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Requirement for *Map2k1* (*Mek1*) in extra-embryonic ectoderm during placentogenesis

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Map2k1$^{-/-}$ embryos die at mid-gestation from abnormal development and hypovascularization of the placenta. We now show that this phenotype is associated with a decreased labyrinth cell proliferation and an augmented cell apoptosis. Although the activation of MAP2K1 and MAP2K2 is widespread in the labyrinthine region, MAPK1 and MAPK3 activation is restricted to the cells lining the maternal sinuses, suggesting an important role for the ERK/MAPK cascade in these cells. In *Map2k1*$^{-/-}$ placenta, ERK/MAPK cascade activation is perturbed. Abnormal localization of the syncytiotrophoblasts is also observed in *Map2k1*$^{-/-}$ placenta, even though this cell lineage is specified at the correct time during placentogenesis. The placental phenotype can be rescued in tetraploid experiments. In addition, *Map2k1*-specific deletion in the embryo leads to normal embryo development and to the birth of viable *Map2k1*$^{-/-}$ mice. Altogether, these data enlighten the essential role of *Map2k1* in extra-embryonic ectoderm during placentogenesis. In the embryo, the *Map2k1* gene function appears dispensable.

KEY WORDS: MAP2K1, Map kinase cascade, Conditional deletion, Placenta, Labyrinthine morphogenesis, Syncytiotrophoblast, Mouse

INTRODUCTION

Mitogen-activated protein kinase (MAPK) signaling pathways consist of protein kinase cascades linking extracellular stimuli with various targets scattered in the cytoplasm, the cytoskeleton, the cellular organelles and the nucleus (Seger and Krebs, 1995). Multiple MAPK pathways have been described in vertebrates, which include the extracellular signal-regulated kinase (ERK), the p38 kinase and the Jun NH2-terminal kinase (Cano and Mahadevan, 1995; Seger and Krebs, 1995; Zanke et al., 1996). The classical pathway, which appears to be the major one in growth factor signaling, involves the extracellular signal-regulated kinases ERK1 and ERK2 (renamed MAPK3 and MAPK1, respectively) and the ERK kinases (MAP2K1 and MAP2K2 also known as MEK1 and MEK2), and it is known as the ERK/MAPK pathway. In this cascade, MAP2Ks are dual specificity kinases that activate MAPK1 and MAPK3 upon agonist binding to receptors (Crews et al., 1992). The ERK/MAPK pathway is implicated in cell fate determination in *Caenorhabditis elegans*, *Drosophila* and *Xenopus laevis* (Hsu and Perrimon, 1994; Kornfeld et al., 1995; Umbhauer et al., 1995; Wu et al., 1995). In mammals, this pathway is proposed to regulate cell growth and differentiation (Johnson and Vaillancourt, 1994).

Although two different MAP2K proteins are present in the ERK/MAPK cascade in mammals, a single *Map2k* gene fulfills this role in *Caenorhabditis elegans*, *Drosophila* and *Xenopus laevis*. Sequence analysis revealed that the murine MAP2K1 is more related to the *Xenopus laevis* MAP2K1 (X-MEK) than to the mouse MAP2K2 (Brott et al., 1993; Crews et al., 1992; Russell et al., 1995). Two regions of MAP2K1 show reduced homology with MAP2K2: (1) the N terminus (33% identical/66% similar), which has been shown in X-MAP2K1 to be involved in the interaction of MAP2K1 with MAPKs; and (2) a part of the MAP2K specific sequence (MSS; 21% identical/36% similar), which is shared by MAP2K1 proteins from different species (Fukuda et al., 1997; Papin et al., 1996; Xu et al., 1997). The MSS domain of MAP2K1 contains a PAK phosphorylation site important for its function (Coles and Shaw, 2002). It is also involved in the interaction with the Raf family members (Catling et al., 1995; Dang et al., 1998; Eblen et al., 2002; Nantel et al., 1998; Papin et al., 1996). The protein sequence differences observed between MAP2K1 and MAP2K2 suggest that they have most probably diverged to achieve unique functions in mammals.

The differential role of MAP2K1 and MAP2K2 in signal transduction during mouse development has been revealed by the characterization of mutant mouse lines in which the *Map2k1* or *Map2k2* gene has been disrupted (Bélanger et al., 2003; Giroux et al., 1999). The null mutation of the *Map2k1* gene results in a recessive lethal phenotype, the mutant embryos dying at 10.5 days of gestation because of an abnormal development of the labyrinthine region of the placenta. *Map2k1*$^{-/-}$ embryos appear morphologically normal, and vasculogenesis and angiogenesis seem to take place normally, as evidenced by the presence of intersomitic vessels and capillaries in the head region. However, the *Map2k1*$^{-/-}$ placenta presents a marked reduction of vascularization in the labyrinth. *Map2k2*$^{-/-}$ mice showed no obvious phenotype, suggesting compensatory effects by *Map2k1* (Bélanger et al., 2003; Giroux et al., 1999). By contrast, the *Map2k1*$^{-/-}$ phenotype is observed in the presence of normal *Map2k2* expression levels in the placenta, providing genetic evidence that *Map2k2* is unable to make up for the absence of *Map2k1* (Giroux et al., 1999). Altogether, these observations indicate that *Map2k1* and *Map2k2* genes accomplish specific functions in mammals.

The placenta is a highly vascularized organ, which allows fetal-maternal exchanges during gestation. It is composed of a vascular network and stroma coming from the embryonic mesoderm, and of trophoblast cells that arise from the extra-embryonic tissue and differentiate to achieve specialized functions (Coan et al., 2005; Cross, 2000; Rossant and Cross, 2001; Simmons and Cross, 2005). In mice, placenta formation initiates around E8.5 with the fusion of the chorion with the allantois, which will give rise to the labyrinthine region. As the labyrinth develops, it becomes highly folded and

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branched, generating an important surface area required for nutrient and gas exchanges. A large part of the labyrinth is composed of one layer of mononuclear trophoblasts and two layers of syncytium (syncytiotrophoblasts) that separate the maternal blood space from the fetal blood vessels. Contribution of the ERK/MAPK pathway to placental development has been highlighted by the characterization of mouse lines carrying mutations in molecules involved in this signaling cascade. Growth factors (HGF), growth factor receptors (MET, FGFR2 and PDFGR) and components of the ERK/MAPK cascade (GRB2, GAB, SOS1, RAF1, MAP2K1 and MAPK1) have been reported to present defects in the development of the placenta labyrinthine region (reviewed by Rossant and Cross, 2001). Hence, signaling via the ERK/MAPK pathway is essential for placental development.

To dissect the physiological role of MAP2K1 in placental development, we have used the *Map2k1* mutant mouse line previously generated to fully characterize the placental phenotype. *Map2k1*-deficient placenta exhibited a reduced proliferation combined to an augmented apoptosis of labyrinth trophoblasts. Moreover, the activation of the ERK/MAPK cascade is dramatically diminished in *Map2k1* mutant placenta extracts indicating the requirement of *Map2k1* function to transduce signals to the MAPKs, MAPK1 and MAPK3. In wild-type placenta, MAPK activation is mainly observed in the cell layers lining the maternal sinuses. Despite the absence of MAPK1 and MAPK3 activation, the *Map2k1* mutation does not affect the determination and the differentiation of the syncytiotrophoblasts. However, the *Map2k1*-deficient syncytiotrophoblasts are unable to invade the placental labyrinth thereby suggesting a role for the ERK/MAPK cascade in this process. Rescue of the *Map2k1* mutation in the extra-embryonic structures allows normal development of *Map2k1*$^{-/-}$ embryos and production of live-born animals. Altogether, these observations indicate that MAP2K1 acts cell autonomously in the extra-embryonic ectoderm to support the development of the labyrinthine region.

MATERIALS AND METHODS

Mouse strain and genotyping

The establishment and the genotyping of the *Map2k1* mutant mouse line in the 129/SvEv background has been previously described (Giroux et al., 1999). All experiments were performed according to the guidelines of the Canadian Council on Animal Care and approved by the institutional animal care committee.

Tissue collection

Embryonic age was estimated by considering the morning of the day of the vaginal plug as E0.5. Specimens were collected and processed for paraffin inclusion or cryosection. For paraffin inclusion, the specimens were fixed overnight in 4% paraformaldehyde at 4°C. Serial sections of 4 μm were deparaffinized, rehydrated and either stained with Hematoxylin and Eosin according to standard procedures, or submitted to immunohistological analyses described below. Frozen sections were prepared from specimens fixed overnight in 8% paraformaldehyde, equilibrated in a 30% sucrose solution in 0.1 M phosphate buffer at pH 7 and then embedded in OCT. Serial sections of 8 μm were mounted on polylysine-treated slides for CD31 and phospho-p38 immunodetection.

Proliferation, apoptosis and immunohistochemical analyses

Proliferation rate was assessed by immunodetection of phospho-histone H3, a mitotic marker (pH3; Upstate Biotechnology) (Aubin et al., 2002). Apoptotic cells were detected by terminal transferase (TdT) DNA end labeling (Giroux and Charron, 1998). Rabbit polyclonal antibodies against: phospho-p38 MAPK, phospho-MAP2K1/MAP2K2 and phospho-MAPK1/MAPK3 (Cell Signaling Technology) were used at a 1/50 dilution. The placental vascular network was revealed with anti-CD31 antibody used at a 1/50 dilution (PECAM; Pharmingen International). Antigen retrieval was performed under pressure in a microwave for 2 minutes in 10 mM sodium citrate buffer. Non-specific binding was blocked by incubation with 10% normal goat serum for 1 hour at room temperature. The Vectastain HRP ABC Reagent (Vector Laboratories) was used for detection and the sections were counterstained with Hematoxylin.

