CORRIGENDUM

Transcription factor AP-2γ is essential in the extra-embryonic lineages for early postimplantation development

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In the printed version of this article, one of the references was listed incorrectly. The correct version is shown below.

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Transcription factor AP-2γ is essential in the extra-embryonic lineages for early postimplantation development

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SUMMARY

The members of the AP-2 family of transcription factors play important roles during mammalian development and morphogenesis. *AP-2γ* (*Tcfap2c* – Mouse Genome Informatics) is a retinoic acid-responsive gene implicated in placental development and the progression of human breast cancer. We show that AP-2γ is present in all cells of preimplantation embryos and becomes restricted to the extra-embryonic lineages at the time of implantation. To study further the biological function of AP-2γ, we have generated *Tcfap2c*-deficient mice by gene disruption. The majority of *Tcfap2c*⁻/⁻ mice failed to survive beyond 8.5 days post coitum (d.p.c.). At 7.5 d.p.c., *Tcfap2c*⁻/⁻ mutants were typically arrested or retarded in their embryonic development in comparison to controls. Morphological and molecular analyses of mutants revealed that gastrulation could be initiated and that anterior-posterior patterning of the epiblast remained intact. However, the *Tcfap2c* mutants failed to establish a normal maternal-embryonic interface, and the extra-embryonic tissues were malformed. Moreover, the trophoblast-specific expression of eomesodermin and *Cdx2*, two genes implicated in FGF-responsive trophoblast stem cell maintenance, was significantly reduced. Chimera studies demonstrated that AP-2γ plays no major autonomous role in the development of the embryo proper. By contrast, the presence of AP-2γ in the extra-embryonic membranes is required for normal development of this compartment and also for survival of the mouse embryo.

Key words: *Tcfap2c* mutant mouse, Postimplantation development, Extra-embryonic, Trophoblast, AP-2γ

INTRODUCTION

Studies in human, mouse, chicken and *Drosophila* have implicated the AP-2 proteins in the control of cell proliferation, morphogenesis and tumor progression (Bosher et al., 1996; Hilger-Eversheim et al., 2000; Kerber et al., 2001; Monge et al., 2001; Shen et al., 1997a). These proteins, which all recognize the same GC-rich consensus sequence (McPherson and Weigel, 1999; Mohibullah et al., 1999), are also distinguished by a basic helix-span-helix motif that is required for DNA binding and dimerization (Williams and Tjian, 1991a; Williams and Tjian, 1991b). Four members of the AP-2 family of transcription factors, AP-2α, AP-2β, AP-2γ and AP-2δ, have been characterized in mammalian species (Bosher et al., 1996; Mitchell et al., 1987; Moser et al., 1995; Oulad-Abdelghani et al., 1996; Zhao et al., 2001). We focus on AP-2γ, which was first isolated in human breast cancer cell lines because of its ability to bind to a critical regulatory element within the ERBB2 promoter (Bosher et al., 1996). Subsequently, AP-2γ was also found to interact with regulatory elements associated

with the estrogen receptor gene, another gene involved in mammary gland development and oncogenesis (McPherson et al., 1997). Moreover, an immunological survey indicated that the presence of AP-2γ protein in breast cancer samples correlated with the expression of ERBB2 and the insulin-like growth factor 1 receptor (Turner et al., 1998). These findings suggest a direct role of AP-2γ in regulating important molecular markers of breast cancer.

The AP-2 proteins have also been linked with cell fate determination, as they are responsive to a number of signaling molecules, including cAMP and the morphogen retinoic acid (RA) (Imagawa et al., 1987; Lüscher et al., 1989). Indeed, the mouse *Tcfap2c* gene (which encodes the protein AP-2γ) was isolated independently, and named *AP-2.2*, in a screen for RA-inducible genes in murine embryonal carcinoma cells (Oulad-Abdelghani et al., 1996).

The mouse *Tcfap2c* gene is expressed in the central and peripheral nervous system, ectoderm, limbs, face and mammary glands during mouse development, in a dynamic spatiotemporal pattern similar to that of the other AP-2 family

members (Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1997b) (J. Zhang and T. W., unpublished). Concurrent with their initial expression in the embryo, all three AP-2 genes are also expressed in the extra-embryonic trophoblast (Chazaud et al., 1996; Moser et al., 1997b). Importantly, *Tcfap2c* has been shown to be uniquely expressed in trophoblast at 6.5 d.p.c. and thus earlier in development than the other members of the family, which are expressed at 8 d.p.c. (Chazaud et al., 1996; Moser et al., 1997b; Shi and Kellems, 1998). Later in embryogenesis, *Tcfap2c* also demonstrates strong and persistent expression in all trophoblast lineages of the chorioallantoic placenta, including the secondary giant cells, spongiotrophoblast and labyrinthine trophoblast (Sapin et al., 2000; Shi and Kellems, 1998).

Previous gene targeting studies have shown that either *Tcfap2a* or *Tcfap2b* (genes encoding AP-2α and AP-2β, respectively) can be mutated without affecting placental function, although both genes have essential functions during development of the embryo proper. *Tcfap2a* is required for limb, eye, craniofacial, cardiovascular, skeletal and body wall development (Brewer et al., 2002; Nottoli et al., 1998; Schorle et al., 1996; Zhang et al., 1996), while *Tcfap2b* is required for renal epithelial cell survival during late embryogenesis (Moser et al., 1997a). Consistent with these findings, several genes expressed in epidermal and neural crest lineages have been found to contain AP-2-binding sites as a component of their regulatory sequences (Leask et al., 1991; Maconochie et al., 1999). Other potential targets of AP-2 action are genes associated with extra-embryonic function, including those involved in steroid hormone biosynthesis and signaling within the placenta (Hu et al., 1996; Johnson et al., 1997; LiCalsi et al., 2000; Pena et al., 1999; Peng and Payne, 2001; Piao et al., 1997; Richardson et al., 2000; Steger et al., 1993). In particular, Shi and Kellems (Shi and Kellems, 1998) have identified a placental-specific footprinted region, matching the AP-2 consensus site, within the placental regulatory element of the murine adenosine deaminase gene (*Ada*). Mutation of this AP-2 site abolished reporter gene expression in the placenta of transgenic mice. Indeed, as *Tcfap2c* is the most highly expressed of the AP-2 family members in the extra-embryonic tissues, it has been proposed that this gene may be required not only for placental *Ada* expression but also for development of the mature placenta (Shi and Kellems, 1998).

Considering the links between *Tcfap2c* and placental function, and its potential importance in both normal development and in tumorigenesis, we investigated its biological function by generating *Tcfap2c*-deficient mice. Our studies demonstrate that *Tcfap2c* is vital to embryonic survival during the early postimplantation period, and that expression of AP-2γ in the extra-embryonic membranes can rescue development of *Tcfap2c* mutant embryos.

MATERIALS AND METHODS

Generation of *Tcfap2c* mutant mice

The isolation of a mouse *Tcfap2c* clone from a 129/Sv P1 genomic library has been described previously (Williamson et al., 1996). A 2.2 kb *Bam*HI fragment derived from this P1 clone was used as a 5′ homology region, and a 2.8 kb *Cla*I/*Hin*dIII fragment was used as a 3′ homology region for a replacement targeting construct. These two fragments, which include exon 6 and a region of exon 7, respectively, were inserted into a vector containing the PGK-*neo* cassette and two herpes simplex virus thymidine kinase expression cassettes (Fig. 1). Linearized targeting vector (25 μg) was electroporated using established procedures (Hogan et al., 1994) into CJ-7 ES cells (Thomas Gridley, Jackson Laboratories). G418-resistant colonies were screened for the correct targeting event by PCR analysis using the enzyme r*Tth* DNA polymerase, XL (Perkin Elmer) according to the manufacturer's instructions in a 25 μl reaction containing 1.2 mM magnesium acetate. Clones were initially screened for the correct homologous recombination event within the 3′ region of the targeting vector using the primer pair neo 3′ KO (5′-AAC GCA CGG GTG TTG GGT CGT TTG TTC G-3′) and GKO2 (5′-CAG CAT ACA GGC AGA GGC TCA CTT CTG TAG-3′). These primers, respectively, occur within the *neo* gene and in the genomic sequences downstream from the region of homology (Fig. 1). The following conditions were used for PCR amplification: 60 seconds at 93°C, 36 cycles of 93°C for 60 seconds, 70°C for 7 minutes 36 seconds, followed by 72°C for 10 minutes. Four positive clones were identified based upon the presence of the expected ~3 kb product in the PCR reaction. Subsequently, the correct 5′ targeting event was confirmed in these clones by PCR analysis using the primer pair GKO1 (5′-CGT GAC TTG TGC TGA AAG GGA CTT GAC AGG-3′) and Neoup (5′-GCG TGC AAT CCA TCT TGT TCA ATG GCC GAT CC-3′). These primers, respectively, occur within the genomic sequences upstream from the region of homology and within the *neo* gene (Fig. 1). PCR amplification was performed as above and a successful recombination event was scored by the presence of 2.7 kb product. Subsequently, Southern blot analysis was used to confirm the presence of correct targeting events in these clones using either a 550 nucleotide *Pst*I/*Nco*I 5′ probe, located in the genomic sequences upstream of the 5′ region of homology, or a 130 nucleotide *Sma*I probe, which is within the 3′ region of homology. Appropriate recombination at the 5′ end produced a *Pst*I fragment of 3.0 kb in comparison with a 5.3 kb wild-type fragment. Correct targeting events at the 3′ end were recognized by the appearance of a 5.0 kb *Nco*I fragment, in contrast to a 7.8 kb wild-type fragment. Following karyotype analysis, three properly targeted euploid ES clones were injected into C57BL/6 blastocysts. Subsequently, *Tcfap2c*+/− offspring were produced from two different lines of resulting chimeras and then interbred to obtain *Tcfap2c*−/− homozygotes. The targeted mutation has been maintained through backcrossing with outbred Black Swiss mice (Taconic).

