Ets2 is necessary in trophoblast for normal embryonic anteroposterior axis development

Pantelis Georgiades1,* and Janet Rossant1,2,†,‡

Although the trophoblast is necessary for the growth, viability and patterning of the mammalian embryo, understanding of its patterning role is still rudimentary. Expression of the transcription factor Ets2 is restricted to the trophoblast in early postimplantation stages and Ets2 mutants have been previously shown to have defects in trophoblast development. We show here that Ets2 is necessary in the trophoblast for fundamental aspects of anteroposterior (AP) epiblast axis initiation, including mesoderm initiation at the primitive streak, establishment of posterior character in the epiblast and appropriate spatial restriction of the anterior visceral endoderm (AVE). Most homozygous Ets2 mutants also show highly reduced development of the trophoblast with an absence of extraembryonic ectoderm (EXE) markers. Embryos in which the EXE has been physically removed before culture in vitro phenocopy the patterning defects of Ets2 mutants. These defects cannot be rescued by providing Ets2 mutants with wild-type epiblast in tetraploid aggregations. Thus, EXE-derived signals are necessary for normal embryonic patterning. Ets2 is likely to be required in the EXE downstream of epiblast signals, such as Fgf, and, in turn, helps to regulate signals from the EXE that signal back to the epiblast to promote proper primitive streak and AVE development. This study provides new insights about the genetic and cellular basis of the patterning role and development of the early trophoblast.

KEY WORDS: Ets2, Trophoblast, Anteroposterior axis, Embryo patterning

INTRODUCTION

The trophoblast of the definitive placenta in mammals mediates fetomaternial interactions promoting growth and viability of the fetus. The definitive placental stage begins around embryonic day (E) 9.5-10.5 in mice with fusion of the chorion and allantois, and establishment of the placental labyrinth where physiological exchange between fetal and maternal blood circulations can occur. Prior to this stage, the trophoblast destined to become definitive placental trophoblast is sometimes referred to as a ‘preplacental trophoblast’ (PPT), although here the terms ‘trophoblast’ and PPT will be used interchangeably (Burton et al., 2002; Georgiades et al., 2002; Rossant and Cross, 2001). At the blastocyst stage, when the trophoblast lineage is first established, the trophoblast plays a key role in promoting implantation in the uterus. Soon after implantation, but still well before the establishment of the definitive placental stage, the PPT can be subdivided into two main regions: the ectoplacental cone (EPC; the half of PPT that is largely in contact with maternal tissues) and the extraembryonic ectoderm (EXE; the half of PPT that contacts the epiblast). However, the role and development of the PPT from implantation until the establishment of the definitive placenta is largely unexplored.

During this period, the embryonic region undergoes the major events of gastrulation and anteroposterior (AP) axis formation. The first morphological sign of mouse AP patterning within the epiblast is the appearance of ingressing mesoderm at its posterior end of the primitive streak (PS) at E6.5. This signifies the onset of gastrulation, which, in turn, sets in motion many of the subsequent embryonic patterning events. However, for the postimplantation conceptus as a whole, the earliest known morphological and gene expression AP asymmetries are evident within the visceral endoderm (VE), which envelops the epiblast prior to and during epiblast AP patterning. At E5.5, the distal VE (DVE) constitutes a localized VE thickening and displays a unique expression profile (Rivera-Perez et al., 2003). By E5.75, the DVE shifts anteriorly to reach the anterior-most epiblast and becomes known as anterior VE (AVE) (Thomas and Beddington, 1996). It is well established that interactions between the epiblast and the VE are necessary for AP axis formation. For example, Nodal expression within the epiblast is required for mesoderm initiation, DVE formation and anterior DVE shift, and Cripto is necessary in the epiblast for the anterior shift of the DVE (Ding et al., 1998; Lu and Robertson, 2004; Yamamoto et al., 2004). Signaling from the DVE/AVE has been proposed to induce anterior and suppress posterior identity in the epiblast, based on gene knockout, tissue ablation and transplantation experiments (Dufort et al., 1998; Hallonet et al., 2002; Kimura et al., 2000; Perea-Gomez et al., 1999; Thomas and Beddington, 1996). Moreover, the anterior AVE shift per se is necessary for epiblast AP axis formation, as its failure results in posterior epiblast characters being localized proximally and anterior ones distally (Ding et al., 1998; Perea-Gomez et al., 2001).

The involvement of the PPT as a possible source of signals for these events was suggested by several recent findings (Beck et al., 2002, 2005; Donnison et al., 2005; Fujiwara et al., 2002; Rodriguez et al., 2005). For example, the Spc1 and Spc4 genes encoding secreted proteases of the subtilisin-like proprotein convertase (SPC) family, were shown to be required in EXE for the elongation of a pre-existing, mesoderm-producing PS (Beck et al., 2002), although it was unclear from this study whether EXE signaling is also required for mesoderm and PS initiation (Beck et al., 2002; Guzman-Ayala et al., 2004). Another study reported that mutation of the
trophoblast-specific Ets-related factor Eif5 resulted in a loss of EXE in all cases, and in varying defects in mesoderm formation and AP patterning (Donnison et al., 2005). Rodriguez and colleagues demonstrated that removal of the EXE at pre-gastrulation stages resulted in failure to express markers of the primitive streak and also in expansion of the AVE (Rodriguez et al., 2005).

