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/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-1α and VEGF levels, whereas all these parameters are normal in Raf-1-deficient placentae. In addition, neither ERK phosphorylation nor development are affected in Braf-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. Placenta 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 placenta, 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 (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-1α 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-rafflo) was generated (22). B-rafflo (Fig. 1A) contains loxp sites cloned 5’ and 3’ of exon 12, which encodes the start of the kinase domain. B-rafΔflo animals were bred to Mox2Δflo mice (23) to obtain Mox2ΔfloB-rafΔflo littermates. Offspring were genotyped by PCR (Fig. 1A). Mox2ΔfloB-rafΔflo animals were bred to WT to obtain B-rafΔflo/+ mice with a WT Mox2 locus. B-rafΔflo/+ crosses did not yield any viable B-rafΔflo−/− offspring after E11.5, at which stage 12% of B-rafΔflo−/− 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; E, 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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Proliferation of primary F control was detected by immunoblotting. (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^+/embryos to Mox2^{+/cre}B-raf^f/f embryos, 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^f/f embryos were born at a Mendelian ratio (n = 135) and were indistinguishable from their B-raf^+/+, B-raf^f/f, or Mox2^{+/cre}B-raf^f/f 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^f/f embryos 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^{+/−} crosses are a consequence of placental failure. Placental anomalies, albeit less pronounced than those caused by Braf 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^-/- 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^-/- 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 apparent in WT organ primordia, and pERK was unimpaired in B-Raf KO embryos (Figs. 1E and 4B). The pERK pattern was similar to 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^-/- 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...
are expressed in the placenta, they cannot compensate the lack of B-Raf in ERK activation and tissue development.

Angiogenesis is regulated by VEGF in both the placenta and the embryo proper. During placentation, VEGF is produced by the giant trophoblast cells (25) in a pattern consistent with paracrine stimulation of the developing vascular endothelium. ERK signaling has been implicated in regulating the VEGF promoter in response to a variety of stimuli under hypoxic or normoxic conditions (26, 27). In particular, ERK activation is required for A isoform of VEGF (VEGF-A) expression by human cytotrophoblast cells in vitro (28, 29), suggesting that lack of VEGF production may correlate with decreased ERK phos-

![Fig. 2. B-raf ablation perturbs embryonic development. (A) Phenotype of an E11.5 /-/+ fetus and a +/+ littermate. /-/- embryos are developmentally retarded and smaller than their +/+ littermates, and the placentae are less vascularized. (B) Placental defects in /-/- embryos. Hematoxylin and eosin-stained radial sections of E11.5 and E10.5 placentae from +/+ and /-/- littermates are shown. In the /-/- placentae, the spongiotrophoblast (Sp) and labyrinth layer (L) are severely underdeveloped and disorganized compared with +/+.(Top and Bottom) The dotted lines mark the boundaries between the labyrinth and the spongiotrophoblast layer and between the giant cell layer and the maternal decidua (De). (Insets) Higher magnifications. Thick filled arrows, giant cells; open arrows, hemorrhages. (Middle) Labyrinth architecture in +/+ and /-/- E11.5 placentae. t, trophoblasts; s, stroma; thin filled arrows, endothelial cells; filled arrowheads, nucleated embryonic RBCs; open arrowheads, maternal RBCs. (C) Massive apoptosis in /-/- placenta at E11.5 revealed by TUNEL staining. (Lower) Adjacent sections stained with hematoxylin to show tissue structure. (D) Proliferating cells (Ki67+, brown staining) are present in E10.5 +/+ and /-/- placentae. (Scale bar, 500 μm.)](#)

![Fig. 3. Epiblast-restricted ablation rescues embryonic lethality due to lack of B-raf. (A) Lack of placental defects in embryos with epiblast-restricted B-Raf or Raf-1 ablation. Radial sections of E11.5 Mox2+/cre;B-raf f/ and Mox2+/cre;c-raf-1 f/ , and the respective WT placentae. De, decidua; L, labyrinth. (B) Complete conversion of the B-raf f to the B-raf f genotype in tissue samples of three E10.5 Mox2+/cre;B-raf-1 f embryos (embryo codes 230, 233, and 234; Upper) and of a Mox2+/cre;B-raf-1 f adult animal as determined by PCR analysis (Lower); B, brain; S, spleen; Li, liver; H, heart; L, lung; K, kidney; St, stomach; N, negative control. (C) Fetal liver apoptosis in Mox2+/cre;c-raf-1 f embryos. Parasagittal sections of E11.5 livers stained with TUNEL are shown. (Scale bar, 500 μm.)](#)
phosphorylation 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-A promoter and is the main player in hypoxia-induced VEGF transcription (30). HIF-1 is composed of two subunits, the constitutively expressed HIF-1β and the unstable HIF-1α subunit. HIF-1α 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 or CoCl2 (Fig. 5C). Thus, B-Raf contributes to HIF-1 regulation with similar or identical substrate specificity may prevent the identification of essential kinase-dependent functions in gene-identification of essential kinase-dependent functions in gene-expression.]}

In addition, the results indicate that the 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<sup>−/−</sup> mice, which express Cre in all endothelial cells and in the majority of hematopoetic cells (35). Tie2-Cre;B-raf<sup>−/−</sup> 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 mutant placenta, rather than to a cell-autonomous defect of endothelial cells.

