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The bHLH-Zip transcription factor *Tfeb* is essential for placental vascularization

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SUMMARY

***Tfeb* is a member of the basic Helix-Loop-Helix-Zipper family of transcription factors. In vitro studies have shown that TFEB can bind DNA as a homodimer or as a heterodimer with three closely related family members: MITF, TFE3 and TFEC. While mutations of *Mitf* have been shown to affect the development of a number of cell types including melanocytes, osteoclasts, and masts cells, little is known about the phenotypic consequences of mutations at *Tfe3*, *Tfeb* and *Tfec*. Here we show that mice with a targeted disruption of *Tfeb* die between 9.5 and 10.5 days in embryonic development and have severe defects in placental vascularization. *Tfeb* is expressed at low levels in the embryo but at high levels in the labyrinthine trophoblast cells of the placenta. While labyrinthine cells are present in the mutant *Tfeb* placenta, they fail to express VEGF, a potent mitogen required for normal vasculogenesis of the embryo and extraembryonic tissues. In *Tfeb* mutant embryos the embryonic vasculature forms normally but few vessels are seen entering the placenta and those that do enter fail to thrive and branch normally. Our results indicate that *Tfeb* plays a critical role in the signal transduction processes required for normal vascularization of the placenta.**

Key words: *Tfeb* transcription factor, Gene targeting, Placenta, Vascularization

INTRODUCTION

Transcription factors of the basic helix-loop-helix leucine zipper (bHLH-Zip) family are DNA binding regulatory proteins that have been implicated in various developmental and cellular processes and some are known to be oncogenes. To date, a total of 18 different family members have been identified in mammals and evolutionary studies suggest that they fall into 6 subfamilies, each with their own characteristics (Atchley and Fitch, 1997). All but one of these proteins regulate gene expression by binding to a DNA sequence known as the E-box (CANNTG) as either hetero- or homodimers; some dimers result in activation of gene expression while others result in repression.

Some members of this family of transcription factors are known to be involved in complex networks of interacting proteins. The best characterized of such networks is the one formed by the MYC, MAX, MAD and SIN3 proteins. The ubiquitous MAX protein is at the heart of this network since it dimerizes with MYC and is required for most activities of that protein (reviewed by Amati and Land, 1994). In addition, MAX can dimerize with each of the five members of the MAD sub-family of bHLH-Zip proteins, resulting in repression of gene expression (Ayer et al., 1993; Hurlin et al., 1997; Hurlin et al., 1995; Zervos et al., 1993). This MAD-mediated repression is due to the interaction of the MAD subfamily of proteins with mSIN3A and mSIN3B, mammalian homologs of the yeast co-repressor SIN3 (Ayer et al., 1995; Hurlin et al., 1997; Hurlin et al., 1995), and recently, it has been shown that the SIN3 proteins in turn interact with histone deacetylases to moderate the repression (reviewed by Pazin and Kadonaga, 1997). The emerging complexity of this network suggests that the specificity of this class of bHLH-Zip proteins is due to protein-protein interactions rather than to specificity in DNA binding, and that their biological effects are determined by their relative activity and concentrations in the cell.

The Mi-Tfe subfamily of bHLH-Zip proteins consists of four related genes, *Mitf*, *Tfe3*, *Tfeb* and *Tfec*, all isolated by different means. The *Mitf* gene was isolated through transgene insertion events at the mouse *microphthalmia* locus (Hodgkinson et al., 1993; Hughes et al., 1993) while the human *TFEB* and *TFE3* genes were both isolated on the basis of their ability to bind the E-box sequence: *TFEB* using a DNA fragment from the adenovirus late promoter and *TFE3* using a fragment from the immunoglobulin heavy chain enhancer (Beckmann et al., 1990; Carr and Sharp, 1990). *Tfec* was subsequently isolated from a rat osteosarcoma cDNA library based on sequence similarity to *TFE3* (Zhao et al., 1993). All four proteins have an identical DNA binding domain and very similar HLH and Zip dimerization domains. In addition, they all contain an amphipathic helix that has been implicated in transcriptional activation (Beckmann et

al., 1990; Yasumoto and Shibahara, 1997) as well as a serine-rich region at the carboxyl-end that serves an unknown function.

Like the other members of the bHLH-Zip family, the Mi-Tfe proteins can bind the E-box sequence as either homodimers or as heterodimers with each other (Hemesath et al., 1994). However, no heterodimer formation has been observed between these proteins and other members of the bHLH-Zip family tested, such as USF1, MYC or MAX or with the bHLH protein E47 (Hemesath et al., 1994). This suggests that the Mi-Tfe proteins form a distinct subfamily of interacting proteins and raises the possibility that, like the MYC, MAX and MAD proteins, they form a protein network whose effects in the cell are determined by the presence of a particular combination of the different family members, at a certain concentration. Such a network would be consistent with the complex genetic behavior of mutations at the mouse *Mitf* locus: many semidominant *Mitf* mutations show coat color phenotypes in the heterozygous condition and some show additional phenotypes in the homozygous condition, phenotypes not seen in loss-of-function mutations in the gene. This indicates that at least some of the phenotypes are caused by interactions of *Mitf* with other factors in the cell. Biochemical experiments support this idea since semidominant mutant *Mitf* proteins act in a dominant negative fashion and interfere with the DNA binding ability of an interacting family member such as *Tfe3* (Hemesath et al., 1994; Steingrímsson et al., 1996). Thus, both the genetics of *Mitf* and the biochemical behavior of *Mitf* mutant proteins indicate that other interacting factors (e.g. *Tfe3*, *Tfeb* or *Tfec*) play an important role in the function of the gene in vivo. Although the in vivo biological roles of the three *Tfe* genes have not been characterized to date, fusions involving the *Tfe3* gene and a novel gene, *PRCC*, have been shown to result in papillary renal cell carcinomas in humans (Weterman et al., 1996).

In order to investigate the biological roles of the *Tfe* genes in development, we have begun to make germline mutations in these genes by homologous recombination in ES cells. Here we report the generation of mice carrying a mutation at the *Tfeb* locus and show that homozygotes die during embryogenesis due to defective vascularization of the placenta. The vascular defects appear to be due to the effects of *Tfeb* on gene expression in the labyrinthine trophoblasts of the placenta and identify a critical role for this transcription factor in vascularization of the placenta.

MATERIALS AND METHODS

Mutant construction and generation of ES cells

Genomic *Tfeb* clones were isolated from a 129/Sv mouse genomic library in lambda FixII (Stratagene) using the human *Tfeb* cDNA as probe (Carr and Sharp, 1990). The genomic structure of clone Tfeb-N1 was determined using PCR and sequencing across exon/intron junctions. A 4.5 kb *Kpn*I/*Bam*HI fragment containing exons 2-5 was isolated from one of the resulting clones and cloned into corresponding sites in pBluescript. A 2 kb *Xba*I/*Bgl*II fragment, which contains sequences 3′ to the *Tfeb* coding region, was endfilled and ligated into the endfilled *Spe*I site of the clone containing the *Kpn*I/*Bam*HI fragment. After confirming the correct orientation of the *Xba*I/*Bgl*II fragment with respect to the *Kpn*I/*Bam*HI fragment, the pGKneo cassette (Soriano et al., 1991) was inserted into the *Bam*HI site in the opposite transcriptional orientation. Finally, the pGKtk cassette (Soriano et al., 1991) was ligated into the *Kpn*I site and the resulting targeting construct linearized with *Not*I and electroporated into CJ7 ES cells as described (Swiatek and Gridley, 1993). DNAs derived from G418/FIAU resistant ES clones were identified by hybridization with probes external to the targeting vector: probe A, a 180 bp *Kpn*I/*Sac*I genomic fragment (which detects a 7.0 kb *Hin*dIII fragment in wild-type DNA and a 7.7 kb fragment in *Tfeb* mutant DNA) and probe B, a 1.2 kb *Bgl*II/*Not*I genomic fragment (which detects a 10.5 kb *Bam*HI fragment in wild-type DNA and a 6.5 kb *Bam*HI fragment in mutant DNA). Eight independent *Tfeb* targeted ES cell lines were isolated and two of those generated chimeras which, when injected into C57BL/6J blastocysts, transmitted the mutant allele to the offspring. Chimeras were identified by the agouti coat color. Male chimeras were mated with C57BL/6J females and agouti colored offspring genotyped by tail clip. Chimeras that transmitted the $Tfeb^{Fcr}$ mutation through the germline were then mated to both 129/Sv and C57BL/6J females to establish lines.