To elucidate further the role of trophoblast signaling in embryonic patterning and to better understand early PPT development, we examined the development of mouse conceptuses homozygous for the previously reported, targeted mutation of Ets2 (Yamamoto et al., 1998). Ets2, which encodes a member of the Ets family of nuclear transcription factors (Sharrocks, 2001), was shown to be expressed exclusively in PPT during the period of embryonic patterning, and Ets2 mutant embryos were reported to show reduced growth of the EPC and to lack the chorion, the derivative of the EXE (Yamamoto et al., 1998). In view of these PPT defects, the possibility that epiblast and VE patterning processes could be affected by the loss of Ets2 function in the trophoblast remained an unexplored possibility.

We report here that loss of Ets2 in the trophoblast can lead to the loss of EXE identity and a failure to produce the appropriate patterning signals from the EXE to the epiblast, thereby resulting in a failure to form a PS or to undergo normal AVE spatial orientation. Our results provide new genetic insights into the pathways of EXE maintenance and function.

MATERIALS AND METHODS

Mice, embryo collection and genotyping
Mice heterozygous for the Ets2 targeted mutation Ets2\(^{+/-}\)/Ets2\(^{+/-}\) on a Swiss Black/129Sv genetic background (Yamamoto et al., 1998) were maintained by crossing to outbred ICR strain mice. Homozygous Ets2\(^{+/-}\)/Ets2\(^{+/-}\) conceptuses were collected from intercrosses of Ets2\(^{+/-}\)/Ets2\(^{+/-}\) heterozygotes. Genotyping was performed on individually isolated embryos, on Richert’s membrane directly, or on isolated embryos following in situ hybridization. This involved two separate PCR reactions with a primer set that produced either the wild-type (220 bp) or mutant (200 bp) sequences, which were resolved on a 1.5% agarose gel as previously described (Yamamoto et al., 1998). The conditions for both PCR reactions were: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 64°C for 30 seconds and 74°C for 4 seconds; then 72°C for 5 minutes. For the detection of the wild-type DNA the primers used were: 5’-CGTCCCTACTGGATGACAGCGG-3’ and 5’-TGGTTTG-GTCAAAATAGGAGCCACTG-3’. For the detection of the mutant DNA the primers used were: 5’-CGTCCCTACTGGATGACAGCGG-3’ and 5’-AATGACAAGACGCTGGGCGG-3’.

Whole-mount in situ hybridization and histology
Single-color whole-mount in situ hybridization, with either one probe or two probes simultaneously, was carried out essentially as previously described (Lickert et al., 2002), with the exception that the proteinase digestion step was replaced by a 15-minute incubation at room temperature with RIPA reagent (150 mM NaCl, 1% Nonidet P-40, 0.5% Na deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 8). Double-color whole-mount in situ hybridization was carried out as above with an additional color development of Ets2 function in the trophoblast remained an unexplored possibility.

Generation of chimeric conceptuses
Wild-type 129S6/B6-F1 hybrid ES cells ubiquitously expressing GFP (Vintersten et al., 2004) were aggregated with tetraploid embryos (one tetraploid embryo per ES cell clump), and were subsequently transferred to recipient ICR females and the resulting chimeras collected at E7.75. The tetraploid embryos were produced from two-cell stage embryos derived from intercrosses of Ets2\(^{+/-}\)/Ets2\(^{+/-}\) heterozygotes, followed by electrofusion, as previously described (Nagy, 2002). Upon embryo isolation, the Richert’s membrane, which is exclusively derived from the tetraploid embryo, was genotyped as described above.

Embryo culture and microsurgery
E5.5 or E6.0 conceptuses, derived from matings between mice homozygous for a GFP transgene under the control of Oct4 regulatory elements (generous gift of A. Nagy, Samuel Lunenfeld Research Institute, Toronto, Canada), were isolated free from their Richert’s membrane and cultured intact or after trophoblast ablation for either 32-36 (for E5.5) or 24 (for E6.0) hours. E5.5 conceptuses were typically isolated between 11 am and 1 pm on the fifth day (plug day=day 0) and were morphologically staged so that only those conceptuses with a strictly distal VE thickening were chosen. E6.0 conceptuses were isolated around midnight of the fifth day. They were cultured in serum-free conditions in a pre-equilibrated medium containing 60% (v/v) serum replacement for embryonic stem cells (GIBCO), and 40% of DMEM, 1 mM Sodium pyruvate, 4 mM l-glutamate and 100 mM β-mercaptoethanol. The conditions were 37°C, 5% CO\(_2\) in atmospheric oxygen. For trophoblast ablation, conceptuses were immobilized and a cut was made along the embryonic-extraembryonic junction using finley pulled 1-mm-thick glass rods (World Precision Instruments). Most trophoblast-ablated conceptuses healed within 30 minutes and only those that healed were kept for further study.

