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Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning

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Anteroposterior (AP) polarity in the mammalian embryo is specified during gastrulation when naive progenitor cells in the primitive ectoderm are recruited into the primitive streak to form mesoderm and endoderm. At the opposite pole, this process is inhibited by signals previously induced in distal visceral endoderm (DVE). Both DVE and primitive streak formation, and hence positioning of the AP axis, rely on the TGFβ family member Nodal and its proprotein convertases Furin and Pace4. Here, we show that Nodal and Furin are initially co-expressed in the primitive endoderm together with a subset of DVE markers such as Lefty1 and Hex. However, with the appearance of extra-embryonic ectoderm (ExE), DVE formation is transiently inhibited. During this stage, Nodal activity is essential to specify embryonic VE and restrict the expression of Furin to the extra-embryonic region. Activation of Nodal is also necessary to maintain determinants of pluripotency such as Oct4, Nanog and Foxd3 during implantation, and to stimulate elongation of the egg cylinder, before inducing DVE and germ layer formation. We conclude that Nodal is already activated in primitive endoderm, but induces a functional DVE only after promoting the expansion of embryonic VE and pluripotent progenitor cells in the epiblast.

KEY WORDS: TGFβ, Oct4, Stem cells, Differentiation, Implantation, Axis specification, Mouse

INTRODUCTION

Early progenitor cells in the mammalian embryo communicate with each other and their extra-embryonic microenvironment. Insights into the underlying molecular mechanisms are essential to understand how the potential of uncoordinated proliferation and differentiation is harnessed in stem cells. Self-renewing embryonic stem (ES) cell lines can be derived from the inner cell mass (ICM) of the blastocyst in the presence of factors that sustain determinants of pluripotency, such as the transcription factors Oct4, Nanog, Sox2 and Foxd3 (Nichols et al., 1998; Hanna et al., 2002; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). However, when the blastocyst implants in the uterus, a subpopulation of ICM cells expresses the zinc finger transcription factor Gata6 and congregates as a layer of primitive endoderm (PrE) facing the blastocoelic cavity, followed by downregulation of Oct4 (Rossant et al., 2003). PrE cells in contact with the ICM subsequently become visceral endoderm (VE) and act as a substrate for the remaining pluripotent cells to firmly adhere and form a rapidly expanding epithelial sheet, or epiblast, which gives rise to the embryo proper. Meanwhile, outer trophectoderm cells abutting the ICM undergo a burst of proliferation to form the extra-embryonic ectoderm (ExE) and its derivatives in the fetal layers of the placenta. Coordinate growth after implantation thus converts the mouse blastocyst within 24 hours into the egg cylinder, a structure peculiar to rodents, with the epiblast and ExE forming the inner layer of an elongated cylinder that is invested by an outer VE layer.

Communication with the VE is essential to control the fate of pluripotent cells in the epiblast, as distal visceral endoderm (DVE) moving along one side of the epiblast restricts mesoderm and endoderm formation to the opposite pole and thereby defines the position of the prospective anteroposterior (AP) axis (Kimura et al., 2000; Perea-Gomez et al., 2002) (for a review, see Rossant and Tam, 2004). Moreover, the VE is essential for gas and nutrient exchange and as a substratum to which the epiblast and ExE adhere (reviewed by Bielinska et al., 1999). However, lineage-tracing experiments have shown that VE cells do not colonize embryonic tissues, and instead give rise to the outer layer of the visceral yolk sac (Lawson and Pedersen, 1987). Irrespective of this common extra-embryonic fate, two morphologically different populations of embryonic VE (EmVE) and extra-embryonic VE (ExVE) cells are distinguished at the egg cylinder stage based on their association with the epiblast or ExE, respectively (Enders et al., 1978). Accordingly, the DVE can be regarded as a specialized derivative of EmVE. Alternatively, it may be set aside as a separate lineage before or in parallel to EmVE.

