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# Placental vascularisation requires the AP-1 component Fra1

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## SUMMARY

***Fra1* is an immediate-early gene encoding a member of the AP-1 transcription factor family, which has diverse roles in development and oncogenesis. To determine the function of *Fra1* in mouse development, the gene was inactivated by gene targeting. Foetuses lacking *Fra1* were severely growth retarded and died between E10.0 and E10.5, owing to defects in extra-embryonic tissues. The placental labyrinth layer, where X-gal staining revealed expression of *Fra1*, was reduced in size and largely avascular, owing to a marked decrease in the number of vascular endothelial cells, as shown by the lack of *Flk1* expression. In contrast, the spongiotrophoblast layer was unaffected and expressed the marker genes *4311* (*Tpbp*) and *Flt1*. Furthermore, mutant foetuses exhibited yolk-sac defects that may contribute to their growth retardation and lethality. Importantly, when the placental defect was rescued by injection of *Fra1*$^{-/-}$ ES cells into tetraploid wild-type blastocysts, *Fra1*$^{-/-}$ pups were obtained that were no longer growth retarded and survived up to 2 days after birth without apparent phenotypic defects. These data indicate that a defect in the extra-embryonic compartment is causal to the observed lethality, and suggest that *Fra1* plays a crucial role in establishing normal vascularisation of the placenta.**

Key words: AP-1, Fra1, Embryonic lethality, Tetraploid blastocyst injection, Gene targeting, Mouse

## INTRODUCTION

AP-1 (activator protein 1) is a dimeric transcription factor composed of the products of the *Jun* and *Fos* proto-oncogenes (Angel and Karin, 1991). The Jun proteins (Jun, Junb and Jund) form either homo- or heterodimers with members of the Fos and ATF protein families, whereas the Fos proteins (Fos, Fosb, Fra1 and Fra2) cannot associate with each other or with ATF proteins, but form stable heterodimers with any of the Jun proteins. AP-1 is crucially involved in a multitude of cellular processes, including development and differentiation, cell proliferation, apoptosis, oncogenic transformation, and the response to genotoxic agents (Angel and Karin, 1991; Devary et al., 1992; Angel and Herrlich, 1994; Schreiber et al., 1995; Karin et al., 1997). AP-1 activity is rapidly induced by a vast number of extracellular stimuli, such as growth factors, cytokines, tumour promoters, carcinogens and specific oncogenes, and AP-1 is thought to play a central role in changing the pattern of gene expression in response to extracellular signals. These extracellular signals activate AP-1 via the ERK and JNK signal-transduction cascades through increased expression, as well as phosphorylation, of pre-existing and newly synthesised AP-1 subunits, which modulate the DNA-binding and transactivation functions of Jun and Fos proteins (Karin, 1995; Karin and Hunter, 1995; Leppä and Bohmann, 1999).

Like all other *Fos* and *Jun* genes, *Fra1* (Fos-related antigen 1; also termed *Fosl1*) is an immediate-early gene (Cohen and Curran, 1988; Cohen et al., 1989). The DNA-binding specificity of Fra1/Jun heterodimers is indistinguishable from that of Fos/Jun heterodimers on several AP-1 binding sites (Cohen et al., 1989). In contrast to Fos, Fra1 lacks a transactivation domain, and the entire Fra1 protein (e.g. when fused to the DNA-binding domain of Gal4) fails to activate transcription (Suzuki et al., 1991; Wisdom and Verma, 1993; Bergers et al., 1995). Therefore, *Fra1* can either increase or decrease total AP-1 activity depending on the status of the other Fos and Jun proteins in the cell, and has been proposed to function as a negative-feedback regulator of AP-1 (Suzuki et al., 1991; Groskopf and Linzer, 1994; Bergers et al., 1995; Welter et al., 1995; Yoshioka et al., 1995; Schreiber et al., 1997). The oncogenic potential of *Fra1* is significantly weaker than that of *Fos*. Nevertheless, overexpression of *Fra1* in established rat fibroblasts leads to anchorage-independent growth and tumour development in nude mice (Bergers et al., 1995). Furthermore, neoplastic transformation of rat thyroid cells requires induction of *Fra1* and *Junb* (Vallone et al., 1997). *Fra1* expression is subject to positive control by AP-1 in several cell types (Grigoriadis et al., 1993; Brüsselbach et al., 1995; Bergers et al., 1995; Schreiber et al., 1997; Matsuo et al., 2000). Interestingly, the basal and AP-1-induced expression of *Fra1* depends primarily on regulatory sequences in the first

intron, which contains three AP-1 binding sites (separated by 8 and 7 bp; Bergers et al., 1995).

Numerous in vitro studies have suggested that the different AP-1 dimers may act as tissue-specific and signal-specific transcriptional activators. Indeed, each individual targeted deletion of an AP-1 gene reported so far leads to specific phenotypes, indicating that the different AP-1 subunits, although highly homologous, are not fully redundant in vivo. Mice lacking *Jund* are viable, but exhibit reduced postnatal growth and multiple defects in male reproductive functions (Thepot et al., 2000), whereas deletion of *Jun* or *Junb* leads to embryonic lethality (Hilberg et al., 1993; Johnson et al., 1993; Schorpp-Kistner et al., 1999). Lack of Jun causes liver and heart defects, resulting in embryonic lethality at E12.5 (Hilberg et al., 1993; Eferl et al., 1999), whereas *Junb*$^{-/-}$ foetuses die between E8.5 and E10.0, owing to multiple defects in extra-embryonic tissues, such as the placental labyrinth (Schorpp-Kistner et al., 1999). In contrast, mice lacking *Fos* or *Fosb* are viable and fertile. In one study, nurturing defects were detected in *Fosb*$^{-/-}$ mice (Brown et al., 1996), whereas this phenotype was not observed in a second study (Gruda et al., 1996). Mice lacking *Fos* develop osteoporosis, owing to a complete differentiation block in bone-resorbing osteoclasts (Wang et al., 1992; Johnson et al., 1992; Grigoriadis et al., 1994; Matsuo et al., 2000). The biological function of *Fra1* in vivo is not yet known, although it is a transcriptional target of Fos in osteoclasts and macrophages (Matsuo et al., 2000). Therefore, it is possible that deletion of *Fra1* might phenocopy, at least in part, the effects of the *Fos* deletion. To address these questions, we have disrupted the *Fra1* gene in embryonic stem (ES) cells and mice. Foetuses lacking *Fra1* were found to be severely growth retarded and died between E10.0 and E10.5. Mutant embryos revealed a highly abnormal yolk sac, and the placenta lacked a properly vascularised labyrinth layer. *Fra1* expression was detected in the yolk sac and the labyrinth layer using the *lacZ* reporter gene integrated into the targeted allele. Injection of *Fra1*$^{-/-}$ ES cells into tetraploid wild-type blastocysts, which form exclusively extra-embryonic tissues, rescued the embryonic lethality and gave rise to *Fra1*$^{-/-}$ foetuses that were no longer growth retarded and survived up to 2 days after birth, suggesting that the observed defects in the extra-embryonic compartment were causal to the lethality.

## MATERIALS AND METHODS

### Construction of the targeting vector

The mouse (strain 129/Sv) *Fra1* gene has previously been cloned and characterised (Schreiber et al., 1997). To generate a targeting construct for homologous recombination, a 603 bp *Bsr*BI-*Nhe*I fragment of *Fra1* consisting of the last 66 nucleotides of exon 3, the entire third intron and the first 165 nucleotides of exon 4 was replaced by a pGNA cassette (Le Mouellic et al., 1992), fusing the *lac*Z gene of pGNA in-frame with the coding sequence of exon 3 of *Fra1*. The pGNA cassette introduces a neomycin phosphotransferase gene driven by a PyEC F9.1 enhancer/RSV LTR promoter, which allows for positive selection in ES cells with G418. The targeting construct (pGNA/*Fra1*) had, as a 5′ arm of homology, a 5170 bp *Nsi*I-*Bsr*BI fragment of *Fra1* containing part of intron 1, exon 2, intron 2 and part of exon 3, whereas the 3′ arm was comprised of a 985 bp *Nhe*I-*Nde*I fragment of exon 4. The introduced deletions in exons 3 and 4 removed the basic region of the DNA-binding domain (except the first three amino acids) and the entire leucine-zipper dimerisation domain of Fra1. In addition, the last splice acceptor site of the gene was deleted to prevent aberrant splicing across the introduced pGNA sequences. A herpes simplex virus thymidine kinase-cassette (HSV-TK) was added to the construct for negative selection against random integration (Mansour et al., 1988), which could be positioned to the long or short arm of homology after linearisation with *Not*I or *Nsi*I, respectively (see Fig. 1B).