RESULTS

Ets2 is exclusively expressed in the trophoblast prior to and during embryonic AP patterning
Although Ets2 was previously shown to be expressed in PPT from at least E6.0, the earliest known AP patterning events begin around E5.5 with the onset of the anterior DVE shift. To investigate whether Ets2 could be involved in these events, we examined its expression from pre-implantation (E3.5) up until the early headfold stage (E7.75). At E3.5 and E4.5, Ets2 transcripts are not detectable in the polar trophoderm (the PPT progenitor), or in the inner cell mass (ICM; the epiblast and VE progenitor; Fig. 1A and data not shown). However, Ets2 is expressed from E5.0 to E6.75 and its expression is PPT specific (Fig. 1B-D). By E7.75, Ets2 expression becomes downregulated from the PPT and begins to appear in the PS (Fig. 1E).

Ets2\(^{+/-}\)/Ets2\(^{+/-}\) conceptuses display two main types of phenotypes
We generated and genotyped Ets2\(^{+/-}\)/Ets2\(^{+/-}\) conceptuses using a PCR-based approach, as previously described (Yamamoto et al., 1998). In accordance with previous observations, heterozygous mutants were indistinguishable from their wild-type littermates. Gross examination of all homozygous mutants from E6.75 onwards revealed small size, reduced accumulation of maternal blood around the implantation site and resorption by E9.5 (Yamamoto et al., 1998). Although all mutant embryos were much smaller than wild types from E6.75, two classes of mutants could be detected. The majority of Ets2\(^{+/-}\)/Ets2\(^{+/-}\) conceptuses, henceforth referred to as type I mutants, had a small PPT region relative to rest of the conceptus, whereas the PPT in type II mutants was in proportion to the size of the rest of the conceptus (Fig. 1F-H; Table 1). Based on histology at E6.75, type I, but not type II, mutants showed an absence of mesoderm and their VE was abnormally thickened in the anterior-distal tip region (Fig. 1F-H).

"Development 133 (6)"
Both type I and type II mutants express the EPC marker Mash2 in their PPT. In both cases, the expression domain of Mash2 does not extend to the EXE-epiblast boundary (Fig. 2A,I), and type I mutants do not express the five independent EXE markers (Cdx2, Bmp4, Spc4, Errβ and Sox2) at either E6.75 or E7.75 (n=3 for each probe and stage; Fig. 2B-G). By contrast, type II mutants do possess EXE and EPC, as judged by Cdx2, Errβ and Mash2 expression at E6.75 and E7.75 (n=3 for each probe and stage) (Fig. 2H,I and data not shown). Double color in situ hybridization with Mash2 and the epiblast marker Oct4 demonstrated that there is a clear extraembryonic domain between the Mash2-positive PPT and the Oct4-positive epiblast of E6.75 type I mutants (Fig. 2J).

Examination of 84 conceptuses at E5.5 derived from Ets2<sup>db1/db1</sup>/Ets2<sup>db2/db2</sup> intercrosses revealed that 12 (14.3%) had a small PPT. Genotyping confirmed that these E5.5 conceptuses were Ets2<sup>db1/db1</sup>/Ets2<sup>db2/db2</sup> (Fig. 2K and data not shown). Double in situ hybridization with either Hex or Cerberus, and either Cdx2 or Bmp4 (n=7), revealed an absence of EXE markers prior to the DVE-to-AVE transition (Fig. 2L,M).

Expression of Nodal and Fgf4 in type-I Ets2 mutants

Our findings demonstrate that, in type I Ets2 mutant embryos, the distal PPT fails to display any EXE identity, but does not adopt an EPC phenotype. Recent experiments suggested that secreted signaling molecules from the epiblast, Nodal and Fgf4, are involved in EXE maintenance, with Fgf4 inhibiting EPC identity and Nodal plus Fgf4 promoting EXE fate (Guzman-Ayala et al., 2004). Examination of Fgf4 expression at E6.75 revealed that in contrast to the wild-type situation, type I mutants show no expression of this gene (n=3; Fig. 2N), perhaps due to the fact that these mutants lack a PS, as Fgf4 at this stage normally marks the distal PS rather than the epiblast. However, Fgf4 expression was detected in the epiblast of mutants (n=3) at E5.5 (Fig. 2O). We next investigated Nodal expression and showed that in type I (n=3), but not type II (n=2) mutants, Nodal expression is reduced and fails to become posteriorized (Fig. 2P). A similar abnormality in the locality of the Nodal expression pattern was seen in a subset of mutants lacking one of the Nodal enhancers that contains binding sites for the

Table 1. Classification of mutant embryos at different stages: frequency of Ets2 homozygous mutant phenotypes