By the time the DVE has moved anteriorly, cells in the epiblast are not committed to specific fates (Tam and Zhou, 1996) and express Oct4 as well as Foxd3 (Hanna et al., 2002). However, they have lost the capacity to give rise to self-renewing ES cells (Rossant, 1977; Beddington, 1983). Possibly this transition reflects a change in the microenvironment caused by the DVE. Alternatively, DVE formation and initiation of differentiation in the epiblast may coincide because they are triggered by a common stimulus after implantation. Consistent with the second hypothesis, both DVE and germ-layer specification rely on the TGFβ-related protein Nodal (Zhou et al., 1993; Conlon et al., 1994; Brennan et al., 2001), and prolonged activation of the Nodal pathway recently has been shown to impose a mesendodermal differentiation program on cultured ES cells (Tada et al., 2005). However, depending on the context, Nodal may also provide anti-differentiation signals, as it is essential to maintain Oct4 expression during gastrulation (Brennan et al., 2001), and activation of its signaling receptors prevents differentiation of cultured human ES cells (James et al., 2005). Nodal signaling in the ExE during gastrulation also stimulates the expression of Bmp4 (Brennan et al., 2001; Beck et al., 2002), another factor inhibiting ES cell differentiation (Ying et al., 2003). This paradox indicates that Nodal may have distinct functions as its activity is dynamically regulated in space and time. Indeed, after a wave of widespread auto-induction throughout the epiblast and EmVE at the egg cylinder stage, *Nodal*

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is swiftly silenced in the VE and anterior epiblast (Collignon et al., 1996; Norris and Robertson, 1999), presumably by secreted feedback antagonists from the DVE such as Lefty1, cerberus 1 homolog (Cer1), and Dkk1, whereas the posterior epiblast maintains *Nodal* expression through positive feedback loops mediated by Wnt3, Nodal itself, and its co-receptor Cripto (Cfc1 – Mouse Genome Informatics) (for a review, see Ang and Constam, 2004). This network of positive and negative feedback signals is activated by the secreted proprotein convertases Furin and Pace4 (Pcsk6 – Mouse Genome Informatics), which are necessary for proteolytic maturation of the Nodal precursor protein (Beck et al., 2002). Thus, *Furin*$^{-/-}$; *Pace4*$^{-/-}$ compound mutants display a delayed onset of Nodal expression and lack both DVE and AP asymmetry in the epiblast. Based on the analysis of chimeric embryos, and as Furin and Pace4 during gastrulation are specifically expressed in the ExE in a pattern that is complementary to that of *Nodal*, we previously proposed that they act cell non-autonomously to cleave Nodal after secretion (Beck et al., 2002). Accordingly, we hypothesized that proNodal is more efficiently processed in the proximal epiblast near the source of its convertases than in distal regions. This model is consistent with the idea that the level of Nodal processing may have to exceed a certain threshold before it induces germ-layer formation. However, it does not explain how Furin and Pace4 activities specify DVE at the apex of the egg cylinder. In one possible scenario, Furin and Pace4 promote DVE formation only indirectly by stimulating *Nodal* auto-induction, while DVE is actually induced by uncleaved Nodal reaching maximal levels far from the source of proprotein convertases. Alternatively, we hypothesized that DVE forms in response to processed Nodal generated locally at a distance from the ExE.

The aim of this study was to determine whether Nodal and proprotein convertases already interact during implantation to pattern the VE, epiblast or ExE. We find that key components of the Nodal pathway are present from the time of implantation at embryonic day (E) 4.5, with *Pace4* mRNA being localized to the ExE, and *Furin* and *Nodal* being expressed together in PrE. Furthermore, we show that Nodal signaling is required initially to specify EmVE and promote growth of the egg cylinder before it can induce DVE. In addition, analysis of Nodal mutants and embryo explants suggests that early Nodal signaling is also essential to sustain the expression of several molecular determinants of pluripotency. We conclude that Nodal activity is already required during implantation to control the fate of early progenitor cells in the epiblast and PrE lineages.

MATERIALS AND METHODS

Nomenclature used for the VE lineage

We use the term primitive endoderm (PrE) only at E4.5, before the overt appearance of extra-embryonic ectoderm (ExE). Thereafter, we refer to visceral endoderm (VE), or more specifically to embryonic VE (EmVE) or extra-embryonic VE (ExVE), depending on whether it abuts the epiblast or the ExE, respectively.

Mouse strains

Mice carrying the HexP-GFP reporter transgene (Rodriguez et al., 2001) were maintained pathogen-free in individually ventilated cages on a mixed genetic background of C57BL6 × NMRI. Mice carrying the Nodal^{LacZ} allele (Collignon et al., 1996) were maintained on a mixed genetic background of 129svEV × NMRI. Both strains were inter-crossed to obtain males carrying the Nodal^{LacZ} allele and homozygous for the HexP-GFP transgene. Outbred diabetes-resistant NMRI mice were from Harlan or Janvier.