### Gene targeting in ES cells

The ES cell lines used in this study, D3 (Doetschman et al., 1985) and R1 (Nagy et al., 1993), were cultured on feeder cells in the presence of LIF as described by Wang et al. (1992). For electroporation, $10^7$ trypsinised ES cells were suspended in 800 μl PBS, mixed with 10-20 μg of linearised targeting vector (pGNA/*Fra1*), and an electric pulse of 260 V at 500 μF was applied with a Gene Pulser (BioRad, Munich). Cells were selected in the presence of 0.3 mg/ml G418 and 2 μM gancyclovir to enrich for clones that had undergone homologous recombination at the *Fra1* locus. Screening of colonies by PCR was performed as described (Wang et al., 1992, 1994) with two sets of nested primers: fra1Y (5′-TGGGGTGGGATTTGAGACGG-3′; 3′ of exon 4 of *Fra1* outside the sequence of the targeting vector) and neo3 (5′-GTCATCTCACCTTGCTCCTGC-3′; neo$^R$ gene), and a set of primers positioned inside of primers fra1Y and neo3, i.e. fra1X (5′-CTAAAGCCCACTGAACCGCC-3′; 3′ of exon 4) and neo2 (5′-CGCCTTCTTGACGAGTTCTTCTGAG-3′; neo$^R$ gene). Colonies positive for the diagnostic approx. 2.0 kb PCR fragment were further analysed by Southern blotting (see below).

### Isolation of genomic DNA and genotyping by Southern blot analysis and PCR

Genomic DNA was isolated from ES cells, embryos, yolk sacs and tail biopsies, as described by Hilberg et al. (1993). Genotyping was performed by PCR and/or Southern blot analysis, which allowed us to distinguish the endogenous and targeted alleles of *Fra1* based on different lengths of amplification products or restriction fragments, respectively. Southern blot analysis was performed using a probe located 3′ of exon 4 outside of the targeting vector (approx. 750 bp *Nde*I/*Sac*I fragment; Fig. 1) and genomic DNA which had been digested with *Apa*I (Fig. 1D), *Sac*I or *Sal*I/*Not*I, and separated by gel electrophoresis (0.8% agarose/TAE). The following primers were used for genotyping by PCR: Pr1, 5′-GGGCTTTGTTGGCATAGTA-GATTG-3′ (derived from intron 2 of *Fra1*); Pr2, 5′-AGCTCCTTTC-TTCGGTTTCTGC-3′ (exon 3 of *Fra1*; deleted in the targeted allele); and Pr3, 5′-AAGCGCCATTCGCCATTCAG-3′ (nucleotides 179-159 of *lac*Z). Primers Pr1 and Pr2 amplify a 567 bp fragment of the endogenous allele, whereas primers Pr1 and Pr3 amplify a 699 bp fragment of the targeted allele. The Ubi-*Junb* and H2K$^b$-*Fra1*-LTR transgenes used in genetic rescue experiments were genotyped as described in Schorpp et al. (1996) and Jochum et al. (2000), respectively. In matings involving the H2K$^b$-*Fra1*-LTR transgene, genotyping of the endogenous and targeted alleles of *Fra1* was performed by Southern blot analysis.

### Generation of chimaeras and derivation of *Fra1*$^{-/-}$ ES cells

Targeted ES cells were injected into C57BL/6 blastocysts that were subsequently transferred into the uteri of pseudopregnant recipients. Tetraploid pre-implantation embryos were generated by electrofusion of the two blastomeres of two-cell stage embryos as described (Nagy et al., 1993; Nagy and Rossant, 1993). The generation of chimaeras by aggregation of diploid with tetraploid morulas, or by injection of ES cells into tetraploid blastocysts was performed as described by Nagy and Rossant (1993) and Wang et al. (1997). To derive *Fra1*$^{-/-}$ ES cells, E3.5 blastocysts were isolated from *Fra1*$^{+/-}$ intercrosses (strain 129/Sv), cultured on feeder layers, and ES cells established as described (Hogan et al., 1994). Out of 80 blastocysts explanted, five

ES cell lines were established (one *Fra1*$^{+/+}$, three *Fra1*$^{+/-}$ and one *Fra1*$^{-/-}$). *Fra1*$^{-/-}$ ES cells were used for injection into diploid or tetraploid blastocysts at passage 5.

### GPI isoenzyme assay

Various tissues of adult chimaeric mice were dissected and minced in distilled water. Samples were lysed by three cycles of freeze-thawing and were subjected to glucose phosphate isomerase (GPI) isozyme analysis as described previously (Hilberg et al., 1993). The proportion of ES-cell- and host-derived cells in the chimaeric tissues was estimated from the ratio of the GPI-1A versus GPI-1B isozyme activity after visualisation in a coupled enzymatic assay.

### Histological methods and β-galactosidase staining

Tissue samples and mouse embryos were fixed in 4% paraformaldehyde, embedded in paraffin wax, cut and counterstained with Haematoxylin and Eosin as described by Aguzzi et al. (1990). Sense and antisense cRNA probes for in situ hybridisations were in vitro transcribed in the presence of $^{35}$S-rUTP as described (Aguzzi et al., 1990). The following probes were used: 4311 (Lescisin et al., 1988), *Flt1* and *Flk1* (Breier et al., 1995). For BrdU-labelling, pregnant mice of heterozygous timed matings were injected intraperitoneally with 60 μg of BrdU (Sigma) per gram bodyweight at day 9.5 of pregnancy. 4 hours after injection, mice were sacrificed and the decidual swellings isolated and fixed in 4% paraformaldehyde at 4°C overnight. Paraffin sections were prepared and incubated with an α-BrdU mouse monoclonal antibody (Calbiochem) at a dilution of 1:200. Thereafter, an ABC staining procedure (Vector) was performed according to the manufacturer's instructions. To determine the percentage of proliferating cells in various tissues, more than 100 stained and unstained nuclei of several sections were counted. Embryos, placentas and decidual swellings were stained with X-gal following fixation in 0.2% glutaraldehyde/100 mM sodium phosphate, as described by Bonnerot et al. (1987). After staining, tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax, cut and counterstained with Eosin.

### Isolation and culture of embryonic fibroblasts

Primary MEFs were isolated and immortalised according to the 3T3 protocol (Todaro and Green, 1963). The yolk sac was used as source of genomic DNA for genotyping. Each primary fibroblast culture was isolated from a single E9.5 embryo of 129/Sv or 129×BL/6 genetic background, and each 3T3 fibroblast line was immortalised from an individual primary culture. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (37°C, 100% humidity and 5% $CO_2$).

## RESULTS

### Generation of mice lacking a functional *Fra1* gene

The targeting strategy used for disruption of the *Fra1* gene is shown in Fig. 1. Following homologous recombination, the *lac*Z gene was fused in-frame to the coding sequence of exon 3 of *Fra1* (Fig. 1C). In addition, part of exon 3, intron 3 and part of exon 4 were deleted, removing almost all of the basic DNA-binding domain, the entire leucine-zipper dimerisation domain and the last splice acceptor site of *Fra1* (Fig. 1C). Thus, the introduced changes should lead to a null mutation. The HSV thymidine kinase gene (HSV-TK) was used for negative selection against random integration. Following electroporation of ES cells, targeted clones were identified by genomic Southern blot analysis (Fig. 1D), and were obtained at a high frequency in D3 and R1 ES cells (one targeted clone per 3 and 3.6 double resistant transfectants, respectively). Interestingly, this high frequency was observed only when the HSV-TK cassette was present on the short arm of homology (following linearisation with *Nsi*I; Fig. 1B). When the HSV-TK cassette was present on the long arm (following linearisation with *Not*I; Fig. 1B), the targeting frequency was approx. 2.5-fold lower, presumably due to lower enrichment following gangcyclovir selection (data not shown). Neither additional random integrations of the targeting construct nor rearrangements of the targeted allele were detected (data not shown). Several D3-derived and R1-derived *Fra1*$^{+/-}$ ES cell clones were microinjected into C57/BL6 blastocysts. Male chimaeras from one R1 clone (Rfr-3) and from one D3 clone (Dfr-52) transmitted the mutated allele to their offspring when crossed to C57BL/6 or 129/Sv females. All following studies were performed on mice derived from each of the two independent clones, and in both genetic backgrounds (129/Sv and 129×BL/6). Offspring were genotyped by PCR or Southern blot analysis (Fig. 1D,E).