Mouse genotyping by PCR

Noon of the day of the copulatory plug was considered 0.5 days post coitum (d.p.c.). Genotyping of embryos (7.5 d.p.c. and younger), yolk sac DNA (8.5 d.p.c. and older) and tail DNA (adults) was performed by PCR using Taq DNA polymerase (Qiagen). DNA isolation from single embryos that were 7.5 d.p.c. and younger, including tissues scraped from histological sections and blastocyst outgrowths, was performed as described (Yamaguchi et al., 1994). Tissues were incubated at 56°C in proteinase K/lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2 mM MgCl$_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 100 μg/ml proteinase K). For blastocysts after indirect whole-mount immunostaining, embryonic tissues scraped from histological sections and for embryonic tissues scraped from in situ hybridization sections, incubations were performed for 5 days in 20 μl of proteinase K/lysis buffer. For blastocyst outgrowths and for 7.5 d.p.c. embryos after whole-mount examination or in situ hybridization, incubations were overnight in 20-100 μl of proteinase K/lysis buffer. After incubation, samples were boiled for 10 minutes and 1-5 μl were subjected to PCR amplification. DNA from yolk sac and tail samples was prepared using the DNeasy tissue kit (Qiagen). Genotyping was performed using a two-allele, three-primer PCR to generate a 190 bp DNA fragment from the wild type *Tcfap2c* allele and/or a 140 bp fragment from the targeted *Tcfap2c* allele. Primers used were: Neo3′KO, *neo*-specific (as above); Xgamma3′, common

to wild-type and targeted *Tcfap2c* alleles (antisense, 5′-TCA TGG CTT TGG CAG CCA GGC CAT C-3′); and Gamwt5, equivalent to a region of exon 7 deleted from the targeted allele (sense, 5′-CTT CTG CTC TCT GGC CTC CTT GCA GCC-3′). The following program was used for PCR amplification: 90 seconds at 95°C; 40 cycles of 95°C for 30 seconds, 72°C for 2 minutes, followed by 72°C for 10 minutes.

Whole-mount immunocytochemistry of blastocysts

Embryos were flushed from uteri at 3.5 d.p.c. and fixed at room temperature for 30 minutes in 4% paraformaldehyde in saline buffer (pH 7.4), preincubated with 0.1% H_2O_2/PBS/0.2% Triton-X-100 for 20 minutes, blocked in 1% BSA for 5 minutes and incubated with 10% normal goat serum for 30 minutes at room temperature. Embryos were incubated overnight at 4°C with the anti-AP-2γ rabbit polyclonal antiserum, γ96, described elsewhere (Turner et al., 1998). Subsequently, embryos were incubated for 30 minutes at room temperature with biotin-SP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) followed by peroxidase-conjugated Streptavidin (Jackson Immunoresearch Laboratories) and the substrate 3,5 diaminobenzidine for visualization (Aldrich). Alternatively, for fluorescent detection, embryos were incubated with AlexaFluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) and counterstained for DNA with Hoechst 33342 (Molecular Probes).

Immunohistochemistry

Uteri or whole decidua were isolated in ice-cold PBS at 4.5-7.5 d.p.c., fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, oriented with respect to the antimesometrial-mesometrial axis, embedded in paraffin and serially sectioned at a thickness of 5 μm. Immunostaining was performed as described (Turner et al., 1998). After thorough washing, the samples were treated with 2.5% hydrogen peroxide in methanol for 30 minutes, then blocked in 1% BSA and 10% normal goat serum. Addition of the anti-AP-2γ polyclonal rabbit primary antiserum at a 1:500 dilution was followed by incubation with Biotin-SP-conjugated goat anti-rabbit IgG, peroxidase-conjugated Streptavidin, and 3,5 diaminobenzidine for visualization (Jackson ImmunoResearch; Aldrich). No staining was observed when the primary antibody was omitted, or when a blocking peptide specific for AP-2γ (Turner et al., 1998) was preincubated with the primary antibody. Note that the antisera used to study AP-2γ expression recognizes an epitope at the C terminus of the protein that should be absent from any product derived from the targeted allele.

Histological analysis

Embryos from *Tcfap2c+/−* intercrosses were processed for histological analysis as described by Kaufman (Kaufman, 1990). Briefly, whole decidua were dissected and fixed overnight in Bouin's fixative, dehydrated, oriented with respect to the antimesometrial-mesometrial axis and embedded in paraffin. Sagittal sections (5 μm) were cut and stained with Hemotoxylin and Eosin (H&E). After histological examination, embryonic tissues were scraped from slides and subjected to PCR analysis as described above.

In situ hybridization

Embryos were staged according to their morphology (Downs and Davies, 1993; Theiler, 1989). In situ hybridization using digoxigenin-labeled RNA probes was performed on whole-mount embryos and histological sections according to the protocol described (Shen et al., 1997b), except that whole embryos were processed in 0.74 μm mesh inserts (Costar). In situ hybridization on sections using [^{33}P]UTP-labeled probes was performed according to the protocol described by Hogan et al. (Hogan et al., 1994) and modified for ^{33}P as described (Biroc et al., 1993). The plasmid CCC36, containing a 200 bp fragment of *Tcfap2c* exon 7, was used to synthesize the AP-2γ sense and antisense probes. This fragment, which extends from the 5′

boundary of exon 7 to the *Cla*I site in the middle of exon 7, is absent from the targeted *Tcfap2c* allele. Other probes used for in situ hybridization analysis were generously provided by the following researchers: *T* (R. Beddington); *Otx2* and *Hnf3b* (T. Gridley); *Pl1* (*Csh1* – Mouse Genome Informatics) *Mash2* (*Ascl2* – Mouse Genome Informatics), *Eomes*, *Cdx2*, *Fgfr2* (J. Rossant); *Ada* (R. Kellems); *Bmp4* (B. Hogan); and *Hand1* (J. Cross).

Blastocyst outgrowths

Blastocysts from *Tcfap2c+/−* matings were flushed from uteri at 3.5 d.p.c. in M2 medium and individually cultured in ES cell medium without LIF containing 15% fetal bovine serum (HyClone), in 5% CO_2 at 37°C, on 24-well tissue culture dishes (Falcon). On the seventh day of in vitro culture, outgrowths were photographed and subsequently removed and genotyped by PCR as described above.

Generation of Rosa26 *Tcfap2c+/−* and *Tcfap2c−/−* ES cell lines and mouse chimera studies

Tcfap2c+/− mice on a 129/Sv background were bred with B6,129 hybrid mice homozygous for the ROSA β-geo 26 gene-trap allele (ROSA 26; Jackson Laboratories) to produce *Tcfap2c* heterozygotes containing the ROSA26 allele. These mice were interbred; blastocysts were collected and cultured in ES medium with LIF on γ-irradiated mouse embryo fibroblasts for generation of ES cell lines (Robertson, 1987). The resulting ES cell lines were genotyped by Southern blotting, stained for β-galactosidase (β-gal) activity and karyotyped (Hogan et al., 1994). *Tcfap2c+/−* and *Tcfap2c−/−* ES cells containing the ROSA26 allele were injected into C57BL/6J blastocysts. Resulting chimeras were either collected at various points during gestation and processed for β-gal staining (*n*=123) or allowed to develop to term (*n*=130). For complementary blastocyst injection experiments, wild-type ES cells containing the ROSA26 insertion (provided by Elizabeth Robertson) were injected into blastocysts obtained from *Tcfap2c+/−* intercrosses. Resulting chimeras were collected 10.5 d.p.c., stained for β-gal activity and examined by whole mount. Yolk sac endoderm DNA from these chimeras was isolated as described (Hogan et al., 1994) and subjected to PCR genotyping as described above. Of all morphologically normal chimeras with high percentages of β-gal staining, 16 were derived from wild type and 41 from *Tcfap2c+/−* blastocysts, respectively, while all six abnormal high percentage chimeras were derived from *Tcfap2c−/−* blastocysts. For tetraploid-diploid aggregations, tetraploid embryos were generated from electrofusion of two-cell-stage CD-1 embryos using an Electro Cell Manipulator 2001 (BTX) (according to the manufacturer's conditions) and aggregated at the four- to eight-cell stage with *Tcfap2c−/−* ES cells (Nagy and Rossant, 1993). Successful aggregates were transferred as blastocysts to foster mothers, and embryos were collected at 8.5, 9.5, 10.5 and 18.5 d.p.c., and processed to detect β-galactosidase activity (*n*=11).

Imaging

Images were captured using 35 mm film or a SPOT II camera (Diagnostic Instruments) and manipulated in Adobe Photoshop.

RESULTS

Targeted disruption of *Tcfap2c*

The open reading frame of *Tcfap2c* contains seven exons and spans approximately 7.5 kb. To produce a null mutation of the gene, a targeting vector was designed in which a *PGK-Neo* cassette replaced a 300 bp region, including the 5′ region of exon 7 that is necessary for the DNA-binding activity of AP-2γ (Fig. 1A; see Materials and Methods) (Williams and Tjian, 1991a). Subsequently, this construct was used in a standard gene targeting approach to derive mice that were

