-Raf as a MEK/ERK activator in the developing placenta *in vivo* and highlight the significance of the B-Raf/MEK/ERK pathway for angiogenesis in this organ.

Results and Discussion

To circumvent early embryonic lethality by *B-raf* inactivation, a conditionally targeted *B-raf* allele (*B-raf^f*) was generated (22). *B-raf^f* (Fig. 1A) contains *loxP* sites cloned 5' and 3' of exon 12, which encodes the start of the kinase domain. *B-raf^{f/f}* animals were bred to *Mox2^{+/-cre}* transgenic mice (23) to obtain *Mox2^{+/-cre};B-raf^{f/+}* mice. Offspring were genotyped by PCR (Fig. 1A). *Mox2^{+/-cre};B-raf^{f/+}* animals were bred to WT to obtain *B-raf^{-/+}* mice with a WT *Mox2* locus. *B-raf^{-/+}* crosses did not yield any viable *B-raf^{-/-}* offspring after E11.5, at which stage 12% of *B-raf^{-/-}* embryos could be recovered (Fig. 1B). Analysis of mouse embryonic fibroblasts (MEFs) showed that exon-12 excision completely abrogated B-Raf expression (Fig. 1C) and B-Raf kinase activity (Fig. 1D). In addition, as described for the ablation of B-Raf exon 3 (10, 11), growth factor-induced ERK phosphorylation was impaired over a

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Abbreviations: En, embryonic day *n*; ERK, extracellular signal-regulated kinase; KO, knockout; MEF, mouse embryonic fibroblast; MEK, mitogen-activated protein kinase kinase; pERK, phosphorylated ERK; VEGF-A, A isoform of VEGF.

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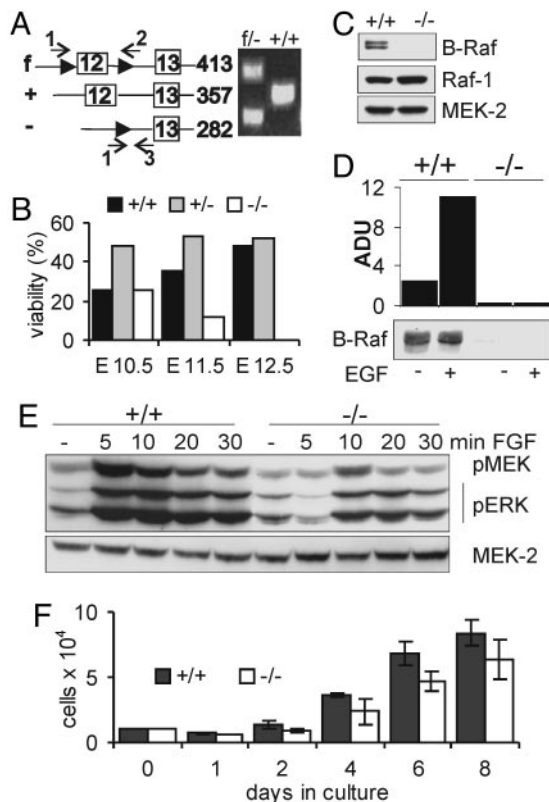


Fig. 1. Exon-12 excision abrogates B-Raf expression and kinase activity and causes lethality at midgestation. (A) Targeting strategy and PCR analysis. Primers 1 and 2 amplify a 357-bp fragment of the endogenous allele and a 413-bp fragment of the floxed allele, and primers 1 and 3 amplify a 282-bp fragment of the targeted allele. Tail biopsies were used for the PCR. (B) The percentage of *B-raf*^{flox} conceptuses of 153 viable embryos recovered from *B-raf*^{flox/+} intercrosses is shown as a function of gestational age. Sixty embryos were recovered at E10.5, 63 at E11.5, and 30 at E12.5. (C) Immunoblot of whole-cell lysates from MEFs. (D) Immunocomplex kinase assay of B-Raf i.p.s from +/+ or -/- MEFs stimulated with EGF (33 nM for 5 min). One representative experiment of three is shown. ADU, arbitrary densitometric units. (E) +/+ and -/- MEFs were stimulated with 30 ng/ml FGF for different time periods before cell lysis. The presence of pMEK, pERK, and MEK2 (loading control) was detected by immunoblotting. (F) Proliferation of primary +/+ and -/- MEFs monitored for 8 days in culture. The plot shows the mean of three independent WT and KO lines, respectively. Vertical bars, standard deviation of the mean.

wide range of concentrations (data not shown) and time points (Fig. 1E) in B-Raf KO MEFs. Regardless of the defect in pERK, however, B-Raf ablation decreased proliferation only marginally in the presence of FCS (Fig. 1F).

