Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta

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Mek is a dual-specificity kinase that activates the extracellular-signal-regulated (Erk) mitogen-activated protein (MAP) kinases upon agonist binding to receptors. The Erk MAP kinase cascade is involved in cell-fate determination in many organisms. In mammals, this pathway is proposed to regulate cell growth and differentiation. Genetic studies have shown that although a single *mek* gene is present in *Caenorhabditis elegans*, *Drosophila* and *Xenopus*, two *mek* homologs, Mek1 and Mek2, are present in the mammalian cascade. In the present study, we describe a mutant mouse line in which the *mek1* gene has been disrupted by insertional mutagenesis. The null mutation was recessive lethal, as the homozygous mutant embryos died at 10.5 days of gestation. Histopathological analyses revealed a reduction in vascularization of the placenta that was due to a marked decrease of vascular endothelial cells in the labyrinthine region. The failure to establish a functional placenta probably explains the death of the *mek1*$^{-/-}$ embryos. Cell-migration assays indicated that *mek1*$^{-/-}$ fibroblasts could not be induced to migrate by fibronectin, although the levels of Mek2 protein and Erk activation were normal. Re-expression of Mek1 in the mutant mouse embryonic fibroblasts (MEFs) restored their ability to migrate. Our findings provide genetic evidence that establishes the unique role played by Mek1 in signal transduction. They also suggest that *mek1* function is required for normal response to angiogenic signals that might promote vascularization of the labyrinthine region of the placenta.

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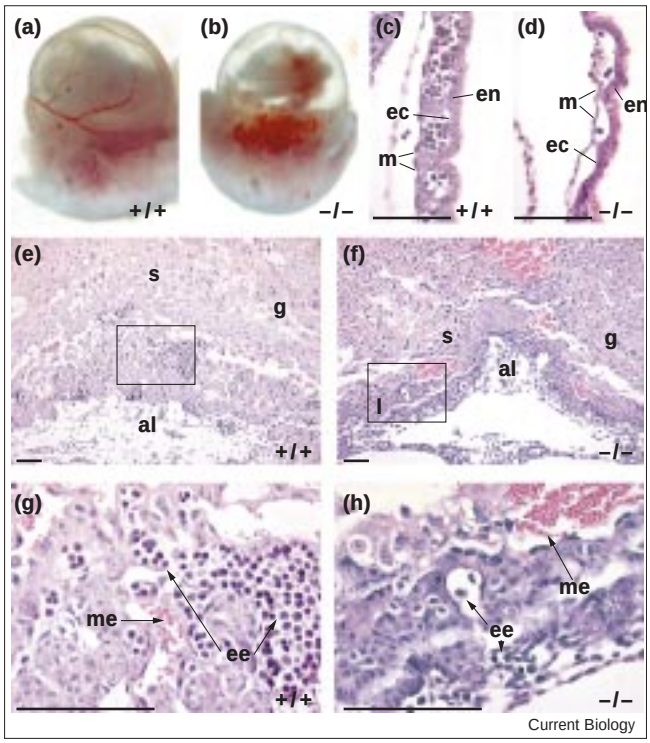
Results and discussion

A mutation in the *mek1* gene was generated by random insertional mutagenesis into embryonic stem cells using the ROSAβ-*geo* promoter-trap retroviral vector [1]. One ROSAβ-*geo* clone (J2), which contained a single insertion, did not generate viable homozygous mutants when heterozygous mice were intercrossed, suggesting that the mutation was embryonic lethal. The integration of the promoter-trap vector in this clone occurred in the *mek1* gene without major rearrangement and generated a *mek1* null allele; see Supplementary material published with this article on the internet for a detailed analysis.

To determine the time of death of *mek1*$^{-/-}$ embryos, 9.5 to 16.5 day old (E9.5 to E16.5) embryos were genotyped. At E9.5 and E10.5, Mendelian ratios of wild-type, heterozygous and homozygous *mek1* mutant embryos were obtained (N = 114 at E9.5 and N = 116 at E10.5). At E10.5, however, 8 of the 25 *mek1*$^{-/-}$ embryos were underdeveloped, while 9 were moribund or in the process of being resorbed. At E11.5, out of 21 embryos obtained, 8 *mek1*$^{-/-}$ embryos were found dead, indicating that the loss of *mek1* function resulted in embryonic lethality at around E10.5. In whole embryos, defects were first observed at E10.5, when the yolk sac of *mek1*$^{-/-}$ embryos appeared pale and apparently devoid of blood circulation (Figure 1a,b). Histological analysis of E10.5 mutant yolk sacs revealed few blood cells, however, and the blood vessels appeared distended (Figure 1c,d). One third of the homozygous mutant embryos were smaller than their wild-type and heterozygous littermates, and hemorrhaging in the embryo proper was observed in a few cases (Figure 1b), suggesting anomalies in blood circulation or blood vessel formation. Histological analyses did not reveal any obvious defects in the heart of *mek1*$^{-/-}$ embryos, however (data not shown). In addition, most of the E10.5 *mek1*$^{-/-}$ embryos exhibited necrosis in various tissues, even though the heart was still beating and no sign of hemorrhaging was detectable at the time of dissection, indicating that the onset of resorption was most likely due to pre-existing problems.