A

B
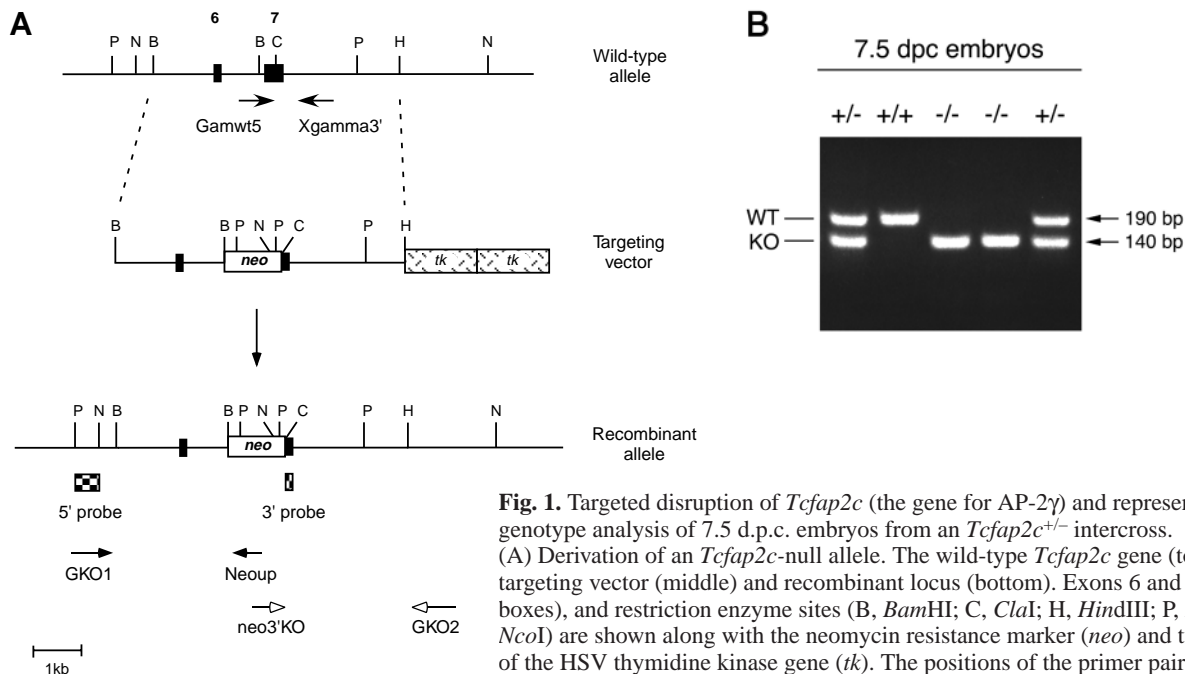
Fig. 1. Targeted disruption of *Tcfap2c* (the gene for AP-2γ) and representative genotype analysis of 7.5 d.p.c. embryos from an *Tcfap2c*^{+/−} intercross. (A) Derivation of an *Tcfap2c*-null allele. The wild-type *Tcfap2c* gene (top), targeting vector (middle) and recombinant locus (bottom). Exons 6 and 7 (black boxes), and restriction enzyme sites (B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; N, *Nco*I) are shown along with the neomycin resistance marker (*neo*) and two copies of the HSV thymidine kinase gene (*tk*). The positions of the primer pairs used for ES colony screening by PCR amplification are shown beneath, as are the positions of the 5′ and 3′ probes used for Southern blot analysis (checked boxes). The position of the primers used for identifying the wild-type allele in subsequent mice are shown towards the top of the figure (Gamwt5 and Xgamma3′). (B) DNA samples were subjected to PCR analysis using a mixture of three primers (see Materials and Methods). Amplification of the wild-type allele produces a fragment of 190 bp (upper band), while amplification of the targeted allele results in a 140 bp product (lower band). Abbreviations: WT, wild type; KO, size of *Tcfap2c*^{−/−} allele PCR product.

heterozygous for the targeted allele; these animals were viable and fertile.

Loss of *Tcfap2c* causes early embryonic lethality

Intercrossing heterozygotes failed to produce live *Tcfap2c*^{−/−} pups out of 50 live offspring, indicating embryonic lethality (Table 1). To determine the period of lethality, gestation sites from heterozygote matings were examined at progressively earlier stages of development. Embryos from timed matings were dissected free of maternal tissues, observed by whole mount and genotyped (Fig. 1B, Fig. 2A). *Tcfap2c* mutants rarely survived beyond 7.5 d.p.c., but the expected ratios of *Tcfap2c*^{−/−} mutant embryos were recovered up until and including this timepoint (Table 1). By whole-mount examination, 7.5 d.p.c. *Tcfap2c*^{−/−} embryos were consistently smaller than littermates and frequently lacked organized structures such as a primitive streak (Fig. 2A, parts a-e). The extra-embryonic tissues were either underdeveloped or disorganized, and the boundary between embryonic and extra-embryonic ectoderm was often poorly defined (Fig. 2 and data not shown). On occasion, the orientation of the *Tcfap2c* mutants within the decidua was atypical (Fig. 2A, part c), a phenotype that is described in more detail later with reference to Fig. 4. The few *Tcfap2c* mutants that survived until 8.5-10.5 d.p.c. were small, often severely disorganized, and/or undergoing resorption (Table 1). One of the more organized and advanced embryos within this category is shown in Fig. 2A (panel f), a 10.5 d.p.c. *Tcfap2c*^{−/−} embryo in which a head fold is apparent, but the overall size and developmental stage is more typical of an 8.0 d.p.c. wild-type embryo. Taken together, these observations indicate that *Tcfap2c* is required

for early postimplantation survival in the mouse with the major period of lethality occurring between 7.5 and 8.5 d.p.c.

Localization of AP-2γ in pre- and peri-implantation wild-type and mutant embryos indicates that there is a maternal AP-2γ component

We next examined the expression of AP-2γ in wild-type and mutant embryos at 7.5 d.p.c. For these experiments, we chose to use riboprobes and antisera that should detect any residual maternal AP-2γ transcripts and protein, but would not react with spurious products produced from the targeted locus (see Materials and Methods). Using a ^{33}P-labeled antisense riboprobe on wild-type embryos, AP-2γ transcripts were detected in the giant cells surrounding the embryo, in the ectoplacental cone, and in the extra-embryonic ectoderm (Fig. 2B). An additional domain of expression was observed in the antimesometrial region of the maternal deciduum. The data for

**Table 1. Genotype analyses of *Tcfap2c* heterozygous intercross progeny**

	Genotype			
Stage	+/+	+/−	−/−	Total
Newborn	20	30	0	50
10.5 d.p.c.	17	53	4*	74
8.5 d.p.c.	10	17	1	28
7.5 d.p.c.	12	45	24	81
3.5 d.p.c.	12	14	6	32

DNA from yolk sacs or whole embryos was isolated and amplified by PCR as described in the Materials and Methods.
*, extremely underdeveloped and/or resorbing.
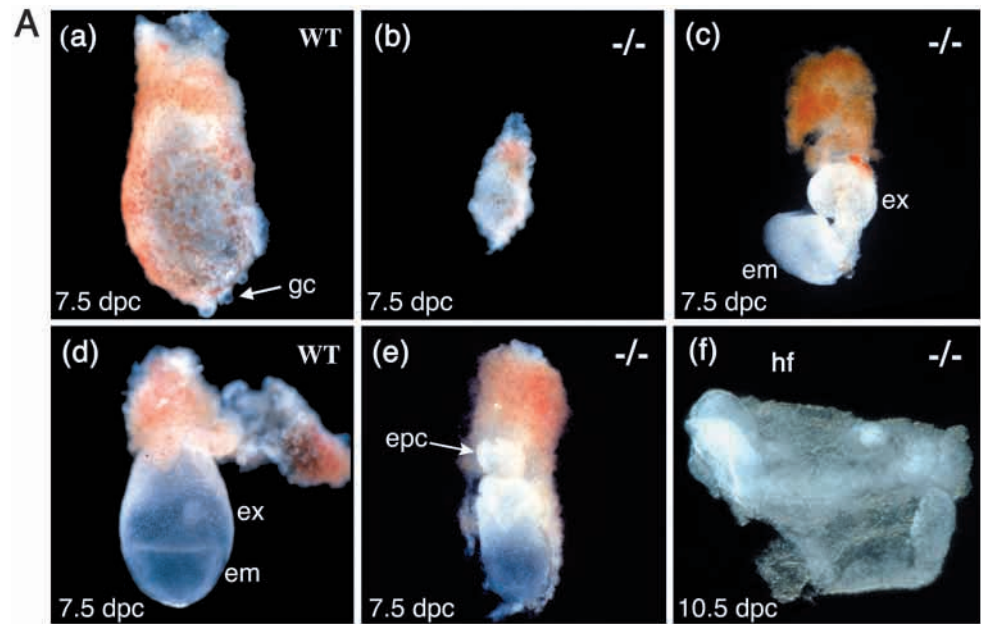
Fig. 2. Analyses of gross morphology (A) and expression of *Tcfap2c* (B) in embryos from *Tcfap2c*^{+/−} intercrosses. (A, parts a, d) Wild type late allantoic bud stage embryo recovered at 7.5 d.p.c., covered by parietal endoderm/Reichert's membrane and associated trophoblast cells (part a) and with Reichert's membrane dissected away (part d). (A, parts b,c,e,f) *Tcfap2c*^{−/−} embryos recovered at 7.5 d.p.c. (b,c,e) and 10.5 d.p.c. (A, part f). (A, part b) *Tcfap2c*^{−/−} mutant with Reichert's membrane intact. Embryos are oriented with mesometrial pole to the top and are of the same magnification. (B) RNA in situ hybridization with an AP-2γ antisense probe on sagittal sections of 7.5 d.p.c. embryos from *Tcfap2c*^{+/−} intercrosses. Mesometrial pole is towards the top. (B, parts a-d) Darkfield images. (B, parts e,f) Brightfield images. AP-2γ transcripts localize to trophoblast derivatives in wild-type conceptuses (a,c,e) but not in *Tcfap2c*^{−/−} conceptuses (b,d,f). AP-2γ transcripts are present in the antimesometrial decidua of both wild type (a,c,e) and mutant (b,d,f) conceptuses. Abbreviations: [−/−], *Tcfap2c*^{−/−}; gc, giant cell; em, embryonic region; ex, extra-embryonic region; epc, ectoplacental cone; hf, headfold; exe, extra-embryonic ectoderm; ee, embryonic ectoderm; de, deciduum.
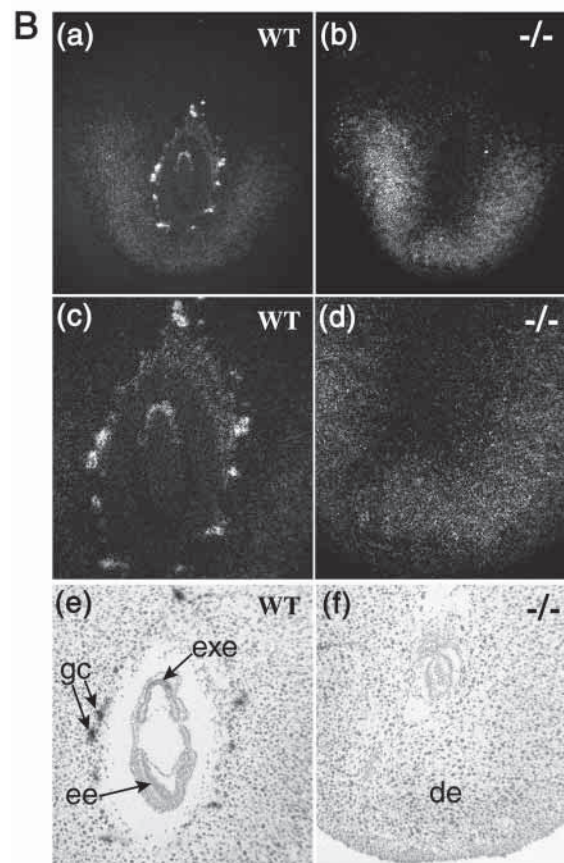
the wild-type embryos agree with previously published results in which expression of AP-2γ mRNA was studied from 6.5 d.p.c. onwards (Shi and Kellems, 1998). The AP-2 protein, detected using the specific γ96 antiserum (Turner et al., 1998), showed a similar pattern of expression in the extra-embryonic lineages and maternal tissues of wild-type embryos between 5.5-7.5 d.p.c. (Fig. 3A-D and data not shown). Sporadic staining for the AP-2γ protein was also observed in cells of

the parietal endoderm, but not in the visceral endoderm, at 7.5 d.p.c. (Fig. 3C,D). By contrast, for conceptuses that were scored as *Tcfap2c*^{−/−} both by morphology and by PCR genotyping, there was no AP-2γ expression apparent in the extra-embryonic membranes or the associated giant cells (Fig. 2B; Fig. 3E,G). The only AP-2γ RNA and protein expression observed was in the antimesometrial portion of the deciduum, reflecting the maternal origin of this tissue (Fig. 2B and data

not shown). The absence of both AP-2γ protein and transcripts in *Tcfap2c⁻/⁻* embryos at 7.5 d.p.c. confirmed that we had disrupted the *Tcfap2c* allele. In addition, our studies ruled out the possibility that maternal AP-2γ transcript or protein were present in mutant embryos at 7.5 d.p.c.

