Loss of the extraembryonic ectoderm in *Elf5* mutants leads to defects in embryonic patterning

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Summary

The extraembryonic ectoderm (ExE) is essential for mammalian placental formation and survival of the embryo in utero. We have obtained a mouse model lacking the ExE, by targeted deletion of the transcription factor *Elf5*. Although *Elf5* mutant embryos implant and form an ectoplacental cone, no trophoblast stem (TS) cells can be derived, indicating that the absence of ExE is a result of the lack of TS cell maintenance. Embryos without ExE tissue are able to form the anterior visceral endoderm but fail to undergo gastrulation, demonstrating an essential role for the ExE in embryonic patterning during a defined window of development.

Key words: Elf5, Extraembryonic ectoderm, Trophoblast stem cells, AVE, Mesoderm

Introduction

Two of the earliest lineages that can be distinguished during mammalian embryogenesis are the inner cell mass (ICM) and the trophectoderm cells of the blastocyst stage (Rossant et al., 2003). Whereas the ICM segregates into epiblast and primitive endoderm, and gives rise to embryonic as well as extraembryonic tissues, the trophectoderm cells will contribute solely to extraembryonic lineages. In particular, the polar trophectodermal (pTE) cells, which overlie the ICM, will form the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC). In the mouse, the ExE makes up the proximal half of the egg cylinder after implantation.

The proliferative potential of the pTE and ExE is dependent on a population of trophoblast stem (TS) cells that can be isolated from these tissues at blastocyst to gastrula stages (Tanaka et al., 1998; Uy et al., 2002). FGF signalling from the ICM, and subsequently from the epiblast, is required for maintaining these TS cells. Removal of Fgf4 in TS cell cultures leads to the differentiation of TS cells into giant cells and the loss of early ExE-specific markers, such as *Eomes* and *Cdx2* (Tanaka et al., 1998). *Eomes* and *Cdx2* appear to be crucial to the formation and maintenance of TS cells within the pTE as no TS cells can be derived from null mutants of *Eomes* (Russ et al., 2000) or *Cdx2* (Rossant et al., 2003), in accordance with the in vivo trophoblast defects in these embryos. The EPC, which overlies the ExE, is devoid of TS cells (Uy et al., 2002). It contains differentiated trophectodermal cells that are thought to form the spongiotrophoblast (Cross et al., 2003).

Towards the end of gastrulation, the ExE forms a bilayer with extraembryonic mesoderm and becomes separated from the epiblast by the exocoelomic cavity (Kaufman, 1995). This bilayer is termed the chorion and is deflected towards the proximal pole of the conceptus. At around embryonic day (E) 8.5, the allantoic mesoderm attaches and fuses with the basal layer of the chorion. Chorionic trophoblast cells begin to differentiate into syncytiotrophoblast cells and villi progenitors, and, in conjunction with allantoic cells, will form the chorioallantoic placenta essential for maternal-foetal nutrient and gaseous exchange and therefore embryonic survival (Cross et al., 2003; Rossant and Cross, 2001).

However, the ExE also fulfils an earlier inductive function by signalling to the subjacent epiblast during germ cell formation (Yoshimizu et al., 2001) and embryonic patterning (Beck et al., 2002). These roles of the ExE are mediated by at least two distinct pathways involving the TGFβ superfamily members Bmp4 and Nodal. *Bmp4* is expressed at gastrulation stages in the ExE adjacent to the epiblast, and, in chimeric loss-of-function mutants in which extraembryonic *Bmp4* expression is selectively ablated, neither primordial germ cells nor extraembryonic mesoderm is formed (Lawson et al., 1999).

Secondly, Nodal activity in the epiblast is necessary for both mesoderm and anterior visceral endoderm (AVE) formation (Brennan et al., 2001). The AVE is formed by migration of a group of distal visceral endoderm cells to one side of the egg cylinder well before gastrulation commences. It secretes antagonists into the adjacent epiblast thereby restricting Nodal activity and thus mesoderm formation to the opposite (posterior) side of the egg cylinder (Lu et al., 2001). However, *Nodal* translation generates Pro-Nodal, which has to be cleaved by endoproteases to generate the fully active Nodal signalling molecule. This cleavage is performed by Furin/Spc1 and Spc4/Pace4, which are expressed in, and secreted from, the ExE (Beck et al., 2002). Double loss-of-function mutants for

these two proteases closely resemble *Nodal*-deficient embryos and do not form AVE or mesoderm (Beck et al., 2002).

We describe here the identification of a novel key gene involved in maintaining the polar trophectoderm/ExE lineage. This gene is *Elf5* (*ESE2* in humans), which encodes a transcription factor belonging to the Ets superfamily. It is characterised by a DNA-binding Ets domain, is able to bind to a subset of Ets-binding sites and can transactivate constructs containing Ets-binding sites upstream from a minimal promoter (Oettgen et al., 1999). *Elf5* has previously been shown to be expressed in foetal and adult epithelial cells of organs such as the mammary and salivary glands, kidney, prostate and lung in mice and in humans (Oettgen et al., 1999; Zhou et al., 1998). Significantly, placenta of pregnant mice at E9.5 and later exhibited *Elf5* expression, as assayed by northern blots (Zhou et al., 1998). Presently no in vivo role for Elf5 has been demonstrated though *Elf5* expression appears to be increased significantly in mouse mammary tumors relative to in normal mammary tissue (Galang et al., 2004).

We report here on the early expression of *Elf5* in the ExE lineage and demonstrate an essential function of *Elf5* for the generation of this tissue. We discuss our findings in relation to trophoblast stem cell maintenance and epiblast-ExE interactions.

Materials and methods

Gene targeting

Elf5 genomic clones were isolated from a 129 mouse λ library (Stratagene) using a 380 bp 5′ probe of the published cDNA sequence. The targeting vector was constructed by ligating the 3′ short arm of 660 bp commencing within exon 2 and isolated by PCR adding *Sal*I sites, and inserted into the *Xho*I site after the polyadenylation signal of the phosphoglycerate kinase promoter-driven, *puromycin*-containing, PGKPuroA cassette (Ridgeway et al., 2000). The *diphthera toxin* gene (*DT-A*) (McCarrick et al., 1993), allowing negative selection, was inserted (blunt/*Sal*I) upstream of *puromycin*. The PCR-derived 5′ flank of 11.3 kbp extending into exon 2 was inserted into the *Sal*I site between *DT-A* and *puromycin*. The *Not*I linearised construct was electroporated into thirteenth passage R1 ES cells (Nagy et al., 2003) (Jackson Laboratory). Puromycin resistant colonies were screened for correct targeting using PCR, and by Southern analysis using a probe external to the short arm. This probe was isolated by PCR with the primers 5′-AATCACCTCCTACCC-CCTAC and 5′-GCAAACATTAGCATAGCATCAG. The correct targeting event resulted in insertion of the *puromycin* cassette into exon 2 of *Elf5* 76 bp downstream of the ATG. Clones 33 and 59 produced germ-line chimeras and were maintained on a 129 background. Diploid embryo-tetraploid embryo aggregation chimeras were produced as described (Nagy et al., 2003). This work was performed under ERMA permit GMD002183 and animal ethics approval RAEC4307.

