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

Martyn Donnison*, Angela Beaton*†, Helen W. Davey‡, Ric Broadhurst, Phil L’Huillier and Peter L. Pfeffer§

AgResearch Crown Research Institute, Ruakura Campus, East Street, Hamilton 2001, New Zealand

*These authors contributed equally to this work
†Present address: Academic Medical Genetics, The Children’s Hospital at Westmead, Locked Bag 4001, Westmead, NSW 2145, Australia
‡Present address: Department of Physiology, University of Otago, PO Box 913, 270 Great King Street, Dunedin, New Zealand
§Author for correspondence (e-mail: peter.pfeffer@agresearch.co.nz)

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. 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 \textit{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 trophoderm/ExE lineage. This gene is \textit{Elf5} \((\text{ESE2 in humans})\), 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). \textit{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 \textit{Elf5} expression, as assayed by northern blots (Zhou et al., 1998). Presently no in vivo role for \textit{Elf5} has been demonstrated though \textit{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 \textit{Elf5} in the ExE lineage and demonstrate an essential function of \textit{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

\textit{Elf5} genomic clones were isolated from a 129 mouse \(\lambda\) 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 the targeting vector was constructed by ligating the \(3'\) 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 \(\mu\)l used in a standard 25 \(\mu\)l 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 littersmates always treated in the same vessels in the same way. Embryos were genotyped after photography by PCR. Mouse \textit{Elf5} cDNA clones were isolated by screening a mammary gland cDNA library with a bovine \textit{Elf5} fragment. The \textit{Elf5} antisense probe used covered nucleotides 9 to 334 of the reference sequence NM_010125, excluding the conserved ETS domain. A 530 bp mouse \(H\text{ex}\) fragment was cloned into pGEM-Teasy (Invitrogen) in the \(5'\)/sense orientation using the primers 5\textsuperscript{-}CCCCTGTTACCCGT-TCCC and 5\textsuperscript{-}CCGATGACGTCATCCAGC and a \(T_{m}\) of 50°C.

Trophoblast stem (TS) cell culture

E6.5 embryos from \textit{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 \textit{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 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\textsuperscript{-}GAGTTTTCCACATTACGAAGAACA-3\textsuperscript{-}and 5\textsuperscript{-}GCTCACATTGACATAGCTCC-3\textsuperscript{-} (1; 301);
- \(Cd2\), 5\textsuperscript{-}CCAAGTGGAAACACCAGACAAAA-3\textsuperscript{-} and 5\textsuperscript{-}AACAGAAGGCCCCAGGAA-3\textsuperscript{-} (1; 669);
- \(Elf5\), 5\textsuperscript{-}CTTGTCTTACGGTATGTGTTG-3\textsuperscript{-} and 5\textsuperscript{-}CATTCTTCTTTGTTCCCC-3\textsuperscript{-} (4; 640);
- \(Eomes\), 5\textsuperscript{-}GCAAAAACAAACACACACAC-3\textsuperscript{-} and 5\textsuperscript{-}GGGCG-AAGGACTATACCCA-3\textsuperscript{-} (3; 583);
- \(Fgf2\), 5\textsuperscript{-}ACCAATTACCAATCTTCCCAAC-3\textsuperscript{-} and 5\textsuperscript{-}ATTCA-TTCCACCAATGCG-3\textsuperscript{-} (3; 592);
- \(Furin\), 5\textsuperscript{-}TGCCAGACACATGTACTC-3\textsuperscript{-} and 5\textsuperscript{-}CAAGGAG- TTGGGTGATGAA-3\textsuperscript{-} (3; 320);
- \(Otx2\), 5\textsuperscript{-}AACACGCGAGAGGAGGA-3\textsuperscript{-} and 5\textsuperscript{-}AGAGGG-AGGAAGTGCCACA-3\textsuperscript{-} (1; 400);
- \(Sp4\), 5\textsuperscript{-}GCGCATCACCCAACTACA-3\textsuperscript{-} and 5\textsuperscript{-}ATCCACACTCCACG-3\textsuperscript{-} (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 specificity 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.
Elf5 mutants lack extraembryonic ectoderm

**Results**

Elf5 is expressed in the extraembryonic 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 129:129BALB/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
embryos were severely reduced in size, consisting of a sack of visceral endoderm and no ExE-EmE constriction (Fig. 3B,C). The EPC was present. By E8.5, Elf5−/− embryos still resembled egg cylinders, composed of ectoderm with no mesoderm derivatives. We therefore probed embryos with Oct4/Pou5f1, a marker for undifferentiated embryonic ectoderm (Scholer et al., 1990). We found that Oct4

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

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

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

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 trophoderm lineage (Rossant et al., 2003), is expressed in the polar trophoderm of Elf5−/− blastocysts, but no expression is evident by E5.5 (Fig. 4A,B). Thus from gastrula 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−/− 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.](image-url)
Elf5 mutants lack extraembryonic ectoderm

Elf5 mutants lack extraembryonic ectoderm 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).
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+/– or H11003 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 (Ceri) was expressed in the presumptive AVE of Elf5+/– embryos (Fig. 7B). Similarly, the AVE/anterior neuroectoderm marker Hexx1 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,
Elf5 mutants lack extraembryonic ectoderm

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 (Chavengsaksophak 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. 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 trophectoderm 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. Fgfr2 and Fgfr4 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 Fgfr2 is expressed in the IC 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 spongiotrophoblasts 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 imply that Elf5 is only required after the formation of trophoblast. 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 extraembryonic 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 organisational 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).

Whatsoever 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/EGC decision at the implanted blastocyst stage.

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Supplementary material

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