The early post-implantation phenotype of the *Tcfap2c⁻/⁻* mutants prompted us to examine the localization of AP-2γ protein at progressively earlier stages in both wild-type and mutant embryos. All morulae and blastocysts derived from *Tcfap2c⁺/⁻* matings were found to contain AP-2γ protein, and this protein was present in the nuclei of all cells of these embryos (Fig. 3H-M). PCR genotyping confirmed that *Tcfap2c⁻/⁻* embryos were present at the expected ratios within this population (data not shown) and an example of a *Tcfap2c⁻/⁻* blastocyst is shown in Fig. 3M. Because the *Tcfap2c⁻/⁻* embryos lacked a functional *Tcfap2c* gene, the observed AP-2γ immunoreactivity must represent maternally derived protein in the preimplantation embryos. As noted above, this maternal protein was exhausted by 7.5 d.p.c. in the *Tcfap2c⁻/⁻* embryos (Fig. 3E,G). Presumably, maternal protein is also depleted in wild-type embryos by this stage, and therefore zygotic transcription would account for the more restricted domain of expression in the extra-embryonic lineages at 7.5 d.p.c.

Tcfap2c⁻/⁻ mutants are defective in embryonic and extra-embryonic development

To further investigate the cause of the early postimplantation lethality, *Tcfap2c* mutants and wild-type or heterozygous littermates were examined in greater detail through histological sections at 7.5 d.p.c. (Fig. 4). Once documented, embryonic tissue was removed from slides and genotyped. Analysis of these morphological and genotypic data indicated that a range of developmental abnormalities was present in the 7.5 d.p.c. *Tcfap2c⁻/⁻* embryos (Table 2). All *Tcfap2c⁻/⁻* embryos exhibited growth retardation, disruption of the maternal-embryonic interface, and defective development of the extra-embryonic tissues. One of the major trophoblast cell types, the trophoblast giant cells, was reduced in number in the mutant embryos (Fig. 4). Whereas 50 to 60 primary giant cells could be present in wild-type conceptuses, in some *Tcfap2c⁻/⁻* embryos, as few as one to two giant cells were observed. In the majority of mutants, the extra-embryonic ectoderm was disorganized and often appeared to be either abnormally elongated or as a series of folds stacked upon one another (Fig. 4D,H, respectively; Table 2). Moreover, the exocoelomic and ectoplacental cavities usually failed to form, although the proamniotic cavity was present in the majority of *Tcfap2c⁻/⁻* mutants (Fig. 4; Table 2). The ectoplacental cone was typically
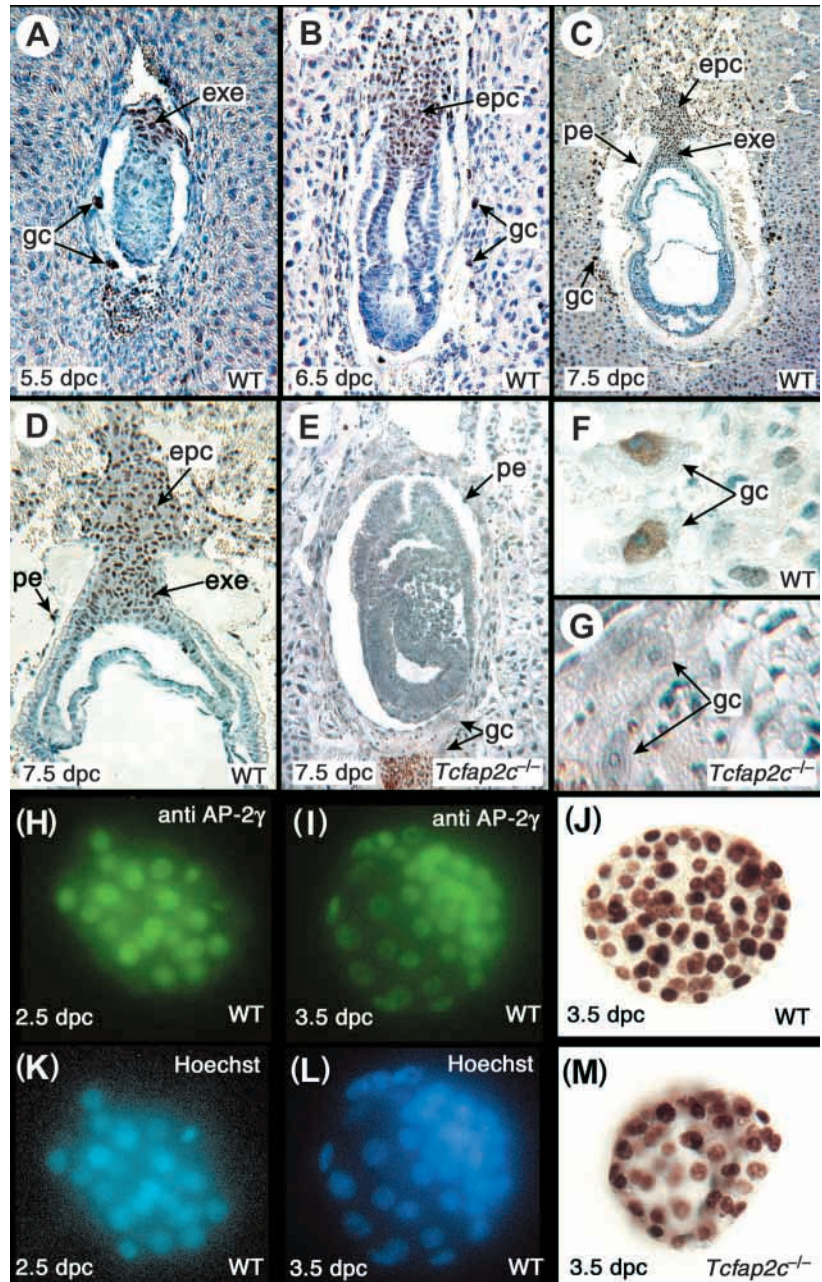
Fig. 3. AP-2γ protein localization from 2.5-7.5 d.p.c. (A-G) Sagittal sections of 5.5-7.5 d.p.c. embryos. The mesometrial pole is positioned towards the top, and the antimesometrial pole is to the bottom. AP-2γ protein is localized to wild-type extra-embryonic tissues and is present in the trophoblast giant cells (gc), extra-embryonic ectoderm (exe), ectoplacental cone (epc) and parietal endoderm (pe). No AP-2γ protein was detected in 7.5 d.p.c. *Tcfap2c⁻/⁻* mutants (E,G). (H-M) Whole-mount analysis of morulae and blastocysts. (H,I) Fluorescent and (J,M) DAB stained immunodetection of AP-2γ protein. (K,L) DNA counterstaining with Hoechst. AP-2γ protein is present in all cells of the morula (H) and in all cells of both wild-type (I,J) and *Tcfap2c⁻/⁻* (M) blastocysts.

reduced in size, compact, and not well-integrated with the surrounding maternal tissues. In most cases, there were large pools of maternal blood in the decidua adjacent to, but not continuous with, the ectoplacental cone of the *Tcfap2c⁻/⁻* conceptuses (Fig. 4). In some developmentally delayed

mutants, the ectoplacental cone was absent altogether. In other examples, the primary embryonic axis (ultimately the dorsoventral axis) was defective, as the embryo was oriented atypically with respect to the extra-embryonic membranes. For example, the ectoplacental cone was frequently positioned nearly perpendicular to the epiblast (Fig. 4G and data not shown), in contrast to the slightly angled alignment observed in wild-type conceptuses (Fig. 4A,C; Table 2). This atypical orientation was also observed in whole-mount preparations of some embryos, evident as abnormal bending of the embryo accompanied by malformation of Reichert's membrane (Fig. 2A, panel c). Although the primary embryonic axis was normal in approximately 30% of mutant embryos, in many instances the extent of the defect was difficult to determine due to general disorganization.

A distinct class of orientation defects, affecting the position of the embryo with respect to the antimesometrial-mesometrial axis of the deciduum, was observed in a small percentage (13%) of mutant conceptuses. Normally a sagittal section of the embryo is obtained when the deciduum is sectioned longitudinally (Fig. 4A,C). Instead, when sectioning mutant decidua in the same plane, in some instances a cross-section of the embryo was obtained (Fig. 4B,F). In the most extreme

Table 2. Morphological analysis of *Tcfap2c*$^{-/-}$ mutant embryos at 7.5 dpc

Morphology	Number of embryos/total number (%)
Abnormal	23/23 (100)
Abnormal egg cylinder stage (TS 8-9)	13/23 (57)
Abnormal primitive streak stage (TS 10)	10/23 (43)
Proamniotic/amniotic cavity formed	20/23 (87)
Exocoelomic and/or ectoplacental cavity formed	6/23 (26)
Extra-embryonic structures disorganized or underdeveloped	23/23 (100)
Ectoplacental cone abnormal or underdeveloped	20/23 (87)
Reichert's membrane and distal (parietal) endoderm defective	10/23 (43)
Extra-embryonic and embryonic structure highly disorganized	4/23 (17)
Embryo misoriented with respect to AM-M axis	3/23 (13)

Tcfap2c$^{-/-}$ mutant embryos display a range of developmental abnormalities at 7.5 d.p.c. Embryos from *Tcfap2c*$^{+/-}$ intercrosses were analyzed in detail by histological examination of serial sections. Afterwards, embryonic tissues were scraped from slides and genotyped. The morphology of 23 *Tcfap2c*$^{-/-}$ embryos is summarized here.