B-Raf KO embryos at E11.5 were significantly smaller than WT or heterozygous littermates (Fig. 2A), and extensive apoptosis could be detected in liver, brain, and heart (data not shown). Mutant placentae were similar in diameter to those of WT or heterozygous littermates, but the fetal part was less vascularized and thinner. E11.5 and E10.5 WT or heterozygous placentae displayed an organized three-layered structure, in which the intensely vascularized innermost layer, the labyrinth, was lined by a continuous layer of spongiotrophoblasts. A third layer of giant trophoblast cells demarcated the boundary to the maternal deciduas (Fig. 2B). In the mutant placenta, the spongiotrophoblast and the giant trophoblast layers were discontinuous, and the labyrinth layer was severely underdeveloped. Whereas the WT labyrinth contained abundant blood vessels of fetal origin (characterized by the presence of nucleated fetal

erythrocytes) in close contact with the maternal sinuses (filled with mature, enucleated RBCs), the KO labyrinth contained few patent blood vessels that did not make proper contact to the maternal blood sinuses and large hypocellular areas filled with stroma (Fig. 2B). Dilated blood vessels and hemorrhage were observed in E11.5 placenta (Fig. 2B), and the embryonic part was characterized by extensive apoptosis (Fig. 2C). Defects in placenta development were already evident at E10.5 (Fig. 2B Bottom), although the density of proliferating (Ki67+) cells at this time was comparable between WT and KO organs (Fig. 2D), and apoptosis was undetectable (data not shown).

To determine whether the severe placental defects caused the death of the B-Raf KO embryos, we performed epiblast-restricted ablation by crossing *B-raf*^{flox} animals to *Mox2*^{+cre};*B-raf*^{Δ/+} animals, completely rescuing the placental phenotype (Fig. 3A Top) as well as the widespread apoptosis observed in the embryo proper (data not shown). *Mox2*^{+cre};*B-raf*^{Δ/+} pups were born at a Mendelian ratio ($n = 135$) and were indistinguishable from their *B-raf*^{flox/+}, *B-raf*^{flox/-}, or *Mox2*^{+cre};*B-raf*^{Δ/+} littermates (data not shown), although complete conversion of flox to null alleles could be observed in E10.5 embryos and in all adult tissues examined (Fig. 3B). However, *Mox2*^{+cre};*B-raf*^{Δ/+} animals showed progressive growth retardation and died around postnatal day 21 of an aggressive neurodegenerative disease (G.G.-K., D.M., and M.B., unpublished work).

The data above establish that the essential function of B-Raf during intrauterine life is the control of placental development and that the defects observed in the KO embryos from *B-raf*^{flox/+} crosses are a consequence of placental failure. Placental anomalies, albeit less pronounced than those caused by *B-raf* ablation, are also a hallmark of embryos lacking Raf-1 (13). However, epiblast-restricted KO did not rescue embryonic lethality due to Raf-1 ablation, and, although *Mox2*^{+cre};*c-raf-1*^{Δ/+} embryos were present at a Mendelian ratio on E10.5, live offspring of this genotype could not be obtained ($n = 50$; data not shown). Consistently, the fetal liver apoptosis caused by Raf-1 ablation (13) was still evident in the *Mox2*^{+cre};*c-raf-1*^{Δ/+} embryos (Fig. 3C), regardless of the rescue of the placental defects (Fig. 3A Lower), indicating that this and possibly other alterations in the Raf-1 KO embryos are not secondary to placental insufficiency and are the cause of embryonic lethality.

To gain some insight into the molecular mechanisms downstream of B-Raf in the placenta, we examined pERK in WT and B-Raf KO organs. At E10.5, massive ERK phosphorylation was detectable in the WT labyrinth, in particular in the vicinity of developing blood vessels and in giant trophoblast cells; in contrast, pERK was virtually absent in *B-raf*^{flox/-} placentae. In contrast, similar levels of pERK could be detected in *c-raf-1*^{flox/-} and control placentae (Fig. 4A). Thus, B-Raf, but not Raf-1, is required for ERK activation during placental development *in vivo* and in MEFs *in vitro* (Figs. 1E and 4A; and see ref. 13).

To assess whether B-Raf was essential for ERK phosphorylation in the embryo proper, we performed whole-mount immunohistochemistry on E10.5 *Mox2*^{+cre};*B-raf*^{Δ/+} embryos to avoid recording effects due to the placental insufficiency. As described in ref. 24, pERK was evident in eye primordia, branchial arches, frontonasal processes, limb buds, and liver primordia, and pERK was unimpaired in B-Raf KO embryos (Fig. 4B). Thus, B-Raf is not required for ERK activation in the embryo proper *in vivo*.