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total number of implantation sites</th>
<th>Type I homozygous mutants</th>
<th>Type II homozygous mutants</th>
<th>Undetermined homozygous mutants</th>
<th>Total number of homozygous mutants</th>
<th>Resorptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6.75</td>
<td>426</td>
<td>48 (11.3%)</td>
<td>35 (8.2%)</td>
<td>7 (1.6%)</td>
<td>90 (21.1%)</td>
<td>14 (3.3%)</td>
</tr>
<tr>
<td>E7.75</td>
<td>332</td>
<td>36 (10.8%)</td>
<td>29 (8.7%)</td>
<td>3 (0.9%)</td>
<td>68 (20.4%)</td>
<td>13 (3.9%)</td>
</tr>
<tr>
<td>E8.50</td>
<td>96</td>
<td>7 (7.3%)</td>
<td>9 (9.3%)</td>
<td>0</td>
<td>16 (16.6%)</td>
<td>7 (7.3%)</td>
</tr>
<tr>
<td>E9.50</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>1 (3.6%)</td>
<td>2 (18%)</td>
<td></td>
</tr>
</tbody>
</table>

Total numbers and percentages (in parentheses) of genotyped homozygous mutants and their morphological grouping into type I or type II, based on the relatively small size of the PPT and the thickened distal-anterior VE, in type I, but not type II, mutants (see text). Mutants were classified as ‘undetermined’ with respect to whether they are type I or type II either because of damage during dissection or because they were necrotic (asterisk). No attempt was made to genotype resorptions because of the uncertainty regarding DNA quality and the impossibility of distinguishing with certainty between zygote-derived and maternal material.

Fig. 1. Ets2 expression profile and morphological classification of Ets2<sup>db1/db1</sup>/Ets2<sup>db2/db2</sup> conceptuses. (A-E) Ets2 expression from pre-implantation (E3.5) to early head-fold stage (E7.75). Note that Ets2 is not expressed in the polar trophectoderm prior to implantation, but its expression is trophoblast specific from E5.0-E6.75, and by the early head-fold stage it is also expressed in the PS (asterisk in E). (F-H) Conceptuses on the left of each image are whole-mount views; those on the right are sagittal semithin sections of the same type of embryo. Type-I (H) and type-II (G) Ets2 homozygous mutants tend to be smaller than their wild-type (F) littermates. Type I mutants also have a short PPT, as judged by the position of the embryonic-extraembryonic junction (small arrows), and an abnormally thickened anterior-distal VE (dashed lines), and show absence of mesenchymal (m) cells. Whole-mount images in A-D, and in F-H are of the same magnification. Sections in F-H are of the same magnification with the exception that the right image in H is an enlargement of the image to its left. Scale bars: 100 μm.
transcription factor Foxh1 (Norris et al., 2002), or in a subset of homozygous Foxh1 mutants (Yamamoto et al., 2001). We therefore examined the expression of the Foxh1 transcription factor, which is known to be involved in Nodal signaling (Norris et al., 2002; Yamamoto et al., 2001), and show that it is also not expressed in type I mutants (n=3; Fig. 2Q). These results indicate that, in the epiblast of type I mutants, the upregulation and posteriorization of Nodal, but not the epiblast expression of Fgf4, are dependent on Ets2.

**Lack of PS and mesoderm initiation in type I, but not type II, Ets2 mutants**

We examined the expression of Oct4 (n=3; an undifferentiated epiblast marker), T (n=4), Hoxb1 (n=3) and Cripto (n=3) (a PS and mesoderm markers) from E6.75 to E7.75. The type I epiblast remained undifferentiated and failed to express any mesoderm markers (Fig. 3A-F). Interestingly, Wnt3, a gene previously shown to be necessary in vivo for both PS and mesoderm initiation (Liu et

---

**Fig. 2. Trophoblast defects in type I mutants. (A-Q)** All conceptuses on the left of each image are either wild type or heterozygous for the Ets2 mutation, and those on the right are either type I or type II homozygous mutant littermates, showing the expression profile (either single- or double-probe in situ hybridization) of the indicated gene transcripts. All are sagittal views and, where applicable, posterior is to the right. The morphologically identifiable embryonic-extraembryonic junction is denoted by arrowheads. (A-E,J,N,Q) E6.75 control conceptuses and their type I mutant littermates, with the exception of Q, where a type II mutant is also included. For A and J, the image on the right is a magnification of the image to its left. (F,G) E7.75 control conceptuses and their type I mutant littermates. The chorion is marked by an asterisk. (H,I) E5.5 control conceptuses and their homozygous Ets2-db1/Ets2-db1 mutant littermates with a reduced trophoblast region (prospective type I mutants). Conceptuses in K are semithin sections. Scale bars: 100 μm. All images without scale bars have the same magnification as A.
al., 1999), is not expressed in type I mutants (n=3) (Fig. 3G). Histologically, type II mutants showed signs of PS and mesoderm formation (Fig. 1H), and this was confirmed molecularly by the expression of T (n=3) at E6.75 in these mutants (Fig. 3H), and by double in situ hybridization using T- and Cdx2-specific probes (n=3; Fig. 3I). In addition, the type I epiblast remains undifferentiated, as judged by the expression of Oct4 at E7.75 (Fig. 3B), and fails to express any anterior neural markers such as Sox1 (n=3; Fig. 3J), Sox2 (n=3; Fig. 2G) and Gbx2, either at E7.75 (n=3) or E8.5 (n=3; Fig. 3K,L).