TS, Theiler Stage; AM-M, antimesometrial-mesometrial.
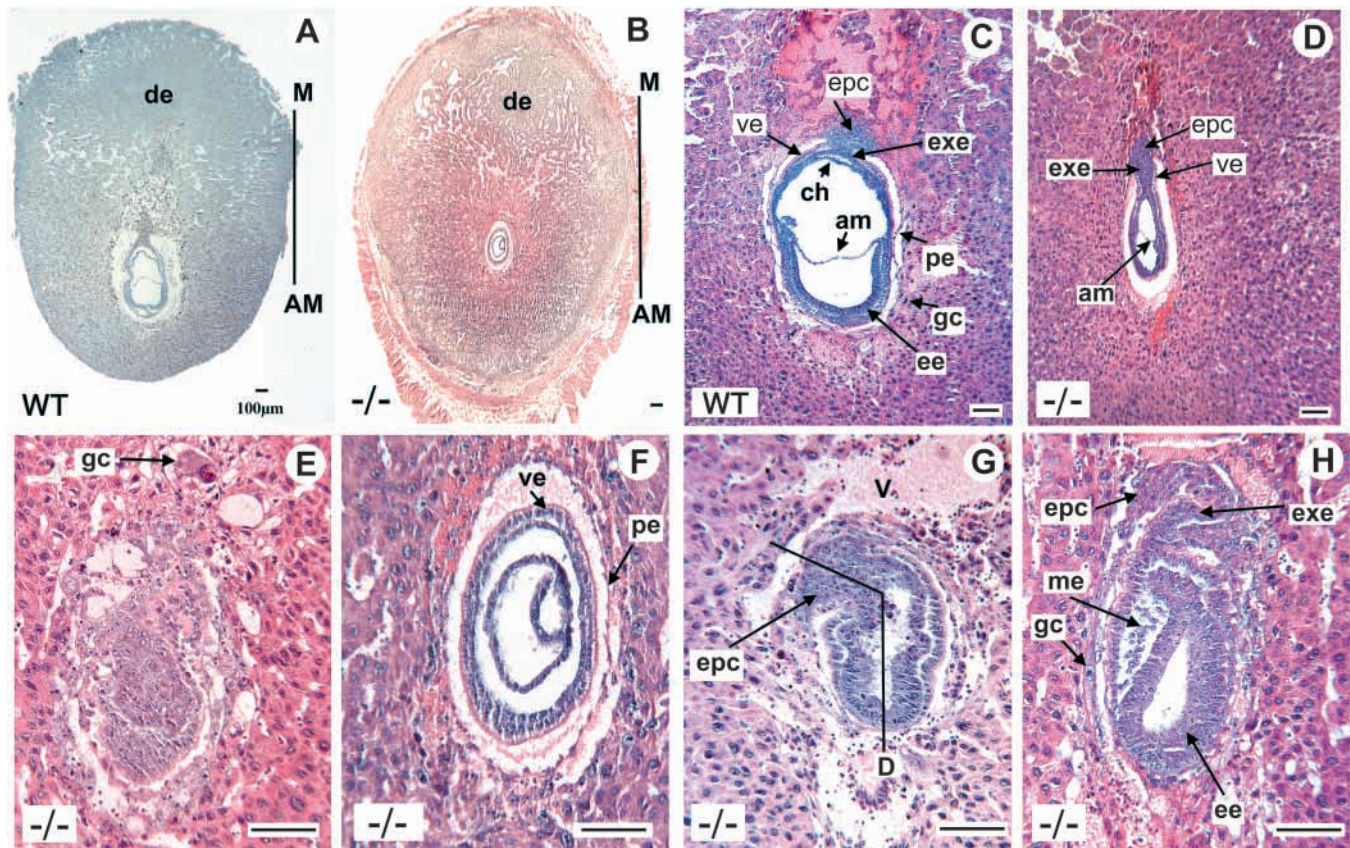
Fig. 4. Histological sections of normal (A,C) and *Tcfap2c*$^{-/-}$ (B,D-H) embryos at 7.5 d.p.c. Conceptuses were sectioned longitudinally along the antimesometrial-mesometrial (AM-M) axis of the deciduum, resulting in sagittal sections of the embryos in A,C-E,G,H. The *Tcfap2c*$^{-/-}$ mutant shown in B,F is misoriented with respect to the AM-M axis, resulting in a cross-section of the embryo. The mesometrial pole is located towards the top. Individual embryos are described in the text. The line in A,B represents the AM-M axis. The line in G represents the abnormal angle of the dorsoventral axis. Scale bars: 100 μm. Abbreviations: WT, wild type; ch, chorion; am, amnion; epc, ectoplacental cone; exe, extra-embryonic ectoderm; ee, embryonic ectoderm; gc, giant cell; pe, parietal endoderm; ve, visceral endoderm; de, deciduum; me, mesoderm; AM, antimesometrial pole; M, mesometrial pole; D, dorsal; V, ventral.

example, the embryo was rotated 180° with respect to its expected positioning within the deciduum, such that the embryo proper was located at the mesometrial pole and the ectoplacental cone was antimesometrial (Table 2; data not shown).

Occasionally, parietal endoderm/Reichert's membrane was observed to intervene abnormally between the extra-embryonic ectoderm and ectoplacental cone of mutants (Fig. 2A, part e; Table 2). Histological sections from this class of mutant indicated that Reichert's membrane appeared to encapsulate the embryo, sometimes as an abnormally thickened and continuous layer (Fig. 3e and data not shown; Table 2). By contrast, visceral endoderm was present and appeared normal in the majority of mutant embryos. The allantoic bud, while sometimes present in wild-type embryos at 7.5 d.p.c., was rarely observed in the *Tcfap2c* mutant embryos.

In addition to the extra-embryonic defects, we also noted abnormalities in the embryo proper. In particular, the mutant epiblast was typically smaller and retarded in development in comparison with littermates (Fig. 4). In some cases, mesoderm formed but accumulated as a large mass of loosely associated cells (Fig. 3E; Fig. 4H). Several of the mutants analyzed lacked mesoderm formation, which meant they failed to undergo gastrulation. Between 15 and 20% of mutants exhibited a more pronounced degree of disorganization, such that separate embryonic and extra-embryonic compartments were not identifiable (Fig. 4E; Table 2).

Molecular marker analysis of *Tcfap2c* mutant embryos

To investigate the defects in the embryonic organization of the *Tcfap2c* mutants, we examined the expression of pertinent marker genes at 7.5 d.p.c. by RNA in situ hybridization. To determine whether mesoderm formation is initiated in the *Tcfap2c* mutants, we examined the expression of the early mesoderm lineage marker *T* (Brachyury) (Wilkinson et al., 1990). In mutants that exhibited *T* expression, transcripts were either confined to a small posterior region of the proximal epiblast or localized in a pattern comparable with that of wild-type littermates, in which the nascent mesoderm was marked along the primitive streak (Fig. 5A,B). Wild-type embryos expressed the later mesoderm marker *Hnf3b* in the node at 7.5 d.p.c., while some *Tcfap2c* mutants expressed *Hnf3b* in a location corresponding to the anterior primitive streak, even in the absence of a distinct primitive streak (Fig. 5C,D) (Sasaki and Hogan, 1993). Otx2, a homeodomain-containing transcription factor that is initially expressed in the anterior visceral endoderm and epiblast and becomes restricted to the anterior epiblast by the end of gastrulation, was also expressed in an anterior pattern similar to that of wild-type embryos (Fig. 5E,F) (Simeone et al., 1993). Thus, mesoderm induction can occur and anteroposterior markers are expressed in appropriate domains, despite the morphological abnormalities in the mutant epiblast. These findings are consistent with the ability of some mutant embryos to develop to the headfold stage (Fig. 2A, part f).

Blastocyst outgrowth assays – AP-2γ is required for proper blastocyst outgrowth in vitro

We next examined the development of the embryonic and
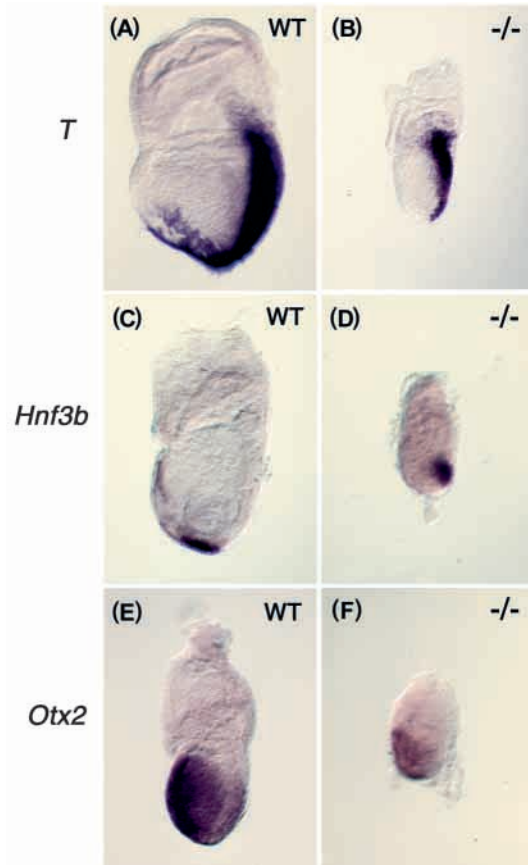
Fig. 5. In situ hybridization analysis of *T* (A,B), *Hnf3b* (C,D) and *Otx2* (E,F) expression in 7.5 d.p.c. wild-type (A,C,E) and littermate *Tcfap2c*$^{-/-}$ embryos (B,D,F). Embryos are shown at the same magnification. Mesometrial pole is towards the top, anterior is towards the left and posterior is to the right.

extra-embryonic compartments in *Tcfap2c*$^{-/-}$ blastocysts in the absence of the uterine environment through in vitro outgrowth assays. When *Tcfap2c*$^{-/-}$ blastocysts were grown on primary mouse embryo fibroblast feeder cells in the presence of leukemia inhibitory factor (LIF), the inner cell mass (ICM) component was able to generate embryonic stem cell lines with normal morphology and growth characteristics (data not shown). Blastocysts from *Tcfap2c* heterozygote matings were also cultured in the absence of a feeder cell layer and without LIF for 7 days, after which they were documented and genotyped. Under these conditions, in outgrowths from wild-type or heterozygous blastocysts, the trophectoderm differentiated into cells with epithelial-like morphology, as well as forming multiple trophoblast giant cells (Fig. 6A). By contrast, there was very little trophectoderm outgrowth from the *Tcfap2c*$^{-/-}$ mutant blastocysts, and giant cells were observed only in small numbers (Fig. 6B-D). As in wild-type outgrowths, a range in the quality of growth was observed (compare Fig. 6C with 6D); however, the average number of giant cells present in mutant outgrowths was 8 (*n*=7), compared with an average of 32 in wild-type outgrowths (*n*=11). With respect to the ICM, both wild-type and mutant embryos could form raised colonies as expected, but frequently those derived from the

Tcfap2c^{–/–} blastocysts were smaller and less coherent. The difference in ICM outgrowth under the two culture conditions (i.e. with or without feeders and LIF) may reflect the defective outgrowth of the trophoblast cells in the latter experimental methodology.