To determine if anomalies in extraembryonic tissues accounted for the embryonic necrosis and lethality, we analyzed placentas of E9.5 and E10.5 embryos. The placentas from *mek1*$^{-/-}$ embryos were abnormal (Figure 1e–h; data not shown). The spongiotrophoblast layer was less defined and the labyrinthine region appeared more compact and had considerably fewer blood vessels (Figure 1e–h, 2e–j). The maternal erythrocytes were located on the maternal side of the labyrinthine area, whereas embryonic erythrocytes remained in the blood vessels of the allantois and the chorion. A detailed *in situ* hybridization analysis of the cell lineages of placenta supported the histological observations (Figure 2). All the placental markers tested were expressed

Figure 1



Morphological and histological analyses of *mek1*$^{-/-}$ embryos. Whole-mount views of E10.5 (a) wild-type (+/+) and (b) *mek1*$^{-/-}$ (–/–) yolk sacs showing a reduction in the number of blood cells in *mek1* mutant yolk sacs. Hematoxylin and eosin staining of E10.5 (c) wild-type and (d) *mek1*$^{-/-}$ yolk sac sections showing the presence of fewer blood cells, and distended blood vessels in mutant specimens. Hematoxylin and eosin staining of E9.5 (e) wild-type and (f) *mek1*$^{-/-}$ placenta sections showing a lack of embryonic blood vessels in the labyrinthine region of mutant placentas. Higher magnifications of the boxed regions in (e,f) are shown in (g,h), respectively. Abbreviations: al, allantois; ec, endothelial cells; ee, embryonic erythrocytes; en, endoderm; g, giant cells; l, labyrinthine region; m, mesoderm; me, maternal erythrocytes; s, spongiotrophoblast cells. Scale bar represents 0.1 mm.

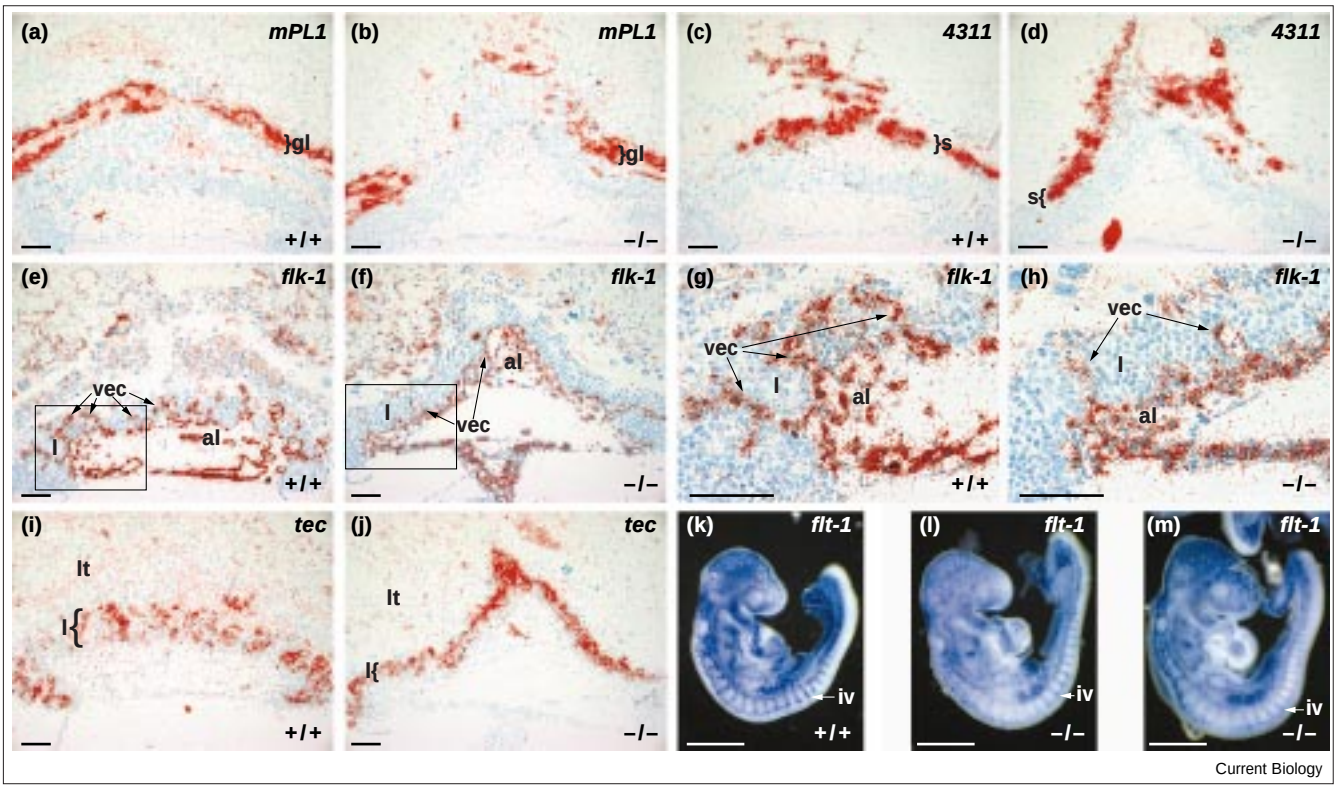
in wild-type and *mek1*$^{-/-}$ embryos, indicating that the lack of *mek1* function did not perturb the normal differentiation of the various cell lineages (Figure 2). In wild-type embryos, however, the labyrinthine trophoblasts (detected using a probe for the *tec* gene) and vascular endothelial cells (detected using a probe for the *flk-1* gene) were intermingled, whereas in *mek1*$^{-/-}$ specimens the vascular endothelial cells were mostly restricted to the chorioallantoic region (Figure 2g,h). Vascular endothelial cells were therefore produced in *mek1*$^{-/-}$ embryos but seemed unable to efficiently invade the labyrinthine region, suggesting a defect in angiogenesis. Whole-mount *in situ* hybridization with a probe (*flt-1*) for the vascular endothelial growth factor (VEGF) receptor showed that vasculogenesis proceeded normally in *mek1*$^{-/-}$ embryos (Figure 2k–m) [2]. Staining of intersomitic vessels and capillaries in the head region suggested that the absence of *mek1* function did not significantly prevent embryonic vasculogenesis or early angiogenic processes [3,4].

