Induction and migration of the anterior visceral endoderm is regulated by the extra-embryonic ectoderm

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Summary

The anterior visceral endoderm (AVE) is an extra-embryonic tissue required for specifying anterior pattern in the mouse embryo. The AVE is induced at the distal tip of the 5.5 dpc embryo and then migrates to the prospective anterior, where it imparts anterior identity upon the underlying epiblast (the tissue that gives rise to the embryo proper). Little is known about how the AVE is induced and what directs its migration. In this paper, we describe an essential role for another extra-embryonic tissue, the extra-embryonic ectoderm (ExE), in patterning the AVE and epiblast. Removal of the ExE in pre-gastrulation embryos leads to ectopic AVE formation, to a failure of AVE cell migration and to the assumption by the entire epiblast of an anterior identity. Ectopic transplantation of ExE cells inhibits AVE formation and leads to an expansion of the posterior epiblast marker *T*. These results demonstrate that the ExE restricts the induction of the AVE to the distal tip of the mouse embryo and is required to initiate the migration of these cells to the prospective anterior. Together, these data reveal a novel role for the ExE in the specification of the anteroposterior axis of the mouse embryo.

Key words: Mouse, Extra-embryonic ectoderm, AP patterning, Anterior visceral endoderm, Migration

Introduction

It is becoming apparent that the anterior-posterior axis (AP) of the mammalian embryo is established by a complex set of interactions between the embryonic and extra-embryonic tissues of the embryo. Just after implantation, at 5.0 days post coitum (dpc), the mouse embryo is composed of three tissues: the extra-embryonic ectoderm (ExE) and visceral endoderm (VE), both of which are extra-embryonic; and the epiblast, which is embryonic. At this stage, the embryo has not developed an AP axis but has a clear proximal-distal (PD) axis, with the extra-embryonic ectoderm situated proximally and the tip of the epiblast situated distally. Two signalling centres are crucial for the establishment of the AP axis, one located in the anterior visceral endoderm (AVE) and the other in the proximal epiblast (Ang and Constam, 2004). At 5.0 dpc, the epiblast induces the AVE to form from the most distal visceral endoderm cells of the embryo (Brennan et al., 2001). The AVE is characterised by the expression of a unique set of molecular markers [such as *Hex* (*Hhex* – Mouse Genome Informatics) *Lhx1*, cerberus-like 1 (*Cer1*) and *Lefty1*] and is required for the correct specification of anterior neural identity (Beddington and Robertson, 1999). Between 5.0 and 5.5 dpc, the second signalling centre is induced in a rim at the boundary between the epiblast and the ExE. Expressed in this centre are signalling molecules such as Nodal and Wnt3. These molecules are responsible for specifying posterior cell fate [characterised by molecular markers of mesoderm such as brachyury (*T*) and *Fgf8*].

Accumulating evidence indicates that reciprocal interactions between the epiblast, VE and ExE are essential for establishing both these signalling centres. For example, recombination experiments (Yoshimizu et al., 2001), analysis of mice mutant for the Tgfβ factor *Bmp4* (Lawson et al., 1999) and for its receptor *Alk2* (de Sousa Lopes et al., 2004), and expression of a constitutively active form of Alk2 in the VE (de Sousa Lopes et al., 2004) have identified a role for the ExE and proximal VE in the induction of primordial germ cells in the proximal epiblast. Similarly, analysis of mutations in the Tgfβ family member *Nodal* and its intracellular signal transducer *Smad2*, have shown that Nodal signals from the epiblast are essential for inducing the AVE in the distal tip of the embryo at 5.0 dpc and for maintaining gene expression in the ExE (Brennan et al., 2001).

Lineage analysis (Thomas et al., 1998) and in vivo time

lapse imaging (Srinivas et al., 2004) have shown that shortly after its induction, and in response to unknown cues, the cells of the AVE migrate from the distal tip to the prospective anterior of the embryo. Once the AVE has migrated to the prospective anterior, it plays a crucial role in restricting proximal epiblast markers to the posterior, either by repressing expression of these markers in the anterior epiblast or by causing epiblast cells to move to the posterior of the embryo (Kimura et al., 2000; Lu and Robertson, 2004; Perea-Gomez et al., 2001). Therefore, AVE migration converts the PD axis of the embryo into an AP axis.

Although *Nodal* has been shown to be essential for AVE induction (Brennan et al., 2001), at the time when the AVE is induced in a localised region of the visceral endoderm, *Nodal* is expressed in a widespread fashion throughout the epiblast and visceral endoderm (Varlet et al., 1997). The mechanism by which AVE induction is restricted to the distal tip of the embryo and what then directs the migration of the AVE cells is unknown. Similarly, how gene expression in the proximal epiblast is established is not well understood. In this paper, we identify several previously unappreciated roles for the ExE: in restricting the induction of the AVE to the distal tip of the 5.5 dpc embryo, in initiating the migration of the AVE cells and in inducing mesoderm markers in the proximal/posterior epiblast. We therefore conclude that by patterning the visceral endoderm and the epiblast, the ExE plays a crucial role in setting up the future AP axis of the mouse embryo.