Analysis of molecular markers of extra-embryonic tissues

As the blastocyst outgrowth studies suggested that the mechanism of action of *Tcfap2c* lies in the extra-embryonic compartment, we wished to examine the expression of genes in the mutant extra-embryonic tissues during the early postimplantation period. Despite fewer giant cells in the mutants, placental lactogen 1 (*Pl1*), adenosine deaminase (*Ada*) and the basic helix-loop-helix transcription factor gene *Hand1* were expressed normally in the remaining trophoblast cells surrounding the gestation site (Fig. 7A and data not shown) (Colosi et al., 1987; Cross et al., 1995; Cserjesi et al., 1995; Knudsen et al., 1988). The number of *Ada*-expressing giant cells in the *Tcfap2c* mutants was greatly decreased in comparison with wild-type or heterozygous littermates, reflecting the overall decrease in the giant cell population (Fig. 7A). Transcripts derived from the achaete-scute homolog *Mash2*, which is normally expressed in the ectoplacental cone and the chorion, were also observed in the ectoplacental cone of mutants in which this tissue was present, although at a reduced level (Fig. 7A) (Guillemot et al., 1994). Similarly, transcripts of both the fibroblast growth factor receptor *Fgfr2*, which marks the extra-embryonic ectoderm of wild-type embryos, and *Bmp4*, which is normally located in the extra-embryonic ectoderm and mesoderm during gastrulation, were expressed in the mutants even though they lacked a normal morphological organization (Fig. 7B, parts b,d) (Lawson et al., 1999; Orr-Urtreger et al., 1991). By contrast, whereas transcripts for the caudal-related homeobox gene *Cdx2* (Beck et al., 1995) were readily detected in wild-type embryos in the extra-embryonic ectoderm and mesoderm (Fig. 7B, part e), they were either significantly reduced (Fig. 7B, parts f,g) or undetectable (Fig. 7B, part h) in all of the mutants examined by whole-mount analysis. Furthermore, in some mutants, very little or no expression was detectable from a second gene expressed in the extra-embryonic ectoderm, the T-box gene eomesodermin (*Eomes*) (Fig. 7B, part k) (Ciruna and Rossant, 1999; Russ et al., 2000). In wild-type embryos, *Eomes* is expressed from 5.0 d.p.c. onwards in the extra-embryonic ectoderm and in the primitive streak and posterior epiblast during the early- to late-streak stages (Fig. 7B, part i) (Ciruna and Rossant, 1999). In *Tcfap2c*^{–/–} embryos, *Eomes* transcripts were either absent (Fig. 7B, part k) or observed only in one domain of expression (Fig. 7B, parts j,l). This single domain presumably corresponds to the primitive streak; however, an alternative possibility is that it represents two domains that are indistinguishable because of disorganization. These findings
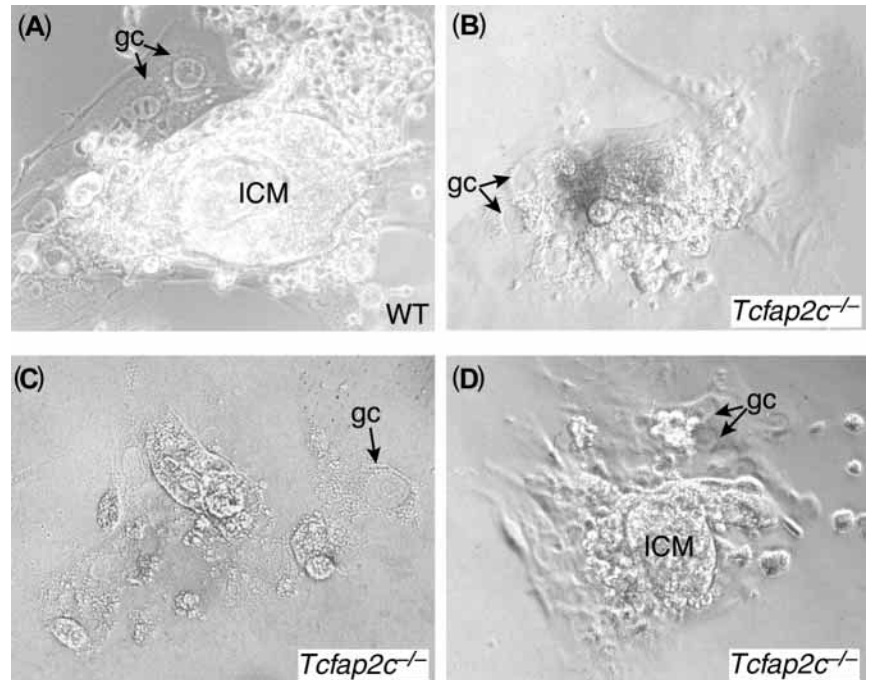
Fig. 6. In vitro outgrowths after 7 days in culture of wild-type (A) and *Tcfap2c*^{–/–} (B-D) embryos collected at 3.5 d.p.c. from *Tcfap2c*^{+/–} intercrosses. Outgrowths are shown at the same magnification. ICM, inner cell mass derivative; gc, giant cell; WT, wild type.

either show that there is a direct correlation between the presence of the transcription factor AP-2γ and extra-embryonic expression of both the *Cdx2* and *Eomes* genes, or that the tissues in which these two genes are expressed are missing or abnormal in the mutants.
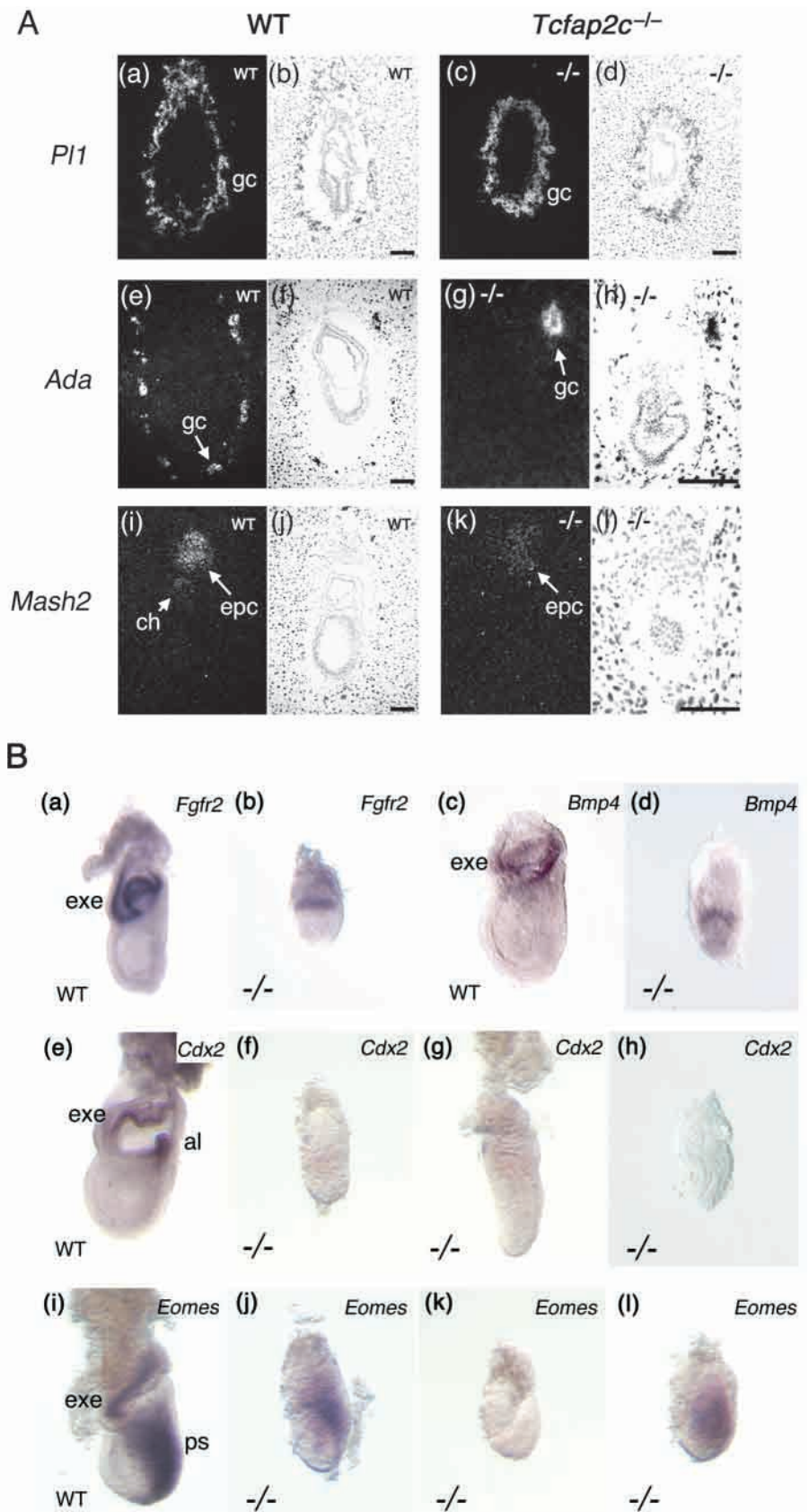
AP-2γ is required in the extra-embryonic compartment