Six specimens were analyzed per genotype. The most representative fields were presented in the figures. Bright-field and dark-field illuminations were photographed on a DM RB microscope (Leica) using a QImaging CCD camera (QICAM) and the Openlab software (Improvision). The photos were processed using Adobe Photoshop CS program. For proliferation and apoptosis studies, ratio of positively stained cells on the total cell number was evaluated for a minimum of five random areas. Repeated measures for the linear mixed model were performed to assess the difference between genotypes at all stages studied when genotype is considered the fixed effect and while area is the random effect. The procedure PROC MIXED from the SAS System was used (Littell et al., 1998).

Western blot analysis

Protein extracts were prepared as previously described (Bélanger et al., 2003). Total protein lysates (20 μg) were resolved on a denaturing 10% SDS-PAGE and probed with rabbit polyclonal anti-MAP2K1, polyclonal anti-MAP2K2 and polyclonal anti-MAPK1 antibodies. Mobility shift assays to resolve phospho-MAP2K1 from phospho-MAP2K2 and the phosphorylated and non-phosphorylated forms of MAPK1 and MAPK3 were performed as described (Bélanger et al., 2003). The phospho-specific antibodies for MAPK1/MAPK3 and MAP2K1/MAP2K2 were the same as those used for immunohistochemistry.

In situ hybridization, β-galactosidase staining and alkaline phosphatase assays

Radioactive in situ hybridization on tissue sections was previously described (Giroux and Charron, 1998). The following murine fragments were used as templates for synthesizing [^{35}S]UTP-labeled riboprobes: a 1.5 kb cDNA fragment for the *Vegf* 120 isoform and a 1.5 kb cDNA *Gcm1* fragment. β-Galactosidase staining was performed on whole *Map2k1*$^{+/-}$ and *Map2k1*$^{-/-}$ placentas (Giroux et al., 1999). Stained specimens were embedded in paraffin wax and sectioned (7 μm) for photography. Alkaline phosphatase activity was assayed by incubating rehydrated E10.5 *Map2k1*$^{+/+}$ and *Map2k1*$^{-/-}$ placenta sections with BM substrate (Boehringer Mannheim).

Generation of tetraploid-aggregation chimeras

Tetraploid embryos of wild-type B6CBAF1 were prepared by electrofusion and aggregated with diploid *Map2k1*$^{+/-}$ embryos or *Map2k1*$^{-/-}$ ES cells as described (Nagy et al., 1993). The resulting aggregates, which had reached the blastocyst stage, were transferred into E2.5 pseudopregnant females, and the embryos were dissected at E11.5 or E13.5.

Generation of the Map2k1 conditional allele

The targeting vector was made by using a 12.4 kb genomic fragment isolated from a 129/Sv mouse strain-derived genomic library. It encompasses exons 2 to 4 of the *Map2k1* gene, which was fused to the herpes simplex virus-thymidine kinase cassette for selection against random integration. To disrupt the *Map2k1* sequences, two *loxP* sequences were inserted into the *Bam*HI sites flanking the third exon of *Map2k1*. A *neo* cassette flanked by *loxP* sites was inserted in the third intron (Fig. 7). Deletion of the *Map2k1* third exon should result in an out-of-frame transcript, creating a *Map2k1*-null allele. Correctly targeted ES clones were injected into MF1 blastocysts to generate chimeras as described (Bélanger et al., 2003). The chimeras were bred with 129/SvEv mice to transmit the targeted *Map2k1* allele (*Map2k1*$^{flox\text{-}neo}$). To generate the *Map2k1* conditional allele (*Map2k1*^{floxed}), we used the *EIIa-Cre* deleter mouse line that can produce mosaic mice with partial excision of *loxP* flanked sequences (Lakso et al., 1996). Specimens were genotyped by Southern analysis of a *Stu*I digestion using a *Map2k1* genomic probe (Fig. 7). The *Map2k1* endogenous allele generates a 2.0 kb fragment, whereas the *Map2k1*^{floxed} and *Map2k1*$^{\Delta}$ alleles produce bands at 2.4 kb and 4.2 kb, respectively.

RESULTS

Map2k1 is required for the expansion of the labyrinthine region of the placenta

Our previous characterization of *Map2k1* mutant conceptuses revealed the essential role played by MAP2K1 in the normal development of the placenta. The absence of *Map2k1* function did not perturb the differentiation of the placental trophoblast cell lineages, as shown by the presence of giant cells, spongiotrophoblasts and labyrinthine trophoblasts (Giroux et al., 1999). However, the labyrinthine region of the *Map2k1* mutant placenta was reduced in size, a phenotype already obvious at E9.5 1 day before the death of the *Map2k1* mutant embryos (Fig. 1A) (Giroux et al., 1999). Given the well-documented role of the ERK/MAPK cascade in cellular proliferation and survival (Huynh et al., 2003; Pages et al., 1993; von Gise et al., 2001; Wu et al., 2004; Yu et al., 2004), we hypothesized that the disruption of *Map2k1* might affect labyrinthine expansion by limiting cell proliferation and/or survival. We first performed immunostaining using phospho-histone H3 antibody to detect mitotic cells in E8.5 to E10.5 placentas (Fig. 1B). In wild-type placentas, the proliferation rate in the labyrinthine region was elevated resulting in extensive labyrinthine development (Fig. 1B,D) (Hemberger and Cross, 2001). By contrast, the proliferation rate was halved in *Map2k1*$^{-/-}$ placenta at E9.5 and E10.5 (P<0.01; Fig. 1D). The decreased proliferation observed in E8.5 *Map2k1*$^{-/-}$ labyrinth was not statistically significant (P>0.09; Fig. 1D). In parallel, TUNEL assays were performed to determine if apoptosis contributes to the reduced growth of the *Map2k1*$^{-/-}$ labyrinthine region. Very few apoptotic cells were detected in E8.5 to E10.5 wild-type specimens (Fig. 1C,E). Conversely, apoptosis was increased by fivefold in E8.5 and E9.5 *Map2k1*$^{-/-}$ specimens and by tenfold at E10.5 when *Map2k1*$^{-/-}$ embryos were dying and in resorption (Fig. 1E).