**Failure in the completion and maintenance of the anterior DVE shift in type I, but not type II, mutants**

Based on histology, the AVE of type I mutants does undergo some anterior shift, but it is incomplete as its anterior edge never reaches the embryonic-extraembryonic junction even by E6.75; this process during normal development is complete by E5.75 (Fig. 1H).

Having already shown that the DVE in E5.5 mutants forms normally in the absence of EXE (Fig. 2L,M), we used Hex and cerberus expression to investigate the subsequent anterior DVE shift in Ets2 homozygous mutants. We confirm by molecular markers (n=5 for Hex; n=3 for cerberus), that the AVE shift is incomplete (Fig. 4G,H). Furthermore, the expression of AVE markers is not well confined to the midline but shows a broad expansion of expression over the surface of the anterior epiblast (Fig. 4G,H). Using either Hex-Cdx2 (n=2) or Hex-T (n=2) double in situ hybridization, we demonstrate that this VE abnormality is present only in type I mutants (Fig. 4J,K). By E7.75, the majority of type I mutants examined (4/6) show a strictly distal Hex expression domain (Fig. 4I), whereas a minority (2/6) display the same abnormal localization of the AVE as is seen at E6.75 (data not shown). The type I AVE defect therefore does not reflect a developmental delay in AVE formation, and, by E7.75, the initially incomplete anterior displacement of AVE becomes distally localized in the majority of cases.

The effect of the abnormal AVE localization in type I mutants on AP patterning in the epiblast was studied by examining the localization of Otx2 expression. As expected from the abnormal localization of the AVE, Otx2 expression in E7.75 type I (n=3) mutants is confined to the distal epiblast; this is in contrast to their wild-type counterparts, where it is expressed anteriorly (Fig. 4M). The positioning of posterior epiblast markers cannot be assessed because the absence of PS, mesoderm and related posterior epiblast genes from type I mutants means that there are no posterior epiblast characteristics to be abnormally localized.

**Ets2 function is solely required in trophoblast for embryo AP patterning**

The type I patterning defects, the early lethality of both types of Ets2 mutant, and the trophoblast-specific expression of Ets2 prior to and during patterning, in conjunction with the previously reported rescue of this lethality in chimeric mice with wild-type extraembryonic tissues and Ets2b1/Ets2b1 embryo proper (Yamamoto et al., 1998), suggest that Ets2 function in PPT is required for all the patterning defects reported here. However, the possibility that the type I phenotype could require defective Ets2 function in both the epiblast and PPT cannot be excluded.
To resolve this issue and to directly show that the disruption of Ets2 expression in the trophoblast is responsible for the epiblast and VE defects observed in type I mutants, we generated chimeric conceptuses with an Ets2$^{db1}$/Ets2$^{db1}$ trophoblast and a wild-type epiblast. The chimeras were derived from the aggregation of green fluorescent protein (GFP)-positive wild-type embryonic stem (ES) cells with tetraploid Ets2$^{db1}$/Ets2$^{db1}$ embryos (2N ES$^{+/+}$ 4N Ets2$^{db1}$/Ets2$^{db1}$). In this type of chimera, the epiblast and all its derivatives are ES-cell derived and GFP positive, whereas the VE and PPT are tetraploid-embryo derived and GFP negative. Chimeras were PCR genotyped using the exclusively extraembryonic parietal endoderm on Reichert’s membrane. 2N ES$^{+/+}$ 4N Ets2$^{db1}$/Ets2$^{db1}$ conceptuses at E7.75 display all the hallmarks of natural mated-derived E7.75 type I mutants. They do not express the PS and mesoderm marker $T$ ($n$=2), their Hex-positive AVE is either distal (2/3) or anterior-distal, resembling that in E6.75 type I mutants (1/3; data not shown), and their epiblast still expresses Oct4 ($n$=2; Fig. 5A-D). Chimeras with a type II phenotype were also detected (P.G., unpublished).

These findings indicate directly that the lack of PS and mesoderm, and the abnormal localization of AVE in type I Ets2$^{db1}$/Ets2$^{db1}$ conceptuses, are solely due to the absence of a functional Ets2 in the PPT.

**Cultured wild-type conceptuses lacking EXE from E5.5 phenocopy the type-I embryonic defects**

To determine whether the loss of epiblast patterning in type I Ets2$^{db1}$/Ets2$^{db1}$ mutants was due to the absence of EXE character, we investigated whether wild-type conceptuses that develop in the absence of EXE, phenocopy the type I patterning defects.