The Erk cascade has been shown to be involved in haptotaxis — cell migration induced by the extracellular matrix (ECM) — as well as in angiogenesis [5,6]. A possible explanation for the failure of endothelial cells to efficiently invade the labyrinthine layer of the placenta of *mek1*$^{-/-}$ mutants is that *mek1*$^{-/-}$ cells are impaired in their ability to migrate. Wild-type and *mek1*$^{-/-}$ MEF cell lines were derived from E9.5 embryos and tested in a transfilter haptotaxis assay [5]. When collagen was used as a substrate, wild-type and *mek1*$^{-/-}$ MEFs showed similar motility (Figure 3a). In contrast, cell migration induced by fibronectin was greatly reduced in mutant MEFs. The failure of *mek1*$^{-/-}$ fibroblasts to migrate was not due to a defect in cell adhesion, because no major difference in attachment and spreading on ECM was observed between the two cell lines (Figure 3b; data not shown). The cell migration defect observed in *mek1* mutant cells might be caused by a lack of Erk activation, because cell migration induced by ECM and angiogenesis is associated with activation of this cascade [5,6]. We analyzed the activation of Erk by mobility shift assay and kinase activity assay when quiescent wild-type and *mek1*$^{-/-}$ MEFs were seeded on fibronectin. Erk activation appeared to proceed normally in *mek1*$^{-/-}$ MEFs (Figure 3c; data not shown). This surprising finding may be explained by the presence of normal levels of Mek2 in *mek1*$^{-/-}$ MEFs.

To confirm that *mek1* function is required for haptotaxis, wild-type and *mek1*$^{-/-}$ MEFs were transfected with a *mek1* expression vector. Individual clones or pools of stable transfectants were isolated and migratory properties of each sample on fibronectin were examined. As shown in Figure 3d, migration was restored by expression of *mek1* in pools of stable transfectants and in individual clones, but not by transfection with empty vector. Restoration of migration also appeared to directly correlate with the level of Mek1 protein produced in the individual clones. In the pool of clones, migration was probably only partially restored because only 20% of the cells expressed Mek1 (this figure is an estimate based on the number of individual Mek1-expressing clones obtained). As the Mek2 protein level was normal in *mek1*$^{-/-}$ MEFs, our rescue experiments indicated that Mek1 was essential for the correct signal transduction required in haptotaxis induced by fibronectin, but not that induced by collagen.

Recent work of Eliceiri *et al.* [6] has shown that the Erk cascade is involved in both angiogenesis and cell migration of vascular endothelial cells and that the sustained activation of Erk MAP kinase by integrin $\alpha v\beta 3$ is necessary for angiogenesis. When wild-type MEFs were preincubated with an anti-$\alpha v\beta 3$ antibody [6], we observed that fibronectin-induced cell migration was blocked,