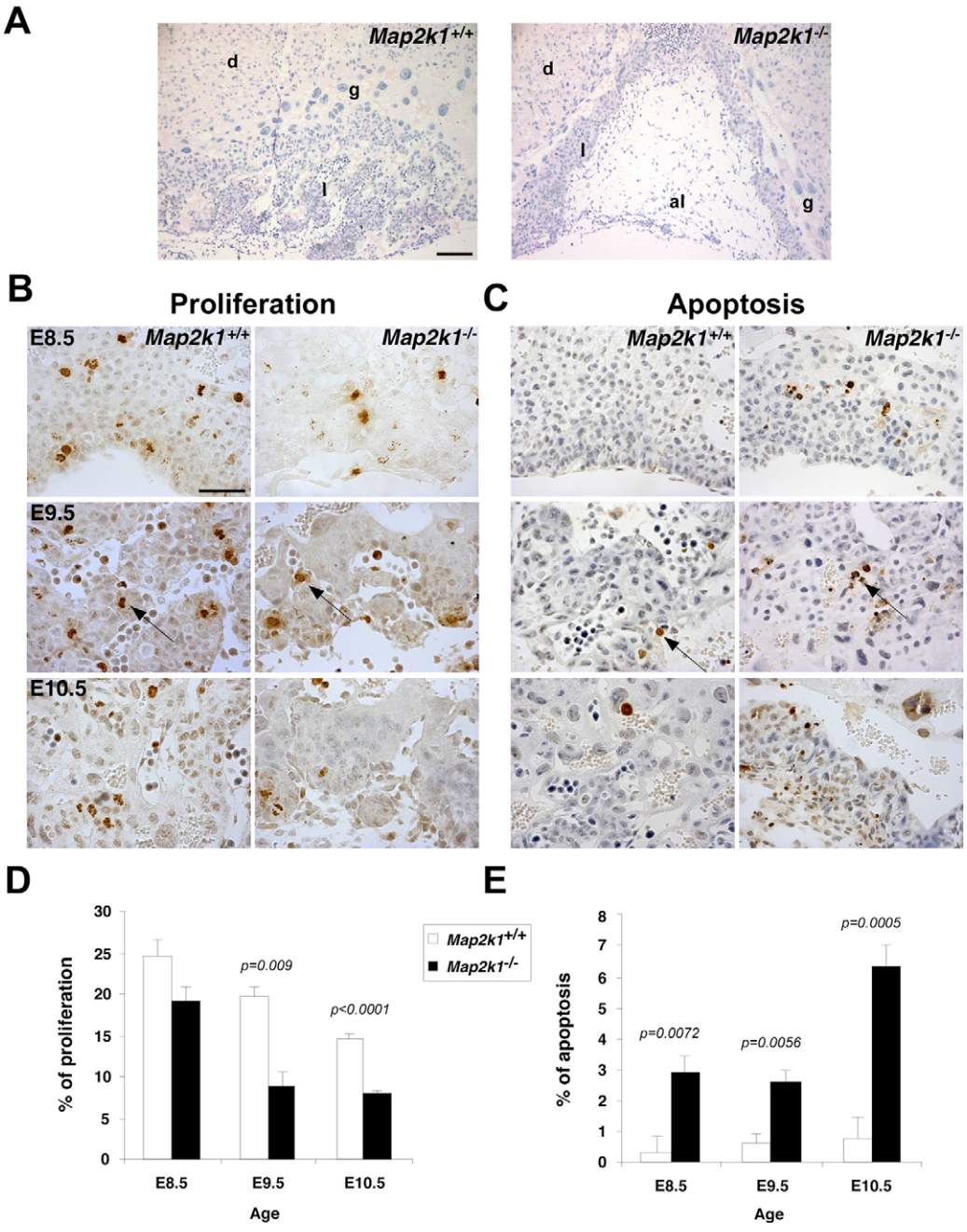

Fig. 1. The underdevelopment of the labyrinthine region in *Map2k1*$^{-/-}$ placenta results from reduced proliferation and increased apoptosis. (**A**) Hematoxylin-Eosin staining of E10.5 wild-type (*Map2k1*$^{+/+}$) and *Map2k1*$^{-/-}$ placental sections. (**B**) Proliferation was detected by immunostaining with phospho-histone H3 antibody and (**C**) apoptosis was detected by TUNEL assays on paraffin sections from E8.5, E9.5 and E10.5 wild-type and *Map2k1*$^{-/-}$ placentas. Arrows indicate examples of mitotic cells (B) and apoptotic cells (C) in the labyrinthine region (or chorioallantoic region at E8.5). The percentage of labyrinthine trophoblasts in proliferation (**D**) and apoptosis (**E**) is represented. A statistically significant reduction of the proliferating cell ratio (D) was observed for E9.5 and E10.5 *Map2k1*$^{-/-}$ placentas. The apoptotic cell ratio was significantly increased in *Map2k1*$^{-/-}$ placenta at all stages analyzed (E). al, allantois; d, deciduum; g, trophoblast giant cells; l, labyrinth. Scale bars: 100 μm in A; 50 μm in B,C.

Therefore, perturbed cell proliferation and apoptosis underlay the reduced expansion of the labyrinthine region in *Map2k1*$^{-/-}$ placenta.

Loss of ERK/MAPK activation in *Map2k1*$^{-/-}$ placenta

Both *Map2k1* and *Map2k2* genes are ubiquitously expressed in the embryo and strongly in the labyrinthine region (Giroux et al., 1999). To define whether the proliferation defect observed in *Map2k1*$^{-/-}$ placenta was due to perturbed ERK/MAPK signaling, we performed western blot analyses with embryonic and placental whole protein extracts from E9.5 and E10.5 wild-type and *Map2k1*$^{-/-}$ specimens using phospho-specific MAPK1/MAPK3 and phospho-specific MAP2K1/MAP2K2 antibodies. The phosphorylation of MAPKs and MAP2Ks at specific residues of the activation loop is usually a good read-out of their state of activation (Gopalbhai et al., 2003). The absence of the MAP2K1 protein and the level of proteins loaded on the gel were controlled with antibodies against MAP2K1 and MAPK1, respectively (Fig. 2A). In E9.5 *Map2k1*$^{-/-}$ embryonic extracts, lack of MAP2K1 did not seem to affect MAPK1 and MAPK3 phosphorylation (pMAPK1/pMAPK3), while in placenta extracts, the mutation caused a diminution of the pMAPK1 and pMAPK3 levels (Fig. 2A). This decrease was more dramatic in E10.5 *Map2k1*$^{-/-}$ placentas, despite the presence of MAP2K2 phosphorylation (pMAP2K2; Fig. 2B). Similarly, an important reduction in pMAPK1 and pMAPK3 levels was detected in *Map2k1*$^{-/-}$ embryos. In *Map2k2*$^{-/-}$ specimens, the levels of pMAPK1, pMAPK3 and pMAP2K1 proteins were unchanged compared with those of wild-type specimens (Fig. 2B). These results suggested that although MAP2K2 was phosphorylated and activated in *Map2k1*$^{-/-}$ placentas, it could not activate the ERK/MAPK pathway and promote cellular proliferation and survival in the placenta. A likely explanation for the difference in pMAPK1 and pMAPK3 levels between E9.5 and E10.5 embryos could be that E10.5 *Map2k1*$^{-/-}$ embryos were dying and in the process of resorption.

Robust activation of the ERK/MAPK pathway in the cells lining maternal sinuses

In order to identify in which cell type of the chorioallantoic region the ERK/MAPK cascade is activated during placental development, we performed immunostaining on E9.5 and E10.5 wild-type, *Map2k1*$^{-/-}$ and *Map2k2*$^{-/-}$ placenta sections with phospho-specific MAPK1/MAPK3 and phospho-specific MAP2K1/MAP2K2 antibodies. At E9.5, the presence of pMAPK1/pMAPK3 and pMAP2K1/pMAP2K2 staining was detected in labyrinthine trophoblasts, in cells lining the maternal sinuses (Fig. 3A) and in cells of the allantois in both wild-type and *Map2k2*$^{-/-}$ specimens (Fig. 3A; see Fig. S1 in the supplementary material). By contrast, in *Map2k1*$^{-/-}$ specimens, even though the MAP2K2 protein was phosphorylated in labyrinthine trophoblasts and in the allantois, pMAPK1/pMAPK3 staining was observed only in cells of the allantois.

One day later, pMAP2K1/pMAP2K2 positive signal was seen in the labyrinthine region, the cells lining the maternal sinuses, the allantoic cells and, in addition, in the endothelium lining the embryonic blood vessels in wild-type and *Map2k2*$^{-/-}$ placentas (Fig. 3B; see Fig. S1 in the supplementary material). In *Map2k1*$^{-/-}$ specimens, the highest staining was restricted to the cells of the allantois and at the chorioallantoic interface (Fig. 3B). Interestingly, despite the widespread MAP2K1 and MAP2K2 activation, pMAPK1/pMAPK3 staining was mainly detected in the cells lining the maternal sinuses, while no signal was observed in the endothelium of the embryonic blood vessels (Fig. 3B). As observed at E9.5, pMAPK1/pMAPK3 staining was restricted to the allantoic cells in E10.5 *Map2k1*$^{-/-}$ placentas.

Altogether, these results indicated that the ERK/MAPK pathway was strongly activated in the cell population lining the maternal sinuses of the placental labyrinth. In *Map2k1*$^{-/-}$ placentas, even though MAP2K2 activation was observed at the chorioallantoic interface, phosphorylation of MAPK1 and

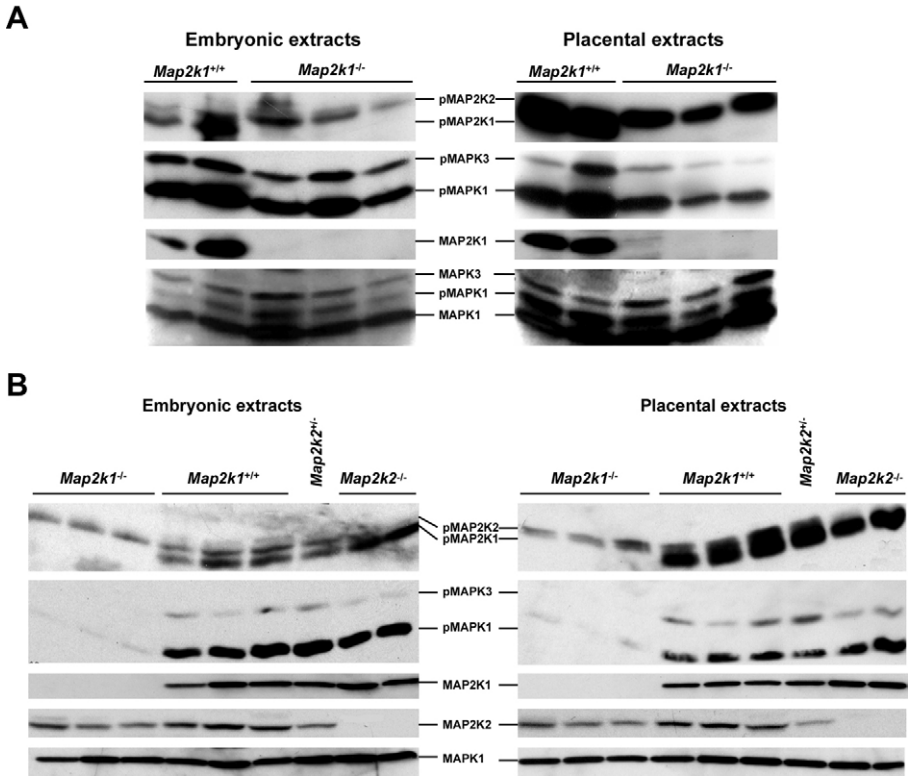

Fig. 2. ERK/MAPK activation in *Map2k1*$^{-/-}$ placentas and embryos. Expression and phosphorylation levels of MAPK1/MAPK3 and MAP2K1/MAP2K2 were evaluated by western blot analysis of total protein extracts from E9.5 (**A**) and E10.5 (**B**) wild-type and *Map2k1*$^{-/-}$ embryos and placentas. Phosphorylation of MAPK1 and MAPK3 was significantly reduced in E10.5 *Map2k1*$^{-/-}$ embryos and placentas, and remained unchanged in E9.5 *Map2k1*$^{-/-}$ embryos. A reduction was also observed in the corresponding E9.5 placentas.