Isolation of embryos and explants

All embryos dissected on the fifth (E4.5) or sixth (E5.0-5.75) day post-coitum were staged according to their size and expression of the HexP-GFP transgene inherited from their HexP-GFP homozygous fathers. 'Post-DVE' stage corresponded to position of the AVE on the lateral side (Rivera-Perez et al., 2003). 'DVE' corresponded to the stage when upregulation of GFP could be observed in the distal tip of the visceral endoderm only. 'Pre-DVE' stage corresponded to younger embryos, in which GFP was only detected at reduced levels and with no specific location. The presence and position of the DVE/AVE correlated with the size of the embryo (Fig. 4A). Epiblast explants from staged heterozygous HexP-GFP transgenic embryos were isolated and cultured essentially as described (Beck et al., 2002) using serum-free DMEM supplemented with glutamine and 15% knockout serum-replacement (Invitrogen). Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Alexis) was used at a concentration of 25 μmol/l. SB-431542 (Sigma) was used at a concentration of 10 μmol/l. Nodal (R&D) was used at a concentration of 50 μg/ml.

Immunofluorescence, in-situ hybridization and β-galactosidase staining

Whole-mount immunofluorescence was performed as described for blastocysts (www.mshri.on.ca/rossant/protocols/immunoStain.html). Rat anti-E-cadherin (Sigma) was used at a concentration of 1/200. Rabbit anti-Laminin (Biodesign) was used at a concentration of 1/100. β-Galacosidase staining and RNA probes for whole-mount in-situ hybridization have been described (Brennan et al., 2001; Beck et al., 2002). A *Bmp2* antisense probe was derived from an *Eag*I-*Eco*RI 988-bp fragment of reference sequence NM_007553. Additional RNA probes for *Foxd3* (Hanna et al., 2002), *Hnf4a* (Morrisey et al., 1998), *Nanog* (Chambers et al., 2003), *Fgf5* (Hebert et al., 1994) and *Ttr* (Makover et al., 1989) have been described. Immunofluorescence images were acquired on a Zeiss LSM510 confocal microscope. HexP-GFP and transmitted light images were acquired on a Leica DC200 fluorescent microscope.

RESULTS

Nodal, Furin and Pace4 are already expressed at the time of implantation

To assess whether Nodal and its proprotein convertases Furin and Pace4 may interact before the egg cylinder stage, we first analyzed their expression patterns by whole-mount in-situ hybridization. Nodal mRNA was detected during implantation (E4.5) in the ICM and PrE of the blastocyst, and subsequently remained expressed until E5.5 in the epiblast and overlying EmVE (Fig. 1A). The proprotein convertases Furin and Pace4 were also present before E5.5, although their expression patterns differed: Pace4 was specifically expressed in the polar trophectoderm and its derivatives at all stages examined. By contrast, Furin was detected initially in the PrE (E4.5), and throughout the VE (E5.0-5.25), before it became restricted to the ExVE and ExE (E5.5) (Fig. 1A; see Fig. S1 in the supplementary material). Thus, the expression patterns of Nodal and Furin overlapped within the PrE and VE until E5.25.

Despite the early presence of Nodal and its convertases, the DVE is not specified before E5.5

The early presence of Nodal and its convertases prompted us to monitor transcription of other known agonists and antagonists of the Nodal signaling pathway. The Nodal co-receptor Cripto was previously detected at the blastocyst stage by RT-PCR and whole-mount staining of a *lacZ* reporter allele (Kimura et al., 2001). Using whole-mount in-situ hybridization, we confirmed that *Cripto* is specifically expressed in the epiblast at all stages between E4.5-5.5 (Fig. 1A). By contrast, the Nodal antagonist Lefty1 was expressed in the PrE (E4.5). After implantation (E5.0-5.25), Lefty1 expression became downregulated before it resumed in the DVE at E5.5 (Fig. 1B). Prompted by this early expression pattern of *Lefty1*, we next asked whether the DVE is already specified at E4.5. Contrary to this idea, mRNA encoding the DVE marker Cer1 was not expressed above detectable levels before E5.5. Moreover, the homeobox transcription factor Hex (Hhex –