We developed a serum-free embryo culture system to culture E5.5 intact or trophoblast-ablated conceptuses for 30-34 hours, by which time intact conceptuses reached the early-to-mid PS stage. Only those E5.5 conceptuses with a strictly distal VE thickening were chosen to ensure that the experiment started prior to the beginning of the anterior shift of DVE (Fig. 6A). This thickening was shown previously to express Hex, a gene that marks the DVE/AVE prior to and during its anterior shift (Rivera-Perez et al., 2003). Conceptuses

---

**Fig. 4. VE defects in type-I mutants.** (A-M) Sagittal views, posterior to the right. The morphologically identifiable embryonic-extraembryonic junction is denoted by arrowheads and the VE area below each arrow denotes the VE of the distal tip. Dashed lines represent the DVE/AVE region and asterisks the definitive endoderm. (A-F) Wild-type conceptuses at the stages indicated showing either the Hex or cerberus expression profile. (G,H,J,K,L) All conceptuses on the left of each image are either E6.75 wild type or heterozygous for the Ets2 mutation, and those on the right are either type I and/or type II homozygous mutant littersmates, showing the expression profile (either single- or double-probe in situ hybridization) of the indicated gene transcripts. The image on the right in H is a sagittal section of the image to its left. (I) E7.75 type I mutant (left) and its sagittal section (right), showing expression of Hex. (M) E7.75 control conceptus (left) and its type I mutant littermate (right), showing expression of Otx2. Scale bars: 100 μm; in A for A-D; in E for E-H,J,K.
transgenic for GFP under the control of the epiblast-specific gene Oct4 (Fig. 6A) were dissected into embryonic (Epi+VE, Fig. 6B) and abembryonic portions (PPT, Fig. 6B) using glass needles. To assess the accuracy of trophoblast-ablation, some conceptuses were examined immediately after surgery for the expression of the EXE marker Cdx2. Ninety-three percent (26/28) of trophoblast-ablated embryos showed no Cdx2 expression (Fig. 6C). Conversely, very few dissected conceptuses showed carry-over of Oct4-GFP expression in the extraembryonic fragments (Fig. 6A). This demonstrates the accuracy of the approach and ensures that any effects of the separation of embryonic and abembryonic regions are not likely to be due to either EXE remnants still attached to the epiblast or the removal of the proximal epiblast during dissection.

E5.5 trophoblast-ablated conceptuses, unlike their intact counterparts, essentially mimic the epiblast phenotype of type I mutants, as judged by histology (n = 4), by the continued expression of Oct4 (n = 3), by the absent expression of T (n = 7), Cripto (n = 5) and Wnt3 (n = 3), and by the reduced expression of Nodal (n = 3; Fig. 6D–I). When the same trophoblast ablation was carried out at E6.0, followed by culture for 24 hours, mesoderm and PS do form, as judged morphologically (n = 2), and by T (n = 6) and Cripto (n = 5) expression (Fig. 6J, data not shown). E5.5 trophoblast-ablated conceptuses do maintain their DVE/AVE, as judged by Hex/cerberus-positive versus Hex/cerberus-negative regions of the VE (Fig. 6K–L).

**DISCUSSION**

We show that the transcription factor Ets2 is required in the trophoblast for the earliest and most fundamental events of embryonic AP patterning, PS and mesoderm initiation from the epiblast, and the completion and maintenance of the anterior shift of DVE. Ets2 is specifically required in trophoblast for the expression of EXE characteristics from E5.5 onwards, as the trophoblast region in contact with the epiblast fails to express EXE markers in type I mutants. Interestingly this trophoblast region does not express markers of the EPC, such as Mash2, either. This is in contrast to the observations made in a subset of Spp4/Spcl and Nodal null mice (Guzman-Ayala et al., 2004), where the entire post-implantation trophoblast region adopted an EPC fate when Nodal signaling from the epiblast was lost.

The epiblast patterning defects seen in type I Ets2 mutants can be solely attributed to the defects in the development of the EXE, thus strongly supporting a direct role for the EXE in signaling to the epiblast for PS and mesoderm initiation. First, Ets2 expression is detectable specifically in the trophoblast before and during the early streak stages of development, when the mutant phenotype starts to become apparent in the epiblast. Second, our tetraploid-ES chimera work shows directly that absence of Ets2 in the trophoblast can lead to epiblast patterning defects. Third, early patterning defects only occur in type I, but not type II mutants, correlating directly with the absence or presence of EXE markers. Fourth, absence of EXE marker gene expression is evident from E5.5 in the prospective type I mutants, well before any of the AP patterning processes affected in type I mutants normally begin, excluding the possibility that the absence of EXE markers is a consequence rather than a cause of type I embryo patterning defects. Fifth, E5.5, but not E6.0, wild-type trophoblast-ablated conceptuses lacking EXE phenocopy the type I epiblast defects.