### Loss of *Fra1* results in embryonic lethality around E10.0

Heterozygous males and females were fertile and phenotypically indistinguishable from wild-type littermates. However, no homozygous mutants were obtained from heterozygous intercrosses, indicating that mice lacking *Fra1* die during embryogenesis (Table 1). To identify the stage of lethality, embryos from timed matings between heteroyzgotes were analysed at different stages of gestation. *Fra1*$^{-/-}$ embryos were obtained with the expected Mendelian frequency at E8.5, E9.5 and E10.0. At E10.5, the number of viable mutant embryos was drastically reduced (1 out of 40; $\chi^2$=11.72; $P$<0.01). Viable mutant embryos were never observed at E11.5 or later (Table 1). Thus, foetuses lacking *Fra1* died in utero around E10 of gestation. This was observed for mice derived from both ES cell clones, and with both mixed 129×BL/6 and pure 129/Sv genetic backgrounds. To confirm that the deletion of *Fra1* alone caused the observed lethality, a genetic rescue experiment was performed by crossing *Fra1*$^{+/-}$ mice with transgenic mice ubiquitously expressing the mouse *Fra1* gene under the control of the H2K$^{b}$ promoter (Jochum et al., 2000). Upon intercrossing, the embryonic lethality was rescued, and viable mice lacking the endogenous *Fra1* gene, but carrying the transgene, were obtained at almost Mendelian frequency, demonstrating that the lethality of *Fra1*$^{-/-}$ mice is due to the absence of a functional Fra1 protein (Table 2).

**Table 1. Offspring obtained from *Fra1* heterozygote intercrosses**

| | Genotype | | | | |
|---|---|---|---|---|---|
| Age | +/+ | +/– | –/– | Resorbed | Total |
| E8.5 | 16 | 23 | 11 | – | 50 |
| E9.5 | 24 | 50 | 26 | 1 | 101 |
| E10.0 | 3 | 8 | 3 | 1 | 15 |
| E10.5 | 8 | 27 | 1 | 4 | 40 |
| E11.5-E16.5 | 31 | 76 | 0 | 39 | 146 |
| At weaning | 109 | 219 | 0 | – | 328 |

DNA was isolated from embryos at day 8.5-16.5 of gestation (E8.5-E16.5) and from approx. 3-week-old mice (at weaning), and analysed by PCR and/or Southern blot. Note the decrease in viable *Fra1*$^{-/-}$ embryos beginning at E10.5 and the parallel occurrence of significant numbers of resorptions.

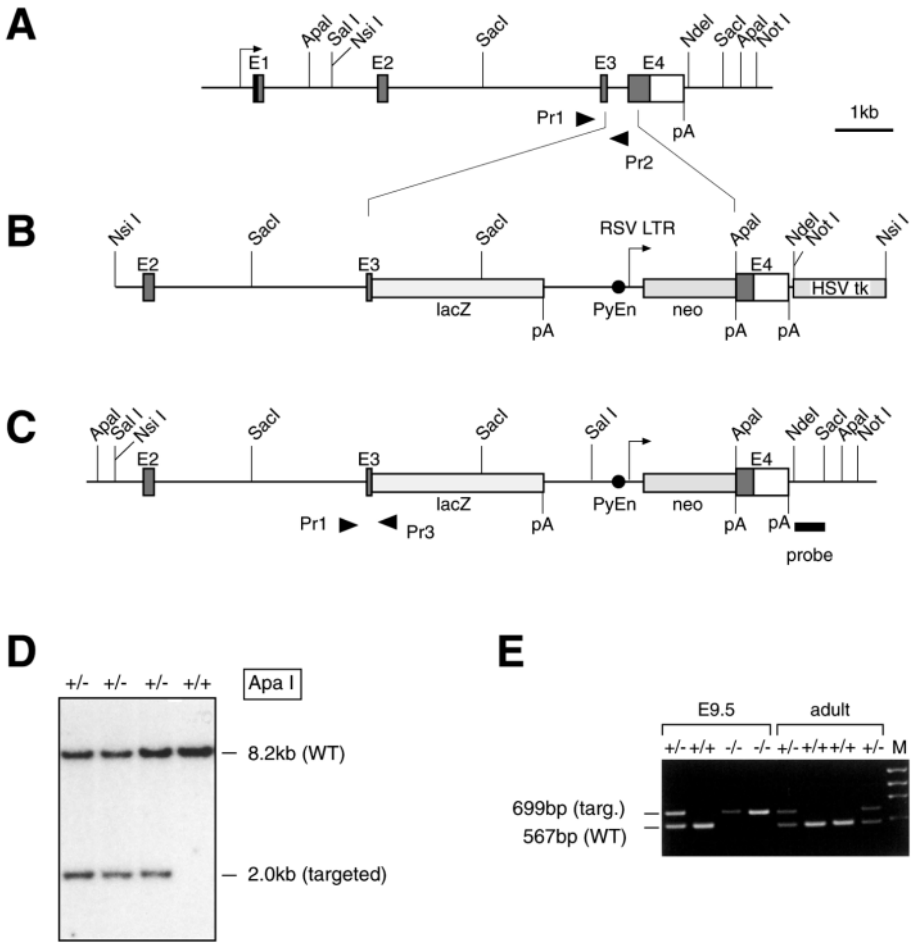


**Fig. 1.** Targeting strategy for inactivation of *Fra1*. (A) Structure and partial restriction map of the complete *Fra1* locus. (B) Targeting construct after linearisation with *Nsi*I. Part of exon 3, intron 3 and part of exon 4 were replaced, and a *lacZ* reporter gene was fused in-frame to the *Fra1* coding sequence. Note that the HSV-TK cassette can also be positioned to the long (5′) arm of homology upon linearisation with *Not*I instead of *Nsi*I. (C) Structure of the targeted *Fra1* allele following homologous recombination. The hybridisation probe and the position of restriction sites used for Southern blot analyses are indicated. Arrows represent promoters, hatched bars and open bars are coding and noncoding sequences of *Fra1*, respectively. E1, exon 1; HSV tk, herpes simplex virus thymidine kinase gene; neo, neomycin phosphotransferase gene; pA, polyadenylation site, PyEn, enhancer of polyoma virus strain PyEC F9.1; RSV LTR, Rous sarcoma virus long terminal repeat. (D) Southern blot analysis of targeted ES cell clones. *Apa*I-digested DNA of the parental ES cell line D3 (+/+) and of three targeted clones (+/–) was analysed by hybridisation with the probe shown in C. E. PCR analysis of E9.5 embryos and adult progeny from heterozygous intercrosses of *Fra1*$^{+/-}$ mice. Primers Pr1, Pr2 and Pr3 shown in A and C were used. The sizes of diagnostic fragments for the wild-type (WT, Pr1 and Pr2) and targeted allele (Pr1 and Pr3), and the genotypes are indicated. M, molecular weight marker.

## Embryos lacking *Fra1* are retarded in growth and development

The most prominent phenotypic alteration of *Fra1*$^{-/-}$ foetuses is their small size, which first becomes apparent at E8.5 and was observed for all mutant embryos at E9.5 and E10.0 (Fig. 2). To determine whether primarily a growth defect or developmental retardation is responsible for this small size, the developmental stage of mutant embryos recovered at E9.0-E10.0 was determined using a series of morphological criteria, e.g. somite number, allantois, heart, hindbrain, otic system, forelimb buds and flexion (Brown, 1990). Mutant foetuses recovered at E9.5 were the size of E8.5 foetuses, but the developmental stage was typical of E9.0-E9.5 foetuses (Table 3; Fig. 2A,C). All major organ systems, the body axis and properly organised somites were present, and the process of turning was completed in mutant embryos (Fig. 2C,D). Histological analysis of E9.5 and E10.0 *Fra1*$^{-/-}$ embryos revealed no obvious structural anomalies (Fig. 2D). Importantly, the hearts of mutant embryos were beating and exhibited the normal looped morphology characteristic of this stage of development (data not shown). Except for the occurrence of a dilated pericardium in several mutants (Fig. 2C), no other morphological alterations were detected. Histological analysis demonstrated the presence of clearly distinguishable myocardial and endocardial layers, which were well connected, and normal trabeculation (Fig. 2E,F). Furthermore, foetal erythroblasts were present in blood vessels of both the yolk sac and the embryo itself (Fig. 2F and see Fig. 5). Taken together, no severe abnormalities in development or early organogenesis that might be causal to the lethality at this early stage or to the growth retardation could be detected in the embryo proper. The *lacZ* reporter gene was used to monitor expression of the targeted *Fra1* allele by staining *Fra1*$^{+/-}$ embryos for β-galactosidase

**Table 2. Offspring obtained from rescue experiments with the H2K$^{b}$-*Fra1*-LTR transgene**

| | *Fra1*$^{+/+}$ | *Fra1*$^{+/-}$ | *Fra1*$^{-/-}$ | Fra1 transgene |
|---|---|---|---|---|
| *Fra1*$^{+/-}$H2K$^{b}$-*Fra1*-LTR | 11 | 28 | 5 | + |
| × *Fra1*$^{+/-}$ crosses | 13 | 27 | 0 | – |
| | | | | |
| *Fra1*$^{-/-}$H2K$^{b}$-*Fra1*-LTR | – | 11 | 9 | + |
| × *Fra1*$^{+/-}$ crosses | – | 8 | 0 | – |

DNA was isolated from from approx. 3-week-old mice and analysed by PCR.