Southern and PCR analysis

DNA was isolated from ES cells according to the method of Laird et al. (1991) and from mouse tails according to the method of Siracusa et al. (1987). DNAs were digested to completion with an excess of restriction enzyme and electrophoresed through 0.8% agarose gels. The DNA was then transferred to Hybond, baked and hybridized according to Jenkins et al. (1982). Probes were as described above and washing was done to a stringency of 0.2-1.0× SSC, 0.1% SDS at 65°C. To genotype embryos, DNA was isolated from embryonic yolk sacs according to the method of Perry (1994) and then PCR amplified using the primers TB35 (5′gtgcctgtctaggccagtggc), Neo7 (5′gcatcgcattgtctgagtaggtgtc) and TB42 (5′gaggaacccgagggatccggctc). PCR amplification of wild-type DNA resulted in a 176 bp fragment while DNA from homozygous mutant animals resulted in a 326 bp fragment; DNA from heterozygous animals resulted in the production of both fragments.

Expression analysis

Northern blots containing RNA from several different mouse tissues and embryonic stages were purchased from Clontech and probed with the mouse *Tfeb* cDNA probe (kindly provided by Colin Hodgkinson and Heinz Arnheiter) according to the manufacturers instructions. To control for loading, the blots were stripped and reprobed with a chicken *Gapd* probe. The expression of *Tfeb* in wild-type and mutant embryos was determined by RT-PCR analysis. Embryos were dissected from mothers at 9.5 dpc, yolk sac DNAs isolated and used for genotyping, and total RNA was also isolated from 9.5 day old embryos using RNAzol (TelTest). The RNA was then reverse transcribed using SuperScript II reverse transcriptase (Gibco BRL) and used in PCR amplification reactions with the following primer pairs: Primer pair A: 5′gcgcatgcagcagcaggctgtc and 5′ctggggatgctgctggggcagg (297 bp fragment); Primer pair B: 5′tcctgccccagcagcatccccag and 5′gcgtgttaggcatctgcatctc (202 bp fragment); Primer pair C: 5′ccctgtctagcagccacctgaacg and 5′gccgctccttggccagggctc (150 bpg fragment); Primer pair D: 5′gcagctctggctccgcatccag and 5′cgcctcccctgggccatcctcactg (147 bp fragment); Actin primers: 5′gtgacgaggcccagagcaagag and 5′aggggccggactcatcgtactc (938 bp fragment).

Histological analysis, in situ hybridization and immunostaining

Postimplantation embryos were collected at various times of gestation. The morning of vaginal plug detection was considered 0.5 dpc. At the desired time, desidua were dissected from the uterus and fixed in 4% paraformaldehyde in PBS. When fixed, the desidua were divided in half by cutting through the placenta, a portion of the yolk sac was removed, washed extensively in PBS and genotyped as described above. The rest of the tissue, both embryo and placenta, was dehydrated and embedded in paraffin for sectioning. Sections, 4-5 μm thick, were mounted on silanized slides (Digene) and every third section stained with H+E. Probe preparation, tissue processing and

hybridization were performed as described by Tessarollo and Parada (1995). The following mouse cDNA probes were used: *Tfeb*, a 1.8 kb probe containing the entire coding region; *Flt1*, a 420 bp probe containing the transmembrane domain and adjacent cytoplasmic region (De Vries et al., 1992); *Tec*, a 2.6 kb probe containing the whole coding region (Mano et al., 1990); *Flk1*, an internal 800 bp *Bam*HI/*Sma*I fragment (Yamaguchi et al., 1993) and *Vegf*, a 1.8 kb probe containing the coding region (Shweiki et al., 1993). Slides were dipped in Kodak NBT emulsion and exposed for 7-9 days. Immunocytochemistry was done using a 1:10 dilution of rat anti-*Pecam* (CD31) antibodies (Pharmingen) after a 6 minute trypsin digestion. The staining was visualized using the rat ABC Elite staining kit (Vector Labs).

RESULTS

Generation of *Tfeb* mutant mice

The targeting vector used to generate the loss-of-function mutation at the mouse *Tfeb* locus is shown in Fig. 1A. The exon-intron structure of the gene was determined by sequencing through the exon-intron boundaries of the genomic clone Tfeb-N1 (E. S., N. G. C. and N. A. J., unpublished data). The first exon shown is equivalent to nucleotides 267 through 501 of the human cDNA which contains an in-frame AUG codon that fits the optimal context of translation initiation in vertebrates (Carr and Sharp, 1990; Kozak, 1987). However, since the reading frame of the human (and mouse) *Tfeb* cDNA is open from the very first nucleotide (Carr and Sharp, 1990), it is unclear whether this AUG codon is used to initiate translation of the TFEB protein. To generate the *Tfeb* mutant construct, a 4 kb *Bam*HI/*Xba*I fragment, which contains the last 5 exons of the gene, encoding for the bHLH-Zip domains as well as the remaining 3′-portion of the *Tfeb* transcription unit, was replaced by the pGKneo cassette in the opposite transcriptional orientation. The resulting construct contains 4.5 and 2 kb of 5′ and 3′ homology, respectively. The linearized construct was introduced into CJ7 embryonic stem cells (ES cells) and recombinant cell lines identified using genomic probes external to the targeting construct (probes A and B in Fig. 1A). Recombinant clones containing the predicted rearranged bands were obtained at a frequency of 8/275 or 2.9%. Four independent recombinant clones were injected into C57BL/6J blastocysts; two of those gave rise to male chimeras that transmitted the mutation through the germline and both showed the same phenotype.

Mice heterozygous for the mutation, designated $Tfeb^{Fcr}$, had no apparent differences in phenotype when compared to wild-type littermates. However, no liveborn homozygous mutant mice were recovered among 144 offspring from heterozygous intercrosses (Table 1), indicating that the $Tfeb^{Fcr}$ mutation is an embryonic lethal. To determine the time of death, embryos derived from intercrosses were fixed for histological analysis at 8.5-12.5 days post coitum (dpc) and their *Tfeb* genotype determined from yolk sac DNAs (Fig. 1B). The results of this analysis show that death of $Tfeb^{Fcr}$ homozygous embryos occurs between 9.5 and 11.5 dpc (Table 1). At 8.5-10.5 dpc, the expected number of homozygous embryos are observed, while at 11.5 dpc, fewer homozygotes are found and all have degenerated to a small mass with no recognizable features (Table 1 and data not shown). No homozygous embryos were detected at 12.5 dpc, although some resorbed tissue was present that could not be genotyped.