Materials and methods

Embryo dissections

Embryos carrying the *Hex*-GFP transgene were derived from *Hex*-GFP mice (Rodriguez et al., 2001) maintained on a mixed CBA/J and C57BL6 background. Embryos used for whole-mount in situ hybridization were from crosses between F1 hybrids (CBA/J×C57BL10). All mice were maintained on a 10-hour light/14-hour dark cycle. Noon on the day of finding a vaginal plug was designated 0.5 dpc. All embryos were dissected in M2 medium as described (Beddington, 1987). *Hex*-GFP embryos were dissected at 5.5 to 5.75 dpc and staged according to the expression of the GFP reporter. In most experiments, embryos in which the GFP expression was observed to be restricted to the distal third of the embryo (i.e. prior to the movement of the AVE cells or when these cells have just shifted in one direction but remain in the distal region of the embryo) were used. For the experiment to test whether ectopic activation of the *Hex*-GFP reporter could occur after the migration of AVE cells, embryos in which the GFP reporter was restricted to the anterior of the embryo were selected.

Microsurgical manipulations and embryo culture

Microsurgical manipulations were carried out as a modification of the methods of Hogan and Tilly (Hogan and Tilly, 1981). Forceps or tungsten needles were used to cut the embryo transversely at the embryonic/extra-embryonic boundary. The embryonic region and control unmanipulated embryos were cultured in DMEM and 50% rat serum at 37°C, 5%CO_2 overnight or over a 40-hour period as described (Thomas et al., 1998). Embryos were photographed before and after culture using the fluorescein epifluorescence filter on a Zeiss Axiophot microscope.

Time-lapse imaging of embryos

Embryos were cultured directly on the stage of an Olympus IX70 inverted microscope as previously described (Srinivas et al., 2004). Phase-contrast and epifluorescence digital time-lapse images were acquired using the Deltavision system from Applied Precision. Images from multiple focal planes were captured at each time point, deconvolved and an extended-focus image projected. Where the cultured embryos drifted in the field of view, projected images from different time points were manually set in register using Adobe Photoshop. Quicktime movies were compiled from individual still images using the program Graphic Converter.

Injection of extra-embryonic ectoderm cells or COS-7 cells into 5.5 dpc embryos

To obtain extra-embryonic ectoderm cells for injection, 5.5 dpc embryos were dissected in M2 medium and washed with PBS. Embryos were then incubated in trypsin-EDTA for 10 minutes at 37°C and 5%CO_2. Visceral endoderm was dissociated from the rest of the embryo using forceps (Nagy et al., 2003). The dissected embryos were then cut transversely at the embryonic/extra-embryonic boundary and the extra-embryonic ectoderm dissociated into small clumps or single cells using an injection pipette. Five to 15 cells were then injected close to the distal tip of 5.5 dpc embryos held securely with a holding pipette. Injected embryos were cultured in DMEM and 50% rat serum at 37°C, 5%CO_2 overnight as described (Thomas et al., 1998), fixed overnight in 4% paraformaldehyde and dehydrated though a graded methanol series.

Whole-mount in situ hybridization

Embryos for whole-mount in situ hybridization were dissected early in the day, before the AVE was likely to have started moving anteriorly. Manipulated and control embryos were cultured for the appropriate time period and then fixed overnight in 4% paraformaldehyde and dehydrated through a graded methanol series. Whole-mount in situ hybridization was carried out following standard procedures (Thomas and Beddington, 1996). The following probes were used as previously described: *Cer1* (Thomas et al., 1997), *Lhx1* (Shawlot and Behringer, 1995), *Afp* (Cascio and Zaret, 1991), cripto (Ding et al., 1998), *Nodal* (Conlon et al., 1994), *T* (Wilkinson et al., 1990), *Pou5f1* (Scholer et al., 1990), *Sox1* (Wood and Episkopou, 1999), *Hesx1* (Thomas and Beddington, 1996) and *Six3* (Oliver et al., 1995).

Results

Removal of the extra-embryonic region leads to ectopic localization of AVE gene markers

Given that the extra-embryonic ectoderm (ExE) has a role in patterning the epiblast (Beddington and Robertson, 1999), we chose to analyse its role in AVE induction and migration. In our first experiments, we microsurgically removed the extra-embryonic region (ExE and the visceral endoderm overlying it) from 5.5 dpc embryos and allowed them to develop in culture for 24 hours (Hogan and Tilly, 1981). We then assayed the induction of AVE markers by looking at the expression of a *Hex*-GFP transgene that is expressed specifically in the anterior visceral endoderm. *Hex* is one of the earliest markers of the AVE and mice carrying a *Hex*-GFP transgene have been shown to recapitulate the expression of endogenous *Hex* in the AVE (Rodriguez et al., 2001).