Genotyping by PCR

PCR genotyping was performed using the two allele, three primer PCR strategy. The common primer A (5′-GAGCAATGGG-AATAAACAGGG) was located within the long arm at the 3′ end of intron 1 and could give a 410 bp product only on the targeted allele with primer B (5′-TGGATGTGGAATGTGTGCGA) located within the puromycin cassette. On the wild-type allele, primer A gave a 578 bp product in combination with primer C (5′-GGAGAAAG-GTGGGGAGGATAA). The inserted *puromycin* cassette prevented the primer A-C combination from yielding a product with the targeted allele as template. Tail tips were digested in Proteinase K buffer [100 mM Tris (pH 8.0), 5 mM EDTA, 0.1% SDS, 200 mM NaCl] at 55°C, with shaking, for 2 hours to overnight, boiled for 5 minutes, centrifuged and then 0.25-1 μl used in a standard 25 μl PCR reaction containing 1 Unit Taq polymerase, 1.5 mM Mg^{2+}, 10 pmol of primer A, and 5 pmol of primers B and C. PCR conditions were 94°C for 4 minutes, followed by 35 cycles of 94°C for 30 seconds, 62°C 45 seconds and 72°C for 1 minute.

Whole-mount in situ hybridisation

The whole-mount in situ hybridisation protocol has been described (Nagy et al., 2003). Staining reactions were carried out for 2 hours up to 5 days, with littermates always treated in the same vessels in the same way. Embryos were genotyped after photography by PCR. Mouse *Elf5* cDNA clones were isolated by screening a mammary gland cDNA library with a bovine *Elf5* fragment. The *Elf5* antisense probe used covered nucleotides 9 to 334 of the reference sequence NM_010125, excluding the conserved ETS domain. A 530 bp mouse *Hex* fragment was cloned into pGEM-Teasy (Invitrogen) in the T7/sense orientation using the primers 5′-CCCTCTGTACCCGT-TCCC and 5′-CCGATGACTGTCATCCAGC and a T_a of 50°C.

Trophoblast stem (TS) cell culture

E6.5 embryos from *Elf5*$^{+/-}$ matings were separated into proximal and distal halves. The distal half was used for genotyping. The proximal half was stripped of visceral endoderm (Nagy et al., 2003) and treated at 37°C for about 10 minutes with 0.25% pronase in Tyrodes Ringer saline. Cells were dispersed by brief pipetting and plated on primary feeder cells (Nagy et al., 2003; Tanaka et al., 1998; Uy et al., 2002). We used 70% conditioned medium (Tanaka et al., 1998) from the outset. Several colonies were observed in wild-type and Elf5$^{+/-}$ cultures by 3 days after dissociation. After the third passage, TS cultures were grown in the absence of feeder cells. For the Fgf4/heparin withdrawal experiment, sixth passage TS cultures were grown in four-well dishes to 30% confluency, whereupon growth was continued for 5 days in conditioned medium with or without Fgf4 and heparin. Cells were pelleted and subjected to real-time PCR as described below.

Real-time RT-PCR

RNA was isolated using TRIZOL (Invitrogen) and reverse transcribed with Superscript3 (Invitrogen) and oligo-dT, according to the manufacturer's instruction. Real-time PCR was performed using SYBR-Green master mix (Applied Biosystems) and the following primers (introns spanned; amplicon size in base pairs):

Bmp4, 5′-GAGTTTCCATCACGAAGAACA-3′ and 5′-GCTCAC-ATCGAAAGTTTCCC-3′ (1; 301);

Cdx2, 5′-CCAAGTGAAAACCAGGACAAAA-3′ and 5′-AAC-GAAGAAGCCCCAGGAA-3′ (1; 669);

Elf5, 5′-CTTGTCTTCACGGTGATGTTGGA-3′ and 5′-CATTC-TTCTTCCTTTGTCCCC-3′ (4; 640);

Eomes, 5′-GCAAACAACAACAACACACA-3′ and 5′-GGGGC-AAGGACTTAATACCA-3′ (3; 583);

Fgfr2, 5′-ACCAAATACCAAATCTCCCAAC-3′ and 5′-ATTCA-TTCTCCACCAGGCA-3′ (3; 592);

Furin, 5′-TGCCAGACCACATGACTAC-3′ and 5′-CAAGGAC-TTGGGGGATGAA-3′ (3; 320);

Otx2, 5′-AAACAGCGAAGGGAGAGGA-3′ and 5′-AGGAGG-AGGAAGTGGACAA-3′ (1; 400);

Spc4, 5′-GGCATCAGACCCAACTACA-3′ and 5′-ATCCACCA-AGCCAAATCCA-3′ (4; 570).

PCR conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and 78°C for 10 seconds, followed by dissociation curve analysis. RT-minus controls were run routinely and representative PCR products analysed on agarose gels to ensure spcecificity of reactions. Amplification efficiencies were monitored by standard curves using serially diluted samples and ranged from 1.6 to 1.9. Relative copy numbers were calculated and normalised against actin.

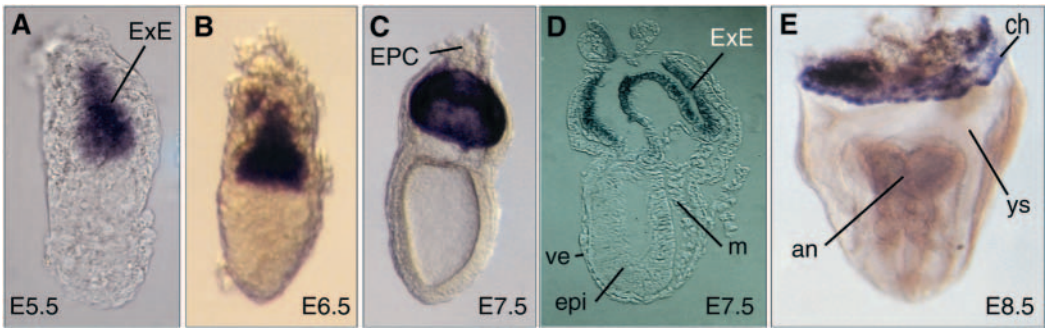

Fig. 1. *Elf5* expression in the extraembryonic ectoderm (ExE). (A-E) Whole-mount in situ hybridisation analysis showing expression of *Elf5* confined to the ExE and its derivative, the chorion, from E5.5 to E8.5. (D) *Elf5* is restricted to the ectodermal layer of the chorion, as seen in a sagittal section. an, anterior neuroectoderm; ch, chorion; EPC, ectoplacental cone; epi, epiblast; m, mesoderm; ve, visceral endoderm; ys, yolk sac.

Results

Elf5 is expressed in the extraembyonic ectoderm lineage

Elf5 is transcribed in adult and foetal epithelial tissues in both humans and mice, but has not been reported to be expressed during early embryogenesis. We noticed using RT-PCR that *Elf5* mRNA is present before somitogenesis, and performed whole-mount in situ hybridisation to determine the spatiotemporal pattern of transcripts. Interestingly, *Elf5* is already expressed before gastrulation in the ExE (Fig. 1A). This pattern of expression is maintained throughout gastrulation with *Elf5* transcripts marking the ExE lineage, which, by E7.5, has formed the chorionic ectoderm (Fig. 1B-D). The chorion still exhibits *Elf5* transcription at E8.5 (Fig. 1E), when chorioallantoic fusion is occurring. Until the start of somitogenesis, we did not detect expression in epiblast derivatives or other extraembryonic tissues, such as ectoplacental cone (EPC) and parietal or visceral endoderm. Low levels of *Elf5* transcripts could be seen by RT-PCR in E3.5 pre-implantation blastocysts (data not shown). This exquisitely specific expression pattern suggested a role for *Elf5* in the ExE lineage.