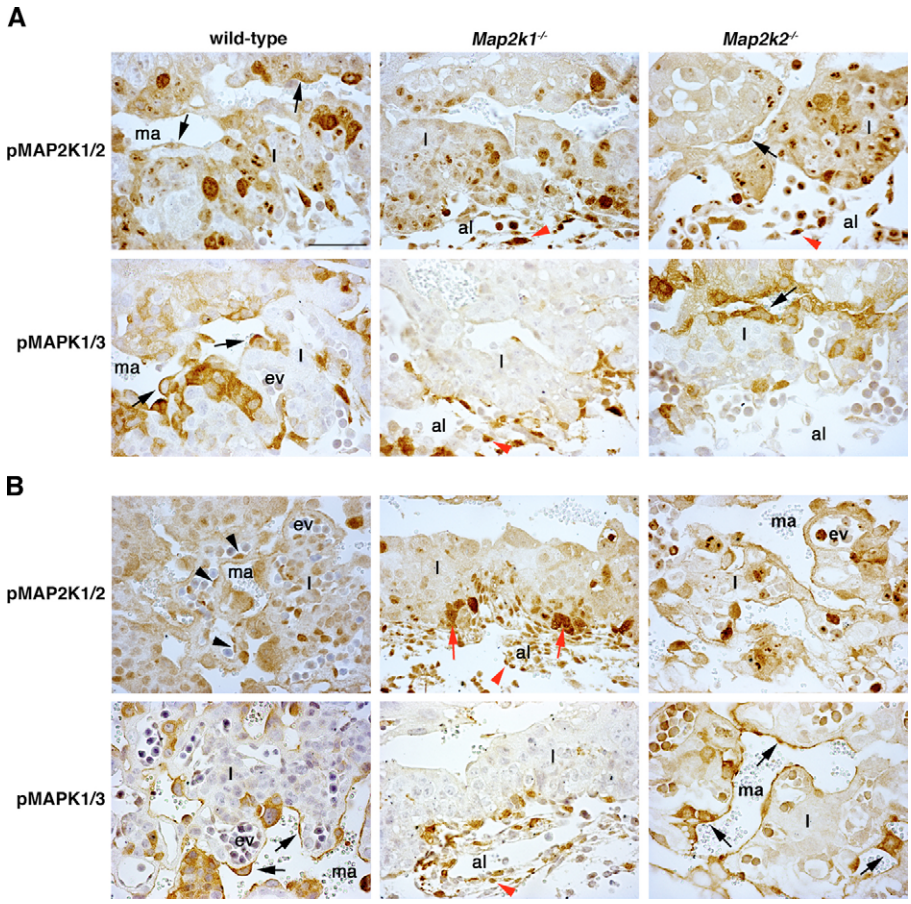

Fig. 3. Localization of phospho-MAPK1/MAPK3 and phospho-MAP2K1/MAP2K2 in wild-type and *Map2k1*$^{-/-}$ placentas. Anti-phospho-MAPK1/MAPK3 and anti-phospho-MAP2K1/MAP2K2 staining of E9.5 (**A**) and E10.5 (**B**) wild-type, *Map2k1*$^{-/-}$ and *Map2k2*$^{-/-}$ placentas. (A) At E9.5, MAP2K1/MAP2K2 were phosphorylated in cells lining the maternal sinuses (black arrows), in cells of the allantois (red arrowheads), and in some labyrinthine trophoblasts of wild-type, *Map2k1*$^{-/-}$ and *Map2k2*$^{-/-}$ placentas. However, phospho-MAPK1/MAPK3 signal was primarily found around maternal sinuses (black arrows) and in some labyrinthine trophoblasts in wild-type and *Map2k2*$^{-/-}$ specimens. In *Map2k1*$^{-/-}$ placentas, MAPK1/MAPK3 were activated only in the allantois (red arrowhead). (B) At E10.5, phospho-MAP2K1/MAP2K2 signal was also observed in the vascular endothelial cells lining the embryonic blood vessels (black arrowheads). Despite the high activation of MAP2K2 at the chorioallantoic interface (red arrows) and in the allantois of *Map2k1*$^{-/-}$ placentas (red arrowhead), phospho-MAPK1/MAPK3 were detected only in the allantois (red arrowhead). al, allantois; ev, embryonic blood vessel; l, labyrinth; ma, maternal sinus. Scale bar: 50 μm.

MAPK3 proteins was greatly reduced, reinforcing the notion of the specific requirement for MAP2K1 in the activation of the ERK/MAPK cascade.

Normal VEGF angiogenic signaling in *Map2k1*$^{-/-}$ placentas

We have previously shown that the fetal vascular endothelial cells are excluded from the chorion in E9.5 *Map2k1*$^{-/-}$ placentas, suggesting that placenta angiogenesis could be defective (Giroux et al., 1999). However, the data presented in Fig. 3 demonstrated that the ERK/MAPK pathway was not strongly activated in the embryonic vascular endothelial cells of the labyrinthine region in wild-type placentas when compared with the activation observed in the cells lining the maternal sinuses, indicating that the ERK/MAPK cascade may not play a direct role in labyrinthine angiogenesis (Fig. 3). The p38/MAPK signaling pathway was shown to be implicated in angiogenesis and activated in response to VEGF (Mudgett et al., 2000; Rousseau et al., 1997). Therefore, we decided to investigate the activation status of the p38/MAPK cascade in *Map2k1* mutant placentas.

To study the vascular endothelial network, we first performed immunostaining on E10.5 placenta sections with an anti-CD31 antibody. In wild-type specimens, the fetal blood vessels invaded the whole labyrinth region, while in *Map2k1*$^{-/-}$ specimens the embryonic blood vessels originating from the allantois were confined to the chorioallantoic plate (Fig. 4A,B). CD31-positive cells corresponding to maternal blood vessels were also detected in the deciduum. To evaluate the VEGF signaling cascade in labyrinthine angiogenesis, we then studied *Vegf* expression and activation of the p38/MAPK cascade by in situ hybridization and immunolocalization, respectively. In wild-type specimens, the p38/MAPK cascade was specifically activated in the fetal vascular endothelial cells of the labyrinthine region and in the allantois, supporting a role for p38/MAPK in angiogenesis (Fig. 4C). In *Map2k1*$^{-/-}$ specimens, p38 remained activated at the chorioallantoic interface, suggesting that the p38/MAPK cascade was still functional (Fig. 4D). *Vegf* expression was detected in discrete islets probably corresponding to vascular endothelial cells in the labyrinth of wild-type specimens (Fig. 4E). In *Map2k1*$^{-/-}$ placentas, a dramatic increase of *Vegf* expression was observed at the chorioallantoic plate and in the allantois, where the vascular endothelial cells were confined and where activation of the p38/MAPK cascade was detected (Fig. 4F), indicating a response to hypoxic stress (Blaschke et al., 2002; Conrad et al., 2000; Fan et al., 2005).

To assess if the increased *Vegf* expression and the p38/MAPK activation coincide with the domain of action of MAP2K1, we took advantage of the *lacZ* cassette of the ROSAβ-geo promoter trap vector used to generate the *Map2k1* mutant allele to visualize X-Gal staining and localize *Map2k1* expression in E10.5 *Map2k1*$^{+/-}$ and *Map2k1*$^{-/-}$ placentas. In *Map2k1*$^{+/-}$ placentas, a widespread X-Gal staining was observed in the labyrinth region, whereas in *Map2k1*$^{-/-}$ specimens, X-Gal stained cells were confined to the chorioallantoic plate, forming a barrier between the embryonic and the maternal blood cells (Fig. 4G,H). Thus, in *Map2k1*$^{-/-}$ placentas, the p38/MAPK cascade was activated in the embryonic vascular endothelial cells blocked at the chorioallantoic plate, indicating a capacity to generate an angiogenic response to the hypoxic stress, the latter most probably resulting from poor blood exchanges between the embryo and the mother caused by

hypovascularization. The defective angiogenesis observed in *Map2k1*$^{-/-}$ placentas may therefore be secondary to a morphogenic problem.

Normal determination and differentiation of *Map2k1*$^{-/-}$ syncytiotrophoblasts

The vascularization of the labyrinth is initiated when the allantois fuses with the chorion around E8.5. At this stage, complex morphogenic and vascularization processes will generate the labyrinth. There is increasing evidence to indicate that the trophoblast epithelium contributes actively to the chorioallantoic branching (Rossant and Cross, 2001). A subgroup of trophoblasts in the chorionic plate starts to express *Gcm1*, an early marker of the syncytiotrophobasts, at the site of allantoic mesoderm evagination before the chorioallantoic fusion, suggesting that the morphogenesis of the labyrinth proceeds in response to instructive signals from the allantois and involved the syncytiotrophoblast cell line. This will then allow the vascularization of the labyrinth by the embryonic blood vessels arising from the allantois (Rossant and Cross, 2001). Syncytiotrophoblasts and mononuclear trophoblasts coat the maternal sinuses to isolate the fetal blood circulation from the maternal one (Coan et al., 2005; Simmons and Cross, 2005). As MAPK1 and MAPK3 are strongly activated in the cells lining the maternal sinuses (Fig. 3), we investigated whether the lack of pMAPK1 and pMAPK3, as well as the vascularization defect observed in *Map2k1*$^{-/-}$ placentas, were due to the misspecification of syncytiotrophoblasts.