In order to determine the effects of the $Tfeb^{Fcr}$ mutation on *Tfeb* gene expression, RT-PCR analysis was used on wild-type (littermate) and homozygous mutant embryos. Embryos were collected at 9.5 dpc from intercrosses of heterozygous $Tfeb^{Fcr}$ animals and their genotypes determined from yolk sac DNA. Three wild-type and three $Tfeb^{Fcr}$ homozygous embryos were pooled, respectively, for total RNA isolation, reverse

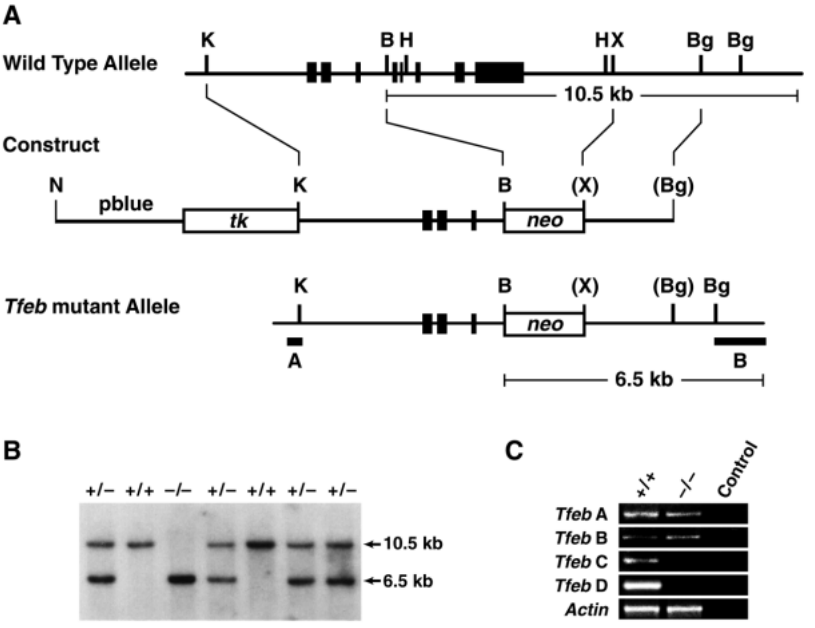

Fig. 1. Generation and analysis of mice with a targeted mutation in the *Tfeb* gene. (A) Targeting of the *Tfeb* gene. The genomic structure of the wild-type allele of *Tfeb* is shown at the top with exons indicated as black boxes. Restriction enzymes used for making the construct and for diagnostics are indicated. A 4 kb *Bam*HI/*Xba*I fragment containing a portion of the basic domain, the HLH and Zip domains as well as the rest of the *Tfeb* coding region was replaced by the PGK*neo* cassette. The *tk* gene was placed at the 5′ end of the *Tfeb* gene to create a mutant allele which, after homologous recombination, lacks the three conserved functional domains of *Tfeb*. Probes A and B are indicated as heavy lines and represent a 180 bp *Kpn*I/*Sac*I genomic fragment and a 1.2 kb *Bgl*II/*Not*I genomic fragment, respectively. The wild-type and mutant *Bam*HI fragments detected with probe B are indicated. (B) Southern analysis of *Bam*HI-digested yolk sac DNAs obtained from 11.5 day old embroys. The Southern blots were probed with probe B and show the expected rearrangement. (C) RT-PCR expression analysis of *Tfeb* in 3 pooled wild-type (+/+) and 3 pooled homozygous $Tfeb^{Fcr}$ embryos (–/–) using primer pairs (see Materials and Methods) from various regions of the *Tfeb* cDNA. The actin primer pair results in the production of a 938 bp fragment and serves as a control for RNA isolation and reverse transcription. The control lane serves to check for contamination of reagents used in the PCR reactions and contains a mix of +/+ and –/– RNA that has not been reverse transcribed. B, *Bam*HI; K, *Kpn*I; H, *Hin*dIII; X, *Xba*I; Bg, *Bgl*II; N, *Not*I. Parenthesis indicate that these sites were destroyed upon cloning.

Table 1. Lethality of *Tfeb*^{Fcr} homozygotes in utero

Tfeb$^{-/+}$ × *Tfeb*$^{-/+}$ Intercrosses				
Age of progeny (dpc)	Genotypes of progeny			
	–/–	+/–	+/+	Total
8.5	16 (32%)	25 (50%)	9 (18%)	50
9.5	29 (29%)	42 (42%)	30 (29%)	101
10.5	14 (26%)	23 (43%)	17 (31%)	54
11.5	3* (15%)	12 (60%)	5 (25%)	20
12.5	3** (23%)	8 (62%)	2 (15%)	13
Adults	0 (0%)	82 (57%)	62 (43%)	144

*All embryos small.
**3 embryos resorbed and presumed to be *Tfeb*^{Fcr} homozygotes.

transcription and PCR amplification with various *Tfeb* primer pairs as well as with an actin primer pair (Fig. 1C). All primer pairs were designed to span exon-intron boundaries in order to eliminate false results from genomic DNA contaminations. Primer pairs A and B are derived from the 5′ portion of the cDNA (exons 1-2 and 2-3, respectively) that are not deleted in the *Tfeb* construct. Primer pairs C and D, on the other hand, are derived from the 3′ portion of the gene (exons 4-5 and 7-8, respectively), that is deleted in the *Tfeb* construct. These results clearly show that although the expression of *Tfeb* is initiated and the first three exons expressed, the 3′ portion of the message is not expressed in mutant embryos. Thus, if expressed, the *Tfeb* mutant protein would be missing essential functional domains of the protein (b, HLH and Zip) and would only contain a fraction of the N-terminal region. There is no report of a separate function of the amino-terminal region of *Tfeb*. These results strongly suggest that the *Tfeb*^{Fcr} mutation represents a null allele.

Analysis of the *Tfeb* lethality

To identify the cause of death of the *Tfeb*^{Fcr} homozygotes, embryos were collected at several stages (9.5, 10.5 and 11.5 dpc), genotyped from yolk sac DNA and fixed for histological analysis. Phenotypic analysis shows that homozygous 9.5 day old *Tfeb*^{Fcr} embryos are indistinguishable from their wild-type littermates (Fig. 2A,C,D; data not shown). The mutant embryos are of normal size and appearance, and sections through various regions (e.g. neural tube in Fig. 2C,D) show no obvious cellular abnormalities. However, a day later (at 10.5 dpc) homozygous *Tfeb*^{Fcr} embryos exhibit clear signs of developmental arrest; although these embryos are of normal size and appearance when compared grossly to wild-type littermates (Fig. 2B), sections through fixed mutant embryos show general signs of cell death in all tissues examined (Fig. 2F,I,J; data not shown). Lysed cells and pycnotic nuclei can be seen in all tissues, including somites, neural tube, ganglia and mesenchyme (Fig. 2F,I,J) from mutant embryos while wild-type embryos show no such signs of cell death (Fig. 2E,G,H). At 11.5 days, homozygous mutant embryos are much smaller than their littermates and have undergone autolysis to the extent that tissues are hard to recognize (data not shown).