The abnormalities in the extra-embryonic tissues of the 7.5 d.p.c. mutants, the expression of *Tcfap2c* in the trophoblast derivatives and parietal endoderm, and the abnormal outgrowth of the trophectoderm in vitro led us to hypothesize that AP-2γ plays a crucial role in the extra-embryonic tissues of the mouse conceptus. To test this hypothesis directly, we generated several classes of mouse chimeras using different techniques, enabling us to distinguish between requirements in the extra-embryonic versus embryonic compartment. The first set of experiments assessed the importance of *Tcfap2c* in the embryo proper and relied upon the generation of *Tcfap2c*^{–/–} ES cells that could be distinguished from wild-type cells. For this purpose, we generated three *Tcfap2c*^{–/–} ES cell lines tagged with both the wild-type *agouti* gene and the ROSA β-geo 26 (ROSA26) insertion (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). The ROSA26 gene-trap allele and *agouti* coat color marker allowed us to follow the contribution of the tagged ES cells in the chimeras. When any of the *Tcfap2c*^{–/–} ES cell lines were injected into wild-type C57Bl/6J blastocysts, morphologically normal chimeras were obtained. This result was seen even when there was a large contribution of the *Tcfap2c*^{–/–} cells in an individual chimera as judged by either the *agouti* coat color (Fig. 8A), or by the extent of β-galactosidase-positive cells within the organs and tissues of the

Fig. 7. RNA in situ hybridization analyses of trophoblast markers in 7.5 d.p.c. embryos from *Tcfap2c+/−* intercrosses. (A) Detection of transcripts for *Pl1* (A, parts a-d), *Ada* (A, parts e-h) and *Mash2* (A, parts i-l) in longitudinal sections of wild-type (A, parts a,e,i) and *Tcfap2c* mutant (A, parts c,g,k) conceptuses. (A, parts b,d,f,h,j,l) The equivalent brightfield images. The mesometrial pole is oriented towards the top, and the antimesometrial pole is towards the bottom. (B) Whole-mount detection of transcripts for *Fgfr2* (B, parts a,b), *Bmp4* (B, parts c,d), *Cdx2* (B, parts e-h) and *Eomes* (B, parts i-l) in wild type (B, parts a,c,e,i) and *Tcfap2c−/−* (B, parts b,d,f-h,j-l) embryos. Mesometrial pole is towards the top. Embryos are at the same magnification. Abbreviations: WT, wild type; −/−, *Tcfap2c−/−*; gc, giant cell; epc, ectoplacental cone; ch, chorion; exe, extra-embryonic ectoderm; al, allantoic bud; ps, primitive streak. Scale bars: 100 μm.

mouse (data not shown). In control experiments, in which *Tcfap2c+/−* ES cells were injected into mouse blastocysts, we obtained comparable results (data not shown).

Many chimeras with a high percentage of *Tcfap2c−/−* cells survived for 1 year until they were sacrificed, suggesting that AP-2γ is not essential for later embryonic development or adult viability. In this class of chimera, cells of the wild-type host blastocyst can contribute to all lineages of the embryo and the extra-embryonic membranes, while the ES cells can contribute to the embryo proper and only a subset of the extra-embryonic tissues, primarily the extra-embryonic mesoderm (Beddington and Robertson, 1989). We thus hypothesized that the wild-type contribution to the extra-embryonic tissues rescued the early embryonic lethality in the *Tcfap2c* mutant. However, because the embryo proper contained a mixture of wild-type and *Tcfap2c* mutant cells, it was not clear whether the rescue was due entirely to the wild type function of AP-2γ in the extra-embryonic membranes or to the small contribution of wild-type cells in the embryo proper. To answer this question, we used the tetraploid-diploid chimera technique, in which wild-type tetraploid cells at the 4- to 8-cell stage were aggregated with *Tcfap2c−/−* ES cells. Because the tetraploid cells contribute almost exclusively to the extra-embryonic membranes, it is possible to generate an embryo that is entirely ES cell-derived using this technique (Nagy et al., 1990). Using this approach, we determined that *Tcfap2c−/−* ES cell-derived embryos collected at mid- or late gestation were morphologically normal (Fig. 8B,C, and data not shown). Contribution of the
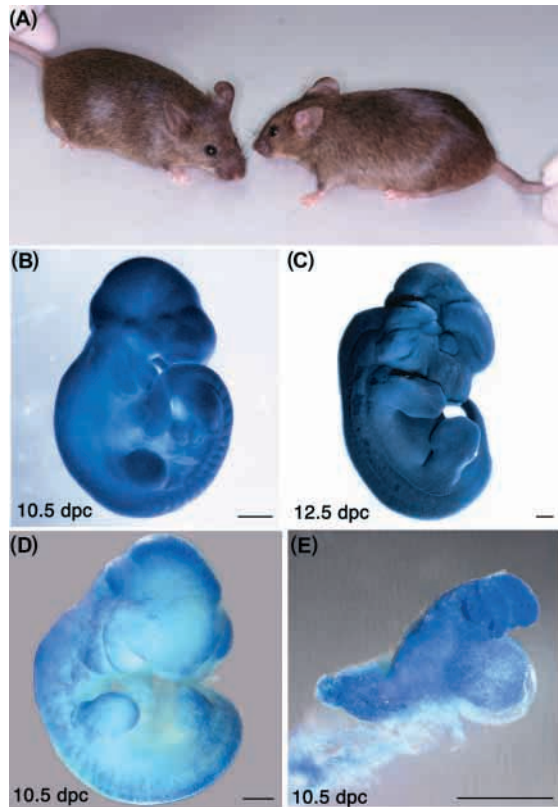
Fig. 8. Chimeras derived from ROSA26-tagged *Tcfap2c*⁻/⁻ ES cells (A-C) and from injection of ROSA26-tagged wild-type ES cells into blastocysts from *Tcfap2c*⁺/⁻ intercrosses (D,E). (A) Adult chimeras generated by injection of *Tcfap2c*⁻/⁻ ES cells into wild-type blastocysts. (B,C) X-gal-stained chimeras at 10.5 d.p.c. and 12.5 d.p.c., respectively, generated by aggregation of ROSA26-tagged *Tcfap2c*⁻/⁻ ES cells with wild-type tetraploid embryos. (D,E) X-gal-stained littermate chimeras at 10.5 d.p.c. generated by injection of wild-type ROSA26-tagged ES cells into an *Tcfap2c*⁺/⁻ blastocyst (D) and an *Tcfap2c*⁻/⁻ blastocyst (E). Scale bars: 600 μm in B-E.

tagged ES cells to all tissues of the embryo was confirmed by staining for β-galactosidase activity in whole-mount and in serial sections (Fig. 8 and data not shown). Note that, as expected, trophoblast cells and trophoblast derivatives in the placenta lacked β-galactosidase expression, indicating that these components were derived from wild-type cells (data not shown). Taken together, these results demonstrate that there is an essential requirement for AP-2γ in the extra-embryonic membranes and indicate that the embryo itself can develop normally in the absence of embryonic *Tcfap2c* expression.

The second set of experiments addressed whether or not the embryonic retardation and disorganization observed in the *Tcfap2c*⁻/⁻ mutants was a secondary consequence of the defects in the extra-embryonic compartment. We thus carried out a converse chimera analysis, in which wild-type, ROSA26-marked ES cells were injected into blastocysts from *Tcfap2c*⁺/⁻ matings, in order to assess the developmental potential of the wild-type ES cells in the context of an *Tcfap2c* mutant host environment. We collected chimeras up to 10.5 d.p.c., as this was the latest timepoint to which the *Tcfap2c*⁻/⁻ embryos had persisted and would allow us to address whether or not early placentation had been rescued by the presence of wild-type

cells. For each chimera, we retrospectively genotyped the host blastocyst by PCR analysis of the DNA from yolk-sac endoderm, which contains little if any contribution from ES cells (Beddington and Robertson, 1989). Chimeras derived from either wild-type or heterozygous host blastocysts were morphologically wild type (Fig. 8D). By contrast, chimeras with *Tcfap2c* mutant extra-embryonic tissues showed variable phenotypes that were morphologically similar to *Tcfap2c* mutants recovered from natural matings, even with extensive contribution from wild-type ES cells, as judged by staining for β-galactosidase activity (Fig. 8E and data not shown). Some of these chimeras were very small and lacked any obvious organization, although one had developed a head structure with an open neural tube (Fig. 8E). Nevertheless, this embryo was considerably smaller than wild-type littermates, and the head and trunk were underdeveloped and malformed for this stage of development, resembling the low percentage of *Tcfap2c*⁻/⁻ mice that survive until 10.5 d.p.c. (Fig. 2A, part f). In all cases, no evidence of a placenta was associated with chimeras derived from *Tcfap2c*⁻/⁻ blastocysts. Together, these findings demonstrate that there is an essential requirement for *Tcfap2c* within the extra-embryonic membranes for normal development of the mouse embryo beyond the early postimplantation period.