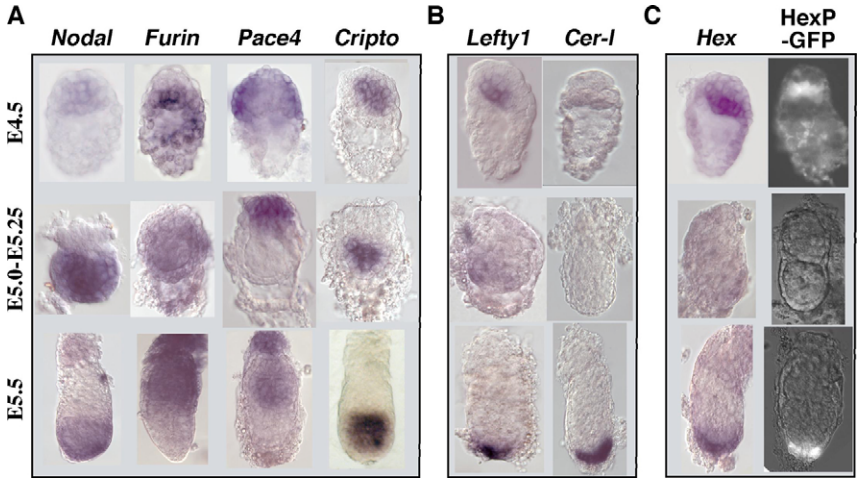

Fig. 1. Expression of Nodal agonists and antagonists between implantation and E5.5. (**A**) *Nodal* is already expressed at E4.5, both in the ICM and PrE of the blastocyst. Furin mRNA is expressed in the PrE lineage until E5.25, before it becomes restricted to the ExE and ExVE (E5.5). Pace4 and Cripto are specifically expressed in the trophectoderm lineage and epiblast, respectively. (**B**,**C**) The PrE also expresses the DVE markers *Lefty1* (B), *Hex* and a HexP-GFP reporter transgene (C), whereas *Cer1* mRNA is below detectable levels (B). *Lefty1* and *Hex* transcripts are clearly downregulated between E5.0 and 5.25 before they reappear together with *Cer1* mRNA in the most distal VE cells at E5.5.

Mouse Genome Informatics), which marks PrE and DVE (Thomas et al., 1998) was clearly induced at E4.5 and 5.5, but undetectable in the VE between E5.0 and 5.25 (Fig. 1C). This expression pattern was confirmed by analyzing a GFP reporter transgene driven by the Hex promoter (HexP-GFP) (Rodriguez et al., 2001). In these transgenic embryos, GFP is induced in the PrE (E4.5) and DVE (E5.5). However, at E5.25, HexP-GFP is only detected at reduced levels throughout the entire VE, probably due to the perdurance of GFP protein in descendants of the PrE. Taken together, the absence of significant expression levels of *Lefty1*, *Cer1* and *Hex* mRNAs between E5.0 and 5.25 strongly suggests that proximal-distal patterning in the visceral endoderm is only determined around E5.5 at the time of DVE formation. This interpretation is corroborated by the fact that a characteristic thickening of DVE cells (Rivera-Perez et al., 2003) is not observed until E5.5 (Fig. 1).

Nodal signaling is required before E5.5 to form an organized egg cylinder

The earliest defect described in Nodal mutants is their inability to form DVE (Brennan et al., 2001). However, we noticed that among embryos from $Nodal^{lacZ/+}$ heterozygous intercrosses, homozygous mutants could already be identified at E5.0 at the expected Mendelian frequency based on the absence of a distinct ExE/epiblast boundary, an abnormally round shape, and frequent detachment of a thickened VE from the epiblast (Fig. 2). In-situ hybridization of a Nodal antisense probe directed against the deleted exon 2 confirmed that these abnormal embryos were $Nodal^{lacZ/lacZ}$ null mutants (Fig. 2A,B). Moreover, β-galactosidase staining revealed that $Nodal^{lacZ/lacZ}$ homozygotes expressed the *lacZ* reporter allele only in the epiblast, whereas heterozygous littermates also induced *lacZ* in the EmVE (Fig. 2C,D). This result is consistent with the existing model that epiblast-derived Nodal activity is essential to induce *Nodal* expression in the VE (Brennan et al., 2001). Moreover, immunofluorescent staining of E-cadherin revealed that cells in the ExE and epiblast were abnormally heterogeneous in size, arranged irregularly, and more rounded than the wild-type controls, especially near the boundary between the epiblast and the ExE, which thus remained diffuse. In addition, the epiblast epithelium of Nodal mutants only loosely adhered to the VE (Fig. 2E,F). Adhesion of the VE to the epiblast is known to depend on laminin alpha 1 in the basement membrane (Smyth et al., 1999; Miner et al., 2004). However, laminin staining was unaffected in Nodal mutants (Fig. 2G,H). Cell attachment thus is not impaired due to a lack of basement membrane, but rather seems to reflect a failure of VE cells to tightly adhere to it (Fig. 2H,P).