Expression analysis

To gain insight into the nature of the *Tfeb* mutation, the expression pattern of the gene was characterized by northern analysis. In wild-type adults, the *Tfeb* gene is expressed at varying levels in all tissues analyzed (Fig. 3A). The major

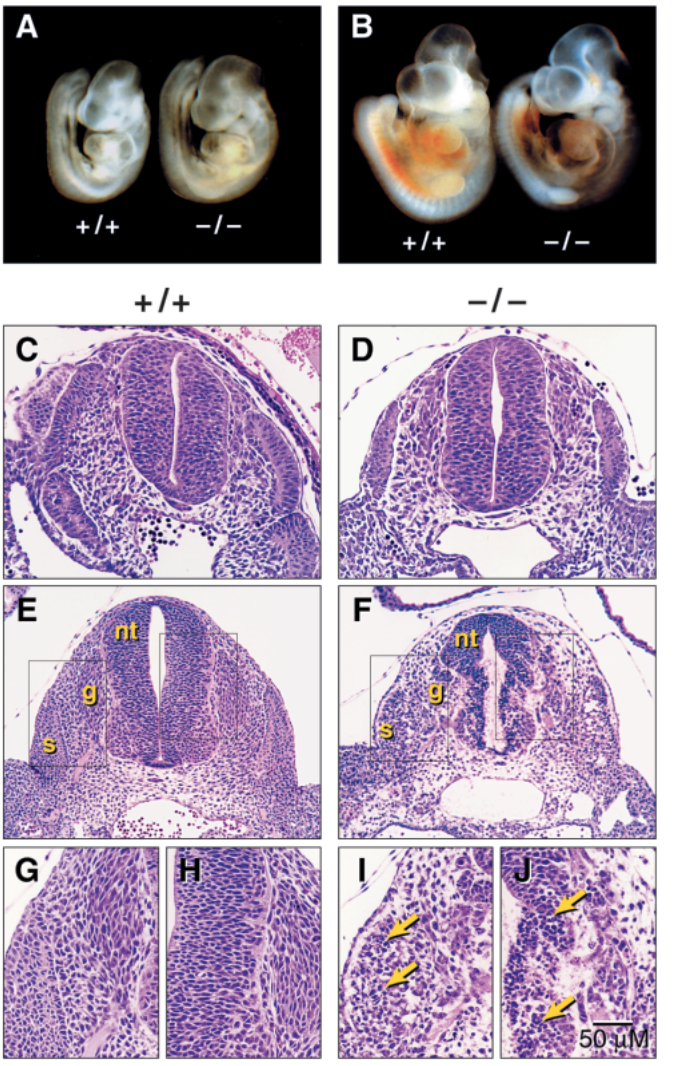

Fig. 2. Developmental arrest in *Tfeb*^{Fcr} homozygotes at 10.5 days of development. (A,B) Wild-type and homozygous *Tfeb*^{Fcr} mutant embryos at 9.5 dpc (A) and 10.5 dpc (B) showing normal size and external appearance of mutant embryos. (C-J) Sections through various regions of 9.5 dpc (C,D) and 10.5 dpc (E-J) wild-type (C,E,G,H) and *Tfeb*^{Fcr} mutant (D,F,I,J) embryos. At 9.5 dpc the tissues are morphologically normal in homozygous *Tfeb*^{Fcr} embryos while at 10.5 days, the mutant embryos show signs of general cell death in all tissues examined. Pycnotic nuclei and lysed tissues (arrows) are clearly visible in *Tfeb*^{Fcr} homozygous embryos (F,I,J) while no such evidence of cell death can be seen in littermate controls (E,G,H). s, somite; g, ganglia; nt, neural tube.

product is 2.5 kb in size and is expressed in all tissues analyzed to date (Fig. 3A). Smaller products of 1.6 and 1.4 kb are also detected in most tissues and appear to be expressed in a tissue specific manner; the 1.6 kb product is expressed in liver, skeletal muscle and kidney while the 1.4 kb message is expressed in heart, spleen, lung, skeletal muscle and kidney. Only kidney and skeletal muscle express all three messages. A 2.0 kb transcript is also seen in the testis (Fig. 3A). Northern analysis shows that a low level of *Tfeb* expression can also be detected during embryogenesis (Fig. 3B). Embryos at 7, 11, 15 and 17 days of development all make the 2.5 kb transcript

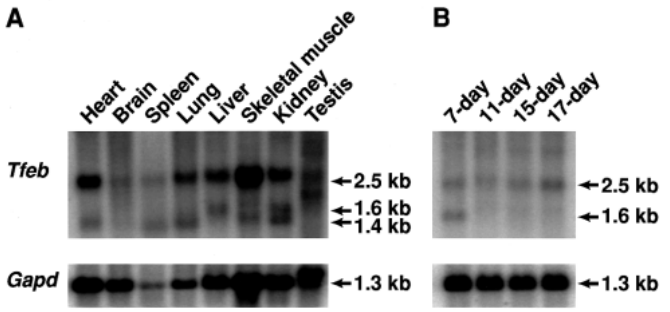

Fig. 3. Northern analysis of *Tfeb* expression in adult tissues (A) and at several stages of embroynic development (B). 2 μg of polyadenylated RNAs from the tissues shown were fractionated on agarose gels, transferred to nylon membranes (Clontech) and hybridized with radiolabeled mouse *Tfeb* cDNA. The filter was rehybridized with a chicken *Gapd* cDNA probe to control for loading.

while the 7-day old embryos also express the 1.6 kb transcript. The exact nature of the smaller alternative *Tfeb* messages has not been characterized. These results suggest that any, or all, tissues could be affected by the *Tfeb* mutation.

The expression pattern of the gene was also characterized by in situ hybridization of embryonic and extraembryonic tissues. This analysis showed that *Tfeb* is expressed at very low levels in 8.5-10.5 day old embryos since similar signals were obtained using both sense and antisense *Tfeb* probes (data not shown). Although the antisense signal is low, the gene must be expressed at some level in the embryo since, as shown above, bands can be detected on northern blots using the *Tfeb* cDNA as probe (Fig. 3B). In sharp contrast to the absence of detectable signal in the embryo, in situ analysis on extraembryonic tissues showed that *Tfeb* is expressed at a very high level in labyrinthine trophoblasts of 9.5 day old placentas (Fig. 4A). The gene is also expressed at a considerable level in 10.5 day old placentas (Fig. 4D) and in 8.5 day old placentas (data not shown). This expression seems to be restricted to the labyrinthine trophoblasts, since no expression is detected in other placental cell types such as spongiotrophoblasts or trophoblast giant cells and no expression is observed in other cell types such as the chorion, embryonic or maternal vasculature or in the yolk sac (Fig. 4A,D and data not shown). The high level of expression of *Tfeb* in the labyrinthine trophoblasts of the placenta suggests that the embryonic lethality of $Tfeb^{Fcr}$ homozygotes might not be due to the requirement of the gene in the embryo itself, rather it may be required for normal placental development.

Tfeb is required in placenta

Consistent with the hypothesis that *Tfeb* acts in the placenta, histological examination of placentas of 10.5 day old homozygous $Tfeb^{Fcr}$ mutants and wild-type and heterozygous littermates revealed a striking difference between the two. In wild-type (and heterozygous $Tfeb^{Fcr}$) placentas of this age, the embryonic vasculature, as evidenced by vessels filled with nucleated embryonic blood cells, has invaded and branched extensively into the labyrinthine trophoblast layer to form a dense network of vessels (Fig. 5B,F). In homozygous $Tfeb^{Fcr}$ mutants, however, the only embryonic vessels present at this stage are seen in the chorion, at the base of the placenta (Fig. 5C,J). No embryonic vessels are seen in the labyrinth layer (Fig. 5C,J). Maternal sinuses still seem to be able to migrate into the placenta, although they are fewer and smaller than normal. No other defects are apparent: the allantois fuses