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activation in the context of the placenta and of MEFs in vitro, but not of the embryo proper in vivo, is unknown. It is conceivable that the stimulation of MEFs in vitro may not be representative of the situation in vivo, where the cells receive a mixture of signals in the context of a tissue. In vivo, the differences observed in the behavior of the placenta and the embryo proper may be cell-autonomous, i.e., some cells may regularly use B-Raf-independent mechanisms for ERK activation production or may up-regulate such mechanisms in the absence of B-Raf, whereas others may not be able to do so. The peculiar distribution of B-Raf and Raf-1 in placenta and embryo (B-Raf higher in the placenta than in the embryo and Raf-1 vice versa) lends some support to this hypothesis. Alternatively, the difference may lie in the nature of the activators (growth factors, adhesion molecules, or hypoxia) that impinge on the pathway in embryo and placenta in vivo.

Stimuli that activate the ERK cascade, notably FGFs and their receptors, have been implicated in extraembryonic ectoderm development and survival (1). FGF4 produced by the epiblast regulates the proliferation and maintenance of trophoblast stem cells (36). FGFR2 (37), the main FGFR in these cells, and its downstream adaptor molecule FRS2α (38) are essential for trophoblast stem cell renewal. FRS2α, in particular, is required for full-fledged ERK activation in the extraembryonic ectoderm. Embryos bearing the FGFR2 mutation or lacking FRS2α die much earlier (<E8.5) than B-Raf KO embryos (E11.5). These data imply that either B-Raf is not the molecule mediating ERK activation through FRS2α at E8.5, or another upstream activator can carry out this function in B-Raf’s absence. Alternatively, impaired ERK activation may not be the essential reason for the reduced survival of FRS2α−/− trophoblast stem cells. Consistent with the latter hypothesis, activation of the PI3-Kinase/Akt survival pathway is also reduced in FRS2α KO fibroblasts treated with FGF (39). At present, we cannot distinguish between these possibilities.

Our data show that B-Raf sustains labyrinth development by regulating the production of VEGF rather than by a cell-autonomous role in survival. This finding may be of significance in the context of tumor development. VEGF is the main inducer of tumor angiogenesis. VEGF is expressed, together with its receptors, in highly vascularized tumors, and expression has been used as an indicator of increased metastatic risk (40). Hence, although the molecular mechanism regulating the “angiogenic switch” by which quiescent endothelium becomes activated by the tumor is unknown, VEGF is regarded as an important therapeutic target. Our data predict that the B-Raf/ERK module may participate in the induction of this angiogenic switch. If so, a drug targeting B-Raf would affect proliferation both directly, by decreasing ERK activation, and indirectly, by reducing VEGF production, thus interrupting the paracrine loop securing nutrient supply to the tumor. Conditional ablation of B-Raf in mouse tumor models whose progression involves an angiogenic switch will help clarify this issue.

Materials and Methods

Mice. B-raf−/− (22) and c-raf-1−/− (41) mice were maintained on a 129/Sv background and crossed to mice expressing the Cre recombinase from the Mox-2 locus (23) for epiblast-restricted ablation. For endothelial-cell-restricted ablation, B-raf−/− animals were bred to transgenic mice expressing Cre under the control of the Tie2 promoter (35) (kind gift of Bernd Arnold, Heidelberg).

PCR Analysis of Offspring and Conceptuses. Tail and embryonic tissue DNA was prepared as described in ref. 13. The following primers were used for genotyping B-raf alleles by PCR: primer 1, 5'-GCATAGCGCATATGCTACA-3'; primer 2, 5'-CCA- TGGCTCTACTAGTGTCT-3'; and primer 3, 5'-GTGGACCT-TGAACCTTCTCC-3'. Primers 1 and 2 amplify a 357-bp fragment of the endogenous B-raf allele and a 413-bp fragment of the floxed allele, whereas primers 1 and 3 amplify a 282-bp fragment of the targeted B-raf allele. Allele-specific PCR genotyping for c-raf-1 was described in ref. 41.

Histology and Immunohistochemistry. Hematoxylin and eosin staining and immunohistochemistry were performed on 3-μm-thick sections of 4% paraformaldehyde-fixed and paraffin-embedded tissues. Staining with the following antibodies was performed according to the manufacturer’s protocol (In Situ Cell Death Detection kit, Roche) and counterstained with propidium iodide. Whole-mount staining of E10.5 embryos was performed as described in ref. 24.

Cell Culture, Transient Transfection, and Luciferase Assay. Primary MEFs were isolated, cultured, and immortalized as described in ref. 13. MEFs were starved in medium containing 0.5% FCS for 18 h before treatment with different stimuli (EGF, Biomedical Tech-
tions; 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

Supplementary Figure 1 - Endothelium-restricted ablation of B-raf is not embryonic lethal and does not affect placental development. A, Lack of placental defects in embryos with endothelium-restricted B-raf ablation. Radial sections of E11.5 Tie2-Cre;B-raf^{+/} and B-raf^{−/−} placentae. Solid arrowheads, fetal blood vessels; open arrowheads, maternal sinuses. B, PCR analysis of B-raf^{+/+}, B-raf^{−/−}, and Tie2-cre;B-raf^{−/−} embryos (embryo codes 218, 222, 223). Partial conversion of the B-raf^{−/−} to the B-raf^{−/−} genotype can be observed in the Tie2-Cre;B-raf^{−/−} embryo. C, The percentage of Tie2-Cre-negative, B-raf^{+/+} or B-raf^{−/−} and of Tie2-Cre-positive, B-raf^{+/+} or B-raf^{−/−} P15 pups recovered from B-raf^{+/+} X Tie2-cre;B-raf^{−/−} intercrosses is shown. A total of 46 pups were analyzed. Partial conversion of the floxed to the null allele was always evident in the biopsies of Tie2-Cre-positive pups. The percentage of Tie2-Cre;B-raf^{−/+} and Tie2-Cre;B-raf^{−/−}+ pups did not differ significantly, indicating that B-Raf ablation in endothelial cells does not lead to embryonic lethality.

Supplementary Figure 1