Figure 2

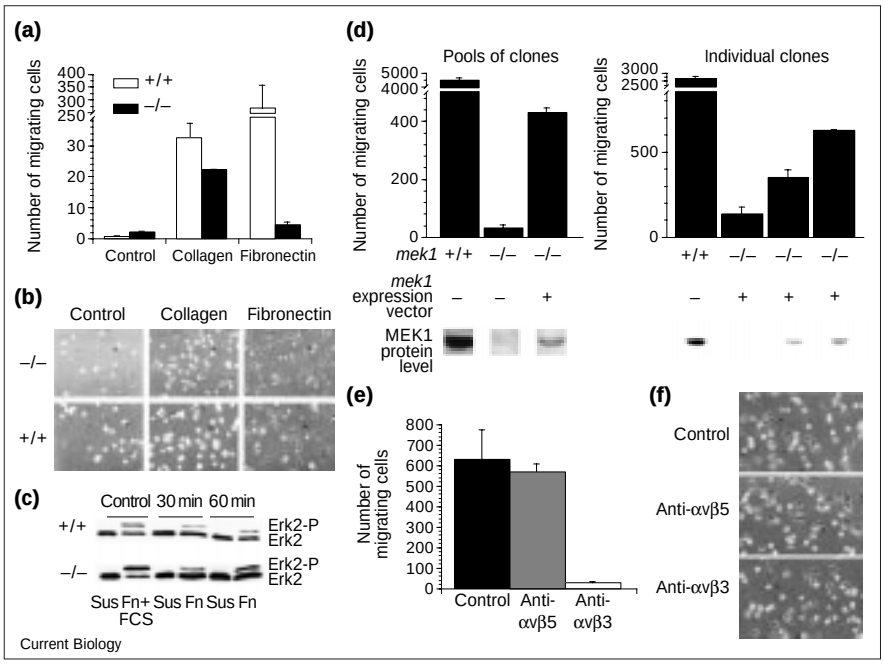


Expression of cell-type specific markers in the placenta. Near adjacent sections of E9.5 (a,c,e,g,i) wild-type (+/+) and (b,d,f,h,j) *mek1*$^{-/-}$ (–/–) placentas were used in *in situ* hybridization experiments with specific placental cell markers: (a,b) mouse placental lactogen, *mPL1* (secondary giant cells); (c,d) *4311* (spongiotrophoblasts); (e–h) VEGF receptor, *flk-1* (vascular endothelial cells); and (i,j) *tec* (labyrinthine trophoblasts) [2,11]. Higher magnifications of the boxed areas from (e) and (f) are shown in (g,h), respectively. (k–m) Whole-mount *in situ* hybridization of *flt-1* expression in E9.5 wild-type and *mek1*$^{-/-}$ embryos. Expression of *flt-1* was widely detected in the whole embryo, with no major difference in the network of blood vessels between (k) wild-type and (l,m) *mek1*$^{-/-}$ embryos. Abbreviations: al, allantois; gl, giant cell layer; iv, intersomitic vessels; l, labyrinthine region; lt, labyrinthine trophoblasts; s, spongiotrophoblast cells; vec, vascular endothelial cells. Scale bar represents 0.1 mm in (a–j) and 0.5 mm in (k–m).

whereas no inhibition occurred when an anti-αvβ5 monoclonal antibody was used (Figure 3e). As was shown for cell attachment to fibronectin, the inability of MEFs to migrate in the haptotaxis assay was not caused by a defect in cell adhesion (Figure 3f). Altogether, these results suggested that cell migration requires the αvβ3 signaling cascade and that, in the absence of Mek1, this pathway may be defective and thereby contribute to the impaired angiogenesis observed for the vascular endothelial cells in mutant embryos.

Our study establishes an essential role for Mek1 in placental development. By contrast, we found no clear phenotype in the *mek1*$^{-/-}$ embryos before midgestation. This suggests both that Mek1 has a specific role in signal transduction and that there is a partial complementation of *mek1* function by *mek2*. The specificity of Mek1 action could be achieved through differential expression of *mek1* and *mek2* genes in particular tissues. This seems unlikely, however, because both genes are widely expressed in wild-type embryonic and extraembryonic tissues at the time of death of the *mek1* mutant embryos (see Supplementary material). Moreover, the presence of Mek2 is not sufficient to compensate for the lack of Mek1 in *mek1*$^{-/-}$ MEFs. Alternatively, it is possible that, although some ligands can use either Mek1 or Mek2 to transduce signals, others might require specific components of the Erk MAP kinase cascade. The two Mek isoforms may be recruited by distinct protein complexes involved in specific responses by scaffolding/adaptor proteins as in yeast [7]. Such proteins have been identified in mammals [8,9]. In some cases, however, Mek2 or another kinase might replace Mek1, similar to the situation in yeast where, in the absence of Fus3p, Kss1p can participate in the protein complex involved in the pheromone response [7]. Some preliminary results support this hypothesis. For instance, activation of Erk by bombesin was dependent on Mek1 and sensitive to the Mek1 inhibitor PD098059 in wild-type MEF cells as reported for Swiss 3T3 cells [10]. In *mek1*$^{-/-}$ MEFs, however, Erk activation still

Figure 3



Migration properties of wild-type, *mek1*$^{-/-}$ and *mek1*-transfected MEFs. (a) Wild-type (+/+) and mutant (–/–) MEFs were serum-starved for 24 h and allowed to migrate for 2 h across a polycarbonate membrane coated with either BSA (control), collagen (10 μg/ml) or fibronectin (10 μg/ml) in a modified Boyden chamber. The results shown are a representative experiment from three independent experiments. (b) Phase-contrast images of wild-type or *mek1*$^{-/-}$ serum-starved MEFs 90 min after seeding on plates coated with BSA (control), collagen or fibronectin. Magnification, 200×. (c) Quiescent MEFs were kept in suspension (Sus) or plated on petri dishes coated with 10 μg/ml fibronectin (Fn) and incubated for 30 and 60 min at 37°C. The control samples were plated on fibronectin in the presence of 20% fetal calf serum (FCS) and incubated for 20 min at 37°C. After incubation, Erk2 phosphorylation was evaluated by mobility shift assay (Erk2-P represents the phosphorylated form of Erk2). The results shown are a representative experiment from three independent experiments. (d) Wild-type and *mek1*$^{-/-}$ MEFs transfected with an empty expression vector or the *mek1* cDNA expression vector were isolated as pool of approximately 100 clones or as individual clones, and used in haptotaxis assays. Pools of clones and individual clones were allowed to migrate for 2 h and 4 h, respectively. Representative results from two separate experiments are shown. The level of Mek1 protein in the transfectants was determined by western blot analysis. (e) Haptotaxis of wild-type MEFs induced by fibronectin can be blocked by anti-αvβ3 antibodies. Wild-type MEFs were serum-starved for 24 h and allowed to migrate for 2 h across a polycarbonate membrane coated with fibronectin after incubation with blocking antibodies against integrins αvβ3 or αvβ5 or without incubation with blocking antibodies (control). (f) Phase-contrast images of wild-type serum-starved MEFs seeded on fibronectin-coated plates for 90 min after incubation with antibodies against the integrins αvβ3 or αvβ5 or without anti-integrin antibodies (control). Magnification, 200×.