Elf5 deficient embryos are embryonic lethal

To determine whether this novel extraembryonic-specific factor does have a function in early development, we inactivated the *Elf5* gene by homologous recombination. The *puromycin* gene was inserted into the second exon of *Elf5*, at the start of the N-terminal pointed domain, leaving only 26 amino acid residues of the Elf5 protein (Fig. 2A-C). Heterozygous $Elf5^{+/-}$ mice derived from two different ES-cell targeting events appeared normal and were fertile. However, no homozygous pups from numerous heterozygous crossings were ever retrieved on either 129 or BALB/c backgrounds, or on mixed 129:BALB/c genetic backgrounds, suggesting embryonic lethality.

Analysis of genotypes of embryos derived from $Elf5^{+/-}$ intercrosses on the 129 background revealed normal Mendelian ratios up to E8.5. Thereafter, an increasing number of embryos were resorbed, as manifested by the retrieval of only dead or resorbing $Elf5^{-/-}$ embryos at E9.5 (Table 1). We noticed an effect of genetic background. BALB/c *Elf5*-deficient embryos showed a more variable phenotype, with the odd developmentally delayed embryo still present at E10.5. Such survivors could not develop much further, as by E12.5 no $Elf5^{-/-}$ embryos were recovered (Table 1). We restricted our further analyses to the more severe phenotype seen on the 129 background.

Morphological and patterning defects in *Elf5* mutant embryos

Elf5-deficient embryos cavitated normally. However, from early streak stages, mutant embryos were consistently smaller than their littermates and exhibited no signs of primitive streak formation (Fig. 3A). The constriction marking the

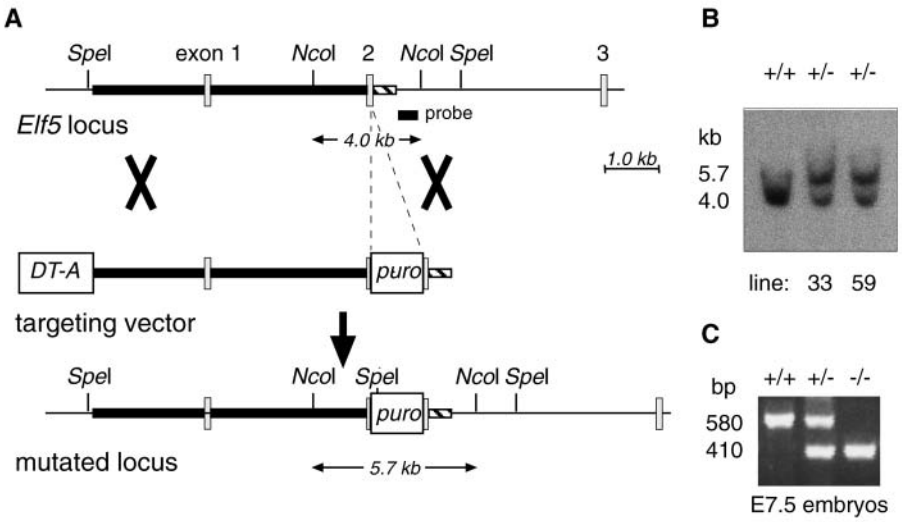

Fig. 2. Loss of *Elf5* function by homologous recombination. (A) Target disruption strategy of the *Elf5* locus. The targeting construct is shown in the middle. The black box represents the long arm, the striped box the short homology arm of the targeting vector. The position of the external probe and the expected fragment sizes upon *Nco*I digestion in a Southern blot are indicated. (B) Southern blot of the *Nco*I-digested genomic DNA of wild-type (+/+) and heterozygous *Elf5* mutant (+/–) mice derived from two separate ES cell lines, probed with the external probe depicted in panel A. (C) Embryos were genotyped by PCR, the 410 bp band corresponding to the targeted allele, the 580 bp to the wild-type locus.

Table 1. Summary of genotypes of embryos from *Elf5* heterozygote matings on two background strains

Age	Strain	Wild type	Heterozygous	Mutant	Total
E6.5	129	19 (26%)	36 (49%)	17 (23%)	73
E7.5	129	11 (25%)	24 (55%)	9 (20%)	44
E8.5	129	10 (26%)	22 (56%)	7 (18%)	39
E9.5	129	9 (30%)	15 (50%)	6 (20%)*	30
E10.5	BALB/c	7 (25%)	14 (50%)	7 (25%)†	28
E12.5	BALB/c	5 (26%)	14 (74%)	0	19

*Dead/resorbing.
†Four dead/extremely underdeveloped; two resembling E8.5, one E9.5 embryo.

extraembryonic-embryonic ectoderm (ExE-EmE) border was absent. Mesoderm had not formed by E7.5, and mutant embryos still resembled egg cylinders, composed of ectoderm surrounded by visceral endoderm and no ExE-EmE constriction (Fig. 3B,C). The EPC was present. By E8.5, *Elf5*$^{-/-}$ embryos were severely reduced in size, consisting of a sack of ectoderm which often had continued to proliferate with folding of the excess epithelium (Fig. 3D,E). This ectoderm displayed an anterior neuroectodermal character in the form of patchy *Otx2* expression, whereas more posterior genes, such as the mid- and/or hindbrain markers *Pax2* and *Krox20*, were not transcribed (Fig. 3E, and data not shown). *T*/brachyury, marking mesoderm from the onset of gastrulation, was seen in half of the E8.5 mutant embryos (Fig. 3D). These *T*-positive embryos, which we term type II *Elf5*-mutants, occasionally contained a mass of mesenchymal cells located at the proximal end of the embryo, indicative of delayed aberrant mesoderm formation (data not shown). *Elf5*$^{-/-}$ embryos did not form a chorion (Fig. 3C), thus precluding chorioallantoic placenta formation. The resulting foetal-maternal loss of nutritional and waste exchange would be expected to lead to the observed midgestational lethality (Cross et al., 2003; Rossant and Cross, 2001).

Elf5 deficient embryos are devoid of extraembryonic ectoderm

The lack of chorion formation in *Elf5*$^{-/-}$ embryos suggested defects in the ExE lineage. We therefore analysed the *Elf5* mutant embryos using a panel of molecular markers that are expressed in the ExE. The genes coding for Cdx2 (Beck et al., 1995), Eomes (Ciruna and Rossant, 1999; Russ et al., 2000), the fibroblast growth factor receptor Fgfr2 (Haffner-Krausz et al., 1999), Bmp4 (Lawson et al., 1999) and the endoproteases Furin and Spc4 (Beck et al., 2002) are primarily or exclusively expressed in the ExE during early gastrulation stages. None of these genes were transcribed in the proximal ectoderm of E6.5 to E7.5 *Elf5* deficient embryos (Fig. 4C-J; Fig. 8D,G), molecularly confirming the absence of ExE. Notably, *Cdx2*, the earliest known marker for the undifferentiated trophectoderm lineage (Rossant et al., 2003), is expressed in the polar trophectoderm of *Elf5*-deficient blastocysts, but no expression is evident by E5.5 (Fig. 4A,B). Thus from pregastrula stages, *Elf5* deficient embryos do not contain ExE.

It should be pointed out that the absence of the ExE does not affect the formation of the EPC, as judged histologically (Fig. 3C) and by the expression of *Furin*, *Spc4* and *Fgfr2*. At E5.5, *Spc4* and *Fgfr2* expression marks both EPC and ExE tissue (Fig. 4E,I) (Beck et al., 2002). In *Elf5* mutants, these genes were robustly expressed only in the EPC overlying the forming egg cylinder (Fig. 4E,I). Similarly, *Furin*, which is normally expressed in the ExE, EPC, proximal epiblast and visceral endoderm at early gastrula stages (Beck et al., 2002), could be detected only in the EPC region of *Elf5*-deficient embryos (Fig. 4H).