Embryos were dissected at 5.5 dpc and only those with GFP expression restricted to the distal tip of the embryo (i.e. prior to the migration of AVE cells) were selected for this experiment. In control un-manipulated embryos, after overnight culture, the expression of the *Hex*-GFP reporter was restricted to the prospective anterior of the embryo (Fig. 1A,B) as a result of the unilateral migration of the AVE cells from the

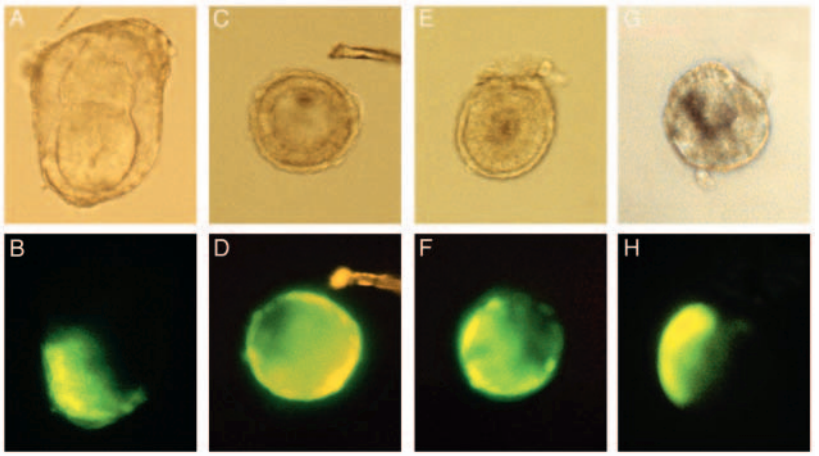

Fig. 1. Removal of the extra-embryonic region leads to ectopic *Hex*-GFP transgene expression. Bright-field (A,C,E,G) and fluorescence (B,D,F,H) images of 5.5 dpc embryos after overnight culture. (A,B) Control unmanipulated embryo. (C-H) Embryos in which the extra-embryonic region was removed before culture. In control embryos cultured overnight, the expression of the *Hex*-GFP reporter is restricted to the anterior visceral endoderm (A,B). In embryos where the extra-embryonic region has been removed prior to the migration of the AVE, the expression of the *Hex*-GFP transgene is observed throughout the visceral endoderm (C-F). However, if the extra-embryonic region was removed after the migration of the AVE, this ectopic expression of the *Hex*-GFP transgene is not observed (G,H).

distal tip to the anterior of the embryo (Srinivas et al., 2004). In contrast, in embryos in which the extra-embryonic region had been removed, after overnight culture, the *Hex*-GFP reporter was no longer restricted to the anterior but was expressed in a widespread fashion throughout the visceral endoderm (Fig. 1C-F; n=19/23). This ectopic expression was confirmed to represent an expansion of the AVE because the AVE markers cerberus-like 1 (*Cer1*) (Belo et al., 1997; Thomas et al., 1997) and *Lhx1* (previously *Lim1*) (Perea-Gomez et al., 1999) were also expressed throughout the visceral endoderm of these embryos (Fig. 3A-D; *Cer1* was expressed ectopically in 17/20 and *Lhx1* in 7/7 embryos). Consistent with this expansion of AVE markers, we also saw a dramatic reduction in the expression of the proximal/posterior visceral endoderm marker *Afp* (Cascio and Zaret, 1991) in embryos lacking the extra-embryonic region (Fig. 3E,F; *Afp* was lost or greatly reduced in 19/23 embryos).

To determine if the expansion in the expression domain of AVE markers in the absence of the extra-embryonic region occurs within a specific window of time, we carried out the microsurgical removal of the extra-embryonic region after the migration of the AVE cells had been completed (at around 5.75 dpc). When such embryos were cultured overnight, expression of the *Hex*-GFP reporter remained restricted to the anterior of the embryo (Fig. 1G,H; n=6/6) suggesting that the ectopic expression of AVE gene markers in the absence of the extra-embryonic region can only occur between 5.5 and 5.75 dpc.

Ectopic AVE formation is due to de novo expression of AVE markers

There are two possible explanations for the expansion in the expression domain of AVE markers in embryos lacking the extra-embryonic region. One is that cells of the proximal visceral endoderm inappropriately upregulate AVE markers de novo, and the other involves aberrant migration of the distal visceral endoderm cells (the AVE progenitors): the distal cells, instead of moving unidirectionally to the prospective anterior of the embryo, might be migrating randomly because they have lost directional cues.

To distinguish between these two alternatives, we examined by time-lapse microscopy 5.5 dpc *Hex*-GFP embryos in which the extra-embryonic region had been removed. In these embryos, we observed that after 2-3 hours in culture, visceral endoderm cells that would not normally express *Hex* start to express the *Hex*-GFP reporter (Fig. 2A-D; see Movies 1 and 2 in the supplementary material; n=3). This indicates that the ectopic expression of the *Hex*-GFP transgene is due to the de novo induction of *Hex* within cells of the proximal visceral endoderm.

