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)

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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 trophoblast/EExE 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 EExE 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-EExE 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′ genomic clones were isolated from a 129 mouse gene targeting-deficient embryos these two proteases closely resemble AVE 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 trophoblast/EExE 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).

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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: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
embryos, which we term type II half of the E8.5 mutant embryos (Fig. 3D). These marking mesoderm from the onset of gastrulation, was seen in transcribed (Fig. 3E, and data not shown).

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>5 (26%)</td>
<td>14 (50%)</td>
<td>7 (25%)</td>
<td>28</td>
</tr>
<tr>
<td>E12.5</td>
<td>BALB/c</td>
<td>7 (25%)</td>
<td>14 (50%)</td>
<td>7 (25%)</td>
<td>28</td>
</tr>
</tbody>
</table>

*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 constriction (Fig. 3B,C). The EPC was present. By E8.5, Elf5–/– embryos still resembled egg cylinders, composed of ectoderm around the proximal end of the embryo, indicative of delayed aberrant mesoderm 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 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-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...
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).

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).
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.

**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+/−/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 (Cerl) was expressed in the presumptive AVE of Elf5−/− embryos (Fig. 7B). Similarly, the AVE/anterior neuroectoderm 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.
Elf5 mutants lack extraembryonic ectoderm

**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
trophoderm 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 trophoderm 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 cannot 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 trophoderm 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 trophectoderm 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 trophoderm. 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).

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

References


Table S1. Statistics on the variability of marker gene expression in \( Elf5^{-/-} \) embryos (type I mutants show no expression; type II showing ectopic expression)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stage (E)</th>
<th>Expression in ( Elf5^{-/-} ) embryos</th>
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<tr>
<td></td>
<td></td>
<td>Mesoderm/epiblast</td>
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<tr>
<td></td>
<td></td>
<td>( Cripto )</td>
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<tr>
<td></td>
<td>6.5</td>
<td>2/4 type I; 2/4 type II</td>
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<tr>
<td></td>
<td>7.5</td>
<td>1/1 type I</td>
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<tr>
<td></td>
<td>6.5</td>
<td>3/5 type I; 2/5 type II</td>
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<tr>
<td></td>
<td>7.5</td>
<td>2/4 type I; 2/4 type II</td>
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<tr>
<td></td>
<td>7.5</td>
<td>2/4 type I; 2/4 type II</td>
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<tr>
<td></td>
<td>8.5</td>
<td>2/5 type I; 3/5 type II</td>
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<td>5.5</td>
<td>2/2 type II</td>
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<td></td>
<td>7.5</td>
<td>2/2 type I</td>
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<td>5.5</td>
<td>2/2 expression up to EPC</td>
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<td>3/3 expression as in Fig. 3E</td>
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<td>4/4 no expression (type I)</td>
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<td></td>
<td>5.5</td>
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<td>1/1 (distal up to 25%)*</td>
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<td></td>
<td>6.5</td>
<td>2/3 (50%); 1/3 (90%)*</td>
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</tbody>
</table>

*For AVE markers, the position of the anterior edge of staining is given as a percentage of the proximodistal length of the egg cylinder, where 100% would indicate the entire length.