occurred but became resistant to PD098059, suggesting the participation of other kinase(s) (M.T. and J.C., unpublished observations).

Supplementary material

Additional data, including sequence and organisation of the disrupted *mek1* gene, characterization of the *mek1* mutant allele, *mek1* and *mek2* gene expression in the developing embryo and methodological details are published with this paper on the internet.

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Supplementary material

Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta

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Supplementary results and discussion

Characterization of the *mek1* mutant allele in the ROSAβ-*geo*-J2 mouse line

Integration of the ROSAβ-*geo* vector generated a fusion gene, the transcript of which was cloned using the rapid amplification of cDNA 5′ ends (5′ RACE) method, and comparison to databases revealed that the integration had disrupted the *mek1* gene (Figure S1). As expected for retroviral integration, no major rearrangement was associated with the integration (Figure S2). The vector had inserted into a 20 kb *Kpn*I fragment that hybridized with a probe located downstream of the insertion site (probe C) and generated a new 15 kb *Kpn*I band. Probes derived from sequences located upstream and downstream of the insertion (probes B and D) detected only endogenous genomic *mek1* fragments. Therefore, the only gene disrupted by the insertion was the *mek1* gene.

Insertion into the *mek1* locus is predicted to interrupt the transcription of the *mek1* gene after codon 26 (Figure S1). To confirm that the insertion generated a null allele, we performed western blot analyses using an antibody directed against the amino terminus of Mek1 [S1] in E10.5 embryos and in established MEFs. Mek1 protein was detected in extracts from wild-type and heterozygous embryos and MEFs but not in homozygous mutant embryos and MEFs (Figure S3; data not shown). The same result was obtained with an antibody against the Mek1 carboxyl terminus [S2], establishing that no full-length or truncated Mek1 protein was produced (data not shown). Western blot analysis using an antibody against Mek2 was also performed. Mek2 protein was present in all embryonic and MEF samples regardless of their genotype and no variation in the level of Mek2 between wild-type and *mek1*$^{-/-}$ samples was observed, suggesting no compensation by

Figure S1

Sequences of the ROSAβ-*geo*-J2 fusion cDNA and comparison with the mouse *mek1* gene. The boxed sequences corresponded to the 5′ primer sequence and the splice acceptor sequence of the promoter trap vector. Interruption of homology in 3′ of the RACE product coincided with the splice acceptor site of the promoter trap vector, indicating that the insertion took place into an intron of the *mek1* gene. The 5′ sequence of the RACE product corresponded to the 5′ untranslated region of the *mek1* mRNA, as shown by the cloning of a full-length *mek1* cDNA clone from an ES cell cDNA library (the 5′ end of the cDNA is indicated by the bold nucleotide C at position 50). The initiation codon of *mek1* is underlined.