Nodal signaling is required to confine ExVE to the extra-embryonic region

To define the early defect in the VE of Nodal mutants at the molecular level, we first monitored the expression of *Gata4*, *Hnf4* and *Ttr*. During gastrulation, these genes are specifically expressed in the ExVE (Makover et al., 1989; Duncan et al., 1994; Morrisey et al., 1998). However, during implantation, they are initially transcribed throughout the PrE and only become downregulated specifically in the EmVE around E5.5 (see Fig. S2 in the supplementary material) (Duncan et al., 1994). An analogous expression pattern has been described for vHnf1β (Barbacci et al., 1999), suggesting that markers of the ExVE generally become excluded from the embryonic region at the egg cylinder stage. By contrast, in Nodal mutants, these markers remained ectopically expressed at elevated levels in the embryonic region (Fig. 2I-N). Interestingly, Nodal mutants also displayed ectopic expression of *Furin*, both in the VE and throughout the epiblast (Fig. 2O,P). These results show that Nodal signaling is essential to downregulate a subset of PrE markers, including the proprotein convertase Furin, in the embryonic region and thus restrict their expression to the ExVE.

Nodal defines an EmVE compartment before it can specify DVE

Nodal mutants may form ectopic ExVE in the embryonic region simply because they lack a DVE. In this scenario, we expected that a functional DVE should appear before the EmVE. Alternatively, the VE may first differentiate into EmVE before it can form DVE. Consistent with the second hypothesis, we identified a panel of EmVE markers that failed to be induced in Nodal mutants at E5.0 long before the DVE would normally appear, including *Lim1*, *Fgf5*, *Fgf8*, *Bmp2*, *Otx2* and *Hnf3b* (*Foxa2* – Mouse Genome Informatics) (Fig. 3, and data not shown). Nodal, Fgf5 and Fgf8 mRNAs are also detected in the epiblast of wild-type, but not of Nodal mutant embryos (Fig. 3; see Fig. S3 in the supplementary material). These data reveal that Nodal is essential to impose an embryonic identity on VE cells that are in contact with the epiblast.

Several possibilities can be considered why Nodal specifies EmVE before the DVE can form at the apex of the egg cylinder. First of all, ExVE is unlikely to be competent to express DVE markers because of dominant inhibitory signals from the ExE. Direct support

for this hypothesis comes from the fact that ExE cells transplanted to distal epiblast repress DVE formation, whereas ablation of the ExE leads to ectopic expression of DVE markers (Rodriguez et al., 2005). Based on this model, we expected that the DVE should only be induced once the egg cylinder has reached a critical size. Confirming this prediction, we found that HexP-GFP expression at E5.5 appeared only once the distance between ExE and the apex of the egg cylinder was larger than 70 μm, and when the conceptus reached a minimal length of 180 μm (Fig. 4A). Moreover, in Nodal mutants lacking DVE and EmVE, the average length of the egg cylinder at E5.5 was reduced by 24% (Fig. 4B). Accordingly, Nodal may primarily be required as a permissive signal to support egg cylinder growth and thereby overcome the prohibitive influence of the ExE. Secondly, processed Nodal in addition may provide instructive cues to induce DVE-specific genes. In this case, the conversion of PrE to EmVE and the concomitant downregulation of *Furin* may also be important to prevent ectopic DVE formation. To distinguish whether Nodal actively induces DVE markers, or whether it only provides a permissive signal, we asked whether it is necessary for HexP-GFP expression in embryo explants. As described, embryo explants severed from the extra-embryonic region ectopically express the HexP-GFP transgene throughout the EmVE (Rodriguez et al., 2005). However, induction of HexP-GFP was abolished in explants treated with SB-431542, a pharmacological inhibitor of Nodal signaling (Fig. 5). Moreover, incubation with the Furin inhibitor decanoyl-RVKR-chloromethylketone (dec-RVKR-CMK) had a similar effect at concentrations that block Nodal processing (Le Good et al., 2005) (Fig. 5). Likewise, *Lim1*, a marker of EmVE and DVE, was lost when Nodal activity was blocked. Thus, Nodal signaling is essential as an instructive cue even in the absence of ExE to induce DVE.