The absence of ExE and a visible ExE-EmE constriction suggested that the ectodermal layer of the *Elf5* mutant egg cylinders consisted only of epiblast derivatives. We therefore probed embryos with *Oct4/Pou5f1*, a marker for undifferentiated embryonic ectoderm (Scholer et al., 1990). We found that *Oct4*

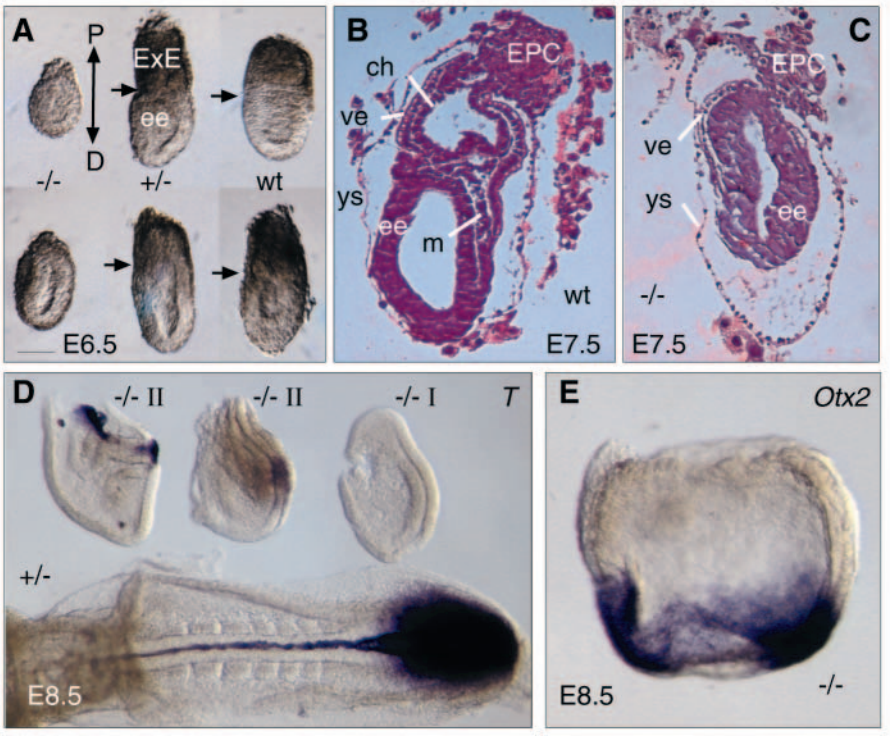

Fig. 3. Morphological defects seen in Elf5 homozygous mutant embryos. (A) *Elf5*$^{-/-}$ embryos could usually be morphologically recognised at E6.5 by their reduced size and the lack of the embryonic-extraembryonic constriction (arrow). (B,C) Haematoxylin and Eosin-stained sagittal cross sections revealed the presence of ectoderm, EPC and visceral endoderm (ve) in homozygous E7.5 mutants (C), but the absence of chorion (ch) and mesoderm (m), which are readily detectable in wild-type littermates (B). (D) At E8.5, approximately half of the *Elf5* mutants did not express the pan mesodermal marker *T* (phenotypically more severe type I mutants), whereas the other half expressed *T* ectopically (type II mutants). (E) By E8.5, *Elf5*$^{-/-}$ embryo epithelium displayed an anterior neuroectodermal character, as shown by whole-mount in situ hybridisation with *Otx2*. D, distal; ee, embryonic ectoderm; P, proximal.