We first performed in situ hybridization experiments with a *Gcm1* probe used as a marker for syncytiotrophoblast precursors on E8.5, E9.5 and E10.5 wild-type and *Map2k1*$^{-/-}$ placentas. As previously reported, *Gcm1* was expressed in cell clusters in E8.5 wild-type chorions (Stecca et al., 2002), and a similar expression profile was detected in *Map2k1*$^{-/-}$ chorions (Fig. 5A,B). In E9.5 and E10.5 wild-type placentas, *Gcm1*-positive cells started to invade the labyrinth (Fig. 5C,E), while in *Map2k1*$^{-/-}$ placentas, *Gcm1*-positive cells were blocked at the chorioallantoic junction (Fig. 5D,F). In order to determine whether the syncytiotrophoblast precursors of *Map2k1*$^{-/-}$ placentas were able to differentiate into syncytiotrophoblasts, we performed alkaline phosphatase assays on E10.5 wild-type and *Map2k1*$^{-/-}$ specimens as differentiated syncytiotrophoblasts lining the maternal sinuses express endogenous alkaline phosphatase activity (Matsubara et al., 1993; Wu et al., 2003). As expected, we observed a strong alkaline phosphatase activity around maternal sinuses in wild-type placentas (Fig. 5G,I). In *Map2k1*$^{-/-}$ specimens, alkaline phosphatase activity was detected in patches and followed a similar pattern to the *Gcm1* signal (Fig. 5H,J). These results strongly suggested that the *Gcm1*-positive cells were correctly determined at the right moment in the *Map2k1*$^{-/-}$ placentas. They were also able to differentiate, as demonstrated by the alkaline phosphatase activity. However, they were unable to invade the chorion, allowing the formation of the vascular network of the labyrinth. Thus, the ERK/MAPK signaling via MAP2K1 is not likely to be required for syncytiotrophoblast differentiation but appears to be necessary for the chorioallantoic branching morphogenesis by the syncytiotrophoblasts.

Tetraploid rescue of *Map2k1*-deficient embryos

Our results suggest that the activation of the ERK/MAPK cascade is greatly reduced in the *Map2k1*$^{-/-}$ syncytiotrophoblasts that originate from extra-embryonic tissues. By contrast, the p38/MAPK pathway was normally activated in *Map2k1*$^{-/-}$ vascular endothelial cells derived from the embryo. These data suggested that the *Map2k1*$^{-/-}$ placental phenotype has an extra-embryonic origin that affects the labyrinthine morphogenesis. The vascularization defect observed might be a consequence of abnormal chorioallantoic branching. To define in which structure, embryonic or extra-embryonic, MAP2K1 was playing an essential role, we performed tetraploid rescue experiments.

Tetraploid embryos develop poorly but they have the potential to contribute to the trophectoderm and its placental derivatives once aggregated with embryonic stem (ES) cells (Nagy et al., 1993). ES

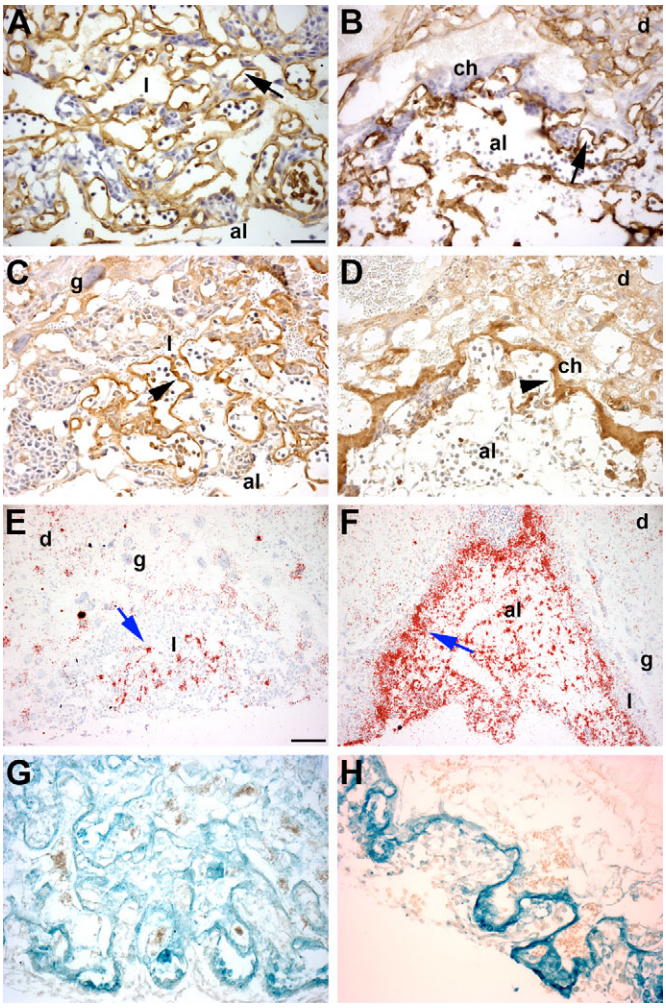

Fig. 4. Abnormal vascularization of *Map2k1*$^{-/-}$ placental labyrinth. The embryonic vascular network of E10.5 wild-type (**A**) and *Map2k1*$^{-/-}$ (**B**) placentas was revealed by anti-CD31 staining (black arrows). In *Map2k1*$^{-/-}$ placentas, the fetal blood vessels were restricted at the chorioallantoic plate (B). Detection of p38 MAPK activation in wild-type (**C**) and *Map2k1*$^{-/-}$ (**D**) placentas revealed a high activation of p38 MAPK in the embryonic blood vessels of the labyrinth of wild-type specimens and at the chorioallantoic interface of *Map2k1*$^{-/-}$ placentas (black arrowheads). *Vegf* expression in wild-type (**E**) and *Map2k1*$^{-/-}$ (**F**) placentas was analyzed by in situ hybridization (red signal and blue arrows). High *Vegf* expression levels were detected in the allantoic region of *Map2k1*$^{-/-}$ placentas, indicating a response to hypoxic stress. For placental structure identification, near adjacent Hematoxylin and Eosin-stained sections of specimens E and F are presented in Fig. 1A. X-Gal staining of *Map2k1*$^{+/-}$ (**G**) and *Map2k1*$^{-/-}$ (**H**) placentas was used to define the *Map2k1* expression profile in normal and mutant placentas. The labyrinthine region is presented. In *Mapk2k1*$^{-/-}$ specimens, X-Gal staining is restricted to the chorioallantoic plate. al, allantois; ch, chorionic plate; d, deciduum; g, tropohoblast giant cells; l, labyrinth. Scale bars: 50 μm in A-D,G,H; 100 μm in E,F.

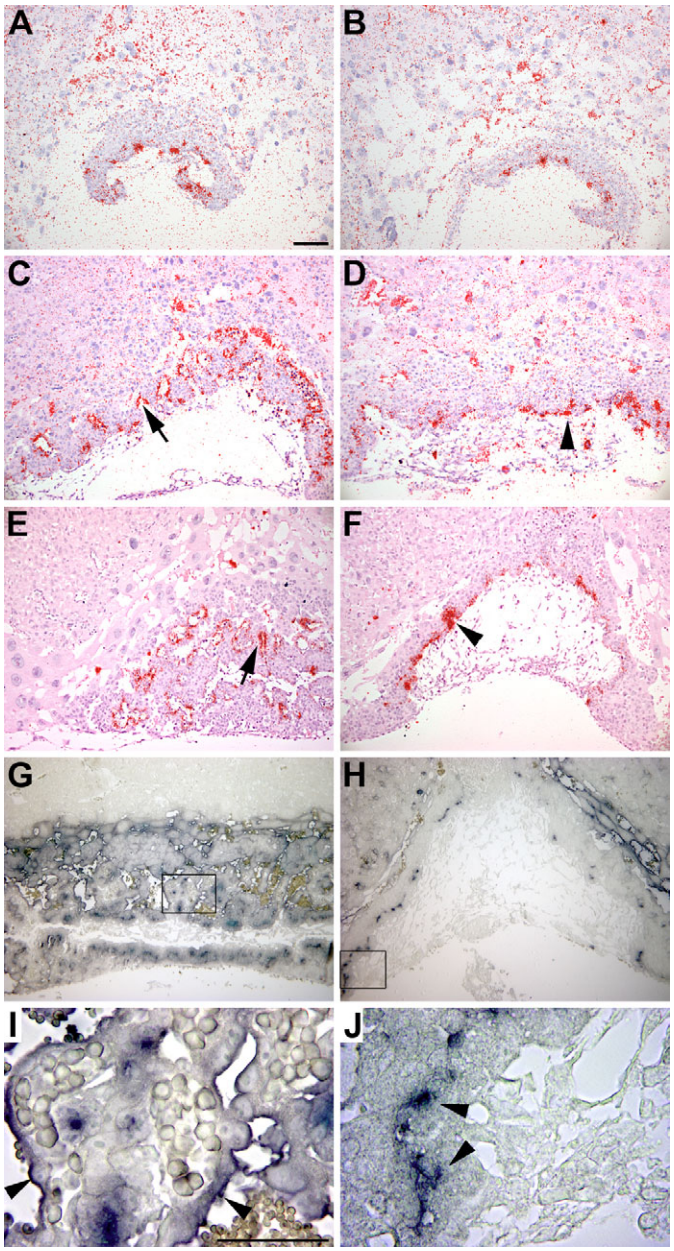

Fig. 5. Determination and differentiation of syncytiotrophoblasts in *Map2k1*$^{-/-}$ placentas. (**A-F**) The functional differentiation of chorioallantoic trophoblasts in syncytiotrophoblasts was assessed by *Gcm1* in situ hybridization (red signal) in E8.5 (A,B), E9.5 (C,D) and E10.5 (E,F), wild-type (A,C,E) and *Map2k1*$^{-/-}$ (B,D,F) placentas. *Gcm1*-positive cells were detected as early as E8.5 in wild-type and *Map2k1*$^{-/-}$ placentas, with no major difference between the genotypes. However, at E9.5 and E10.5, *Gcm1* labeling remained restricted to the chorioallantoic interface in *Map2k1*$^{-/-}$ specimens (D,F; arrowheads), whereas in control placentas, *Gcm1*-positive cells invaded the labyrinth to line the maternal sinuses (C,E; arrows). (**G-J**) Alkaline phosphatase activity in E10.5 wild-type (G,I) and *Map2k1*$^{-/-}$ (H,J) placentas. In wild-type specimens (I), cells surrounding the maternal blood sinuses produced high levels of alkaline phosphatase activity (arrowheads). There is a correlation between the punctuated localization of alkaline phosphatase activity (J; arrowheads) and the *Gcm1* expression in the chorioallantoic interface of *Map2k1*$^{-/-}$ placentas (F; arrowhead). Scale bars: 100 μm in A-H; 50 μm in I,J.