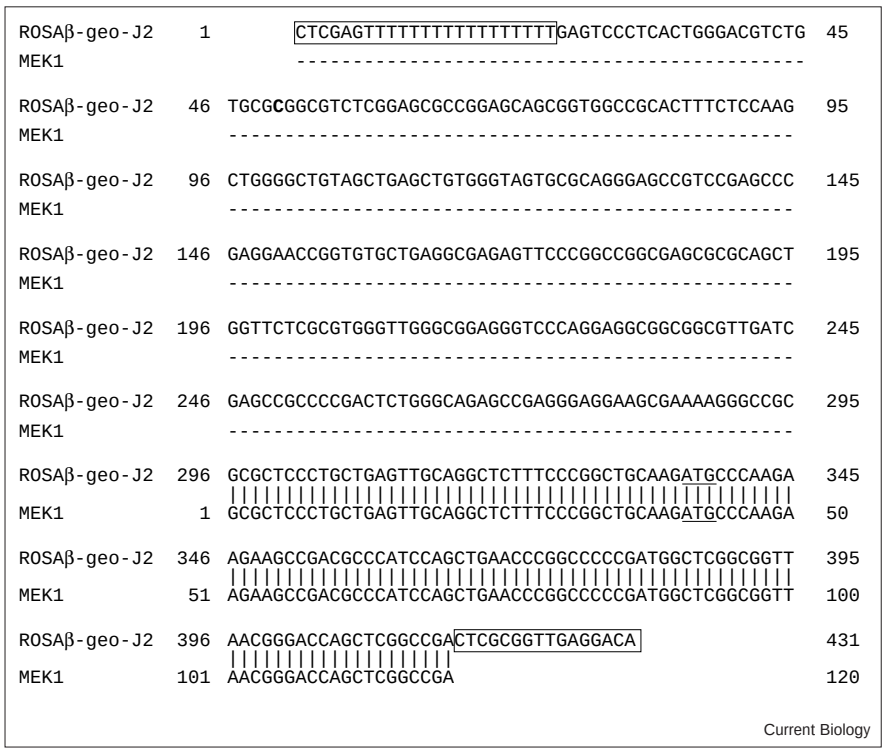
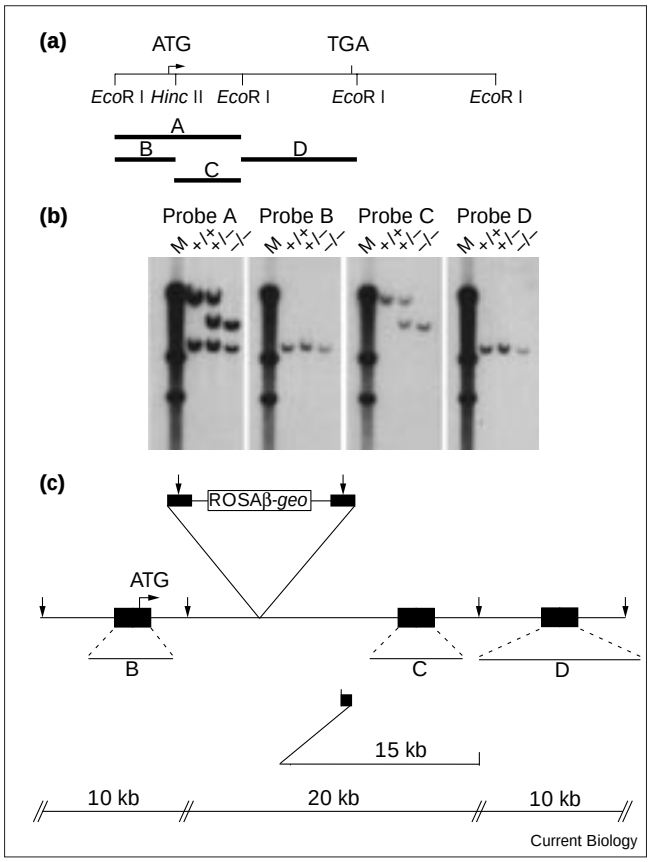


Figure S2



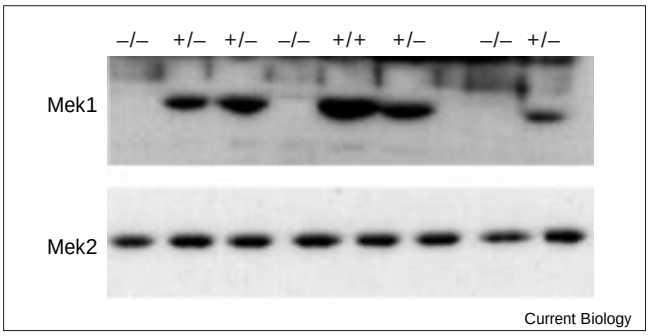
Characterization of the *mek1* mutated allele in the ROSAβ-*geo*-J2 mouse line. (a) Schematic representation of the *mek1* mRNA with the different probes (A–D) used to characterize the *mek1* mutated allele. The initiation and termination codons are indicated. (b) Southern blot analysis of yolk sac DNAs isolated from E10.5 wild-type (+/+), heterozygous (+/–) and homozygous (–/–) *mek1* mutant embryos. Yolk sac DNA was digested with *Kpn*I, blotted, and hybridized with the various *mek1* cDNA probes indicated. Lane M contained the molecular size marker λ *Hind*III (23.1, 9.4, 6.6 kb from top to bottom). (c) Schematic diagram of the organization of the *mek1* gene at the site of insertion. The arrows indicate the positions of the *Kpn*I sites.

overexpression of Mek2 (Figure S3; data not shown) [S3]. These results clearly indicate that the retroviral insertion generated a *mek1* null mutation.

Expression of the *mek1* and *mek2* genes in the developing embryo

To determine whether the structural defects observed in *mek1* mutant embryos correlate with the normal *mek1* expression profile, we analyzed the expression of the gene during development. We first looked at the β-galactosidase staining pattern in *mek1* heterozygous mutant embryos. From E7.5 to E12.5, β-galactosidase activity was detected throughout the embryo indicating a widespread expression (Figure S4a; data not shown). *In situ* hybridization experiments were also performed on sections from

Figure S3



Disruption of the *mek1* gene in the ROSAβ-*geo*-J2 mouse line generated a null allele. Proteins were extracted from E10.5 wild-type (+/+), heterozygous (+/–) and homozygous (–/–) *mek1* mutant embryos to determine the presence of Mek1 (upper panel) and Mek2 (lower panel) proteins by western analysis.