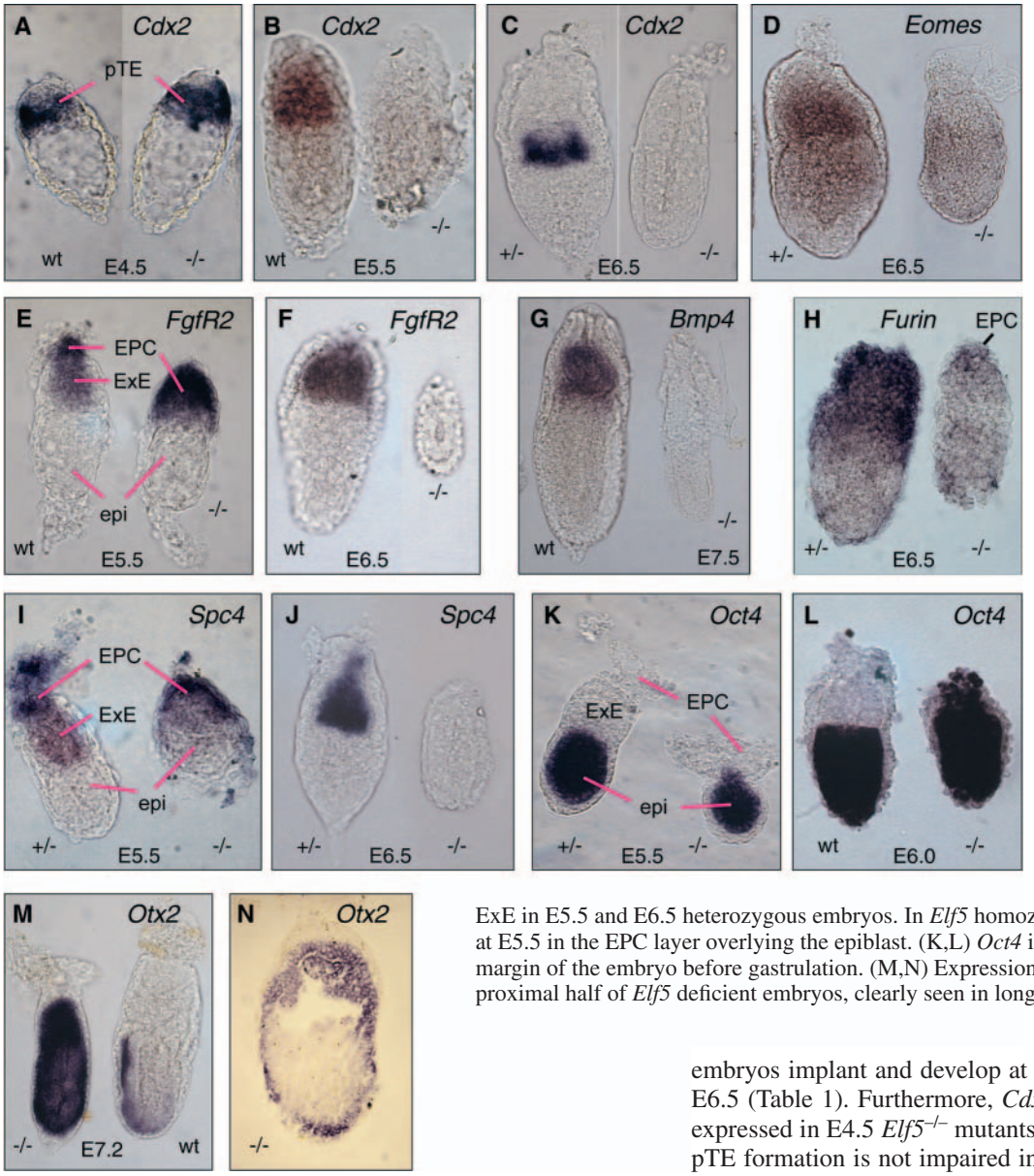

Fig. 4. *Elf5* deficient embryos lack the ExE. (A-N) Embryos are orientated with their proximal end at the top. Whole-mount in situ hybridisation of wild-type/heterozygous (on left side of all panels except M) and *Elf5*$^{-/-}$ embryos for the ExE markers *Cdx2*, *Eomes*, *Fgfr2, Bmp4*, *Furin* and *Spc4*, and the epiblast markers *Oct4* and *Otx2*, as indicated. (A) At implantation stages, *Cdx2* expression is seen in the polar trophectoderm (pTE) in both wild-type and *Elf5*$^{-/-}$ embryos. (B-J) None of the six ExE markers is expressed in the proximal region of mutant egg cylinders, indicating the absence of ExE tissue from as early as E5.5. (E) At E5.5, *Fgfr2* is expressed more strongly in EPC than ExE tissue of wild-type embryos. In *Elf5*$^{-/-}$ counterparts, only the strong expression in the EPC capping the egg cylinder is seen. (H) *Furin* expression is detected in the EPC of E6.5 *Elf5*$^{-/-}$ mutants. (I,J) *Spc4* is expressed in both EPC and ExE in E5.5 and E6.5 heterozygous embryos. In *Elf5* homozygotes, it can only be detected at E5.5 in the EPC layer overlying the epiblast. (K,L) *Oct4* is expressed up to the proximal margin of the embryo before gastrulation. (M,N) Expression of *Otx2* extends into the proximal half of *Elf5* deficient embryos, clearly seen in longitudinal sections (panel N).

expression in mutants extended to the proximal end of the egg cylinder (Fig. 4K,L), confirming the absence of ExE tissue well before the commencement of gastrulation. Similarly *Otx2*, normally restricted to the anterior embryonic epiblast (Perea-Gomez et al., 2001), was expressed across the entire mutant ectoderm, reaching the EPC region (Fig. 4M,N). We conclude from these marker studies that in the absence of Elf5 activity, the ExE is not formed, resulting in embryos composed of EPC directly abutting the embryonic ectoderm.

Elf5 is required for the maintenance of trophoblast stem cells

Why were *Elf5*$^{-/-}$ embryos depleted of ExE by E5.5? The ExE is a direct derivative of the polar trophectoderm (pTE) of the blastocyst. Yet unlike *Cdx2* and *Eomes* deficient embryos, which die around the implantation stage as a result of defects in trophectoderm and pTE cells, respectively (Chawengsaksophak et al., 1997; Russ et al., 2000), *Elf5*$^{-/-}$ embryos implant and develop at expected Mendelian ratios to E6.5 (Table 1). Furthermore, *Cdx2* marking the pTE was still expressed in E4.5 *Elf5*$^{-/-}$ mutants (Fig. 4A). This suggests that pTE formation is not impaired in *Elf5* deficient embryos.

However, both pTE and ExE contain trophoblast stem (TS) cells (Tanaka et al., 1998; Uy et al., 2002), and a failure to maintain these stem cells after implantation would be expected to affect ExE formation. Indeed, our observations of the absence of expression of the undifferentiated TS cell markers *Cdx2*, *Eomes* and *Fgfr2* in *Elf5*$^{-/-}$ embryos suggested that TS cells are not maintained in mutant embryos. We therefore attempted to isolate TS cells from *Elf5*$^{-/-}$ embryos by culturing E6.5 dissociated proximal ectoderm tissue on primary feeder cells in the presence of Fgf4 and heparin (Tanaka et al., 1998; Uy et al., 2002). Notably, TS-like colonies were only formed from wild-type and *Elf5*$^{+/-}$ proximal ectoderm (n=18/18), whereas *Elf5*$^{-/-}$ tissue formed no colonies (n=0/4; Fig. 5A,B).

Analysis of TS cell colonies by RT-PCR indicated that these cells expressed, not only *Cdx2*, *Eomes*, *Fgfr2*, *Errb* (*Esrrb* – Mouse Genome Informatics), *Bmp4*, *Furin* and *Spc4*, but also *Elf5* (Fig. 5C). Fgf4 withdrawal causes the differentiation of TS cells into secondary giant cells and spongiotrophoblasts, both found in the EPC (Tanaka et al., 1998; Yan et al., 2001).

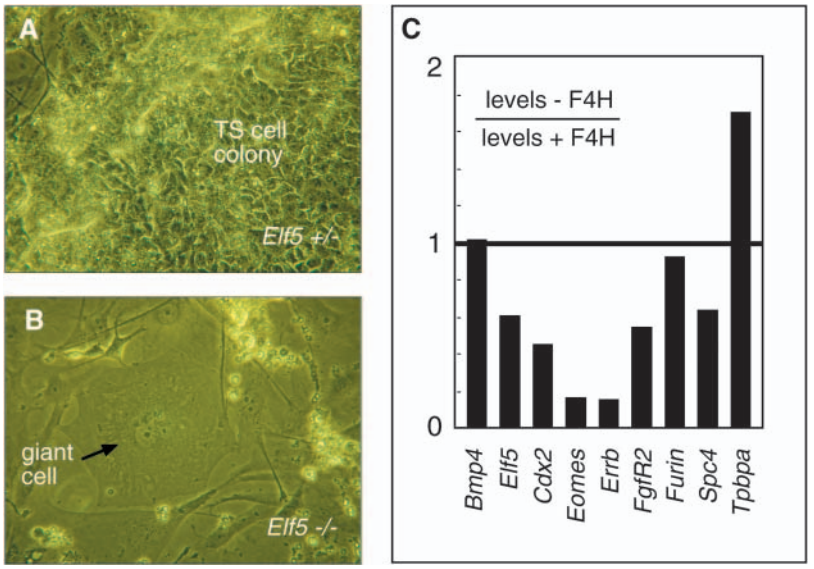

Fig. 5. TS cells cannot be isolated from *Elf5*$^{-/-}$ embryos. (A,B) Cultures from *Elf5*$^{+/-}$ or wild-type E6.5 proximal ectoderm show distinct TS-like colonies (A), whereas *Elf5*$^{-/-}$ cultures yield no colonies. (B) Fifteen days after embryo dissociation only some giant cells remain. (C) Change in gene expression levels in wild-type TS colonies grown for 5 days in the absence versus presence of Fgf4/heparin, as measured by real-time RT-PCR.

We observed that Fgf4 withdrawal resulted, within 5 days, in a downregulation not only of the TS cell markers *Cdx2*, *Eomes*, *Fgfr2* and *Errb* but also of *Elf5*. Concomitantly, the spongiotrophoblast and EPC marker *Tpbp/4311* (Lescisin et al., 1988) was upregulated, indicative of TS cell differentiation (Fig. 5C). We conclude that *Elf5* is a marker for undifferentiated TS cells. In *Elf5*$^{-/-}$ embryos, TS cells no longer self renew but differentiate into EPC progenitors, leading to the observed absence of the ExE by E5.5.

Extraembryonic ectoderm is required for patterning of the embryo proper

Elf5 deficient embryos not only exhibit defects in the ExE lineage but also display severe patterning defects in the embryo proper. Although we observed expression of *Elf5* solely in the ExE and chorion using whole-mount in situ hybridisation, low levels of expression in other regions might have escaped our detection and be partly or wholly responsible for the phenotype seen in the embryo proper. We therefore wished to determine whether patterning defects occurred in embryos lacking Elf5 function only in the epiblast. To this end, we performed a tetraploid rescue experiment. Wild-type eight-cell-stage tetraploid cells known to contribute only to extraembryonic tissue were aggregated with diploid cells derived from four- to eight-cell-stage embryos of *Elf5*$^{+/-}$×*Elf5*$^{+/-}$ matings and allowed to develop to E10.5, when *Elf5*$^{-/-}$ embryos are either dead or severely retarded and malformed. Statistically, one quarter of the chimeric embryos would be expected to contain epiblast with an *Elf5*$^{-/-}$ genotype. Genotyping revealed that four out of 15 embryos were composed solely of *Elf5*$^{-/-}$ cells (Fig. 6A). These four embryos were morphologically normal (Fig. 6B,C), proving that *Elf5* is not required in embryonic tissues for development up to this developmental stage.