While this work was in progress, two recent papers have also provided evidence that trophoblast signaling is required for inducing posterior epiblast identity. All conceptuses with a targeted deletion of another trophoblast-specific Ets-related gene, Elf5 (Donnison et al., 2005), were reported to lack EXE from E5.5, although only about half of them were shown to fail to form mesoderm as judged by T and Cripto expression. This work therefore left open the question of whether EXE signaling is required for mesoderm formation, because those Elf5 mutants with mesoderm, were reported to also lack EXE. Our work, however, using double in situ hybridization with mesoderm and EXE markers on both types of Ets2 homozygous mutants, plus our culture of wild-type trophoblast-ablated conceptuses lacking EXE, provides strong evidence that EXE signaling is indeed required for mesoderm formation. Consistent with our results, Rodriguez and colleagues (Rodriguez et al., 2005) have also demonstrated that removal of the EXE at pre-gastrulation stages following culture in the presence of serum results in failure to express markers of the PS, and that the ectopic transplantation of EXE cells could induce ectopic T expression.

Rodriguez et al. also reported an expansion of the AVE in trophoblast-ablated conceptuses (Rodriguez et al., 2005). Our results with such cultured EXE-deleted embryos do not show such
a dramatic expansion of DVE/AVE markers. This difference may be a result of the serum-free conditions we used, which may not promote full expansion of the DVE/AVE. However, we do see defects in the localization of the AVE in type I mutant Ets2 embryos in vivo, supporting for the first time an in vivo role for the EXE in the proper localization of the AVE.

All of these results strongly suggest that the EXE is a key source of signals to pattern the early embryo. Our work, taken together with the findings of others, provides evidence for a bidirectional signaling model in which Fgf4 and Nodal from the epiblast signal to maintain the EXE, which in turn produces direct signals to the epiblast, such as Bmp4, and indirect signals, such as the Spc proteases, which act on the epiblast to promote mesoderm development and posterior epiblast identity, probably largely by modulating Nodal activity.

Fgf4 is known to be required for maintenance of the trophoblast stem cells of the EXE both in vivo (Goldin and Papaioannou, 2003) and in vitro (Tanaka et al., 1998). Recent evidence from the Constam Laboratory suggests that the default state of pre-gastrulation PPT is EPC, and that the EXE is maintained by Fgf4-mediated inhibition of EPC formation in cooperation with active Nodal signaling (Spc-mediated processed Nodal) (Guzman-Ayala et al., 2004). Our type I PPT phenotype is consistent with this model because Fgf4 expression is still present in the pre-gastrulation epiblast of type I Ets2 mutants, suggesting that correct Fgf4 signaling from the epiblast can still inhibit the distal PPT from adopting an EPC fate. The reduced Nodal expression in type I mutants provides an explanation for the apparent absence of EXE identity in the distal PPT of type I mutants: Fgf4 alone would be sufficient to block EPC markers but not sufficient to induce EXE markers if Nodal signaling is defective (Guzman-Ayala et al., 2004). The barely detectable Nodal expression in Ets2 mutants suggests defective Nodal signaling from the epiblast to the trophoblast, as well as within the epiblast, in view of the known autoinductive function of Nodal for amplifying its own expression (Beck et al., 2002; Guzman-Ayala et al., 2004; Norris et al., 2002). The reduced Nodal expression in type I mutants can be attributed to an intrinsic requirement for Ets2 in PPT for the induction of the EXE signals responsible for elevating Nodal expression in the epiblast. These signals would include the secreted proprotein convertases Spc1/Spc4. Spc1/Spc4 in the EXE are required for the post-translational activation of the secreted form of Nodal, which is necessary for elevating functional Nodal levels in the epiblast and Bmp4 levels in the EXE (Beck et al., 2002; Guzman-Ayala et al., 2004), and their expression is lost in Ets2 mutants. Because it is known that Nodal plays a key role in establishing AP patterning in the embryo (Brennan et al., 2001; Liu et al., 1999; Lu and Robertson, 2004), most of the effects on epiblast