The data above suggested a differential expression of Raf kinases in the E11.5 embryo and placenta. Indeed, immunoblot analysis revealed that, at E11.5, B-Raf was expressed at higher levels in the placenta, more precisely in the embryonic part of this organ, than in the embryo proper. Raf-1 showed the opposite distribution, whereas A-Raf was expressed at very similar levels in both placenta and embryo (Fig. 4C). The distribution of B-Raf is in line with its prominent role in ERK activation and placental development, yet it is surprising that, although Raf-1 and A-Raf

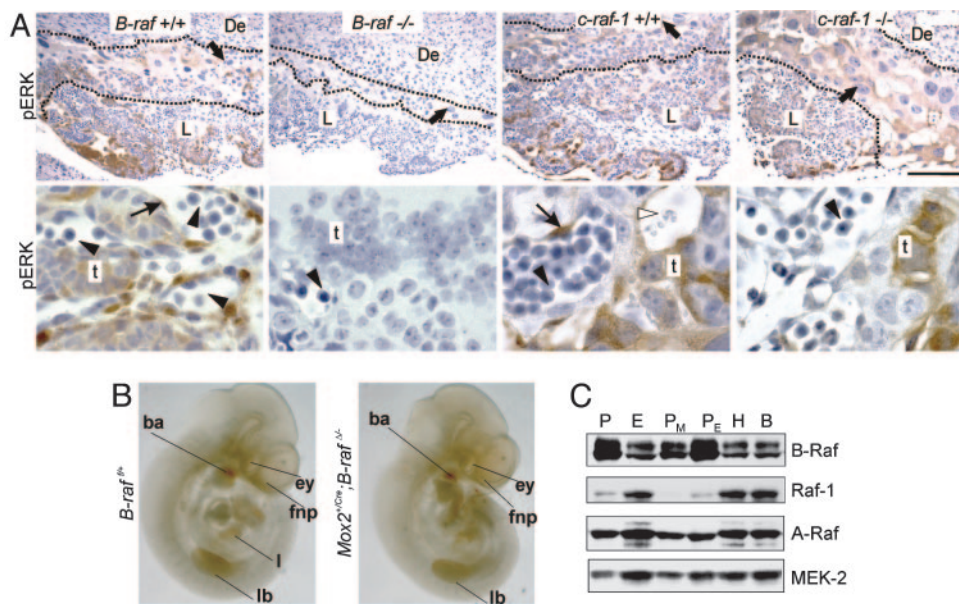


Fig. 4. B-Raf is essential for ERK activation in the developing placenta but not in the embryo proper. (A) pERK immunohistochemistry of E10.5 WT, *B-raf*^{-/-}, and *c-raf-1*^{-/-} placentae. pERK reactivity can be readily visualized as brown staining in WT and Raf-1 KO but not in B-Raf KO placenta. Dotted lines, boundaries between the labyrinth (L) and the spongiotrophoblast layer and between the giant cell layer and the maternal decidua (De). Thick filled arrows, giant cells. (Lower) Higher magnifications of the labyrinth. t, trophoblast; filled arrowheads, developing embryonic blood vessels with nucleated RBCs, open arrowheads, maternal sinuses; thin filled arrows, endothelial cells. (B) Whole-mount pERK staining of E10.5 WT and *Mox2*^{+/-};*B-raf*^{-/-} embryos. pERK staining in eye primordia (ey), branchial arches (ba), frontonasal processes (fnp), limb buds (lb), and liver primordia (l). One representative pair of three is shown. (Scale bar, 250 μ m.) (C) Expression of B-Raf, Raf-1, and A-Raf in E11.5 WT placenta (P) and embryo (E). P_M, maternal, P_E, embryonic part of the placenta; H, head; B, body of the embryo. Fifty micrograms of organ lysates were loaded in each lane. A MEK-2 immunoblot is shown as a loading control.

phorylation in B-Raf KO placentae. Indeed, immunohistochemical analysis of E10.5 B-Raf-deficient placentae revealed a dramatic decrease in VEGF-A expression compared with control littermates (Fig. 5A).