The extraembryonic ectoderm is not required for AVE formation

The ExE has been shown to be involved in reciprocal interactions with the subjacent embryonic ectoderm, activating Nodal, which, in turn, orchestrates AVE development and axis formation in the epiblast (Beck et al., 2002; Brennan et al., 2001; Lu et al., 2001). We therefore investigated whether AVE formation was impaired in *Elf5* mutants. The AVE is formed from a group of distal visceral endoderm cells expressing the homeobox gene *Hex*, which migrate towards the future anterior side between E5.7 and E6.5 (Yamamoto et al., 2004). We found that, in E5.8 *Elf5*$^{-/-}$ embryos, *Hex* expression was confined to the visceral endoderm on one side of the embryo, similar to in controls (Fig. 7A). Furthermore, the Nodal/BMP inhibitor *Cer-1* (*Cerl*) was expressed in the presumptive AVE of *Elf5*$^{-/-}$ embryos (Fig. 7B). Similarly, the AVE/anterior neurectoderm marker *Hesx1* could still be detected in the anterior endodermal region of mutants by E7.5 (Fig. 7C). In addition, we could confirm *Nodal* expression in *Elf5* deficient embryos at E5.5 (Fig. 7D), a prerequisite for AVE formation. These results indicate that the AVE can be specified and established in the absence of the ExE.

Extraembryonic ectoderm is required for gastrulation

In marked contrast to AVE formation, mesoderm formation is impaired in *Elf5* null mutants. At the start of gastrulation,

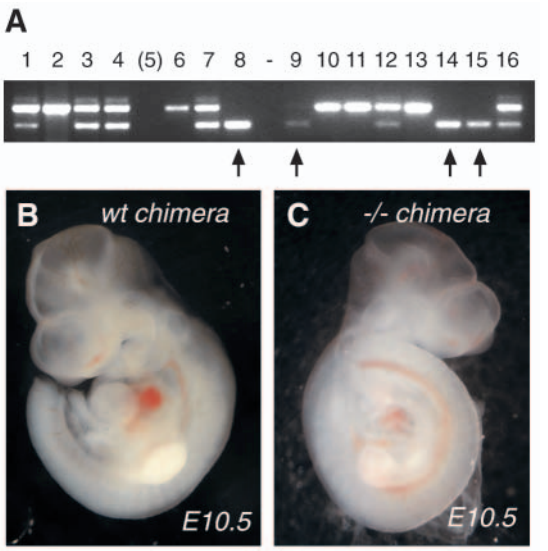

Fig. 6. Tetraploid aggregation chimeras composed of *Elf5*$^{-/-}$ epiblast are developmentally normal. (A) PCR genotyping of the tail tips of chimeric embryos reveals the presence of four embryos, indicated by arrows, composed solely of *Elf5*$^{-/-}$ cells. Embryo 5 had died and was not genotyped. (B,C) Chimeric embryos (embryo 13 shown) containing only wild-type (or wild-type and *Elf5*$^{+/-}$) cells in embryonic and extraembryonic compartments (B) were morphologically indistinguishable from chimeric embryos (embryo 14 shown) containing only *Elf5*$^{-/-}$ diploid embryonic cells (C).

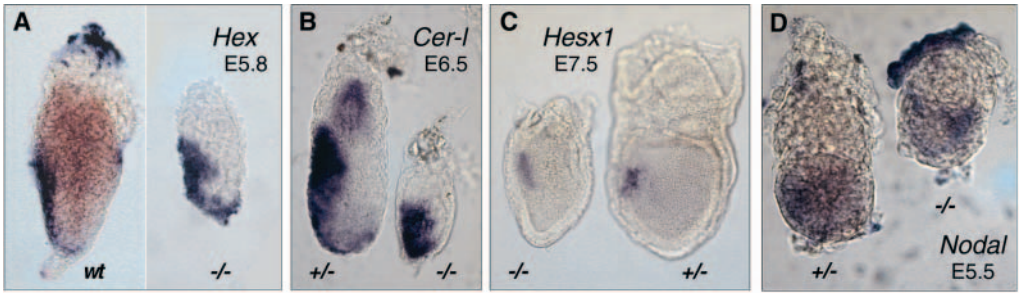

Fig. 7. AVE formation in *Elf5*$^{-/-}$ mutant embryos. (A-C) Whole-mount in situ hybridisation analysis demonstrating that the AVE, as marked at E5.8 by *Hex* (A) and at E6.5 by *Cer-l* (B) expression, is formed and migrates to one side in *Elf5*$^{-/-}$ embryos. (C) By E7.5, *Hesx1* transcripts are still apparent. (D) *Nodal* expression could consistently be detected in pregastrulation *Elf5* null mutants.

Nodal expression marks the epiblast where the primitive streak will form. In *Nodal* mutants, posterior epiblast/mesoderm markers, such as *Cripto*, *Eomes*, *T* and *Fgf8*, fail to be expressed and no primitive streak is formed (Brennan et al., 2001). *Elf5*$^{-/-}$embryos either showed no expression of *Nodal* and its downstream markers (type I mutants), or exhibited ectopic expression abutting the EPC (type II) (Fig. 8A-E: see Table S1 in supplementary material for statistics). Conversely, transcription of *Otx2,* normally showing a reciprocal pattern to Nodal, was not restricted in *Elf5* deficient embryos (Fig. 4J,K). *Cdx2* and *Bmp4* transcription, characteristic of nascent extraembryonic mesoderm, was not seen in mutant embryos (Fig. 8F,G). Thus the lack of ExE leads to a secondary defect in mesoderm formation, the severity of which can vary and may be due to heterogeneity in the 129 background (Simpson et al., 1997).

Discussion

Elf5 as a lineage-determining gene for the extraembryonic ectoderm

The polar trophectoderm (pTE) overlying the inner cell mass in the implanted blastocyst at E4.5 proliferates and differentiates in the following 24 hours to yield the morphologically distinct extraembryonic ectoderm (ExE) and overlying ectoplacental cone (EPC) regions of the egg cylinder stage embryo. We report here the expression of the transcription factor Elf5 specifically in the ExE from E5.5 onward. Furthermore, in our *Elf5* loss-of-function mouse model this tissue is specifically lost. This phenotype of normal implantation followed by the loss of the ExE but not the EPC region is unique. Several other genes have been implicated in the formation or maintenance of trophectoderm-derived lineages but show quite distinct phenotypes when disrupted by homologous recombination (Cross et al., 2003; Rossant and Cross, 2001). *Cdx2* loss-of-function mutants die before implantation, rarely forming expanding blastocysts (Chawengsaksophak et al., 1997). *Cdx2* is already differentially expressed at the morula stage and is believed to be one of the earliest lineage determining genes for the trophectoderm (Kunath et al., 2004; Rossant et al., 2003). *Eomes* appears to be required at a slightly later stage, as mutants do implant but arrest shortly thereafter, failing to undergo trophoblast differentiation in vitro (Russ et al., 2000). Whereas mural