DISCUSSION

The appropriate development of the trophectodermal lineages is crucial for implantation and placental formation in mammals. Failure of extra-embryonic membrane function probably contributes to the high percentage of pregnancy loss that occurs during the peri-implantation period (Copp, 1995). We have now generated mice deficient in *Tcfap2c*, the third member of the AP-2 family to be characterized, and we have shown that it is required soon after the time of implantation. Our results indicate that the appropriate interactions between the embryo, the extra-embryonic tissues, and the maternal environment have not been established and maintained in these mice, which probably leads to the early embryonic lethality. The fact that AP-2γ is specifically required early in the development of the extra-embryonic tissues is consistent with its expression pattern during the pre- and peri-implantation period, which is unique among the AP-2 family members characterized to date. The AP-2γ protein is present during the preimplantation period in all cells of the morula, preceding the specification of the trophectoderm lineage that occurs with the formation of the blastocyst. Subsequently, *Tcfap2c* is expressed in both the ICM and trophectoderm of the blastocyst, before it becomes restricted to the extra-embryonic lineages by 5.5 d.p.c. Taken together with the knockout phenotype, these findings indicate that *Tcfap2c* may be involved in the establishment and/or maintenance of the extra-embryonic lineages. This hypothesis was confirmed through the generation and analysis of chimeric mice. Two experimental strategies were used for these studies: the injection of tagged *Tcfap2c*-null ES cells into wild-type blastocysts, and the aggregation of these ES cells with wild-type tetraploid embryos. In both cases, the majority of the extra-embryonic membranes of the conceptus are derived from wild-type cells, while *Tcfap2c*-null cells are largely confined to the embryo proper. Under both circumstances, development of the embryo

proper was rescued to term by the presence of wild-type cells in the extra-embryonic lineages, demonstrating that there is an absolute requirement for *Tcfap2c* in these latter tissues. Conversely, when wild-type ES cells were injected into blastocysts from *Tcfap2c*^{+/−} intercrosses, embryos derived from *Tcfap2c*^{−/−} hosts were not rescued to term, further confirming the indispensability of *Tcfap2c* in the extra-embryonic tissues. These extra-embryonic lineages include the derivatives of both the primitive endoderm (visceral and parietal endoderm) and the trophectoderm [for a review of mouse extra-embryonic lineages, see Rossant (Rossant, 1995)]. We suggest that the primary role of *Tcfap2c* lies in the trophoblast lineage, owing to the high level of AP-2γ expression in these cells, and the specific defects within these tissues in the absence of *Tcfap2c* both in vivo and in vitro. It is less likely that AP-2γ is required in either visceral or parietal endoderm, as RNA in situ hybridization studies (Shi and Kellems, 1998) (this paper) have failed to detect AP-2γ transcripts in these tissues. We note, however, that AP-2γ protein may be present sporadically in cells of the parietal endoderm, so we cannot absolutely exclude a role for AP-2γ in this tissue.

The process of implantation begins with the attachment and integration of the embryonic trophoblast cells into the maternal endometrium (reviewed by Cross et al., 1994; Rinkenberger et al., 1997). During the early postimplantation period, access to the maternal circulation is vital to the growth and survival of the embryo prior to the formation of the placenta. This connection is facilitated by the trophoblast cells, Reichert's membrane and the parietal endoderm, which form a primitive diffusion barrier to allow nutrients and gases to nourish the embryo without direct exposure to maternal blood. The absence of AP-2γ impacts upon the development of all of these vital extra-embryonic structures during this critical period. *Tcfap2c* joins a small group of molecules that are essential for development during the early postimplantation period and have been proven to act within the extra-embryonic membranes (for reviews, see Copp, 1995; Kupriyanov and Baribault, 1998; Rinkenberger et al., 1997). Several of these are likely to act within the visceral endoderm, including Smad4, HNF4, GATA6 and huntingtin (Chen et al., 1994; Dragatsis et al., 1998; Duncan et al., 1997; Koutsourakis et al., 1999; Sirard et al., 1998). Others, such as HAND1, Ets2 and merlin, appear to affect specific aspects of trophoblast development, such as the differentiation of giant cells or the formation of extra-embryonic ectoderm (McClatchey et al., 1997; Riley et al., 1998; Yamamoto et al., 1998). *Tcfap2c* would fall into this latter category; however, the particular combination of defects in all trophoblast lineages, the changes in trophoblastic gene expression, and the defective parietal endoderm makes the *Tcfap2c*^{−/−} mutants distinctive.

A striking aspect of the *Tcfap2c* knockout mice is the paucity of giant cells present either in utero or in blastocyst outgrowths. Despite the smaller population of giant cells in the *Tcfap2c* mutants compared with wild-type conceptuses, the mutant embryos are nonetheless able to attach to the uterine lining, induce a decidual reaction and implant. The *Tcfap2c* mutant trophoblast cells still produce transcripts for placental lactogen 1, a hormone that is characteristically secreted by giant cells. Moreover, expression of the putative AP-2 target gene *Ada* can also be detected in the *Tcfap2c*-null primary giant cells. Previous studies have shown that an *Ada* transgene was dependent on the presence of an AP-2 binding site for placental expression and implicated AP-2γ in this regulation (Shi and Kellems, 1998). We speculate that other AP-2 family members could compensate for the loss of zygotic AP-2γ and thus maintain *Ada* expression. Alternatively, it is possible that the regulation of the endogenous *Ada* gene in its normal chromosomal context is more complex than that of the AP-2-dependent transgene. Although the *Tcfap2c*^{−/−} primary giant cells express expected markers, there may be subtle differences in the properties of the mural trophectoderm that affect implantation among some mutants, as 10-15% of mutant embryos are misoriented in the decidua. In this regard, the atypical positioning of the *Tcfap2c* mutants with respect to the AM-M axis is reminiscent of the phenotype observed in *Fgfr2*^{−/−} embryos, which are positioned randomly as implanting blastocysts within the uterine crypt (Arman et al., 1998).

Following implantation, the trophectoderm overlying the ICM continues to proliferate and gives rise to the extra-embryonic ectoderm. Precursors in the extra-embryonic ectoderm will populate the ectoplacental cone, which expands into the deciduum to establish intimate connections with the maternal vasculature. In the majority of *Tcfap2c* mutants, the extra-embryonic ectoderm is either underdeveloped or disorganized. In addition, the ectoplacental cone is usually small and compact, and it does not appear to extend normally into the maternal tissues. Often, the ectoplacental cone is positioned inappropriately such that it alters the linearity of the primary axis, or the future dorsoventral axis. The abnormal phenotype in the extra-embryonic ectoderm may be mechanically related to the defects in the ectoplacental cone. For example, if the ectoplacental cone fails to migrate, the extra-embryonic ectoderm may continue to grow but be confined from elongating and collapse upon itself, resulting in the folded sheet appearance. In some cases, Reichert's membrane may serve to further obstruct the expansion of the ectoderm, as when the membrane is removed during dissection, the ectoderm rapidly expands as though under pressure (O. L., unpublished).

Studies in cell culture and in mouse chimeras provide evidence that a stem cell population exists in the extra-embryonic ectoderm, giving rise to precursor cells in the ectoplacental cone that form secondary giant cells (Rossant et al., 1978; Tanaka et al., 1998). These stem cells express the FGFR2 receptor, and proliferate partly in response to an FGF4 signal originating in the epiblast. We find that although *Fgfr2* expression is present in the *Tcfap2c*-null extra-embryonic compartment, the putative FGF-responsive effector molecules Cdx2 and Eomes are downregulated. The uncoupling of signaling between FGFR2 and these effectors by the absence of AP-2γ could reflect either the loss or reduction of specific cells, or that AP-2γ has a direct effect on the expression of these transcription factors. Notwithstanding, lowered expression of *Cdx2* and *Eomes* would be expected to alter the functioning of the stem cell population and limit the number of cells contributing to the extra-embryonic ectoderm and the ectoplacental cone, a situation that typifies the *Tcfap2c*-mutant phenotype. A smaller pool of stem cells in the mutant would be consistent with the paucity of secondary giant cells within or around the ectoplacental cone that also occurs in the *Tcfap2c* mutants.

We have shown that AP-2γ is present as a maternally derived protein in preimplantation embryos and have also determined that maternal AP-2γ transcripts are present in unfertilized oocytes (Q. W., unpublished). The extent to which maternal mRNAs and proteins are involved in the development of the early mouse embryo is unknown. However, it has been known for some time that maternal transcripts can be translated into proteins that persist beyond the time of initiation of zygotic transcription (West and Flockhart, 1989). More recently, two maternal effect genes have been identified, *Hsf1* and *Mater*, whose products are required for mouse development beyond the zygote and the two-cell stage, respectively (Christians et al., 2000; Tong et al., 2000). Rescue by maternal proteins or transcripts has been proposed in the study of several mutants that show later defects than expected based on expression patterns (Haegel et al., 1995; Larue et al., 1994; Meagher and Braun, 2001; Reithmacher et al., 1995; Riley et al., 1998; Shen-Li et al., 2000). Similarly, it is possible that maternal contribution in the *Tcfap2c* mutant mouse masks an earlier requirement for AP-2γ during pre- or peri-implantation development. Indeed, the lethality in the *Tcfap2c*⁻/⁻ embryos occurs soon after maternally derived protein is exhausted, when zygotic expression would normally be expected to replenish AP-2γ protein levels. Based on these findings, we predict that maternal AP-2γ mRNA and/or protein will have an important role in early embryogenesis. We also hypothesize that maternal stores of AP-2γ could account for the variability we observe in the *Tcfap2c*-mutant phenotype during the postimplantation period. Specifically, the phenotype might vary depending upon the quantity of AP-2γ within a particular fertilized oocyte, and its rate and extent of depletion prior to implantation. An alternative possibility is that phenotypic variability is due to the outbred genetic background of the *Tcfap2c* mutant mice.

The early lethality of *Tcfap2c* mutants precluded a direct assessment of whether *Tcfap2c* played a role in later embryonic or extra-embryonic development. Through the generation of chimeras by blastocyst injection, we found that *Tcfap2c*⁻/⁻ ES cells could widely contribute to many tissues of morphologically normal adults, suggesting that it is not required for later embryonic or adult viability. Although *Tcfap2c* does not appear to have a major unique function in the embryo proper, we predict that there will be a continuing requirement for this gene in the development of the extra-embryonic membranes, particularly within the chorioallantoic placenta. The unique and abundant expression of AP-2γ in the trophoblast derivatives throughout placental development and its ability to regulate genes important in later placental function lend support to this idea. The extra-embryonic tissues are affected early in the mutant, before the formation of the chorioallantoic placenta, which begins at 9 d.p.c. with the fusion of the allantois to the chorion. Future targeted mutagenesis studies will be required to test the later requirement of *Tcfap2c* in the mature placenta. The fact that *Tcfap2c* plays no role individually in the embryo proper highlights the divergent functions among the members of the AP-2 transcription factor family. The disruption of either the *Tcfap2a* or *Tcfap2b* gene results in perinatal lethality in the mouse embryo; while *Tcfap2b* has a primary role in kidney morphogenesis, *Tcfap2a* affects multiple developmental programs, including formation of the neural tube, eye, face,

forelimbs, body-wall and cardiovascular system (Brewer et al., 2002; Moser et al., 1997a; Nottoli et al., 1998; Schorle et al., 1996; Zhang et al., 1996). The divergent developmental events influenced by the individual AP-2 family members contrasts with the observation that they bind to the same consensus sequence to activate transcription. Moreover, these transcription factors share overlapping patterns of gene expression during embryogenesis, including within the trophoblast lineages from 8 d.p.c. onwards. Therefore, it is possible that there are also redundant roles for the AP-2 gene family in the regulation of embryonic and extra-embryonic development that remain to be uncovered.

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