Previous work has shown that the cells of the AVE move from the distal tip of the embryo to the boundary between the epiblast and ExE by a process of migration, and in response to cues from their environment (Srinivas et al., 2004). We observed that in embryos where the extra-embryonic region has been removed, in addition to de novo induction of AVE markers, the original AVE progenitor cells that are located at

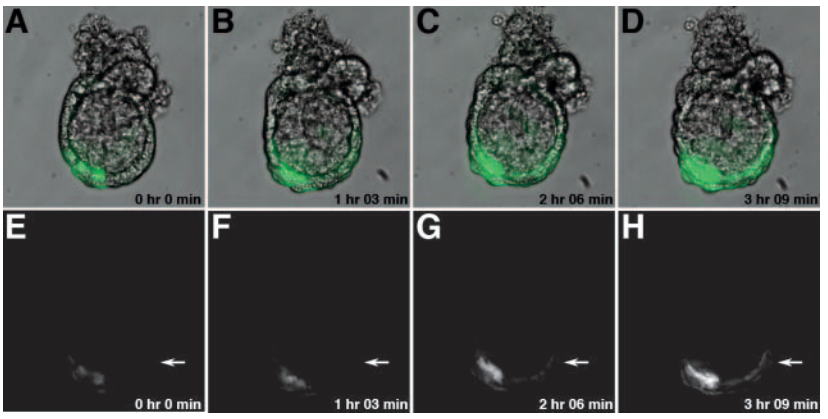

Fig. 2. Ectopic *Hex*-GFP transgene expression is due to de novo expression of the GFP reporter. Representative frames from a movie (supplementary data) of a cultured 5.5 dpc embryo after removal of the extra-embryonic region, showing de novo expression of the *Hex*-GFP reporter in the visceral endoderm. The time from start of culture is indicated in hours and minutes at the bottom right of each frame. The embryo was imaged every 7 minutes with phase-contrast and fluorescence optics. An overlay of both bright-field and fluorescent images is shown in the top panels and, for clarity, just the fluorescence images below. EGFP fluorescence (green) marks the expression of the *Hex*-GFP transgene. Arrows in E-H indicate de novo expression of the *Hex*-GFP reporter.

the distal tip of the embryo do not migrate unilaterally to the prospective anterior of the embryo, but instead remain stationary, in a distal position (Fig. 2A-D; see Movies 1 and 2 in the supplementary material). This suggests that in addition to being required for the induction of the AVE, the extra-embryonic region is required for the proper migration of AVE cells.

The extra-embryonic region is required for the expression of proximal/posterior markers in the epiblast

To address how the absence of the extra-embryonic region affects the patterning of the epiblast, we analysed the expression of the posterior markers cripto (Ding et al., 1998), *Nodal* (Varlet et al., 1997) and *T* (Wilkinson et al., 1990). At 5.5 dpc, these genes are expressed throughout the proximal epiblast, in a ring at the embryonic/extra-embryonic boundary. By 6.5 dpc, their expression refines to the posterior epiblast, where the primitive streak will form (Lu et al., 2001). In control embryos, after overnight culture, the expression of cripto, *Nodal* and *T* is nearly or completely resolved to the posterior epiblast, indicating that these embryos have reached a stage between 6.0 and 6.5 dpc (Fig. 3G,I,K). However, in embryos lacking the extra-embryonic region, the expression of cripto, *Nodal* and *T* is completely lost from the epiblast after overnight culture (Fig. 3H,J,L; cripto was lost in 14/16, *Nodal* was lost in 7/7 and *T* was lost in 9/10 embryos), indicating that induction of posterior epiblast pattern has not occurred in these embryos. This loss of posterior epiblast markers suggests that the extra-embryonic region is playing a role in primitive streak induction (Beddington and Robertson, 1999). The loss of posterior epiblast markers in our explants is unlikely to be due simply to the ectopic AVE repressing these markers, because a similar loss of the posterior marker cripto is observed in epiblast explants separated from both the ExE and the surrounding visceral endoderm (Beck et al., 2002). The striking absence of any posterior markers in embryos lacking the extra-embryonic region is also not due simply to a general failure of patterning in the epiblast, as embryos lacking the extra-embryonic region express the ectoderm marker *Pou5f1* (previously *Oct3/4*)