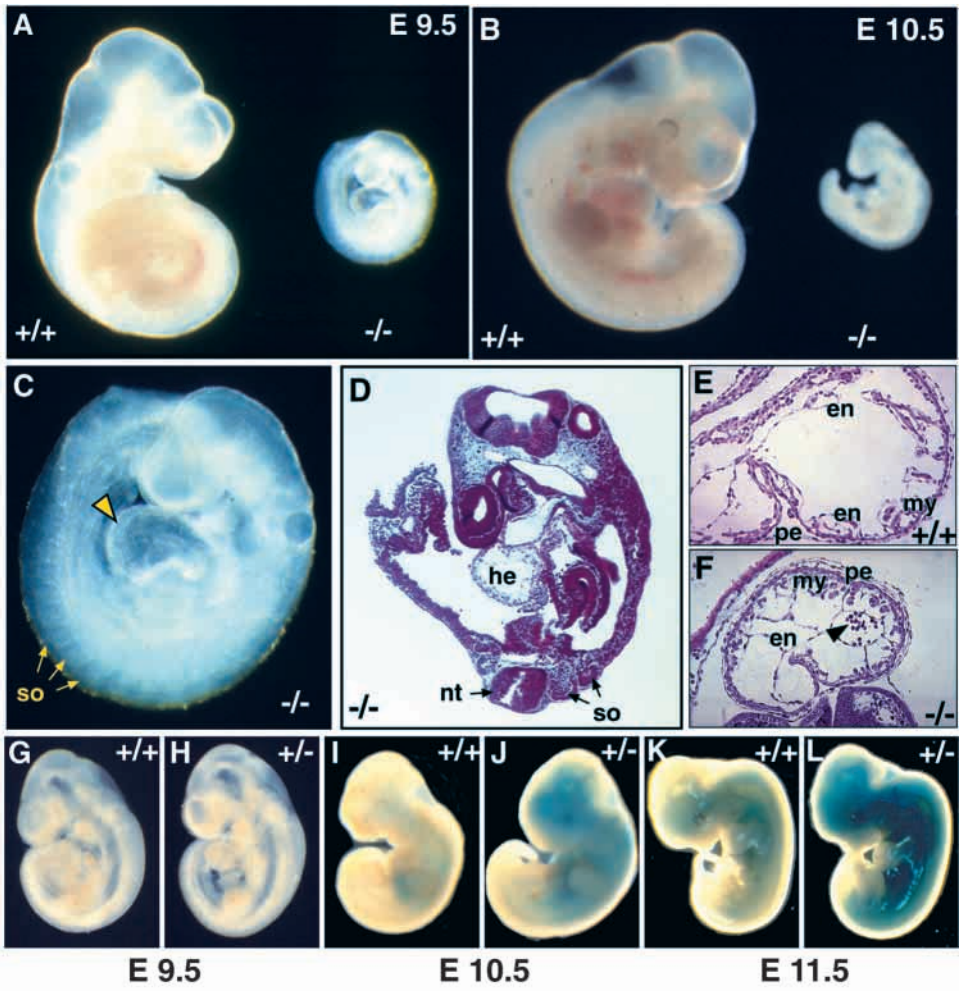


**Fig. 2.** *Fra1*$^{-/-}$ embryos are severely growth retarded. E 9.5 (A) and E10.5 (B) wild-type (+/+) and mutant (–/–) littermate embryos. (C) Higher magnification of a mutant E9.5 embryo. Note the presence of an enlarged pericardium (arrowhead), well-structured somites (so), and that the embryo has almost completed turning. (D) Sagittal section of an E9.5 mutant embryo revealing the presence of a well developed heart (he), somites (so) and neural tube (nt). (E,F) Sagittal section of the heart of E9.5 wild-type (E) and mutant (F) embryos demonstrating the presence of well-connected myocardial (my) and endocardial (en) layers, and the pericardium (pe). Note the presence of erythroblasts in the heart (arrowhead) and adjacent blood vessels of the mutant foetus. (G-L) X-gal staining of *lacZ* expression, which reflects *Fra1* promoter activity, in E9.5 (G,H), E10.5 (I,J) and E11.5 (K,L) wild-type (+/+) and *Fra1*$^{+/-}$ embryos.

activity. Expression of *lacZ* was absent in E9.5 embryos, but was widespread in E10.5 and E11.5 embryos and apparently not restricted to specific organ systems (Fig. 2G-L). Expression was significantly weaker in E10.5 than in E11.5 embryos (Fig. 2I-L).

## No cell-autonomous proliferation defects in the absence of *Fra1*

Embryos lacking *Fra1* are severely growth-retarded before they die. To explore the possibility of a proliferation defect of mutant cells in vivo, we measured the number of S-phase cells by 5-bromo-2′-deoxyuridine (BrdU) immunohistochemistry. Pregnant mice from *Fra1*$^{+/-}$ intercrosses were injected with BrdU at E9.5, and offspring were analysed 4 h after injection. No significant difference in the number of BrdU-positive nuclei was observed between mutants (48.7±3.6%) and controls (51±3.9%) in the neural tube (Fig. 3A). Similar results were obtained in the placenta and other tissues (data not shown).

To further analyse the proliferation and developmental potential of cells lacking *Fra1*, *Fra1*$^{-/-}$ ES cells were derived from E3.5 blastocysts isolated from *Fra1*$^{+/-}$ intercrosses (129/Sv genetic background), and injected into wild-type C57BL/6 blastocysts to generate chimaeric mice. Most chimaeras showed high ES cell contribution, as judged by agouti coat colour, and transmitted the mutant allele to their offspring, indicating that cells lacking *Fra1* contributed to male and female gametogenesis. The degree of ES cell contribution to various organs of 3 adult chimaeric mice was determined by glucose phosphate isomerase isozyme and Southern blot analysis. Except in the pancreas and calvaria, *Fra1*$^{-/-}$ cells contributed efficiently to most organs, indicating a normal proliferation capacity of these cells in vivo, even when in direct competition with wild-type cells (Fig. 3B).

Next, primary fibroblasts were isolated from E9.5 embryos derived from *Fra1*$^{+/-}$ intercrosses, and cell lines were immortalised according to the standard 3T3 protocol (Todaro and Green, 1963). The immortalisation curve of wild-type, heterozygous and mutant cells is shown in Fig. 3C. During the first 5-8 passages (i.e. the first 20-30 days), cells of all three genotypes divided at a significant rate, but subsequently proliferation slowed down until cells entered a senescent state marked by morphological alterations and a cessation of cell proliferation. The crisis of wild-type and mutant fibroblasts lasted approx. 4-5 weeks after which part of the population resumed proliferation and continued to grow as stable cell lines. There was no significant difference between wild-type, *Fra1*$^{+/-}$ and *Fra1*$^{-/-}$ cells in the initial proliferation rate of pre-crisis primary fibroblasts, the onset and duration of crisis, and the post-crisis proliferation rate of immortalised cells, indicating that *Fra1* is not essential for fibroblast proliferation and immortalisation in vitro.

Next, the proliferation of *Fra1*$^{-/-}$ ES cells was analysed. Proliferation curves of *Fra1*$^{-/-}$ and wild-type ES cells were comparable, indicating that the absence of Fra1 did not affect the in vitro proliferation capacity of ES cells (Fig. 3D). Most importantly, rescued *Fra1*$^{-/-}$ foetuses generated via tetraploid blastocyst injection (see below) were of normal size,