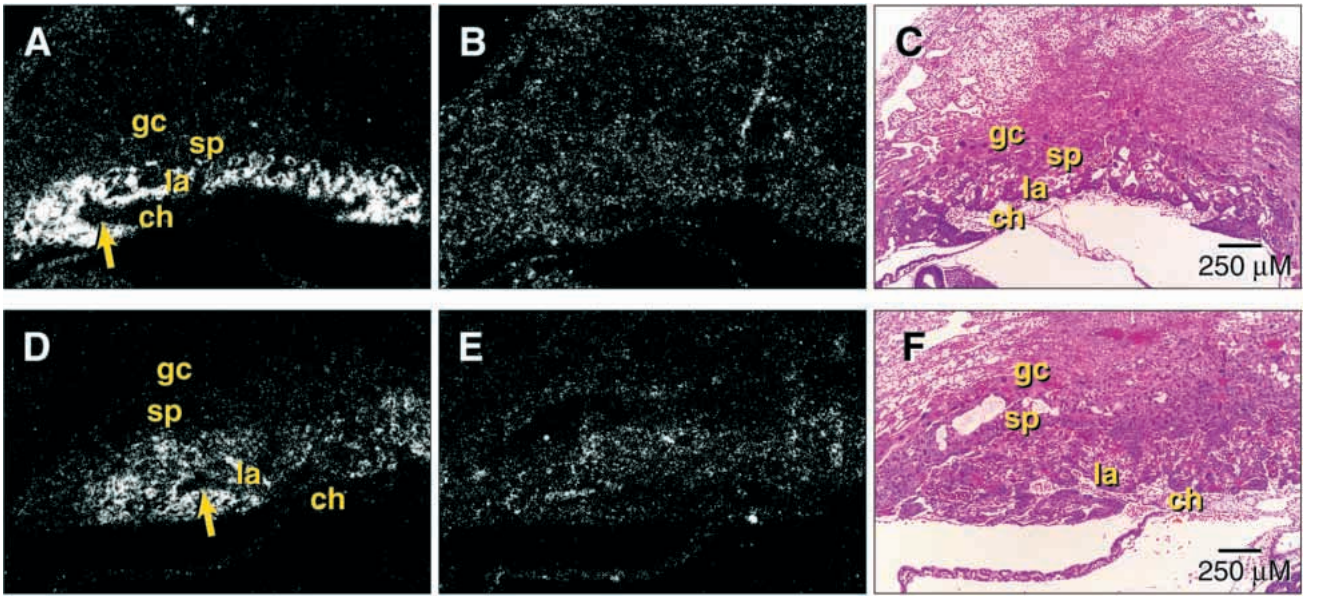

Fig. 4. Expression of *Tfeb* by in situ analysis. *Tfeb* expression in 10.5 dpc (A-C) and 11.5 dpc (D-F) wild-type placentas using both antisense (A,D) and sense (B,E) *Tfeb* probes. The *Tfeb* message is highly abundant in the labyrinthine trophoblasts of the placenta and is not detectable elsewhere. Areas highly abundant in embryonic vessels are indicated with arrows in A and D in order to show the absence of signal in endothelial cells. Adjacent sections stained with H+E are shown in C and F. gc, giant cells; sp, spongiotrophoblasts; la, labyrinth; ch, chorioallantois.

normally with the chorion, the yolk sac and desidua are normal in appearance histologically and no defects are apparent in either the spongiotrophoblasts or giant cells (Fig. 5 C,J and data not shown).

The molecular processes that regulate vascularization of the placenta are not well understood. From histological analysis it is known that after the fusion of the allantois to the chorion, embryonic vessels (filled with embryonic nucleated erythrocytes and lined by endothelial cells) enter the placenta from the allantois and subsequently invade the placenta and branch out to form a dense vascular network (Fig. 5A,B,D-G). At the same time, maternal sinuses (filled with anucleated maternal blood cells, yet not lined by endothelial cells) are forming or invading the desidua from the opposite (uterine) side (Theiler, 1989 and Fig. 5A,B,D-G). Eventually, the labyrinth of the placenta becomes tightly packed with juxtaposing embryonic and maternal blood vessels, allowing the embryonic vasculature to intermingle with maternal blood sinuses to provide efficient transport of oxygen and nutrients into the embryo (Fig. 5F,G).

Part of this process is normal in homozygous *Tfeb*^{Fcr} placentas. The allantois fuses with the chorion in a normal fashion (data not shown) and the embryonic vasculature is formed and seems to be able to initiate invasion of the labyrinth. In 8.5 and 9.5 dpc *Tfeb*^{Fcr} mutant placentas, vessels appear to be entering the labyrinth from the chorion, although fewer vessels are seen entering mutant placentas than wild-type placentas (Fig. 5H,I). Twelve hours later (at 10 dpc), very few