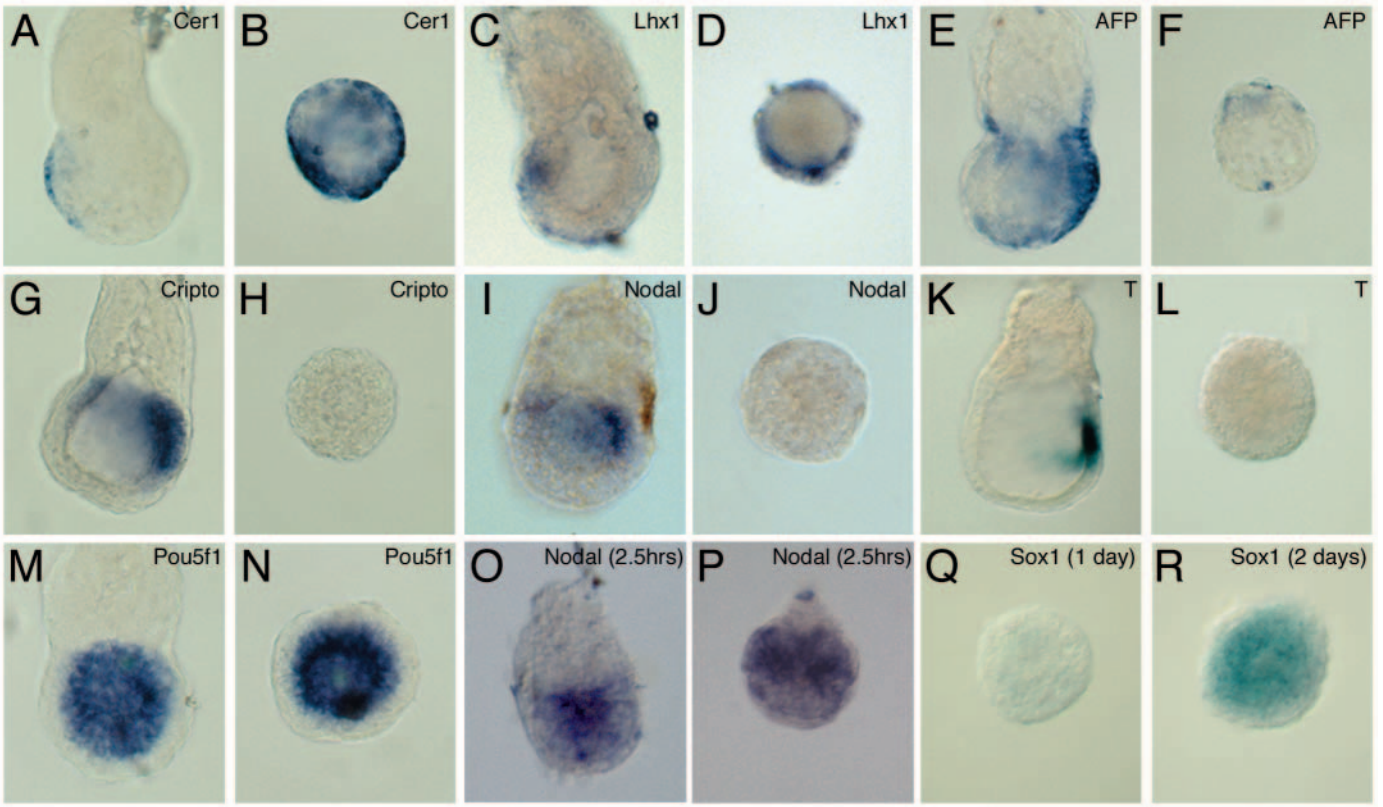

Fig. 3. Analysis of how the removal of the extra-embryonic region changes the patterning of the visceral endoderm and epiblast. Whole-mount in situ hybridization analysis of 5.5 dpc embryos cultured overnight. (A,C,E,G,I,K,M,O) Control unmanipulated embryos. (B,D,F,H,J,L,N,P,Q,R) Embryos in which the extra-embryonic region was removed before culture. In control embryos *Cer1* (A) and *Lhx1* (C) are restricted to the anterior visceral endoderm, but in the absence of the extra-embryonic region, both *Cer1* (B, *n*=17/20) and *Lhx1* (D, *n*=7/7) are expressed ectopically throughout the visceral endoderm. *Afp* expression is observed in the proximal and posterior visceral endoderm in control embryos (E) but is strongly downregulated after removal of the extra-embryonic region (F, *n*=19/24). Expression of cripto (G) *Nodal* (I) and *T* (K) is restricted to the proximal/posterior epiblast in control embryos but is lost after removal of the extra-embryonic region and overnight culture (H, *n*=14/16; J, *n*=7/7; and L, *n*=9/10). Expression of *Pou5f1* is observed in the epiblast of both control (M) and manipulated embryos (N, *n*=11/14), indicating that the downregulation of posterior epiblast markers is a specific effect. No difference is observed in the pattern of *Nodal* expression in controls (O) and embryos lacking the extra-embryonic region (P, *n*=6/6) after only 2.5 hours culture. The expression of the anterior neural marker *Sox1* is detected only at low levels in embryos lacking the extra-embryonic region after overnight culture (R, *n*=4 weak and 11 no expression out of 15) but is strongly upregulated throughout the epiblast of these embryos after 42 hours of culture (S, *n*=7/7).

(Rosner et al., 1990) in the epiblast (Fig. 3N; *n*=11/14) at levels comparable with control embryos (Fig. 3M; *n*=5/5).