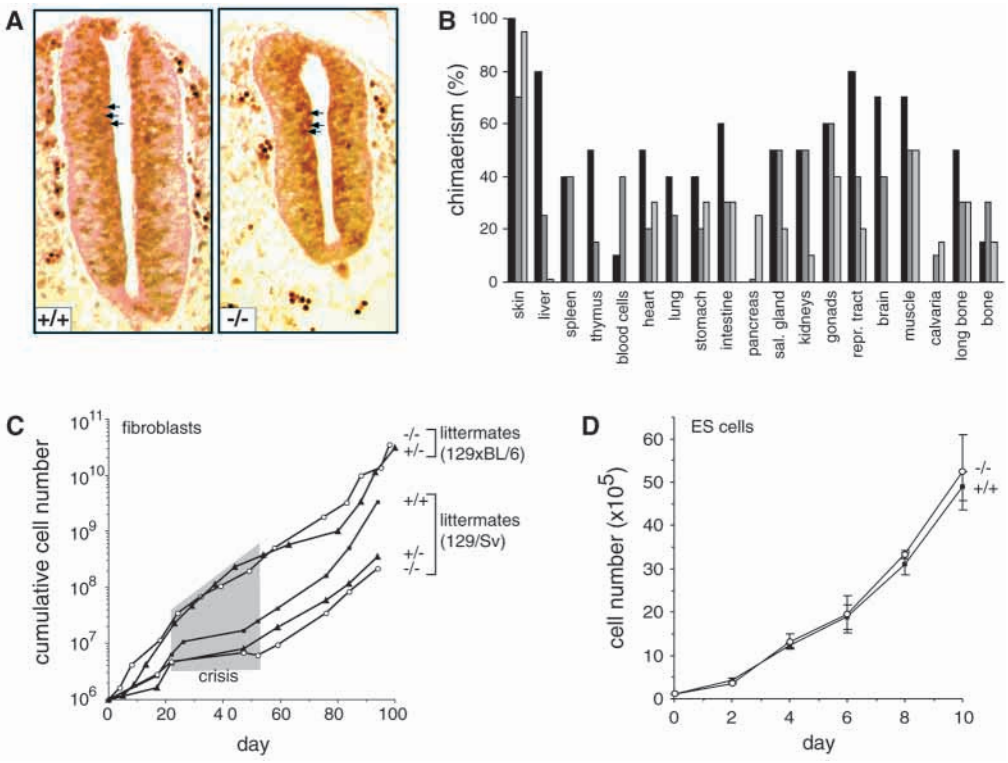


**Fig. 3.** *Fra1* is not essential for cell proliferation in vitro and in vivo. (A) Immunohistochemical detection of BrdU incorporation in the neuroepithelial cells of E9.5 wild-type (+/+) and mutant (–/–) neural tubes. Some BrdU-positive nuclei are indicated by arrows. Note the regular distribution of BrdU-positive cells in both *Fra1*$^{-/-}$ and wild-type neural tubes. (B) Contribution of *Fra1*$^{-/-}$ cells to different tissues of adult chimaeric mice derived via injection of *Fra1*$^{-/-}$ ES cells into wild-type blastocysts. Contribution to the skin was judged from the proportion of agouti versus black fur. ES cell contribution to all other tissues was determined by glucose phosphate isomerase (GPI) isozyme assays for two mice (black and dark-grey bars), and by Southern blot analysis and quantitation of the ES-cell derived *Fra1* mutant band and the host blastocyst derived *Fra1* wild-type band for the third mouse (light-grey bars). (C) Immortalisation curves of *Fra1*$^{-/-}$ and control (*Fra1*$^{+/-}$ and *Fra1*$^{+/+}$) embryonic fibroblasts. Fibroblasts of the indicated genotypes were immortalised according to the 3T3 protocol and cumulative cell numbers were determined. (D) Proliferation curves of wild-type (+/+) and *Fra1*$^{-/-}$ ES cells (passage 7). The average±s.d. of triplicate measurements are shown.

demonstrating that the growth retardation of mutant embryos was not due to a cell-autonomous proliferation defect.

## Defects in *Fra1*$^{-/-}$ placentas and yolk sacs

The absence of severe developmental defects in mutant foetuses together with the observed growth retardation suggests that placental defects may be responsible for the lethality. At E9.0-E9.5, the labyrinth layer of the placenta develops, in which extensive intermingling occurs between maternal blood sinuses and foetal blood vessels (Cross et al., 1994). The first step in the development of the placenta is the fusion of the allantois to the chorion; this process appeared to be unaffected in mutant embryos (Table 3). Thereafter, allantoic vessels invade into the chorionic plate, which is then converted into the labyrinth layer of the definitive placenta. In mutant embryos, this invasion process was drastically reduced, with no embryonic blood vessels found in the rather compact labyrinth layer (Fig. 4E). The vascular endothelial cells remained mostly restricted to the chorioallantoic region (Fig. 4E). The expression of *Flk1*, a marker for the endothelial cells in the labyrinth (Breier et al., 1995) was almost absent in mutant labyrinth layers, whereas significant expression was

**Table 3. Classification of the developmental stage of foetuses lacking *Fra1* by morphological criteria**

| | Morphological characteristics | Developed at stage* | Developed in E9.5 littermates‡ +/+ | Developed in E9.5 littermates‡ –/– |
|---|---|---|---|---|
| 1 | Foetal erythrocytes in embryo | E9.0-E9.5 | + | + |
| 2a | Allantois fused to chorion | E8.75-E9.0 | + | + |
| 2b | Umbilical vessels present | E9.0-E9.5 | + | + |
| 3a | Embryo dorsally convex | E9.25 | + | + |
| 3b | Tailbud dorsal to forebrain | E9.5 | + | – |
| 4 | Heart beating | E8.5 | + | + |
| 5 | Anterior neuropore closed | E9.0 | + | + |
| 6 | Transparent roof of 4$^{th}$ ventricle | E9.5-E10.25 | + | – |
| 7 | Otic vesicle present | E9.5 | + | + |
| 8 | Forelimb bud present | E9.5 | + | – |
| 9 | Number of somites | E9.5: 21-29 | 18-27 | 13-20 |
| 10 | Crown-rump length | E9.5: 1.5-2.2mm | approx. 2 mm | approx. 0.8 mm |

The indicated morphological characteristics were scored on three pairs of wild-type and mutant littermates by gross examination of freshly isolated embryos. The presence of foetal erythrocytes in the embryo proper and of umbilical vessels in the allantois were in addition scored from histological sections.

*According to Brown (1990).

‡+ or –, morphological characteristic observed (+) or not observed (–) in all three embryos scored.

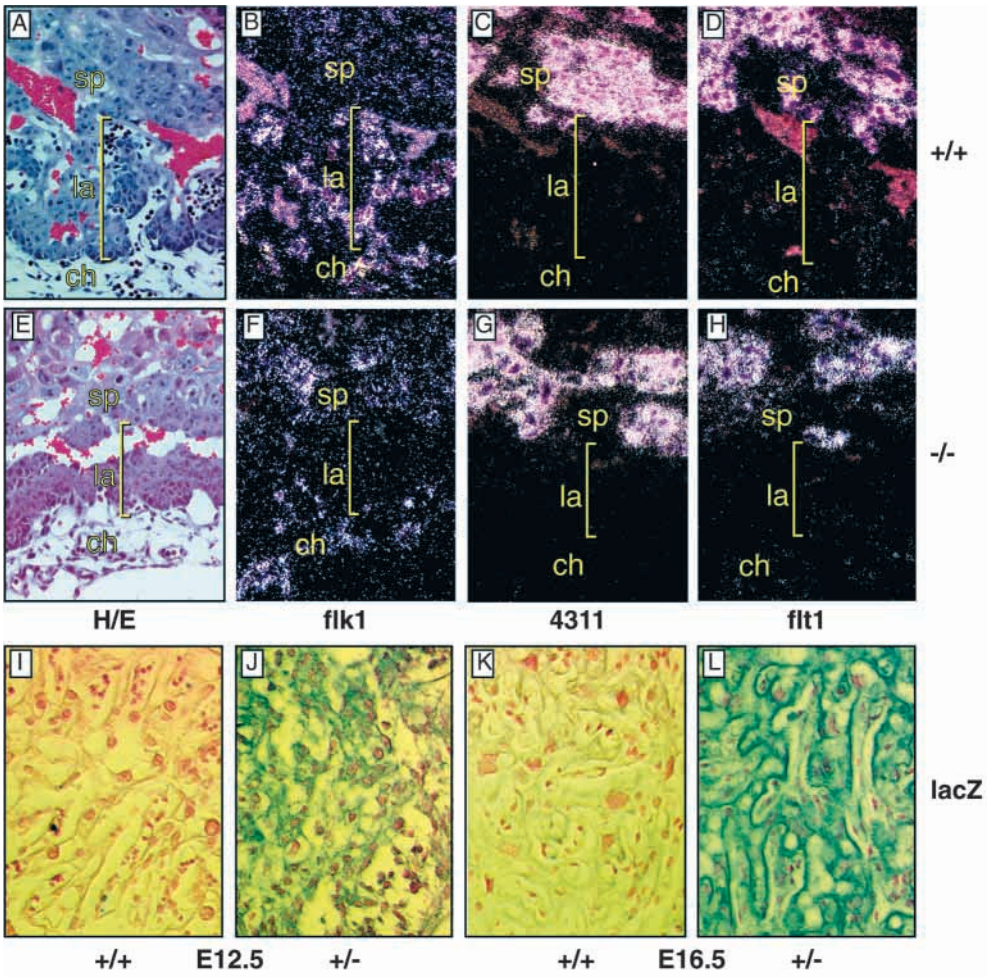


**Fig. 4.** *Fra1*$^{-/-}$ embryos lack a vascularised placental labyrinth layer. Histological analysis at E9.5 reveals a narrow and largely avascular labyrinth layer in the mutant (E) compared with the wild-type (A) placenta. In situ hybridisation analysis using *Flk1* as a marker for endothelial cells shows the absence of expression in the labyrinth layer of the mutant (F) compared with the wild-type (B) placenta. In contrast, the expression of the spongiotrophoblast markers 4311 (C,G) and *Flt1* (D,H) is comparable between mutant (G,H) and wild-type (C,D) placentas (dark field). (I-L) *lacZ* expression, which reflects *Fra1* promoter activity, in the labyrinth layer of E12.5 (I,J) and E16.5 (K,L) wild-type (I,K) and *Fra1*$^{+/-}$ (J,L) placentas (X-gal staining). ch, chorio-allantois; la, labyrinth; sp, spongiotrophoblasts.