cells have essentially the reciprocal developmental potential compared with tetraploid cells in chimeras. They can participate to all inner cell mass derivatives but not to trophectoderm or primitive endoderm lineages. Based on these characteristics, aggregation of *Map2k1*$^{-/-}$ ES cells to wild-type tetraploid embryos should lead to the identification of the tissue responsible for the placental abnormality. The combination of *Map2k1*$^{-/-}$ ES cells and tetraploid wild-type embryos allowed the survival of *Map2k1*$^{-/-}$ embryos up to E11.5 and E13.5 (four and one chimeric embryos, respectively; Fig. 6C,E). Gross morphology and histological staining of the chimeric placentas revealed that the labyrinth developed normally (Fig. 6H,J,M,O,R,T). Anti-CD31 staining showed that in *Map2k1*$^{-/-}$ tetraploid-rescued embryos, fetal blood vessels migrated into the labyrinth and intermingled with the maternal sinuses similarly to those observed in wild-type placenta (Fig. 6U-W). The *Map2k1*$^{-/-}$ tetraploid chimeras obtained at E11.5 were well developed, whereas the gross morphology and the histology of the chimera obtained at E13.5 suggested that the rescue was incomplete. The E13.5 chimera was slightly underdeveloped and presented signs of hemorrhage (Fig. 6E). Moreover, even though the vascularization of the labyrinth was normal, no fetal blood cell was detected in the blood vessels conversely to those seen in *Map2k1*$^{+/-}$ tetraploid chimeras (Fig. 6S,T). These observations raised the possibility that the tetraploid-aggregation experiments rescued partially the *Map2k1*$^{-/-}$ mutant phenotype. Alternatively, as one *Map2k1*$^{-/-}$ ES cell clone was tested in the tetraploid rescue experiments, we cannot exclude a clonal effect.

Altogether, these data indicated that *Map2k1* might be dispensable for the normal development of the embryo, and that MAP2K1 is required in extra-embryonic-derived structures like the trophoblasts for the normal development of the labyrinthine region.

Conditional deletion of *Map2k1* gene in the embryo

To circumvent the placental phenotype and determine whether *Map2k1* gene function is required for normal mouse embryo development, we generated mice carrying a conditional *Map2k1* mutation. The third exon sequences, encoding the ATP-binding site of the MAP2K1 catalytic domain, were flanked by *loxP* sites (Fig. 7A; see Materials and methods). *Map2k1*^{floxed} mice were bred with *Sox2Cre* transgenic mice to specifically delete *Map2k1* exon 3 sequences in embryonic derivatives (Hayashi et al., 2002). To do so, *Sox2Cre*;*Map2k1*$^{+/\Delta}$ males were bred with *Map2k1*$^{+/floxed}$ females. With this breeding, normal Mendelian ratio of live-born *Map2k1*$^{\Delta/\Delta}$ animals was obtained (Table 1; Fig. 7B). To verify that the *Map2k1*$^{\Delta/\Delta}$ mice were devoid of MAP2K1 protein, western blot analyses of protein extracts from different organs of *Map2k1*$^{+/+}$ and *Map2k1*$^{\Delta/\Delta}$ adult mice were performed. No MAP2K1 protein was detected in *Map2k1*$^{\Delta/\Delta}$ organs, demonstrating that the *Map2k1*$^{\Delta}$ allele is a null allele (Fig. 7C). Both *Map2k1*$^{\Delta/\Delta}$ female and male mice were fertile and when they were intercrossed, they generated litters that died at E10.5, exhibiting the same phenotype as the one observed in the *Map2k1*$^{-/-}$ embryos (Fig. 8A). Altogether, these results confirmed the primordial role of *Map2k1* in placenta development, whereas, in the embryo, it can probably be replaced by its homologue, *Map2k2*.

Normal activation of MAPK1and MAPK3 in rescued *Map2k1*$^{\Delta/\Delta}$ embryos

The activation of the ERK/MAPK cascade has been shown to be required for cell proliferation and survival in mammals (Ballif and Blenis, 2001; Brunet et al., 1995; Zhang and Liu, 2002).

In E9.5 *Map2k1*$^{-/-}$ embryos, the cascade was normally activated, whereas 1 day later, no pMAPK1- and pMAPK3-specific bands were detected in *Map2k1*$^{-/-}$ embryos, despite the presence of activated MAP2K2 (Fig. 2B). These data suggested that the inability of MAP2K2 to activate the ERK/MAPK pathway at E10.5 was due to the fact that the *Map2k1*$^{-/-}$ embryos were dying from underdevelopment of the placenta. To address this issue directly, we analyzed the phosphorylation status of MAPK1 and MAPK3 in E10.5 *Map2k1*$^{\Delta/\Delta}$ embryos and their corresponding *Map2k1*$^{\text{floxed}/\Delta}$ placentas obtained from the breeding of *Sox2Cre*;*Map2k1*$^{+/\Delta}$ males with *Map2k1*$^{+/\text{floxed}}$ females. Western blot analyses revealed no decrease in pMAPK1 and pMAPK3 in *Map2k1*$^{\Delta/\Delta}$ embryos, and in the corresponding placental extracts (Fig. 8B). Therefore, in E10.5 conditionally rescued *Map2k1*$^{\Delta/\Delta}$ embryos, MAP2K2 can activate MAPK1 and MAPK3. Thus, in absence of a normal placenta, E10.5 *Map2k1*$^{-/-}$ embryos were dying and hence could not achieve ERK/MAPK activation.