Given that *Nodal* is required for AVE induction, and we observe a loss of *Nodal* expression after removal of the extra-embryonic region and overnight culture, it was important to analyse *Nodal* expression in such embryos at the time when the upregulation of AVE markers is occurring. Time-lapse analysis of *Hex*-GFP embryos lacking the extra-embryonic region indicates that ectopic reporter expression is first observable after 1 hour in culture and robust after 3 hours (Fig. 2). We therefore analysed *Nodal* expression in embryos that had been cultured for 2.5 hours or 5.0 hours after removal of the extra-embryonic ectoderm. We observed no obvious difference in the level or pattern of *Nodal* expression between embryos lacking the extra-embryonic region and control embryos after 2.5 hours of culture (Fig. 3O-P; normal *Nodal* expression was observed in 6/6 embryos lacking the extra-embryonic region). This suggests that *Nodal* signalling is probably responsible for the de novo induction of the AVE markers we observe. However, after 5 hours in culture, we observe a downregulation of *Nodal* expression in embryos lacking the extra-embryonic region (data not shown; downregulation was observed in 8/10 embryos), consistent with the extra-embryonic ectoderm being responsible for maintaining *Nodal* expression in the epiblast.

The AVE is required for anterior patterning. Given the ectopic expression of AVE markers in embryos lacking the extra-embryonic region, we tested whether anterior neural induction could occur in these embryos. To this end, we analysed the expression of the anterior neuroectoderm marker *Sox1* (Aubert et al., 2003; Wood and Episkopou, 1999) in embryos from which the extra-embryonic region had been removed. *Sox1* expression was undetectable (*n*=11/15) or detectible only at very low levels (*n*=4/15) after overnight culture (Fig. 3Q), but readily apparent throughout the epiblast after 42 hours of culture (Fig. 3R; *Sox1* expression was observed in 7/7 embryos). This timing of onset of *Sox1* expression in explants is similar to that observed in embryos in utero or in unmanipulated embryos after 42 hours culture (data not shown). This observation indicates that in the absence of the extra-embryonic region, the epiblast will adopt an early anterior neural character. However, when we analysed the expression of the later neural markers *Hesx1* (Thomas and Beddington, 1996) and *Six3* (Oliver et al., 1995) in these embryos, we failed to see any expression (data not shown; 8/8 embryos lacking the extra-embryonic region showed no *Hesx1* expression and 7/7 showed no *Six3* expression). Bearing in mind that the expression of *Hesx1* and *Six3* is initiated in the anterior neural ectoderm after that of *Sox1*, this indicates that in the explants, either the epiblast initiates anterior neural patterning but does not differentiate further, or the onset of expression of these markers is delayed.

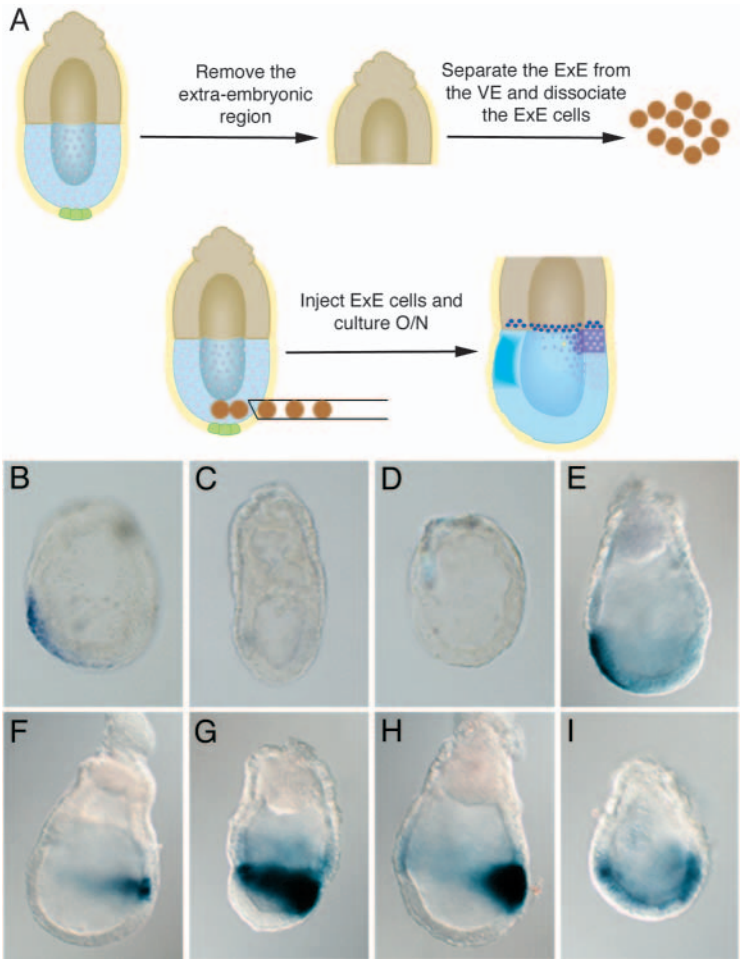

Fig. 4. Injection of ExE cells inhibits AVE formation and leads to an expansion of posterior epiblast markers. (A) Diagrammatic representation of the approach used to isolate and inject ExE cells adjacent to the AVE of 5.5 dpc embryos. (B-E) *Cer1* expression is observed in the AVE of control embryos (B) and of embryos injected with COS-7 cells (E) but is lost in embryos injected with ExE cells (C,D). (F-I) *T* expression was observed in the posterior epiblast of control embryos (F) but its expression was expanded (G,H) or observed ectopically (I) after the injection of ExE cells.