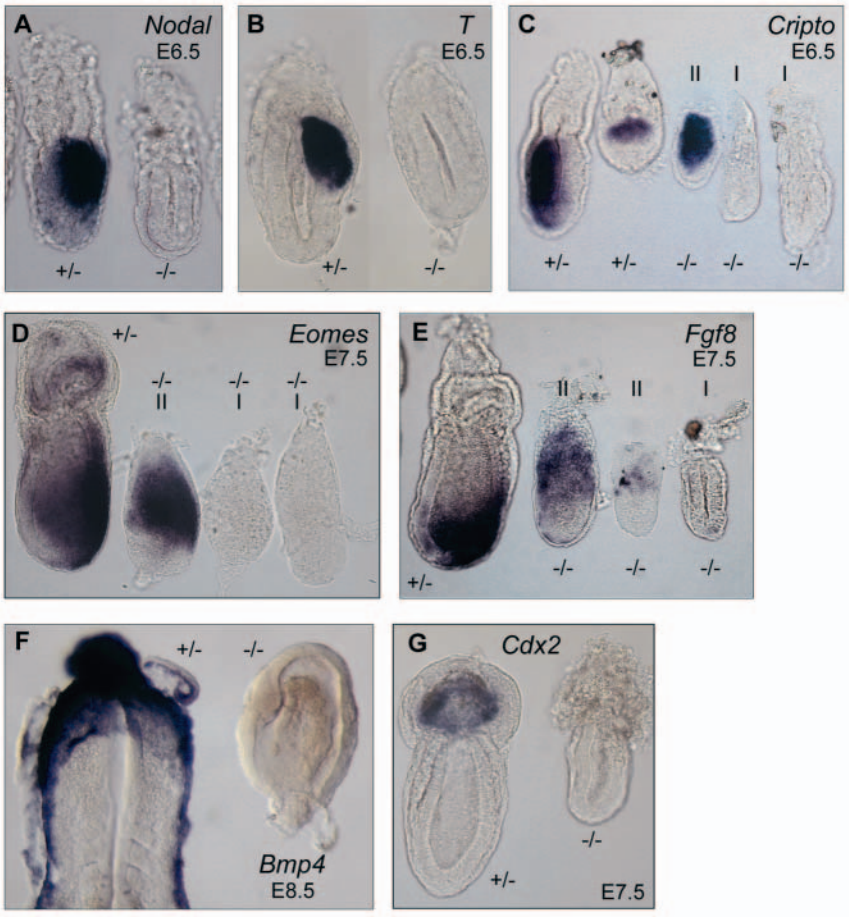

Fig. 8. Expression of markers for mesoderm and posterior epiblast in *Elf5*$^{-/-}$ embryos. (A) *Nodal* transcripts can no longer be detected in (type I) *Elf5* null mutants by E6.5. (B) *T*/brachyury expression marking nascent mesoderm was never seen in E6.5 *Elf5* deficient embryos. (C-E) The posterior epiblast/nascent mesoderm markers *Cripto* (C), *Eomes* (D) and *Fgf8* (E) were either absent (type I mutants) or seen in the proximal region of the embryo, adjacent to the EPC (type II mutants). (F) No lateral mesoderm or extraembryonic mesoderm was formed in *Elf5*$^{-/-}$ embryos, as noted by the absence of *Bmp4* transcripts (dorsal view, posterior at top of panel). (G) *Cdx2*, which in heterozygous E7.5 embryos marks the extraembryonic mesoderm and chorion, was not detected in *Elf5* deficient embryos.

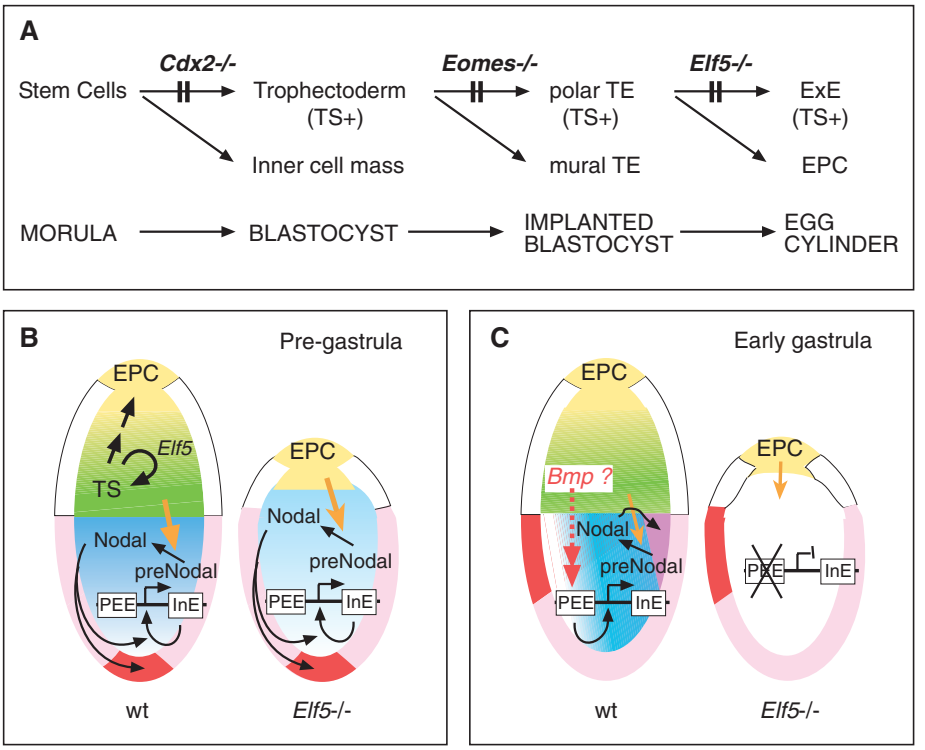

Fig. 9. The role of Elf5 in the TS cell lineage and the effects of its absence on embryonic development. (A) Model for the specification and maintenance of the TS lineage during early development. The effect of loss of function of the transcription factors Cdx2, Eomes and Elf5 is indicated (see text for discussion; TE, trophectoderm). (B,C) Model depicting the effect of the absence of ExE on embryonic development. (B) In wild-type pregastrula embryos, Furin and Spc4 protein produced by the ExE (green) diffuses (orange arrow) into the epiblast (blue), causing proteolytic activation of precursor Nodal (preNodal), which at these stages is produced from the intronic enhancer (InE) (Brennan et al., 2001; Norris et al., 2002). Activated Nodal protein positively acts (curved arrows) on its own transcription, as well as inducing the formation of the AVE (red). The lack of *Elf5*-dependent TS cell renewal in *Elf5*$^{-/-}$ mutants leads to the absence of ExE at E5.5. The EPC, now adjacent to the epiblast, produces Spc4, which is sufficient to activate preNodal in the epiblast, thereby leading to correct AVE formation in *Elf5* null mutants. (C) By E6.0 to 6.5, when gastrulation commences, *Nodal* transcription has come under the control of the Nodal-independent PEE enhancer (Norris et al., 2002; Vincent et al., 2003). In *Elf5* mutants, the absence of the ExE results in the loss of the factor(s) required for *Nodal* transcription from the PEE enhancer. Candidates for such factors are Bmps (dashed red arrow; see text). The loss of *Nodal* transcription in Elf5$^{-/-}$ embryos results in the absence of posterior gene expression and thus mesoderm (purple) is not formed.

trophectoderm generates only primary giant cells, which are essential for implantation, further development requires the pTE which gives rise to the ExE, the EPC, as well as secondary giant cells (Copp, 1979). Eomes could thus be involved in the mural versus polar trophectoderm lineage decision (Fig. 9A).