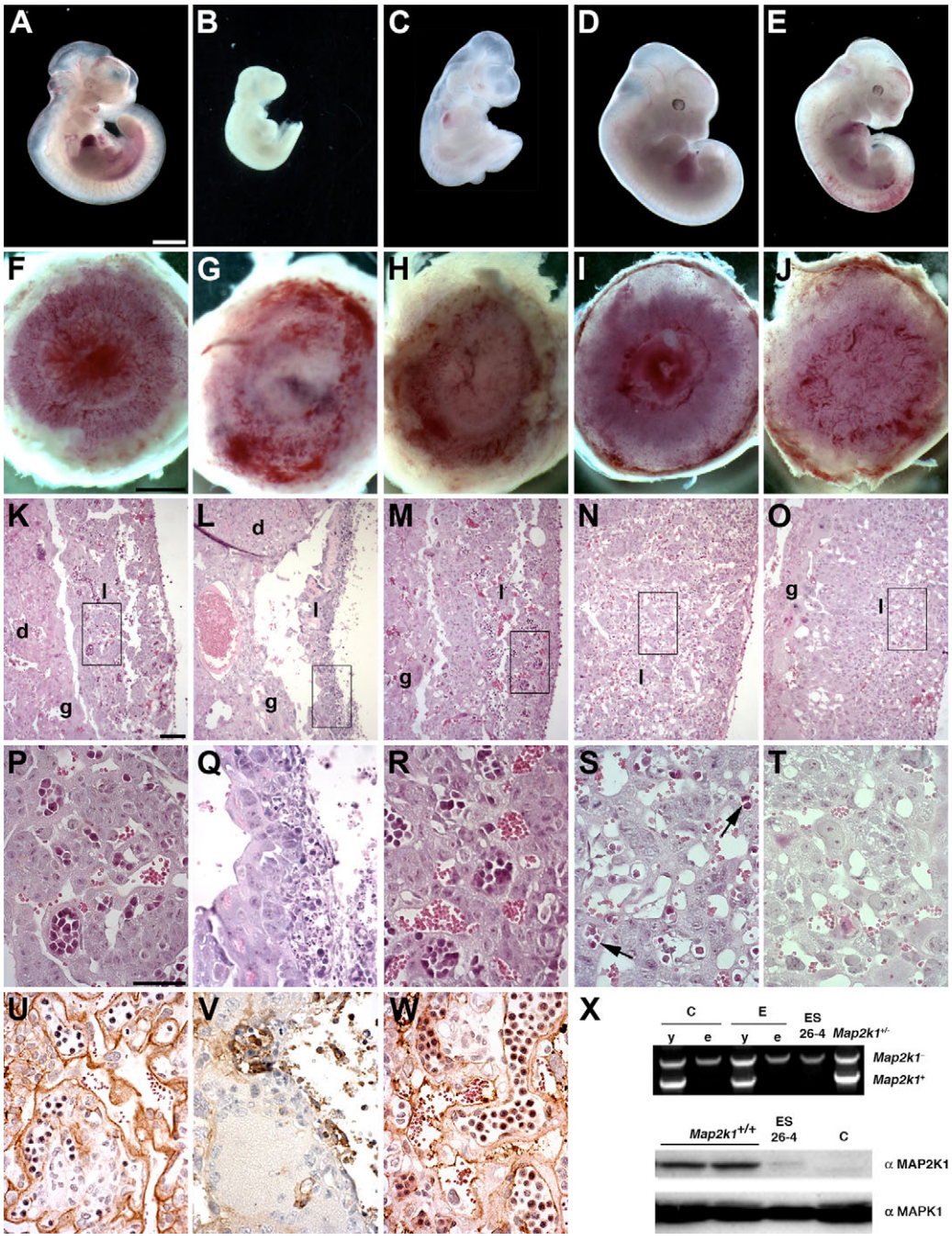

Fig. 6. Tetraploid rescue of *Map2k1*-deficient placentas and fetuses. (**A**,**B**) Wild-type and *Map2k1*$^{-/-}$ embryos are presented for comparison. (**C-E**) Tetraploid-aggregation chimeras were prepared from tetraploid wild-type embryos and diploid *Map2k1*$^{+/-}$ embryos (D) or *Map2k1*$^{-/-}$ ES cells (C,E). After transfer into foster mothers, chimeras were recovered at E11.5 (C) or at E13.5 (D,E) for analysis. Corresponding placentas (**F-J**) were analyzed by Hematoxylin and Eosin staining. (**K-O**) Higher magnification images of F-J. (**P-T**) Higher magnification images of K-O. (C,H,M,R,W) E11.5 chimeras from wild-type tetraploid embryo and *Map2k1*$^{-/-}$ ES cells. For comparison, E10.5 wild-type (A,F,K,P,U) and *Map2k1*$^{-/-}$ (B,G,L,Q,V) specimens are shown. The normal appearance of E11.5 *Map2k1*$^{-/-}$ tetraploid-aggregated embryos (C) and the histology of the placenta (M,R,W) indicated the tetraploid rescue of the *Map2k1*$^{-/-}$ phenotype. The tetraploid chimera obtained at E13.5 (E) also showed normal vascularization of the labyrinth region of the placenta (T) when compared to tetraploid control (D,S). However, the gross morphology of the embryo (E) and the absence of embryonic blood cells in the labyrinth (arrows in S; T) suggested some developmental defects. (**U-W**) Anti-CD31 immunostaining. (**X**) PCR (upper panel) and western blot (lower panel) analyses were performed to confirm the genotype of the tetraploid embryos shown in C,E. Genotype of the tetraploid embryos and yolk sacs revealed that the embryos were *Map2k1*$^{-/-}$, while the yolk sac, which received a contribution from the embryonic and the extra-embryonic tissues contained both *Map2k1* alleles. *Map2k1*$^{-/-}$ ES cells (ES 26-4) and *Map2k1*$^{+/-}$ embryo DNAs were included as control. No MAP2K1 protein was detected in *Map2k1*$^{-/-}$ ES cells (ES 26-4) or in *Map2k1*$^{-/-}$ tetraploid chimera presented in C. d, deciduum; g, tropohoblast giant cells; l, labyrinth. y, yolk sac; e, embryo. Scale bars: 1 mm in A-J; 100 μm in K-O; 50 μm in P-W.

DISCUSSION

The disruption of the murine *Map2k1* gene leads to an embryonic lethal phenotype caused by the underdevelopment of the labyrinth associated with its hypovascularization and revealing the essential role of *Map2k1* during embryonic development (Giroux et al., 1999). By contrast, the absence of phenotype in *Map2k2* mutants suggests that *Map2k2* is dispensable, as *Map2k1* can compensate for the lack of *Map2k2* gene function (Bélanger et al., 2003). We have previously shown that both *Map2k1* and *Map2k2* genes are widely expressed in the placenta (Giroux et al., 1999). Immunostaining of the active forms of MAP2K1 and MAP2K2, as well as their downstream effectors (MAPK1 and MAPK3), revealed that, although MAP2K1 and MAP2K2 can be activated in several cell types of the placenta, their action is limited to specific lineages. For example, detectable levels of pMAPK1 and pMAPK3 were observed in the cells lining the maternal sinuses, the allantoic cells and some labyrinthine trophoblasts of wild-type placentas. In *Map2k1*$^{-/-}$ placentas, pMAPK1 and pMAPK3 were restricted to the allantoic cells at the chorioallantoic interface, suggesting that the activation of the ERK/MAPK cascade in the labyrinth may specifically require *Map2k1*. It has recently been shown that the catalytic activity of activated MAP2K1 and MAP2K2 proteins can be repressed by the phosphorylation of Ser^{212} (Gopalbhai et al., 2003), suggesting that the phosphorylation of MAP2K2 at Ser^{212} may account for the lack of MAPK1/MAPK3

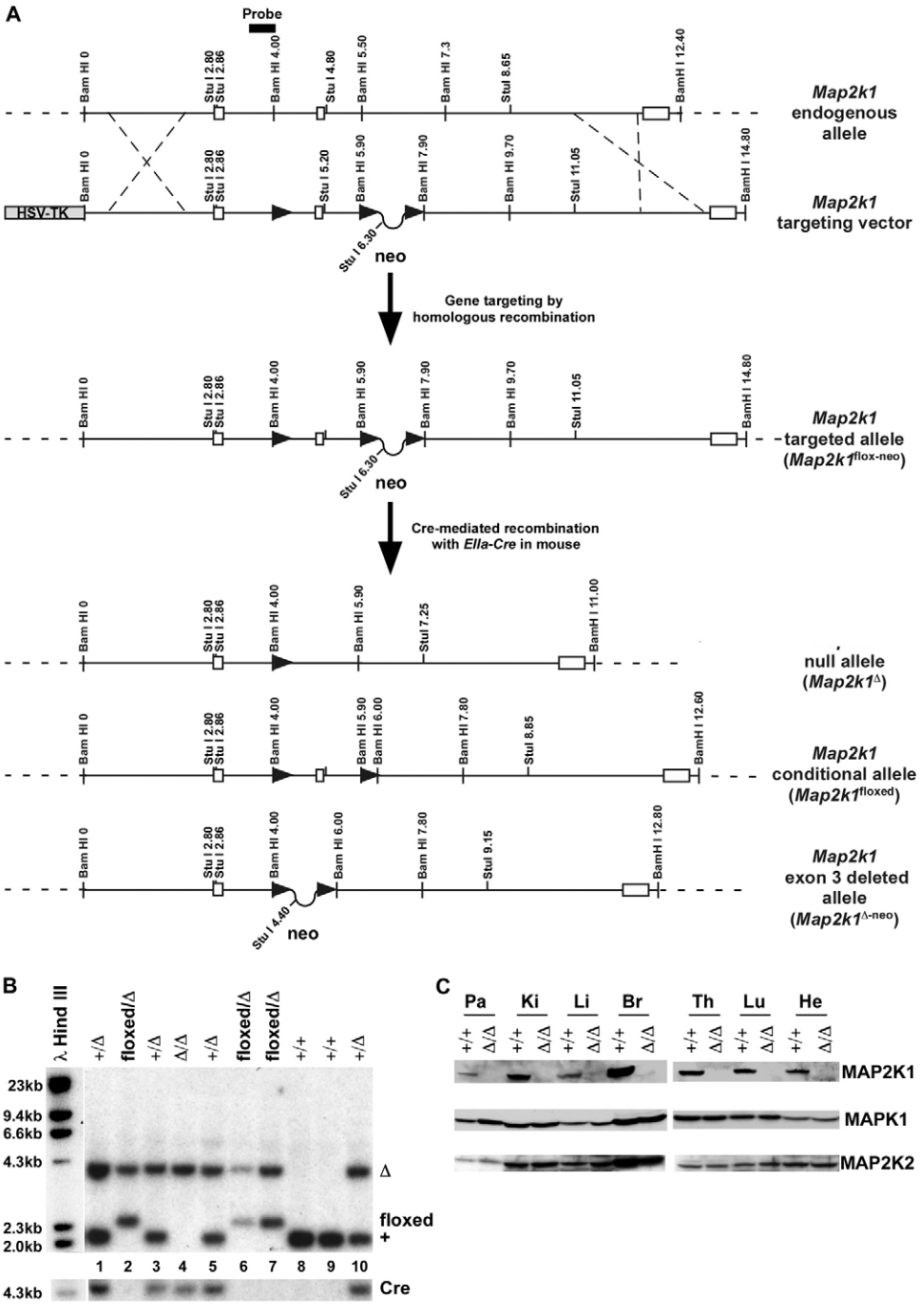

Fig. 7. Generation of the *Map2k1* conditional allele. (**A**) *Map2k1* gene targeting strategy for the generation of the conditional allele. *Map2k1* translated exons 2 to 4 are represented by the white boxes. A *neo* selection cassette flanked by *loxP* sites was inserted between the third and fourth *Map2k1* exons. A third *loxP* site was inserted between the second and the third exons. The targeting vector contains 5.5 kb and 6.9 kb of *Map2k1* homologous genomic sequences on the 5′ and 3′ sides of the *neo* insertion, respectively. The herpes simplex virus-thymidine kinase (HSV-TK) selection cassette (gray box) was added at the 5′ end of the vector. (**B**) Southern blot analysis of tail DNA from a litter obtained after breeding a *Map2k1*$^{+/floxed}$ female with a *Sox2Cre*$^{+/-}$;*Map2k1*$^{+/\Delta}$ male. Tail DNA was digested with *Stu*I, blotted and hybridized with a *Map2k1* genomic probe (A; upper panel) and a *Cre* probe (lower panel). The position of the different alleles is indicated on the right side of the panels. (**C**) Western blot analyses of proteins extracted from different organs of 8-week-old wild-type (+/+) and *Map2k1*$^{\Delta/\Delta}$ (Δ/Δ) littermates. Triplicate blots were probed with antibodies directed against MAP2K1, MAPK1 and MAP2K2. Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Pa, pancreas; Th, thymus.