The extra-embryonic ectoderm can repress AVE gene expression and induce proximal/posterior epiblast markers

The upregulation of AVE markers in embryos lacking the extra-embryonic region suggests that this region may repress AVE gene expression. In order to identify the specific tissue of the extra-embryonic region that is responsible for this repression, we tested the ExE for inhibition of AVE formation. For this purpose, we developed a technique to inject small, dissociated clumps of extra-embryonic ectoderm cells into the distal region of 5.5 dpc embryos (Fig. 4A). Consistent with our previous data, we found that the injection of extra-embryonic ectoderm cells caused the downregulation of the AVE marker *Cer1* in a significant proportion of embryos (four out of seven; Fig. 4B-D). Control injection of COS-7 cells did not cause a similar loss of *Cer1* expression, indicating that this downregulation is not due simply to the injection procedure – every one of the 19 embryos injected with COS-7 cells showed normal *Cer1* expression (Fig. 4E). This observation argues that a signal from the ExE may be inhibiting the proximal and lateral visceral endoderm from initiating AVE gene expression and that the ExE is sufficient for this repression.

In recombination experiments, the ExE is capable of ectopically inducing markers of primordial germ cells in the distal epiblast at 6.5 dpc (Yoshimizu et al., 2001). To test whether the ExE could induce proximal/posterior markers at earlier stages, we analysed the expression of *T* in embryos injected with ExE cells using the assay described above. In two out of nine injected embryos, we observed a significant expansion of the normal domain of T expression (Fig. 4F-H), while a third showed ectopic expression of *T* (Fig. 4I). This ability of ExE cells to induce ectopic T expression after their injection into 5.5 dpc embryos indicates that the ExE may be inducing posterior markers in the epiblast.

Discussion

We have analysed the role of the ExE in establishing the AP axis of the mouse embryo and have shown that it patterns the two other tissues of the early post-implantation embryo, the visceral endoderm and the epiblast, in different ways. In the visceral endoderm, the ExE represses distal/anterior fates by inhibiting AVE formation, and is also required for AVE cell migration, while in the epiblast, the ExE induces proximal/posterior fates (Fig. 5).

Signalling by the Tgfβ factor Nodal has been shown to be required for the induction of the AVE (Brennan et al., 2001); however, *Nodal* expression is widespread in the epiblast and visceral endoderm at the time of AVE induction (Varlet et al., 1997), suggesting that further signals must be required to restrict the formation of the AVE to the distal tip of the embryo. In our experiments, we observe de novo ectopic expression of AVE gene markers upon removal of the ExE and a loss of AVE markers when ExE cells are transplanted adjacent to the presumptive AVE domain, indicating that the ExE must be acting to restrict AVE induction to the distal tip of the embryo.

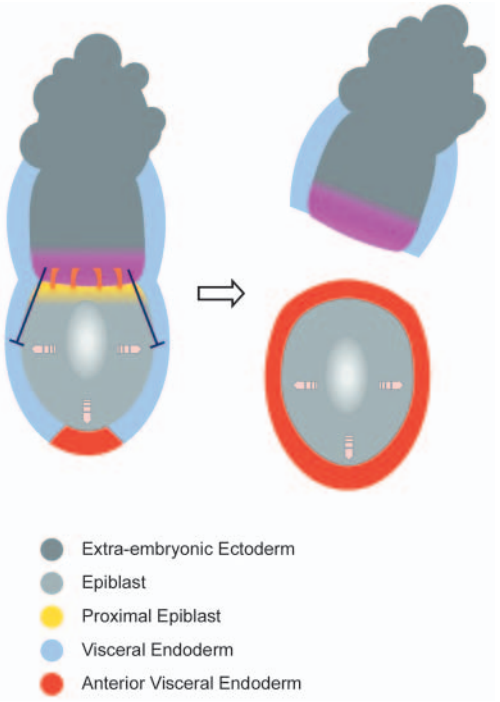

Fig. 5. Proposed model for the role of the ExE in patterning the pre-gastrulation mouse embryo. Signalling by the epiblast induces the AVE (pink arrows) and inhibitory signals from the ExE restrict this induction to the distal tip of the embryo (inhibitory arrows), The ExE also induces the expression of proximal/posterior markers in the epiblast (orange arrows). In embryos where the ExE has been removed, the epiblast induces AVE markers throughout the visceral endoderm and no expression of proximal/posterior markers is observed in the epiblast.