E9.5 to E11.5 mouse embryos and placentas with RNA probes specific for either *mek1* or *mek2*. At these stages, both *mek1* and *mek2* gene expression showed a ubiquitous pattern in the embryo and the placenta (Figure S4b–e; data not shown), but some differences can be observed. For instance, *mek1* was strongly expressed in the placenta, whereas the highest level of *mek2* expression was detected in the embryo. The absence of *mek1* function did not seem to affect the pattern of *mek2* expression in the placenta (Figure S4f). It therefore appeared that the effects of the lack of Mek1 in the placenta could not be rescued by Mek2 expression, causing a profound perturbation of the vascularization of the labyrinthine region. The role of Mek1 in vascularization of the placenta may thus involve transduction of signals specific for a Mek1 cascade.

Supplementary materials and methods

Cell culture and generation of germline chimeras

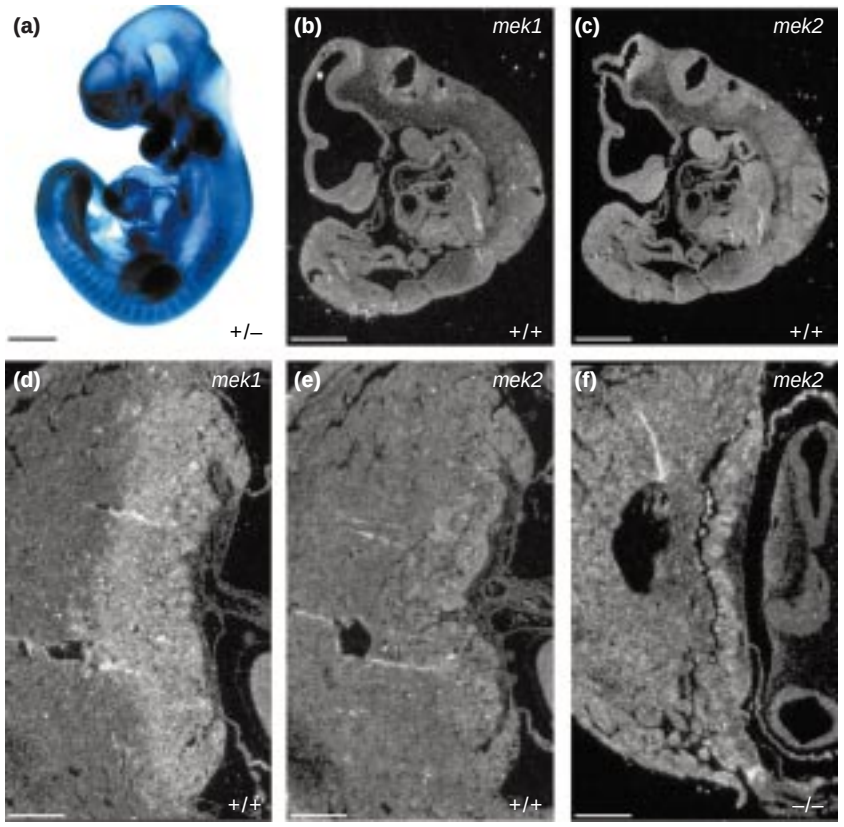
WW6 ES cells were grown and infected with ROSAβ-*geo* retrovirus as described previously [S4]. Embryos were manipulated as described previously [S5]. Chimeric males were first bred with MF1 females to identify germ-line transmitters by coat color. The germ-line chimeras were then mated to 129/SvEv inbred mice to establish the promoter-trap mouse lines.

cDNA cloning

The 5′ RACE technique was performed essentially as described by Frohman *et al.* [S6]. Total RNA was extracted from E10.5 *lacZ*-positive embryos using the LiCl/urea method. Poly(A) RNA was prepared from total RNA by one-step chromatography on oligo(dT)-cellulose [S7]. The *lacZ* primer (5′-TGTTGGGAAGGGCGATCGGT-3′) was used to reverse transcribe the RNA with the Superscript II reverse transcriptase according to the manufacturer's instructions (Gibco BRL). Excess primer was removed by filtration on Microcon-100 (Amicon). The single-stranded cDNA was tailed using terminal transferase and dATP. The anchor primer (5′-CATCGATGTCGACTCGAGTTTTTTTTTTTT-TTTT-3′) was used to initiate the synthesis of the second strand. The double-stranded cDNA was then amplified for 35 cycles with 45 sec at 94°C, 45 sec at 52°C, and 1 min at 72°C using the 5′ adaptor primer (5′-CCATCGATGTCGACTCGAG-3′), and the 3′ promoter trap primer (5′-GATCCGCCATGTCACAGATC-3′). The PCR product was purified

Figure S4

Expression of *mek1* and *mek2* in the placenta and the embryo at E10.5. (**a**) X-gal staining of a *mek1* heterozygous (+/–) embryo showing the widespread expression pattern of *mek1*. Near adjacent sections of E10.5 (**b**,**c**) embryos and (**d–f**) placentas were used in *in situ* hybridization experiments for (b,d) *mek1* expression or (c,e,f) *mek2* expression. (b–e) Wild-type (+/+); (f) *mek1*$^{-/-}$ (–/–). Both *mek1* and *mek2* genes are widely expressed in the embryo, confirming the result obtained by X-gal staining for the *mek1* gene. In the placenta, *mek1* is more strongly expressed in the embryonic portion when compared with the signal detected in the rest of the embryo or placenta. Scale bar represents 0.5 mm.