detected in the underlying allantoic mesoderm (Fig. 4B,F). In situ hybridisation with gene *4311* and *Flt1*, which are marker genes for the spongiotrophoblast layer of the placenta, revealed no significant differences between mutants and controls (Fig. 4). Similarly, no defects were apparent in the chorio-allantoic and giant cell layers of the placenta (Fig. 4A,E). Consistent with these observations, analysis of E12.5 and E16.5 *Fra1*$^{+/-}$ placentas by X-gal staining revealed expression of *Fra1* specifically in the labyrinth layer (Fig. 4J,L).

In addition, mutant embryos exhibited an abnormal yolk sac (Fig. 5). The parietal yolk sac consists of the Reichert's membrane lined by parietal endoderm cells and of an intermittent layer of trophoblast giant cells situated between the maternal decidual cells and the Reichert's membrane. In controls, this single layer of trophoblast cells forms a network of channels in close proximity to the Reichert's membrane that distributes maternal blood throughout the parietal wall of the yolk sac (Fig. 5A). In mutant embryos this network appeared highly disorganised, with trophectoderm cells arranged in several layers and large maternal blood sinuses, which were not in close proximity to the Reichert's membrane and the parietal endoderm cells (Fig. 5B). Whereas a continuous Reichert's membrane with normal parietal endoderm cells was found, the visceral yolk sac of mutant embryos was considerably separated from the parietal yolk sac (Fig. 5B). Since these layers are not physically linked, the retraction of the visceral yolk sac might be due to reduced hydrostatic pressure in the amniotic cavity. *Fra1* expression was analysed by X-gal staining and was found in trophoblast giant cells, parietal endoderm cells and the visceral endoderm, but not in the mesoderm of the visceral yolk sac (Fig. 5G). Histological analysis of the visceral yolk sac revealed the presence of endodermal and mesodermal layers; however, the visceral endoderm appeared more compact and unstructured compared with controls (Fig. 5C-F). Although blood vessels were present and contained foetal erythrocytes, they were reduced in numbers, and some of them were abnormally enlarged, suggesting that the blood supply to the foetus is impaired (Fig. 5B,D).

### Rescue of the lethality of *Fra1* mutants by wild-type extra-embryonic tissues

As a definitive test of whether the lethality of mutant foetuses is due to defects in the extra-embryonic compartment, *Fra1*$^{-/-}$ ES cells were injected into wild-type tetraploid blastocysts (Wang et al., 1997). Tetraploid cells can contribute efficiently to most extra-embryonic tissues but not to the embryo proper (Nagy et al., 1990, 1993; James et al., 1995), and thus should complement defects in the extra-embryonic compartment of mutant foetuses. Following tetraploid blastocyst injection, mutant foetuses were isolated at E13.5, E16.5, E18.5 and P1 (Table 4). In these 'ES foetuses', the foetus was exclusively formed by *Fra1*$^{-/-}$ cells, whereas the placenta contained predominantly wild-type cells, and the yolk sac consisted of both wild-type and mutant cells (Fig. 6A). Rescued *Fra1*$^{-/-}$ foetuses were also obtained by aggregating morulae derived from *Fra1*$^{+/-}$ intercrosses with wild-type tetraploid morulae

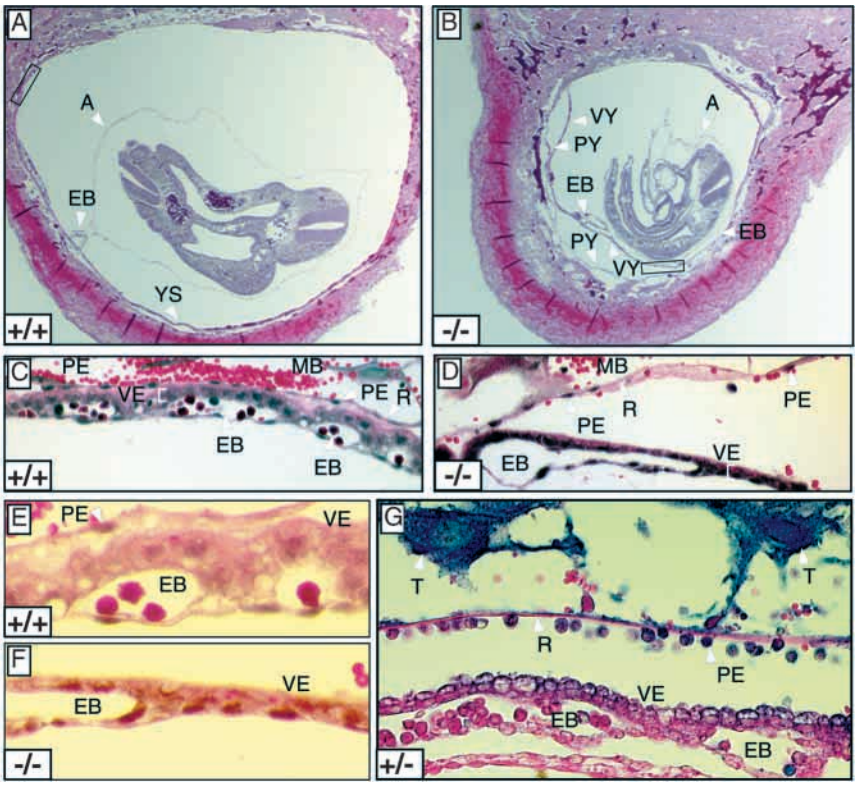


**Fig. 5.** Yolk-sac defects in *Fra1*$^{-/-}$ embryos. Histological analysis of wild-type (A,C,E) and mutant (B,D,F) E9.5 yolk sacs. (A,B) Sections of the exocoelomic cavity containing the embryo and surrounded by the maternal decidua. (C,D) Sections of the visceral and parietal yolk sac. The boxed areas in A and B are shown. (E,F) Sections of the visceral yolk sac at higher magnification. (G) Section of the yolk sac of an X-gal stained E9.5 *Fra1*$^{+/-}$ embryo. Cells stained blue express *lacZ*, which reflects *Fra1* promoter activity. A, amnion; EB, embryonic blood vessel of the yolk sac; MB, maternal blood sinus; PE, parietal endoderm cell of the parietal yolk sac (PY); R, Reichert's membrane; T, trophoblast giant cell; VE, visceral endoderm of the visceral yolk sac (VY); YS, yolk sac.

(Table 4). At the indicated times of isolation, all rescued mutant foetuses were viable, appeared phenotypically normal and were not growth retarded, indicating that the lethality and growth retardation of *Fra1*$^{-/-}$ foetuses can be rescued by providing wild-type extra-embryonic tissues (Table 4; Fig. 6).

Since there is good evidence from *Fra1* transgenic mice for a role of *Fra1* in bone cell development (Matsuo et al., 2000; Jochum et al., 2000), skeletons of E18.5 *Fra1*$^{-/-}$ rescued foetuses were analysed (Fig. 6B). These had ossification centres of normal size and distribution, and chondrocytes, bone-forming osteoblasts and TRAP-positive multinucleated osteoclasts were present in *Fra1*-deficient bones (Fig. 6B-D). Thus, normal bone cell differentiation does apparently not require *Fra1*, at least up to E18.5.