Table 1. Rescue of the placental phenotype in *Sox2Cre* transgene-mediated recombination

	Genotype of live born pups						
	Map2k1$^{+/+}$; Tg$^{+/+}$	*Map2k1*$^{+/\Delta}$; Tg$^{+/+}$	*Map2k1*$^{floxed/\Delta}$; Tg$^{+/+}$	*Map2k1*$^{+/floxed}$; Tg$^{+/+}$	*Map2k1*$^{+/+}$; Tg$^{+/Sox2Cre}$	*Map2k1*$^{+/\Delta}$; Tg$^{+/Sox2Cre}$	*Map2k1*$^{\Delta/\Delta}$; Tg$^{+/Sox2Cre}$
Number of pups	6	8	4	4	6	6	6
% obtained	15%	20%	10%	10%	15%	15%	15%
% expected	12.5%	12.5%	12.5%	12.5%	12.5%	25%	12.5%

Male *Map2k1*$^{+/\Delta}$;Tg$^{+/Sox2Cre}$ were crossed with female *Map2k1*$^{+/floxed}$.

phosphorylation in the labyrinth. Alternatively, it is also possible that the levels of activated MAPK1 and MAPK3 sufficient for MAP2K1 function in the labyrinth trophoblasts are under the detection levels.

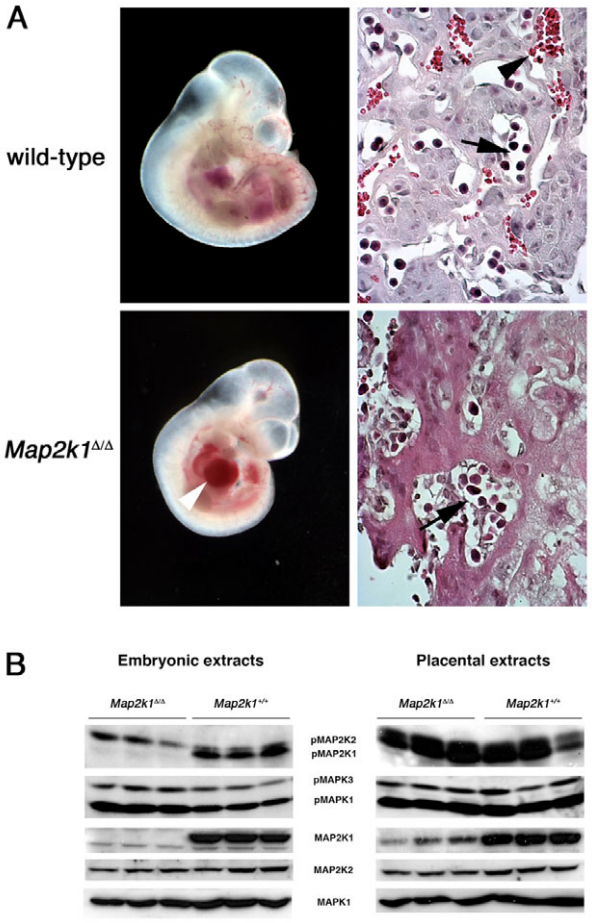

Fig. 8. Normal activation of the ERK/MAPK cascade in rescued *Map2k1*$^{\Delta/\Delta}$ embryos. (**A**) Placental phenotype of *Map2k1*$^{\Delta/\Delta}$ embryos. Gross morphology of *Map2k1*$^{\Delta/\Delta}$ embryos revealed hemorrhages (white arrowhead) characteristic of the *Map2k1*$^{-/-}$ null phenotype. Comparative Hematoxylin-Eosin staining of E10.5 wild-type and *Map2k1*$^{\Delta/\Delta}$ placenta sections showed the absence of maternal sinuses and fetal blood vessels intermingling in mutant specimens. The black arrows and arrowheads indicate the fetal blood vessels and the maternal sinuses in the labyrinthine region, respectively. (**B**) ERK/MAPK cascade activation in *Sox2Cre*;*Map2k1*$^{\Delta/\Delta}$ embryos having a normal development of their extra-embryonic structures. E10.5 embryos produced from mating between *Sox2Cre*;*Map2k1*$^{+/\Delta}$ males with *Map2k1*$^{+/floxed}$ females were used for protein extracts. Western blots were probed with antibodies against MAPK1, MAP2K1, MAP2K2, phospho-MAPK1/MAPK3 and phospho-MAP2K1/MAP2K2.

A similar placental phenotype has been reported for the *Mapk1* mutants, whereas the *Mapk3* mutation does not cause any developmental anomaly (Hatano et al., 2003; Pages et al., 1999; Saba-El-Leil et al., 2003). Together, these results support a model in which placenta development requires that the transduction of specific signals transits via distinct protein kinase isoforms such as MAPK1 and MAP2K1 through the ERK/MAPK cascade. The presence of various isoforms at different levels of the pathway reflects the complexity of the controls required for the regulation of diverse mammalian cellular processes.

Using specific MAP2K1 and MAP2K2 inhibitors, the ERK/MAPK cascade was shown to be required for in vitro trophoblast stem (TS) cell establishment and maintenance (Rossant, 2001). In agreement with this, a strong activation of MAPK1 and MAPK3 was observed in regions of the extra-embryonic ectoderm known for the presence of FGF signaling and TS cell niches (Corson et al., 2003; Tanaka et al., 1998). However, the lack of phenotype in *Map2k2*$^{-/-}$ mutants strongly indicates *Map2k1* as a key player in TS cell establishment and growth. The role of *Map2k1* in TS cell proliferation and maintenance is further supported by the fact that we were able to generate TS cells from wild-type and *Map2k2*$^{-/-}$ embryos but not from *Map2k1*$^{-/-}$ blastocysts (V.B., M.G. and J.C., unpublished; data not shown). Even though the placenta growth is perturbed in *Map2k1*$^{-/-}$ specimens (Fig. 1), the different trophoblastic cell lineages derived from the TS cells (including trophoblast giant cells, spongiotrophoblasts, syncytiotrophoblasts and other trophoblasts from the labyrinth) were present in *Map2k1*$^{-/-}$ placentas (Fig. 5) (Giroux et al., 1999). These results suggest that even if the ERK/MAPK cascade is required in vitro for TS establishment and maintenance, other factors independent of MAP2K1 may contribute in vivo to these processes, allowing the partial development of the *Map2k1*$^{-/-}$ placenta. In the absence of *Map2k1* function, proliferation and survival of the trophoblasts in the chorion and the labyrinthine region were impaired, leading to the underdevelopment of the labyrinth and ultimately to the death of the embryo.

The characterization of the activation of the ERK/MAPK cascade in *Map2k1*$^{-/-}$ embryos and placentas indicates that MAPK1 and MAPK3 are normally phosphorylated in E9.5 *Map2k1*$^{-/-}$ embryos, whereas in their placenta, activation of the ERK/MAPK cascade is reduced. At E10.5, activation of the ERK/MAPK cascade is almost completely abolished in both *Map2k1*$^{-/-}$ embryos and placentas. However, in *Map2k1*$^{\Delta/\Delta}$-rescued embryos, activation of MAPK1 and MAPK3 is comparable with that seen in wild-type specimens (Fig. 8B), indicating that MAPK1 and MAPK3 phosphorylation in the embryo does not require MAP2K1 protein. The absence of ERK/MAPK activation in E10.5 *Map2k1*$^{-/-}$ conceptuses (Fig. 2) was most probably due to the fact that these embryos were dying and undergoing resorption.

The importance of the ERK/MAPK cascade in placental development has been suggested by the identification of placental defects in mouse mutants for several intermediates of the

ERK/MAPK cascade, as well as in mutants affecting other MAPK pathways such as the p38/MAPK cascade (Rossant and Cross, 2001; Watson and Cross, 2005). For example, the *Map3k3* and *Mapk14* mutants (known as *Mekk3* and *p38α*, respectively) present a reduced labyrinthine layer. The development of embryonic, but not of maternal, blood vessels in *Map3k3*$^{-/-}$ labyrinth is dramatically impaired, suggesting a role for the p38/MAPK pathway in the vascularization of the placenta by angiogenesis (Adams et al., 2000; Mudgett et al., 2000; Tamura et al., 2000; Yang et al., 2000). In the case of *Mapk14* mutants, placenta development can be rescued in tetraploid-aggregation experiments, suggesting that the vascularization defect associated with this mutation is non-cell autonomous. We showed that the p38/MAPK cascade was strongly activated in the endothelial cells lining the embryonic blood vessels in wild-type placentas. In *Map2k1*$^{-/-}$ specimens, the p38/MAPK cascade was still activated in the endothelial cells blocked at the chorioallantoic interface. This incapacity of endothelial cells as well as syncytiotrophoblasts to invade the labyrinthine region may reflect the abnormal morphogenesis of the labyrinth in absence of *Map2k1* function. As the p38/MAPK cascade is activated in absence of *Map2k1* function, this suggests that both pathways act independently during placenta formation, with p38 being involved in angiogenesis and MAP2K1 in labyrinthine morphogenesis.

Altogether, our results indicate that the *Map2k1* gene is required in the extra-embryonic ectoderm for the morphogenesis of the labyrinth leading to its vascularization by the embryonic blood vessels derived from the allantois but it is not essential for syncytiotrophoblast cell determination and differentiation. The cell migration deficiency observed in *Map2k1*$^{-/-}$ mouse embryonic fibroblasts might reflect the lack of migration of the syncytiotrophoblasts (Giroux et al., 1999). However, we cannot rule out the possibility that the syncytiotrophoblast defect might be due to the undergrowth of the labyrinth. Indeed, the lack of a sufficient number of labyrinthine trophoblasts in *Map2k1*$^{-/-}$ placenta might explain why the syncytiotrophoblasts are stalled at the chorioallantoic interface. The specific deletion of *Map2k1* in the syncytiotrophoblasts should allow the definition of its role in syncytiotrophoblast cell differentiation and/or function.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/17/3429/DC1

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