We propose that at around 5.25 dpc, Nodal signalling from the epiblast has the ability to induce an AVE cell fate in all the adjacent visceral endoderm, but only the distal most visceral endoderm maintains the expression of AVE markers because of the direct inhibitory action of the ExE upon the proximal visceral endoderm. Two observations support the view that the ExE acts directly, by secreting an inhibitor that represses AVE gene expression in the proximal visceral endoderm. First, the short time-frame between the removal of the ExE and the upregulation of AVE markers (ectopic expression is seen after only 1 hour) suggests a direct interaction. Second, we observe no difference in the expression pattern of the proximal epiblast gene marker *Nodal* 2.5 hours after the removal of the ExE, suggesting that the epiblast remains properly patterned at the time ectopic AVE induction is occurring, and therefore, that the removal of the ExE does not act on the visceral endoderm by altering the patterning of the epiblast. However, an alternative possibility exists for an indirect mode of action of the ExE on the visceral endoderm. By removing the ExE, we remove the activity of the Spc proteases that are responsible for processing Nodal protein from an inactive precursor to the active form (Beck et al., 2002). This lack of the Spc proteases would lead to a decreased level of Nodal activity in the proximal epiblast. It has been shown that different levels of Nodal activity specify different cell fates in the mouse embryo (Norris et al., 2002; Vincent et al., 2003), and it is proposed that the lowest level of Nodal signalling is responsible for specifying the AVE (Robertson et al., 2003). Therefore, it is possible that a widespread 'low' level of Nodal signalling, as a result of removal of the ExE, leads to the observed ectopic AVE induction. *Spc1/Spc4* double mutants lack *Hex* and *Cer1* expression altogether (Beck et al., 2002), probably owing to the complete absence of processed Nodal in such embryos. By comparison, embryos in which the ExE is removed at 5.5 dpc, would be expected to have low residual levels of processed Nodal, sufficient for the ectopic induction of AVE markers that we observe.

The expansion of AVE markers and the loss of proximal visceral endoderm markers after removal of the extra-embryonic region also indicates that, during a specific window of time, all the cells of the visceral endoderm surrounding the epiblast are competent to assume an AVE character. This suggests that the small population of cells at the distal tip of the embryo that normally do become AVE are not exclusively specified for this fate at an early stage. Therefore, at 5.5 dpc the extra-embryonic region mediates the choice of visceral endoderm cells to adopt an AVE or a proximal visceral endoderm cell fate.

Given that, in the absence of the ExE, we observe no migration of AVE cells, we have identified a second requirement for the ExE in the early post-implantation embryo, for AVE cell migration. We suggest that the ExE either directly secretes a factor that initiates AVE cell migration, with the directional cues being provided by the epiblast (Srinivas et al.,

2004), or, alternatively, is essential for maintaining the levels of Nodal signalling in the epiblast that are required for AVE migration to occur (Norris et al., 2002; Yamamoto et al., 2004). Nodal signalling has been shown to promote proliferation within the VE and it has been proposed that increased proliferation within the posterior VE relative to the anterior VE may drive the anterior movement of the AVE (Yamamoto et al., 2004). However, time-lapse movies of AVE movement show that AVE cells actively migrate and that this movement is completed relatively quickly, within ~4 hours, making it unlikely that differential proliferation is the primary driving force for the AVE movements (Srinivas et al., 2004). To reconcile these two sets of data, we suggest that AVE movement is achieved by a rapid migration, but the initial impetus and directionality for this migration might be provided by Nodal-mediated differential proliferation between the posterior and anterior VE. As suggested above, removing the ExE may remove Spc1/Spc4 activity and consequently cause a decrease in the level of Nodal signalling. This lowering in the level of Nodal signalling could cause a decrease in proliferation bellow the crucial threshold required for migration to occur and thereby disrupt AVE cell movements.

Finally, our observation that in the absence of the ExE we lose proximal/posterior epiblast gene markers indicates that the ExE is required to induce proximal/posterior cell fates in the epiblast, probably by maintaining *Nodal* expression in this tissue. The ability of ExE cells to induce ectopic T expression after their injection into 5.5 dpc embryos and the ability of the ExE to ectopically induce primordial germ cells in recombination experiments between distal epiblast and the ExE (Saitou et al., 2002; Ying et al., 2001; Yoshimizu et al., 2001) supports the view that the ExE may be inducing posterior markers in the epiblast. The ExE is likely to fulfil this patterning role both directly, by BMP signalling, and indirectly, by the modulation of Nodal signalling. In support of a direct role is the fact that analysis of *Bmp4* mutants (Fujiwara et al., 2001; Lawson et al., 1999) has shown that BMP4 is required in the ExE for extra-embryonic mesoderm and primitive germ cell development. In support of an indirect role, there is a requirement for SPC proteases to be secreted from the ExE for the correct processing of Nodal and the induction of mesoderm markers in the epiblast (Beck et al., 2002). We propose that both the BMP and SPC activity of the ExE are required to maintain *Nodal* expression in the proximal epiblast, and in turn this expression will be essential to induce primitive streak formation. Therefore, proximal/posterior gene markers will initially become induced all along the boundary between the epiblast and the ExE before becoming restricted to the posterior of the embryo by the migrated AVE. By restricting the site of AVE induction, modulating its migration and inducing proximal/posterior epiblast markers, the ExE plays a pivotal role in coordinating AP patterning in the mouse embryo.

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Supplementary material

Supplementary material for this article is available at
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