---

**Fig. 6. Patterning defects in wild-type cultured trophoblast-ablated conceptuses.** (A) Live view of a typical wild-type E5.5 conceptus transgenic for an epiblast-specific GFP transgene used for trophoblast ablation. Note the strictly distal VE thickening (dashed lines) and the morphologically identifiable embryonic-extraembryonic junction (arrowhead). (B) Outline of the microsurgery procedure used for trophoblast ablation: the preplacental trophoblast (PPT) was separated from the epiblast-visceral endoderm (Epi-VE) by a cut along the embryonic-extraembryonic junction. This was followed by culture of the Epi-VE for approximately 30 hours under serum-free conditions. To control for the accuracy of the ablation, only those Epi-VE whose PPT had no GFP positivity were used. (C) To further control for the accuracy of ablation, several PPT and Epi-VE fragments were fixed immediately after ablation and subjected to in situ hybridization with the EXE-specific Cdx2 probe, to ensure that any ‘trophoblast contamination’ left on the Epi-VE to be cultured was minimized. (D-I, K, L) All conceptuses on the left are intact stage E5.5 and those on the right are trophoblast-ablated at the same stage, then cultured for 30 hours. The expression profile of the indicated gene transcripts is shown. All are sagittal views (where applicable, posterior is on the right). Note, in the semithin sections (D), the PS-derived mesenchyme (m) in the intact, but not in the trophoblast-ablated, conceptus. (J) Semithin section (left) and whole-mount in situ hybridization with T (right) of conceptuses trophoblast-ablated at E6.0, then cultured in serum-free conditions for 24 hours. Scale bars: 100 μm; in E for E-I, K, L.
patterning in Ets2 mutants can be explained by the reduced signaling of activated Nodal in the epiblast. Given that Bmp4 is also known to induce certain posterior mesoderm cell types, including extraembryonic mesoderm and germ cells (Fujiiwara et al., 2001; Lawson et al., 1999), the major defects in posterior development seen in Ets2 mutants are probably caused by a combination of the loss of both Bmp4 and Nodal signaling. A reduction in active Nodal in the epiblast would also lead to a loss of signaling back to the trophoblast for the induction and/or maintenance of EXE identity. Thus the initial cell intrinsic loss of EXE markers in the trophoblast of Ets2 mutants would be compounded by a subsequent loss of the Nodal signaling in the epiblast that would normally act to help maintain the EXE region.

The data presented here show that trophoblast signaling is also necessary for the completion and maintenance of the anterior DVE shift. The developmental consequences of this on epiblast AP patterning could be important in view of the fact that the AVE has been proposed to induce anterior epiblast characters and to suppress posterior ones (Hallonet et al., 2002; Perea-Gomez et al., 2001) (Fig. 4M).

One scenario to explain the DVE/AVE defect of type I mutants (localized beneath the distal tip epiblast and the distal half of the anterior epiblast, as opposed to beneath the proximal half of the anterior epiblast) could be that the reduced levels of Nodal observed in these mutants as a result of the lack of EXE [caused by the apparent absence of EXE-derived Spc1/Spc4, previously shown to be required for the elevation, but not for the initial induction of Nodal levels in the epiblast (Beck et al., 2002; Guzman-Ayala et al., 2004)] could be sufficient for DVE formation and for some limited anterior DVE shift, and that EXE-dependent Cripto expression could be responsible for the completion of this DVE shift so that it becomes correctly localized. This scenario is consistent with the following findings. (1) Although a complete lack of Nodal signaling results in an absence of the DVE (Brennan et al., 2001), reduced Nodal signaling [as is the case for the majority of double mutants lacking both functional alleles of Foxh1 and one allele of Nodal (Yamamoto et al., 2001; Yamamoto et al., 2004)] is sufficient for DVE formation, albeit abnormally localized. (2) Low Nodal levels could be sufficient for an initial anterior DVE shift, because during normal development, between E5.5 and E5.7, when the DVE just could be sufficient for an initial anterior DVE shift, because during DVE formation, albeit abnormally localized. (3) An absence of Cripto could be responsible, in a genetic background-dependent fashion, for either no effect on AVE formation, a complete failure of AVE formation, or a partial failure of AVE formation, as in one genetic background Cripto nulls displayed normal AVE formation (Xu et al., 1999), whereas in a different, but mixed, genetic background the Cripto mutation resulted in either a distally localized AVE, or a ‘slightly askew’ or ‘broad and asymmetric’ DVE/AVE localization (Ding et al., 1998). The proximal, as opposed to posteriorized, expression pattern of Nodal in type I mutants is consistent with the proposed role of Nodal in correctly localizing the DVE/AVE by having a proliferative/anti-migratory effect on the VE cells close enough to ‘experience’ the Nodal signal (Yamamoto et al., 2004) because it provides an explanation (not mutually exclusive with the absence of Cripto) as to why the incomplete shift of type-I DVE/AVE does not progress to reach the anterior embryonic-extraembryonic junction.

The growth retardation observed in both types of Ets2 mutant embryos could be attributed to the previously shown abnormal interactions between trophoblast and maternal cells, as judged by the defective anatomy and gene expression at the implantation site within both the EPC and the maternal decidua. The apoptosis detected within the Ets2 mutants was also suggested to contribute to this (Yamamoto et al., 1998).

In conclusion, type I Ets2 mutant mice provide new insights about early trophoblast development and how the trophoblast influences fundamental embryonic AP patterning events.

We thank all members of the Rossant Lab (current and former) for helpful discussions and technical advice. We are especially thankful to Yoijiro Yamanaka for advice on embryo isolation and culture, Heiko Lickert for advice on in situ hybridization, Jorge Cabecas for invaluable assistance with animal husbandry and technical advice, and Ken Harpal and Dough Holmyard for embryo sectioning. We also thank Andreas Nagy for the Oct4-GFP transgenic mice. We thank Dr Robert Oshima for providing the Ets2 mutant mice. This work was supported by a grant and a Distinguished Investigator award from the Canadian Institutes of Health Research to J.R.

References


Luo, J., Sladek, R., Bader, J. A., Mattheyssen, A., Rossant, J. and Giguere, V.