Little is known about the molecular events leading to the generation of the ExE and the EPC region from the pTE. However, it has emerged that trophoblast stem cells can be isolated from the ExE but not the EPC (Rossant and Tamura-Lis, 1981; Uy et al., 2002). These TS cells require FGF as well as Nodal/Activin signalling for their maintenance (Erlebacher et al., 2004; Guzman-Ayala et al., 2004; Tanaka et al., 1998). Upon FGF withdrawal, TS cells differentiate either into polyploid giant cells or spongiotrophoblast cells. This is reminiscent of the EPC, the outer edge of which differentiates into secondary giant cells, whereas the main part generates spongiotrophoblast cells (Cross et al., 2003). The requirement of FGF signalling is also supported by in vivo results. *Fgf4* and *Fgfr2* loss-of-function embryos die shortly after implantation (Arman et al., 1998; Feldman et al., 1995), and inactivation of Erk2, an important downstream effector of FGF receptors, leads to a specific loss of both the ExE and EPC, indicating a primary proliferative defect at the level of the pTE (Saba-El-Leil et al., 2003). As *Fgf4* is expressed in the ICM of the blastocyst, as well as by the epiblast of the egg cylinder, current models suggest that the tissues in close proximity to the FGF signals, namely the pTE and later the ExE, maintain TS cells, whereas the more distant mural trophectoderm and EPC form differentiated trophoblast cell types (Kunath et al., 2004; Tanaka et al., 1998). Thus the 'lineage decision' between ExE and EPC at post pTE stages may simply reflect differences in the ability to maintain a population of undifferentiated proliferative TS cells. This interpretation places the emphasis on the maintenance of TS cells. In line with this, TS cells cannot be isolated from *Cdx2* nor *Eomes* defective blastocysts (Rossant et al., 2003; Russ et al., 2000). Both genes can be considered to be trophoblast lineage determining genes by virtue of specifying or maintaining TS cells at the morula and blastocyst stages, respectively.

We suggest that Elf5 acts at the next step, being required for maintaining trophoblast stem cell potential beyond the implanted blastocyst stage (Fig. 9A). Thus in the absence of Elf5, TS cells within the pTE are no longer maintained, instead differentiating into EPC precursors that will differentiate finally into sphongiotrophoblasts and giant cells. By egg cylinder stages, the ExE is absent whereas the EPC trophoblast is still seen. Several lines of evidence support this scenario. The normal implantation rates of *Elf5* deficient embryos implies that Elf5 is only required after the formation of trophectoderm. The presence of an EPC in mutants means that the pTE must have formed correctly, as it gives rise to this lineage. This is supported by the correct pTE-specific expression of *Cdx2* at E4.5 in *Elf5*$^{-/-}$ embryos. That TS cells are no longer maintained in *Elf5* mutants past the pTE stage is supported by the absence of the TS-cell containing ExE and the observation that TS cells could not be derived from the proximal half of the egg cylinder of *Elf5* mutant embryos. Moreover, TS cell markers such as *Cdx2*, *Eomes* and *Fgfr2* were no longer expressed in mutant embryos. Lastly, *Elf5* is expressed in TS cell lines and lost upon their differentiation

after FGF removal. Elf5 can therefore be considered to be a lineage-determining factor, required for the formation of the ExE lineage by virtue of maintaining TS cells.

The role of the extraembyonic ectoderm in patterning the embryo

The absence of ExE from its inception in *Elf5* deficient embryos has provided a novel mouse model allowing an examination of the role of this tissue in the development of the embryo proper. Patterning of the embryo involves two distinct steps, the first being the establishment of the AVE signalling centre thereby preparing the adjacent epiblast for anterior induction by subsequent mesendodermal signalling. Thereafter, the primitive streak with its associated organiser activities is formed on the opposite side of the egg cylinder. Both events require Nodal signalling within the epiblast (Brennan et al., 2001; Lu et al., 2001). In turn, Nodal activity has been shown to be dependent on the ExE, which secretes the proteases Furin and Spc4 to cleave and thereby activate the Nodal precursor in the epiblast. In the absence of both proteases, mesoderm and AVE formation are abolished (Beck et al., 2002). Why do *Elf5*$^{-/-}$ embryos, which do not have an ExE, still form the AVE? We propose (Fig. 9B) that in the absence of ExE, Nodal cleavage is mediated by secretion of Spc4, which is strongly expressed in the EPC located adjacent to the epiblast in *Elf5*$^{-/-}$ pregastrula embryos. Once proteolytically activated, Nodal can then amplify its own expression (Fig. 7D, Fig. 9B) via its autoregulatory intronic enhancer, leading to AVE formation and migration (Brennan et al., 2001; Norris et al., 2002).

Although the EPC may substitute for the ExE at stages preceding gastrulation, this is not the case at later stages. The loss of *Nodal* transcription at E6.5 in type I *Elf5* mutants explains the absence of expression of *Eomes*, *Fgf8*, *T* and *Cripto*, all of which are downstream targets of Nodal activity and required for posterior patterning and mesoderm formation. Significantly, the absence of transcription from the *Nodal* locus at E6.5 in *Elf5* mutants differs from ExE-containing *Nodal* null mutants and *Furin/Spc4* double mutants, which do exhibit transcripts in the proximal epiblast (Beck et al., 2002; Brennan et al., 2001). These mutants differ from *Elf5* deficient embryos in two fundamental ways – they have no AVE and they do contain ExE. Could the continued presence of the AVE in *Elf5* mutants result in the absence of posterior markers? We consider this to be unlikely as the Nodal-repressive AVE is restricted to only one side of the mutant egg cylinders. We would thus favour the alternate hypothesis (Fig. 9C), that a protease independent signal emanating from the ExE (absent in *Elf5* but present in *Nodal* mutants) is required for *Nodal* transcription at E6.5. Potential candidates for such signalling are Bmp4 and/or Bmp8b, which are expressed in the ExE, but not the EPC (Fujiwara et al., 2002; Ying et al., 2000). ExE-derived Bmp4 has been shown to induce posterior genes in the epiblast and is required (in a mouse background strain-dependent fashion) for the generation of a normal primitive streak (Beck et al., 2002; Fujiwara et al., 2002; Winnier et al., 1995). In *Nodal*-null mutants, Bmp4 is present in the ExE at E6.5 (Brennan et al., 2001) and thus could theoretically contribute to transcription from the *Nodal* locus, as it does in other contexts (Fujiwara et al., 2002; Piedra and Ros, 2002; Schlange et al., 2002).

Whatever the identity of the signals emanating from the ExE are, our ExE-deficient mouse model and tetraploid rescue experiments strongly support the proposed inductive role of this extraembryonic tissue in primitive streak formation, thus suggesting that this tissue is equivalent to the avian posterior marginal zone (Bachvarova et al., 1998).

Conclusion

We have found a novel factor exquisitely restricted to and required for the formation of the ExE, and have created a mouse model that clearly separates two temporally distinct requirements for the ExE in instructing the patterning of the epiblast. Whereas the early ExE function in anterior patterning via AVE establishment can be replaced presumably by Spc4 secreted from the EPC, there is an essential requirement for ExE in initiating gastrulation and posterior patterning of the embryo proper. Furthermore, we now can identify three genes that consecutively function to determine cell choices in the maintenance of TS cells. First, *Cdx2* for the trophectoderm/ICM choice at the morula stage; second, *Eomes* for the polar/mural trophectoderm choice at the blastocyst stage; and third, *Elf5* in the ExE/EPC decision at the implanted blastocyst stage.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/10/2299/DC1

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