Four *Fra1*$^{-/-}$ newborns obtained by Caesarean section or natural delivery survived up to 2 days after birth. Histological analysis revealed no abnormalities of organs derived from rescued E18.5 *Fra1*$^{-/-}$ foetuses, including skin (Fig. 6E), brain, heart, cardiac outflow tract, liver, intestine, spleen, kidneys and adrenal glands (data not shown). In addition, no defects were observed in organs to which *Fra1*$^{-/-}$ ES cells inefficiently contributed in chimaeric mice (Fig. 3B) such as calvaria, thymus and pancreas. The peripheral blood of E18.5 *Fra1*$^{-/-}$ foetuses contained cells of all haemopoietic lineages. However,

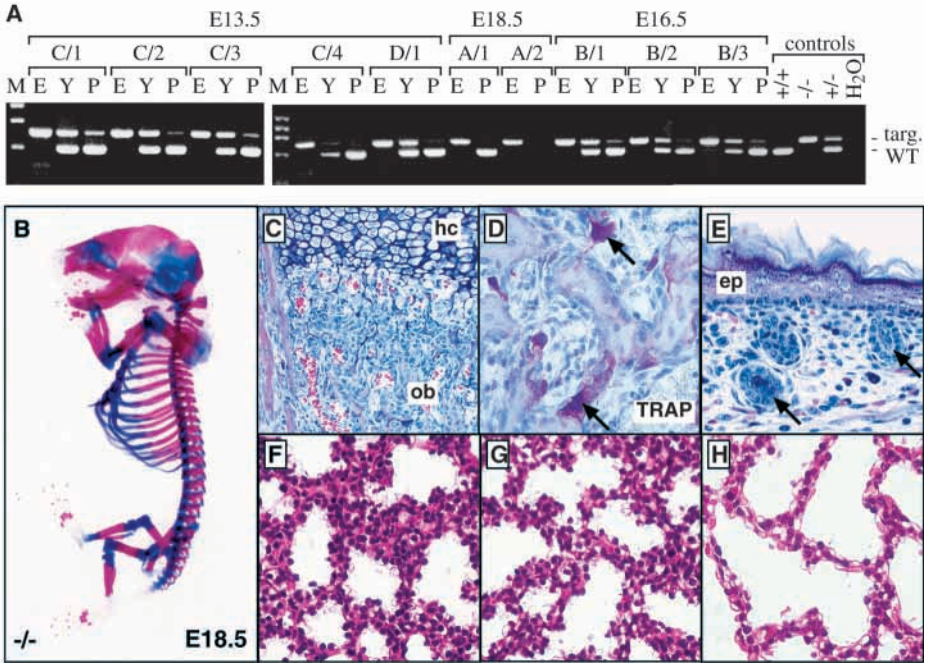


**Fig. 6.** Rescue of the *Fra1*$^{-/-}$ lethality by tetraploid wild-type extra-embryonic tissues. (A) PCR analysis of *Fra1* alleles in the placenta (P), yolk sac (Y) and embryo proper (E) of chimaeras generated by injection of *Fra1*$^{-/-}$ ES cells into tetraploid wild-type blastocysts. C/1, etc. are individual rescued embryos. (B) Skeleton of a rescued E18.5 *Fra1*$^{-/-}$ foetus stained with Alcian Blue and Alizarin Red. (C) Histological analysis of a rescued *Fra1*$^{-/-}$ femur demonstrating the presence of hypertrophic chondrocytes (hc) and bone-forming osteoblasts (ob) at E18.5. (D) TRAP-stained section of a *Fra1*$^{-/-}$ femur showing the presence of multinucleated bone-resorbing osteoclasts (arrows). (E) Skin of a rescued E18.5 *Fra1*$^{-/-}$ foetus displaying differentiating keratinocytes in the epidermis (ep) and developing hair follicles (arrows). (F-H) Lung immaturity in E18.5 foetuses generated by tetraploid blastocyst injection. Lungs of both *Fra1*$^{-/-}$ (F) and wild-type (G) foetuses contain wide and cell rich alveolar septa. The lung of a normal wild-type E18.5 foetus is shown for comparison (H).

**Table 4. Offspring obtained from tetraploid rescue experiments**

| | | Genotype | | | |
|---|---|---|---|---|---|
| | Stage | +/+ | +/– | –/– | Total |
| Crosses | E11.5-E12.5 | 25 | 59 | 0 | 84 |
| +/– × +/– | P1 | 109 | 219 | 0 | 328 |
| Morula aggregation | E13.5 | 1 | 3 | 1 | 5 |
| +/– × +/– ↔ tetraploid +/+ | E18.5 | 6 | 5 | 1* | 12 |
| Blastocyst injection | E13.5 | – | – | 5 | 5 |
| –/– ES cells → tetraploid +/+ | E16.5 | – | – | 3 | 3 |
| | E18.5/P1 | – | – | 15‡ | 15 |

Genotypes of progeny of normal *Fra1*$^{+/-}$ intercrosses, tetraploid aggregation chimaeras from *Fra1*$^{+/-}$ intercrosses, and chimaeras generated by injection of *Fra1*$^{-/-}$ ES cells into tetraploid wt blastocysts.

*This foetus was already dead for an estimated 24 hours at the time of isolation.

‡Four of these rescued pups survived up to 2 days after birth.

the lungs of *Fra1*$^{-/-}$ rescued foetuses were immature, as indicated by wide and cell-dense alveolar septa, which might be a cause of the early postnatal lethality (Fig. 6F). A similar lung immaturity was present in age-matched foetuses obtained by injection of wild-type ES cells into wild-type tetraploid blastocysts, indicating that the lung defect is likely to be due to the technical procedure (Fig. 6G; compare with Fig. 6H). Thus, the early postnatal lethality of *Fra1*$^{-/-}$ rescued mice may be attributable to the experimental approach rather than the lack of *Fra1*.

## DISCUSSION

The results reported here provide the first genetic evidence that *Fra1* plays an essential role in the placentation process. Embryos lacking *Fra1* die between E10.0 and E10.5, when the chorio-allantoic placenta has become the principal route of nourishment for the embryo (Cross et al., 1994; Copp, 1995). Morphological analysis suggests that extra-embryonic defects, particularly the failure to establish a functional placenta, are the cause of this lethality. *Fra1*$^{-/-}$ placentas lack a properly vascularised labyrinth layer, although the fusion of the allantois to the chorion, which initiates the formation of the labyrinth, is not affected. However, the subsequent differentiation of the allantoic mesoderm into vascular channels is impaired in *Fra1*$^{-/-}$ foetuses and leads only to the formation of large vessels in the chorionic plate, which do not invade and sprout into the labyrinth trophoblast. As a consequence, embryonic blood vessels do not intermingle closely with maternal blood sinuses, presumably resulting in an inefficient exchange of nutrients and gas between the maternal and embryonic vascular systems. In agreement with these extra-embryonic defects, *Fra1* was found to be expressed in extra-embryonic tissues such as the placental labyrinth layer and the parietal and visceral endoderm, and trophoblast giant cells of the yolk sac, whereas expression was not detectable in the embryo proper before E10.5.

One of the most striking features of *Fra1*$^{-/-}$ embryos is their severe growth retardation. Interestingly, this retardation does not seem to be due to a cell-autonomous proliferation defect. Primary fibroblasts and ES cells isolated from mutant embryos exhibit normal proliferation rates in vitro. BrdU labelling and generation of chimaeras via blastocyst injection of *Fra1*$^{-/-}$ ES cells did not reveal reduced proliferation capacity of mutant cells in vivo, even when in direct competition with wild-type cells. Most importantly, rescued *Fra1*$^{-/-}$ embryos generated via tetraploid blastocyst injection were not growth retarded. These data demonstrate that *Fra1*$^{-/-}$ embryos grow to normal size if provided with wild-type extra-embryonic tissues, and indicate that cells lacking Fra1 have a normal proliferative capacity in vitro and in vivo. Thus, the observed growth retardation of *Fra1*$^{-/-}$ foetuses is most likely due to the embryo 'starving to death' in utero caused by an inadequate supply of nutrients and oxygen, or by poisoning resulting from a lack of outward exchange via the extra-embryonic foeto-maternal exchange organs.

Importantly, the defect in the placental labyrinth alone may not fully explain the severe growth retardation of *Fra1*$^{-/-}$ foetuses. A number of other mouse mutants with lethality at the same stage and very similar placentation defects exhibit only mild or no growth retardation, such as embryos lacking Mash2 (Ascl2), Gata2, Gata3 or Ets2 (Guillemot et al., 1994; Ma et al., 1997; Yamamoto et al., 1997). Even some mutant foetuses in which the allantois does not fuse to the chorion are less growth retarded than foetuses lacking *Fra1* (Stott et al., 1993; Li et al., 1992; Gurtner at al., 1995; Yang et al., 1995). However, cessation of blood flow within the yolk sac plexus frequently causes foetal growth retardation, such as in embryos lacking HNF4 or trombomodulin (Chen et al., 1994; Healy et al., 1995). In addition, defects in the yolk-sac blood circulation are often associated with the dilatation of the pericardium as an indication of osmotic imbalance within the embryo. Indeed, a large proportion of *Fra1* mutant embryos exhibited an enlarged pericardium. Thus, in addition to the placental defects, structural and functional defects in the yolk sac may also contribute to the severe growth retardation and embryonic lethality of *Fra1*$^{-/-}$ embryos.