ERK can regulate VEGF transcription through a number of elements contained in the proximal region of the VEGF-A promoter (27). Activated ERK helps recruit the transcription machinery to the AP-2/Sp1 site (26). In addition, ERK can modulate the activity of the transcription factor HIF-1, which binds to the hypoxia-responsive element in the VEGF promoter and is the main player in hypoxia-induced VEGF transcription (30). HIF-1 is composed of two subunits, the constitutively expressed HIF-1 β and the unstable HIF-1 α subunit. HIF-1 α stabilization is the rate-limiting event in HIF-1 activation and is strongly induced by low oxygen tension but also, under normoxic conditions, by a variety of growth factors and cytokines (31). Functional HIF-1 is required for mouse placental development, and it has been implicated in murine (32, 33) and human trophoblast differentiation (34). In human cytotrophoblasts, inhibition of ERK activation impairs both HIF-1 α accumulation and VEGF synthesis induced under normoxic conditions by IL-1 β and TGF- β (28, 29). In good correlation with the lack of ERK phosphorylation, HIF-1 α expression was clearly reduced in E10.5 B-Raf KO placentae (Fig. 5A). In addition, nuclear accumulation of HIF-1 α was reduced in *B-raf*^{-/-} MEFs treated with CoCl₂ to mimic hypoxia (Fig. 5B). Consistent with these results, transcription of a luciferase construct driven by the VEGF-A promoter was severely impaired in B-Raf KO cells stimulated with either growth factors or CoCl₂ (Fig. 5C). Thus, B-Raf contributes to VEGF expression *in vivo* and *in vitro*. At this point, it is unclear whether the profound deficiency in VEGF expression observed in the KO placenta is due to reduced HIF-1 α expression, or to the impaired activation of the AP-2/Sp-1 promoter element, or to a combination of both.

In addition to its effects on VEGF production (Fig. 5), B-Raf may play a role in endothelial cell survival (18). Hence, defects in endothelial cell differentiation/survival might contribute to the failure to form a proper labyrinth in the absence of B-Raf. To test this idea, we generated *Tie2-Cre;B-raf*^{fl/fl} mice, which express Cre in all endothelial cells and in the majority of hematopoietic cells (35). *Tie2-Cre;B-raf*^{fl/fl} mice were born at a Mendelian ratio, were of normal size, and were healthy and fertile. The architecture of the placenta was normal at E11.5 (see Fig. 6, which is published as supporting information on the PNAS web site). Thus, B-Raf is not required for endothelial cell proliferation, differentiation, or survival in the embryo and/or in adult mice. Together with the data mentioned above, the results indicate that the defects in placentation of B-Raf KO embryos are due to the reduced amount of VEGF produced by the mutant placenta, rather than to a cell-autonomous defect of endothelial cells.

The data above identify B-Raf as the nonredundant ERK activator in mouse placenta *in vivo*. This result, corroborated by the concomitant investigation of Raf-1 KO mice, was unexpected, given the number of kinases that are able to activate the MEK/ERK module and the selective pressure imposed by the technique. Up to now, in fact, the genetic reconstruction of the Raf/MEK/ERK pathway could be achieved only in simpler organisms like *Drosophila*, which expresses only one Raf form. Although A-Raf and Raf-1 activate MEK less efficiently than B-Raf, they are expressed in the placenta (Fig. 4C), and one might have expected them to take over in the absence of B-Raf. Indeed, this argument has been regarded as a likely explanation for the unimpaired ERK activation in Raf-1 KO cells and tissues and has suggested that the presence of enzymes with similar or identical substrate specificity may prevent the identification of essential kinase-dependent functions in gene-ablation experiments. The lack of ERK activation in the B-Raf-deficient, but not in the Raf-1 KO, placenta demonstrates that this is not always the case. The reason why B-Raf is necessary for ERK

nologies; and FGF and CoCl₂, Sigma). Cells were transfected by using ExGen 500 (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. MEFs in 60-mm dishes were transfected with 4 μg of reporter plasmid (the luciferase gene driven by the human VEGF-A promoter -1176/+54) together with 1 μg of pSV-β-galactosidase plasmid to control for transfection efficiency. Luciferase/β-galactosidase activity was assayed as described in ref. 26. The luciferase activity of each sample was normalized to the β-galactosidase value.

Immunoprecipitation, Assay of Raf Kinase Activity, and Immunoblot Analysis. Immunoprecipitation and i.p. kinase assays were performed as described in ref. 13, except that phosphorylation

of GST-ERK was used as a readout. The primary antibodies were: αB-Raf IS11 (42), kind gift of J. V. Barnier (Institut de Neurobiologie Alfred Fessard, Gif sur Yvette, France); αCREB, αpMEK, and αpERK (Cell Signaling Technology); αRaf-1 and αMek-2 (Transduction Laboratories); αB-Raf (C-19) and αA-Raf (C-20) (Santa Cruz Biotechnology); and anti HIF-1α (Novus Biologicals).

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