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by agarose gel electrophoresis and cloned in BlueScript vector (Stratagene). The 5′ RACE product was also used as probe to screen an ES-cell cDNA library to obtain a full-length *mek1* cDNA [S8].

β-galactosidase staining and RNA in situ *hybridization analyses*

β-galactosidase staining was performed as described [4]. The whole-mount *in situ* hybridization protocol was based on that described in Wilkinson and Nieto [S9]; radioactive *in situ* hybridization on tissue sections has been described [S10]. The following murine fragments were used as templates for synthesizing either digoxigenin or [^{35}S]UTP-labeled riboprobes: a 750 bp *4311* cDNA clone, a 600 bp placental lactogen1 (mPL1) cDNA clone, a *tec* cDNA clone, a 1.6 kb *flt-1* cDNA clone, a 800 bp *flk-1* cDNA clone, the 5′ RACE product containing the 5′ untranslated and translated regions of the *mek1* cDNA, and a 312 bp *Ava*I–*Pst*I cDNA fragment corresponding to the 3′ untranslated sequences of the *mek2* cDNA [S11,S12]. Bright-field and dark-field illuminations were digitized using a DAGE-MTI model DC330E video camera and a Scion CG7 capture board. Lower magnification images were photographed using a Leica Wild M8 binocular. Figures were processed using Adobe Photoshop 4.0 program and printed using a Kodak DS 8650 PS printer.

Isolation of mouse embryonic fibroblasts

Intercrosses of *mek1* heterozygous mutants were used to derive MEFs from E9.5 embryos. Embryos were first dissected to remove the heart and the head, washed in PBS, and the cells were then dissociated in 0.5% trypsin/EDTA for 5 min. Cell suspensions were seeded in 4-well culture plates in DMEM, 10% FCS, 2 mM L-glutamine, 50 units/ml Penicillin and 50 μg/ml Streptomycin. MEFs became established cell lines after a few months in culture.

Haptotaxis cell migration and adhesion assays

Cell migration was assayed using a modified Boyden chamber as described [S13,S14]. Briefly, the underside of a 5.0 μm pore size polycarbonate membrane was coated for 2 h at 37°C with either 10 μg/ml collagen type I or fibronectin prepared in PBS, rinsed once with PBS, and then placed into the lower chamber containing 500 μl of migration buffer (DMEM, 10 mM HEPES pH 7.4, 1.0 mM $MgCl_2$, 0.5% BSA). Serum-starved cells were harvested with trypsin – which was then blocked with trypsin inhibitor – counted, centrifuged and resuspended in migration buffer. Around 50,000–150,000 cells were used in the migration assay. After 2 h, the number of migrated cells on the lower face of the membrane was counted in five independent fields under 100× magnification. When anti-integrin antibodies were used, a pre-incubation of 15 min at 37°C of wild-type MEFs with the antibody was performed prior to the migration assay on fibronectin. Monoclonal antibodies against integrins αvβ3 (clone LM609) and αvβ5 (clone P1F6) were purchased from Chemicon International. All assays were done in triplicate and repeated at least twice.

Adhesion assays were performed as described [S15]: 24-well tissue culture plates were coated overnight at 4°C with either collagen type I or fibronectin (500 μl at 100 μg/ml in PBS). Before the test, the wells were blocked with 2% BSA in PBS at 37°C for 1 h. Serum-starved cells were harvested as described above, except that the cells were washed once, and suspended in DMEM supplemented with 2% BSA before seeding in the wells. Cells were incubated for 30 or 90 min at 37°C in 5% CO_2. Non-adherent cells were gently washed away twice in PBS at room temperature, and the remaining adherent cells were fixed in 3.7% formaldehyde in PBS at 4°C. They were examined and photographed under a phase-contrast microscope (Leica).

Western blot analysis and mobility shift assay

Total cell lysate (20 μg) was resolved on a denaturing 10% polyacrylamide gel, transferred to nitrocellulose and probed with polyclonal antibodies specific for Mek1 [S16,S17], or M24520, a monoclonal antibody specific for Mek2 (Transduction Laboratory). Antibody binding was revealed with chemiluminescence using the LumiGlo substrate kit (Kirkegaard and Perry Laboratories). The MAP kinase mobility shift assay was performed as described by Chen *et al.* [S18], except that the total cell lysate was electrophoresed on a 10% polyacrylamide gel with a 150:1 acrylamide:bis-acrylamide ratio, and the Erk2 polyclonal antibody was used to probe the western blot [S14].

Plasmid construction and fibroblast transfection

The *mek1* expression vector was constructed using the pRc/RSV plasmid (Invitrogen Corporation) using standard cloning procedures. The PGK-Hygro selection marker was first cloned into the *Bgl*II site of pRc/RSV to generate the pHyg/RSV selectable expression vector. Then, the mouse *mek1* cDNA was excised from pBluescriptII (Stratagene) as a *Hind*III–*Xba*I fragment and subcloned into the corresponding sites of the pHyg/RSV vector, generating the pHyg/RSV-*mek1* plasmid. Wild-type and *mek1* mutant fibroblast cell lines were transfected with either 5 μg pHyg/RSV or pHyg/RSV-*mek1* plasmids using the calcium-phosphate co-precipitation method. Stable transfectants were selected in media supplemented with 400 μg/ml hygromycin.

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