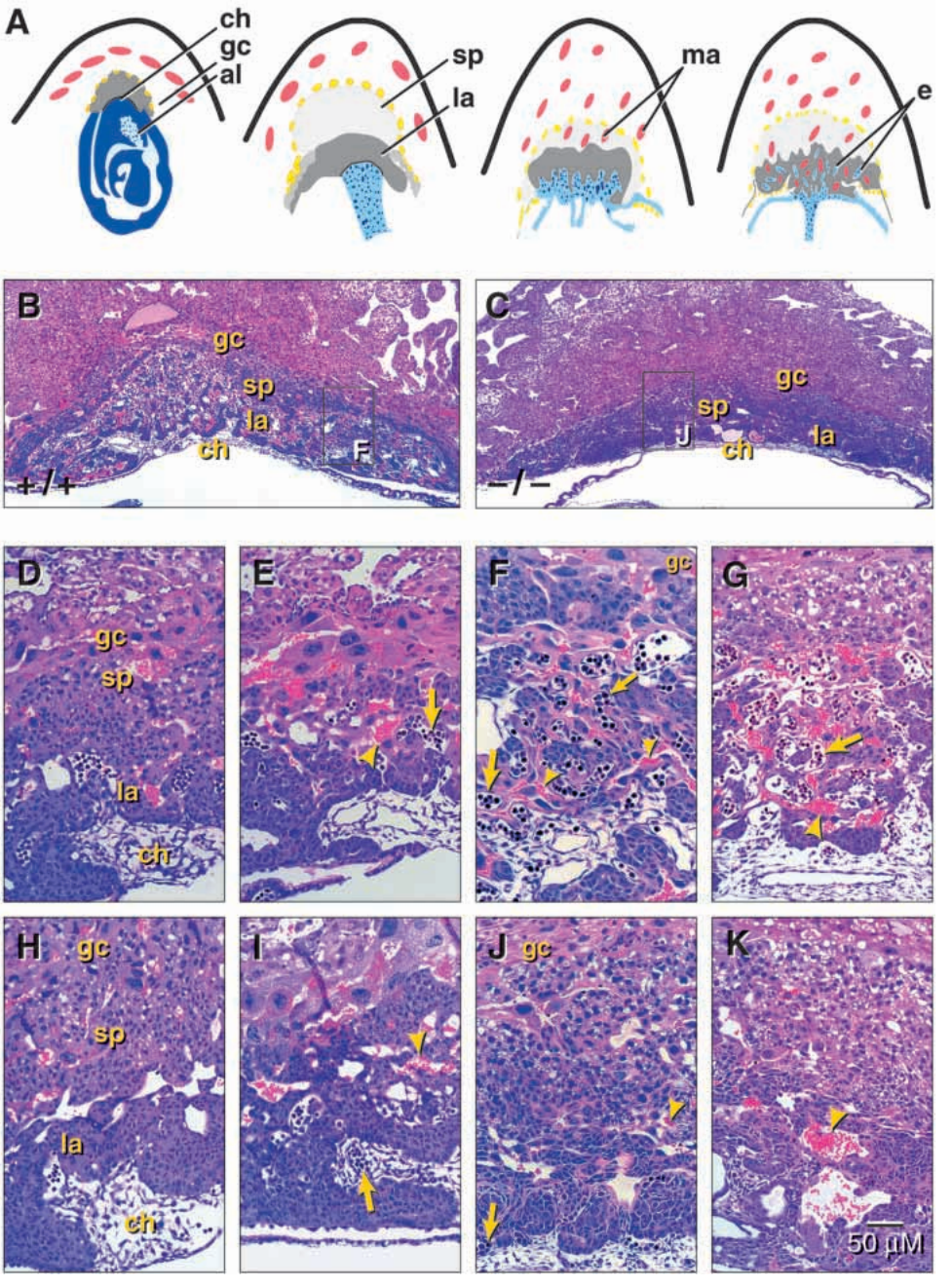

Fig. 5. The *Tfeb*^{Fcr} mutation affects vascularization of the placenta. (A) A schematic drawing outlining the vascularization process in the mouse placenta. The embryonic blood vessels (e) are shown in light blue with nucleated blood cells indicated by darker blue, maternal blood sinuses (ma) are shown in pink and giant cells (gc) in yellow. As development proceeds, the allantois (al) fuses with the chorion (ch) to form the future umbilical cord and its connection to the placenta. The developing embryonic vasculature migrates along the allantois and, after the fusion event, branches into the labyrinth (la) of the placenta. The maternal vascular lagunas grow in from the opposite direction and the two different vascular systems meet to form a dense network. (B,C,F,J) Histological analysis of 10.5 dpc wild-type (B; boxed area enlarged in F) and *Tfeb*^{Fcr} mutant (C; boxed area enlarged in J) placentas. In the mutant placenta the embryonic vasculature (filled with nucleated embryonic blood cells, indicated with arrows) appears unable to invade or branch into the labyrinthine trophoblast layer while maternal sinuses are still present (filled with pink anucleated maternal blood cells, indicated with arrowheads). Note the near complete absence of embryonic vessels in the labyrinth of mutant placentas. (D-K) Temporal profile of placental vascularization in wild-type (D-G) and *Tfeb*^{Fcr} mutant (H-K) placentas at 8.5 (D,H), 9.5 (E,I), 10.5 (F,J) and 11.5 (G,K) days of development. Notice the abundance of embryonic blood vessels (arrows) and their close proximity to maternal blood sinuses (arrowheads) in wild-type placentas at 10.5 and 11.5 days (F,G) while in the mutants the embryonic blood vessels are not visible or lie at the base of the chorion (J,K). At 8.5 and 9.5 days of development, few embryonic vessels are seen entering the labyrinth of mutant placentas and they never travel far (compare the numerous embryonic vessels in the labyrinth in D and E to the few in H and I). Labels are as in Fig. 4.

vessels are seen in the labyrinth (data not shown) and at 10.5 dpc, the only remaining embryonic vessels are in the chorion, at the base of the placenta and have never traveled far into the labyrinth layer itself at this stage of development (Fig. 5J). The same is true in 11.5 day old placentas (Fig. 5K). Since the embryonic vessels initially seem able to enter the labyrinth, yet later fail to sustain further branching into the placenta, it is likely that the presence of *Tfeb*-expressing labyrinth cells is necessary for supporting normal branching or survival of the vascular system in the placenta and not for their initial establishment or migration. As just explained, the defects observed in placenta are visible at 9.5 days of development as fewer vessels seem able to enter the labyrinth (Fig. 5I and data not shown). This is one day before obvious histological defects are visible in the embryo (Fig. 2D), supporting the idea that the embryonic lethality is caused by placental defects which subsequently result in hypoxia and cell death in the embryos.

Tfeb affects gene expression in the placenta

In order to determine the fate of the labyrinthine trophoblasts in *Tfeb*^{Fcr} mutant placentas, the *Flt1* and *Tec* genes were used as markers in in situ experiments. The receptor tyrosine kinase *Flt1* is expressed at a high level in spongiotrophoblasts of 10.5 and 12.5 day old wild-type placentas (Dumont et al., 1995; Fig. 6A). It is also detected at a high level in 10.5 day old *Tfeb*^{Fcr} homozygous mutant placentas (Fig. 6B), suggesting that the expression of this gene in placental spongiotrophoblasts is unaffected by the *Tfeb*^{Fcr} mutation. However, the tyrosine kinase *Tec* (Mano et al., 1990), which normally is expressed specifically in the labyrinthine trophoblasts of the placenta (Fig. 6E), was not expressed in *Tfeb*^{Fcr} mutant placentas (Fig. 6F). This indicates that either the labyrinthine trophoblasts are missing from *Tfeb* mutant placentas, or that they are present, yet do not express genes such as *Tec* which are required for normal function of these cells. The latter explanation is more likely since, in *Tfeb*^{Fcr} mutant placentas, a layer of cells is present between the *Flt1*-positive cells and the chorion, just like in the wild-type situation (indicated by brackets in Fig. 6). In addition, by histological analysis, these cells have characteristics of labyrinthine trophoblasts; their cytoplasm is small when compared to the cytoplasm of spongiotrophoblasts and giant cells (Fig. 5F and J). The apparent difference in thickness of the labyrinthine trophoblast cell layer between wild-type and mutant placentas (as evidenced by the difference in the height of the brackets in Fig. 6) can be accounted for by the presence of embryonic vessels in wild-type placentas and not in the *Tfeb*^{Fcr} mutant.

Placental vasculature

In order to determine if the embryonic endothelial cells appear normal in the mutants, antibodies against the endothelial marker *Pecam* (platelet-endothelial cell adhesion molecule) (Vecchi et al., 1994) were used to stain 10.5 dpc wild-type and mutant placentas. The results show that in wild type, endothelial cells surround the embryonic blood cells and can be found all over the labyrinth of the placenta (Fig. 7A); the maternal blood sinuses are not lined by endothelial cells. While endothelial cells still surround the embryonic blood cells of *Tfeb*^{Fcr} mutant placentas, they have not ventured far beyond the chorion into the placenta or formed the extensive networks seen in wild type (Fig. 7A,B). The same results were obtained using the receptor tyrosine kinase *Flk1* as a probe in in situ experiments (data not shown). This shows that the endothelial cells form normally in the mutant and their function is normal in the sense that they