To verify that extra-embryonic defects are causal to the lethality, *Fra1*$^{-/-}$ ES cells have been generated and injected into tetraploid wild-type blastocysts (Wang et al., 1997). Since tetraploid cells can efficiently contribute to extra-embryonic tissues but not to the embryo proper, this technique (Wang et al., 1997) has allowed the provision of *Fra1*-deficient embryos with a wild-type placenta. Unlike *Fra1*$^{-/-}$ embryos derived from heterozygote intercrosses, *Fra1*$^{-/-}$ foetuses rescued by tetraploid blastocyst injection did not die at E10.0, but completed embryonic development and survived up to 2 days

after birth. Thus, the embryonic lethality was fully rescued, demonstrating that the presence of functional *Fra1* in the embryo proper is not required for survival during embryogenesis, and that defects in extra-embryonic tissues are most likely to represent the primary cause of lethality. All tetraploid rescued mice died within two days of birth, presumably owing to a pronounced lung immaturity. Importantly, a similar lung immaturity was present in age-matched foetuses obtained by tetraploid blastocyst injection of wild-type ES cells, and is frequently observed with most wild-type or mutant ES cell lines (Wang et al., 1997). Although the early postnatal lethality of *Fra1*$^{-/-}$ rescued mice is thus presumably due to the experimental approach, rather than the lack of *Fra1*, it is possible that this early lethality masks additional important functions of Fra1 in postnatal development.

Interestingly, deletion of *Junb*, another gene of the AP-1 family, leads to embryonic lethality between E8.5 and E10.0 with a phenotype remarkably similar to that of *Fra1*$^{-/-}$ foetuses (Schorpp-Kistner et al., 1999). Some *Junb*$^{-/-}$ embryos die around E8.5 and exhibit yolk-sac defects, as well as additional extra-embryonic defects that are more severe than in the *Fra1* mutant. *Junb*$^{-/-}$ embryos that escape these initial defects die around E10.0 and, like *Fra1*$^{-/-}$ embryos, are severely growth retarded, exhibit defects in the yolk sac and a non-vascularised placental labyrinth. These strikingly similar phenotypes suggest that *Fra1* and *Junb* might have overlapping functions during extra-embryonic development, an idea supported by the observations that the temporal and spatial expression patterns of *Junb* and *Fra1* during embryogenesis are partly overlapping (Schorpp-Kistner et al., 1999). Moreover, Junb/Fra1 dimers can regulate the tissue-specific expression of AP-1 target genes (Vallone et al., 1997), and *Fra1* is a potential transcriptional target of *Junb*. It has been found that *Junb* can repress transactivation of some promoter constructs with single AP-1 binding sites, but activate analogous reporter constructs containing multimeric AP-1 sites (Deng and Karin, 1993). Intriguingly, a major DNA element regulating *Fra1* expression, which is located in the first intron, consists of a cluster of 3 AP-1 sites (Bergers et al., 1995), which might be a target for activation by Junb. The embryonic lethality of *Junb*$^{-/-}$ embryos can be rescued by crosses with transgenic mice ubiquitously expressing *Junb* under the control of the human ubiquitin C promoter (Ubi-*Junb* transgene; Schorpp et al., 1996; Schorpp-Kistner et al., 1999). Since the phenotypes caused by *Junb* or *Fra1* deficiency are very similar, we attempted to rescue the lethality of *Fra1* mutants by using the Ubi-*Junb* transgene. Interestingly, out of 284 offspring, 4 *Fra1*$^{-/-}$ mice carrying the Ubi-*Junb* transgene were obtained, whereas 102 offspring were *Fra1*$^{+/+}$ and 178 were heterozygous; 57.5% of the *Fra1*$^{+/+}$ and *Fra1*$^{+/-}$ mice carried the transgene. The four rescued *Fra1*$^{-/-}$ mice were healthy, phenotypically normal and had a normal lifespan. Thus, deregulated expression of *Junb* can complement at low frequency for the lack of *Fra1*, further suggesting that *Fra1* and *Junb* have overlapping functions during embryonic development.

The extra-embryonic defects of *Fra1*$^{-/-}$ foetuses could be due to defective tissue remodelling and/or cell-cell and cell-matrix interaction. The expression of several extracellular matrix (ECM) proteinases appears to be regulated in part by AP-1 (Angel and Karin, 1991; Hennigan et al., 1994; Schorpp-Kistner et al., 1999). Some of these proteases are expressed during early embryogenesis and play an important role in the invasive processes involved in establishing foeto-maternal interactions, such as MMP-9 and uPA (Birkedal-Hansen et al., 1993; Groskopf and Linzer, 1994). Similar to *Junb* (Schorpp-Kistner et al., 1999), deletion of *Fra1* might alter AP-1 activity and consequently expression of ECM proteases, which in turn might lead to a lack of invasive processes required for the formation of the labyrinth layer, and to the observed aberrant structure of the parietal and visceral yolk sac of mutant conceptuses. Alternatively, defects in vasculogenesis or angiogenesis could explain the dilated yolk-sac blood vessels, as well as the lack of invasion and sprouting of vascular channels into the placental labyrinth. Targeted mutagenesis has confirmed the crucial roles of VEGF and its receptors Flk1 (Kdr) and Flt1 in these processes (Fong et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). In *Fra1*$^{-/-}$ foetuses, a vascularisation defect leads to a non-vascularised labyrinth layer as demonstrated by the lack of *Flk1* expression. Large, unusually shaped foetal blood vessels are present in the chorionic plate, but are unable to invade and sprout into the labyrinth trophoblasts. This defect, as well as the defect in yolk-sac vascularisation closely resembles the defects in *Junb*$^{-/-}$ extra-embryonic tissues (Schorpp-Kistner et al., 1999), which may involve the same molecular mechanism.

Perhaps the most surprising result of the present study is that deletion of *Fra1* has significantly more deleterious effects than inactivation of *Fos*, the 'prototype' *Fos* family member. Mice lacking Fos are osteopetrotic, but most survive to adulthood (Wang et al., 1992; Johnson et al, 1992; Grigoriadis et al., 1994). Since Fra1 lacks a transactivation domain (Wisdom and Verma, 1993; Bergers et al., 1995), it is presumed to have only a subset of the functions of the potent transcriptional activator Fos. Why then does *Fos* not compensate for the lack of *Fra1*? *Fos* is expressed in most extra-embryonic tissues, including the spongiotrophoblast and the labyrinth layer of the placenta, visceral endoderm and mesoderm, and amnion, at levels that are several-fold higher than in the embryo proper (Müller et al., 1983). Thus, *Fos* is apparently expressed in the right tissues; however, it is conceivable that the onset of expression is too late to rescue the lethality of *Fra1*$^{-/-}$ embryos. Significant expression of *Fos* in the placenta was detected at E9.5, whereas extra-embryonic membranes were only analysed at E12.5 and later (Müller et al., 1983). Alternatively, a crucial function for *Fra1* in embryonic development might be transcriptional inhibition rather than activation. Because Fos, unlike Fra1, has a potent transactivation domain, a putative inhibitory role of Fra1 could not be compensated for by Fos.

In addition, *Fra1* is a transcriptional target of *Fos* (Bergers et al., 1995; Schreiber et al., 1997; Matsuo et al., 2000). However, *Fra1* expression during embryonic development must be, at least in part, *Fos* independent, as *Fos*$^{-/-}$ mice develop to term, survive to adulthood and do not have the same embryonic lethal phenotype as *Fra1*$^{-/-}$ foetuses. In agreement with this idea, an early *Fos*-dependent and a late *Fos*-independent phase of *Fra1* expression were observed in 3T3 fibroblasts upon serum stimulation (Schreiber et al., 1997). In contrast, *Fos*$^{-/-}$ osteoclast precursors are viewed as 'virtual double knockouts' of *Fos* and *Fra1*, as *Fra1* expression is largely *Fos* dependent in these cells (Matsuo et al., 2000). Therefore, endogenous *Fra1* does not compensate for the lack

of *Fos* in *Fos*$^{-/-}$ osteoclasts, although ectopically expressed *Fra1* can fully rescue the *Fos*$^{-/-}$ osteopetrotic phenotype and osteoclast differentiation block (Matsuo et al., 2000). Furthermore, *Fra1* potentiates osteoclastogenesis (Owens et al., 1999). It was therefore of interest to determine whether *Fra1* is necessary for osteoclast development. Using E18.5 rescued *Fra1*$^{-/-}$ embryos obtained by tetraploid blastocyst injection, we could show the presence of multinucleated osteoclasts and the formation of normal bone marrow cavities in the absence of *Fra1*. Thus, unlike *Fos*, *Fra1* is not essential for osteoclast formation, at least in newborn mice. In the future, it will be interesting to determine, by tissue-specific conditional inactivation of the *Fra1* gene in bone and endothelial cells, whether lack of *Fra1* leads to skeletal and vascular defects in adult mice.

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