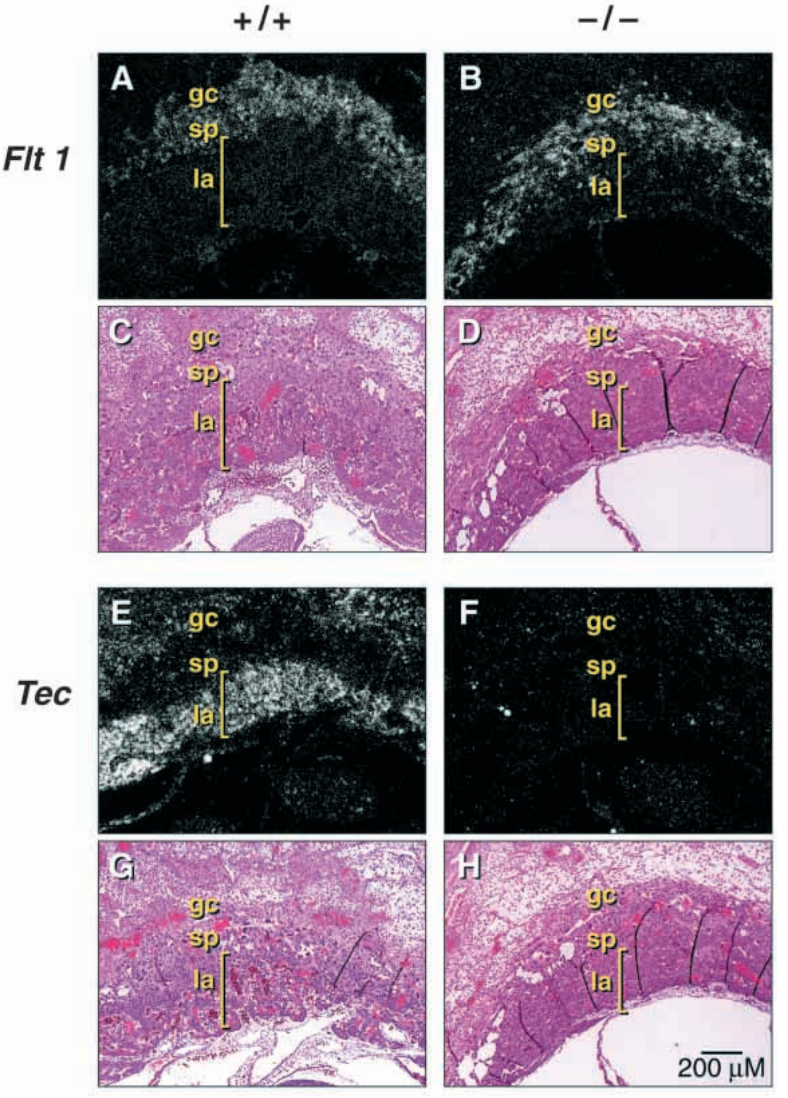

Fig. 6. The *Tfeb*^{Fcr} mutation affects gene expression in the labyrinth layer. Expression analysis of the spongiotrophoblast marker *Flt1* (A,B) and the labyrinthine trophoblast marker *Tec* (E,F) in 10.5 dpc wild-type (A,E) and *Tfeb*^{Fcr} mutant (B,F) placentas. While *Flt1* expression is normal in mutant embryos, no *Tec* expression was detected in the labyrinthine trophoblasts of *Tfeb* mutant placentas (F), even after overexposure of the in situs (data not shown). The presence of non-expressing cells between the *Flt1*-expressing cells and the lumen of both wild-type and *Tfeb*^{Fcr} mutant placentas is indicated by brackets, and suggests that the labyrinthine trophoblasts are still present. Adjacent sections stained with H+E are shown in (C,D,G,H). Labels are as in Fig. 4. The lines observed in D and H represent folds in the tissue that are also present, although not visible, in the dark-field images in B and F.

still express the endothelial markers *Pecam* and *Flk1* and they still form vessels which surround the blood cells. However, as observed previously, they are either unable to branch into the placenta or unable to survive, once in the placenta.

The *Tfeb* gene does not appear to be expressed in endothelial cells, only in the labyrinthine trophoblasts (Fig. 4A,D), suggesting that the effects of the *Tfeb*^{Fcr} mutation on endothelial cells is not due to direct effects of *Tfeb* in that cell type. The vascular endothelial growth factor (*Vegf*) is known to be expressed in labyrinthine trophoblasts of 12.5 day old placentas (Dumont et al., 1995) and to be required for normal vasculogenesis throughout both the embryo and extraembryonic tissues; heterozygotes carrying a null mutation in the gene die during early embryogenesis due to a general failure of vasculogenesis (Carmeliet et al., 1996; Ferrara et al., 1996). *Vegf* is expressed at a fairly low level in labyrinthine trophoblasts of 10.5 day old placentas and at a much higher level in giant cells at the other pole of the embryo as well as in the embryo itself (Fig. 7C, Dumont et al., 1995 and data not shown). No *Vegf* expression was detected in the labyrinth of 10.5 dpc *Tfeb*^{Fcr} homozygous placentas, whereas the giant cells still express the gene (Fig. 7D). This suggests that the endothelial effects of the *Tfeb*^{Fcr} mutation are due to the absence of *Vegf* expression in the mutant which, in turn, suggests that *Tfeb* may be directly or indirectly involved in regulating the expression of *Vegf* and other genes needed for the proper branching of the embryonic vasculature in the placenta. In the absence of *Tfeb*, these genes are not properly expressed and normal vascularization of the placenta does not occur.

DISCUSSION

We have created mice that carry a null mutation in the bHLH-Zip transcription factor *Tfeb* and shown that mice homozygous for this mutation die around mid-gestation, or around 10 dpc. This lethality is most likely due to defects in placental vascularization; the embryonic vasculature is unable to invade the placenta and the exchange of oxygen and nutrients that normally takes place between embryonic and maternal blood cells cannot take place. The embryos degenerate due to hypoxia. All the data are consistent with this explanation: first, vascular defects in the placenta are apparent at 9.5 dpc, a day before defects are visible in the embryo proper. Second, due to their growing size, mouse embryos start depending on the chorio-allantoic circulation around 10 days of development, the time of death of the *Tfeb*^{Fcr} homozygotes. Third, the *Tfeb* message is expressed at a very high level in the labyrinth of the placenta and only at a low level in the embryo itself. In fact, expression cannot be detected in wild-type embryos by in situ hybridization, only by northern and RT-PCR analysis. And fourth, the expression of other genes known to be expressed (and required) in the labyrinth such as *Tec* and *Vegf* is affected by the *Tfeb*^{Fcr} mutation.

The vascular defects observed in the *Tfeb*^{Fcr} mutant can be attributed to a requirement for *Tfeb* in labyrinthine trophoblasts of the placenta, where this factor is expressed at a high level

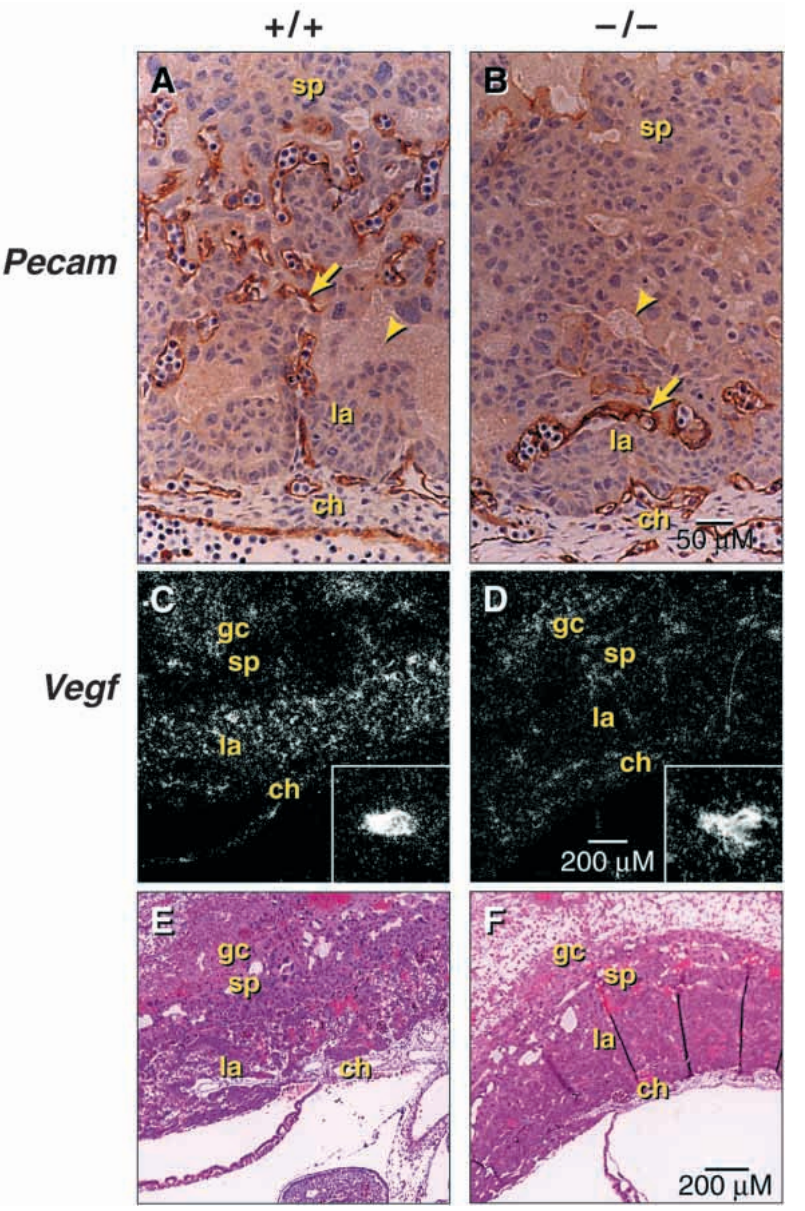

Fig. 7. The embryonic vasculature forms normally but is unable to invade the placenta. (A,B) Immunostaining of wild-type (A) and mutant (B) placentas with anti-*Pecam* antibodies which recognize endothelial cells specifically. In wild-type placentas (A), endothelial cells have branched extensively into the placenta and surround the nucleated embryonic blood cells. However, in mutant placentas (B), the only visible embryonic blood vessels are at the base of the placenta and have not ventured far into the labyrinth. (C,D) In situ hybridization of wild-type (C) and mutant (D) placentas using *Vegf* as a probe. In wild type, *Vegf* is expressed in giant cells at the other pole of the embryo (insert in C) as well as in the labyrinth of the placenta. However, although *Vegf* is still expressed in the giant cells at the other pole of *Tfeb* mutant embryos (insert in D), no expression is detected in the labyrinth. Adjacent sections stained with H+E are shown in E and F. Labels are as in Fig. 4. The lines observed in F represent folds in the tissue that are also present, although not visible, in the darkfield image in D.

and needed for the proper activation of genes, including *Tec* and *Vegf*. Other cells in the placenta appear unaffected and the embryonic vasculature forms normally although it does not invade the labyrinth. At the histological level, the defects observed in *Tfeb*^{Fcr} homozygous placentas are similar to the defects observed in *Vegf* heterozygous mutant placentas (Ferrara et al., 1996), supporting the notion that *Vegf* expression is regulated by *Tfeb* in this tissue. However, in vitro co-transfection studies using approximately 2.5 kb of human *Vegf* upstream regulatory sequences (Forsythe et al., 1996) have not revealed activation by the human *TFEB* gene (E. S. and D. E. Fisher, unpublished information), suggesting either that humans are different from mice in their activation of *Vegf* or that *Tfeb* is acting on sequences located further upstream or in introns of the gene. An alternative explanation is that the effects on *Vegf* and *Tec* expression are secondary to effects on the regulation of other genes; perhaps the transcriptional activity of *Tfeb* triggers a cascade that then results in the activation of *Tec* and *Vegf* expression. At present, the in vivo role of *Tec* in the placenta has not been characterized. Similarly, no other downstream targets of *Tfeb* have so far been identified.

Mutations in the hepatocyte growth factor (*Hgf*) gene also affect the labyrinth of the placenta (Schmidt et al., 1995; Uehara et al., 1995). However, these mutations result in gradual disappearance of the trophoblasts of the labyrinth, starting around day 10.5 and the embryos are still viable and histologically normal at 13.5 days (Uehara et al., 1995). The effects of *Tfeb* and *Hgf* mutations are therefore different and not likely to be involved in the same pathway. Several other genes have been identified that are involved in the establishment and function of extraembryonic tissues and some, such as the transcription factors *Mash2* and *Estrrb*, are specific for the placenta. However, the effects of mutations in these genes differ significantly from the *Tfeb*^{Fcr} phenotype in that they result in more general defects within the placenta. The *Mash2* gene is expressed in both labyrinthine- and spongiotrophoblasts as well as in the chorion (Guillemot et al., 1994), and in homozygous *Mash2* mutants, all trophoblast cell types are affected; the spongiotrophoblasts are absent, the labyrinthine layer is reduced in area compared to wild type, while the layer of giant cells is enlarged (Guillemot et al., 1994). However, chimera studies show that the gene is only required for the formation of spongiotrophoblasts and not for labyrinthine trophoblasts or giant cells (Tanaka et al., 1997). Although the labyrinth layer is compact in *Mash2* mutants, the embryonic vasculature seems to be able to invade in a normal fashion, suggesting that the role of *Mash2* in these cells is very different from that of *Tfeb*. Expression of the oestrogen-receptor-related receptor β, *Estrrb*, can be detected in early extraembryonic ectoderm and later becomes restricted to the chorion and its free margin (Luo et al., 1997). In spite of this restricted expression, homozygous *Estrrb* mutants show more general defects and are nearly devoid of diploid trophoblasts while giant cells are increased in number (Luo et al., 1997). Since the allantois of mutant embryos does not fuse with the chorion, the embryonic vasculature is unable to invade the labyrinth. The general effects of the *Estrrb* mutation suggest that the wild-type ERRβ orphan receptor results in the production of a long-range signal that is necessary for the support of placental cells other than just the cells that express the gene. Again this is different from the *Tfeb*^{Fcr} phenotype which only exhibits defects in the very cells that express the gene. However, since only structural differences between wild-type and *Tfeb*^{Fcr} mutant placentas have been tested, it is possible that effects of the mutation can be felt outside the labyrinth although not seen histologically.

At present, we do not know if the *Tfeb* gene acts alone in placenta or whether it requires a partner such as another bHLH-Zip protein for its function. Since homozygotes carrying mutations at the *Tfe3*, *Tfec* and *Mitf* loci are all viable and fertile (Steingrímsson et al., 1994; ES, LT, NGJ and NAJ, unpublished observations), it is unlikely that these close relatives of *Tfeb* are required as partners for its placental function. However, it is of course possible that *Tfeb* interacts with other bHLH-Zip family members in the placenta, or with yet uncharacterized proteins.

Due to the lethality of *Tfeb* embryos during early development, we currently do not know whether *Tfeb* is required for the establishment or function of adult tissues or cells. In addition, we do not know if *Tfeb* is a functional partner of *Mitf* in melanocytes, retinal pigment epithelial cells, osteoclasts, mast cells or any of the other cell types affected by *Mitf* mutations (reviewed by Moore, 1995), as suggested by the in vitro DNA binding data (Hemesath et al., 1994). Genetic experiments have shown that mice carrying combinations of the *Tfeb* and *Mitf* mutations (e.g. *Tfeb*–/+; *Mi*^{wh}/+ and *Tfeb*–/+; *Mi*^{wh}/*Mi*^{wh} mice) only exhibit the normal hetero- or homozygous *Mi*^{wh} phenotypes and show no additional coat color, eye, or bone defects (ES, NGC and NAJ, unbublished observations). Similar double combinations with mutations at the *Tfe3* and *Tfec* loci also show no additional defects (E. S., N. G. C. and N. A. J., unbublished observations), indicating that these genes are not important functional in vivo partners of *Tfeb*. However, final proof of *Tfeb* function in adult tissues will only come from performing chimera or tetraploid embryo experiments (Nagy et al., 1990) on *Tfeb*^{Fcr} mutant embryos. And to test for in vivo interactions with *Mitf*, *Tfe3* and *Tfec*, chimera experiments must be done in the respective double mutant backgrounds.

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