If the Furin inhibitor dec-RVKR-CMK primarily blocks Nodal processing, its negative effect on HexP-GFP expression might be neutralized by exogenous recombinant Nodal. As shown in Fig. 5, incubation with exogenous mature Nodal was not sufficient to restore HexP-GFP expression in explants treated with dec-RVKR-CMK, presumably because the Nodal co-receptor Cripto is downregulated under the conditions examined (Beck et al., 2002), and cannot access the apical membrane of the VE. However, HexP-GFP was rescued upon addition of recombinant activin, a Cripto-independent activator of the Nodal pathway (Fig. 5). Likewise, activin also restored expression of Lim1. These results confirm that the SPC inhibitor dec-RVKR-CMK is specific and blocks expression of EmVE markers by inhibiting Nodal processing.

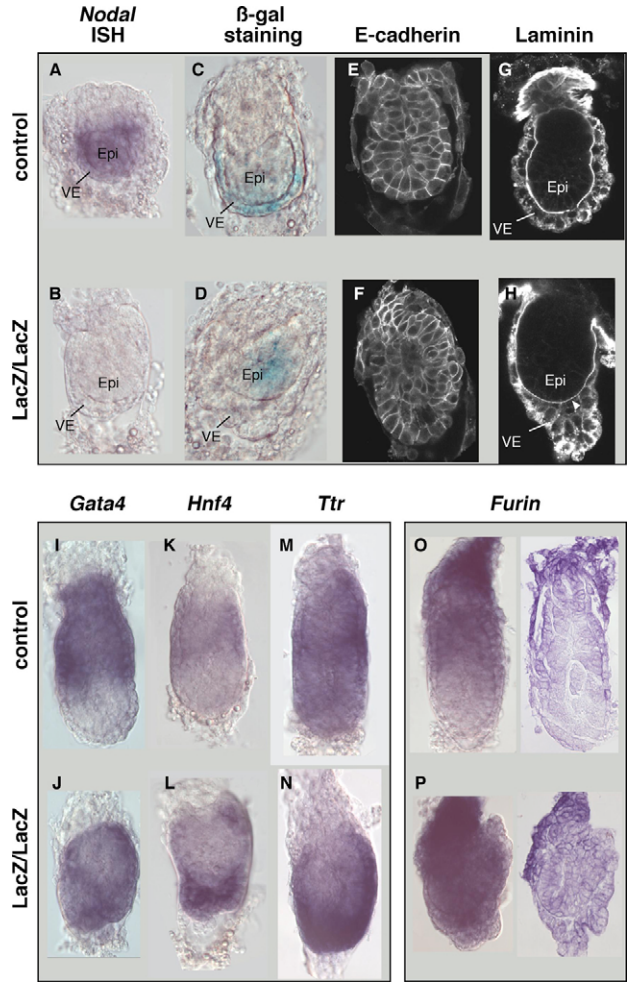

Fig. 2. Early Nodal signaling is required for normal epithelialization of the epiblast and to prevent ectopic expression of extra-embryonic VE markers and Furin in distal regions. (**A-D**) Whole-mount in-situ hybridization (ISH) of an antisense probe directed against the deleted exon 2 identifies *Nodal*$^{lacZ/lacZ}$ null mutants (B) and reveals specific expression of *Nodal* mRNA in the epiblast and EmVE of wild-type embryos (A). Nodal expression is also detected by β-galactosidase staining of *Nodal*$^{+/lacZ}$ heterozygotes (C). In E5.25 *Nodal*$^{lacZ/lacZ}$ homozygotes, *Nodal* expression independent of Nodal activity is detected only in the epiblast (D). (**E-H**) Immunostaining of E-cadherin (E,F) and laminin (G,H) reveals irregularities in the epithelial organization of mutant epiblasts (F), and detachment of distal embryonic visceral endoderm cells from the laminin-positive basement membrane (arrowhead in H). (**I-P**) In wild-type and Nodal$^{+/lacZ}$ heterozygous embryos (control), *Gata4*, *Hnf4*, *Ttr* and Furin adopt a restricted expression in the ExVE around E5.5 (I,K,M,O). In sharp contrast, ectopic distal expression of these markers is observed in Nodal$^{lacZ/lacZ}$ null mutants (J,L,N,P).

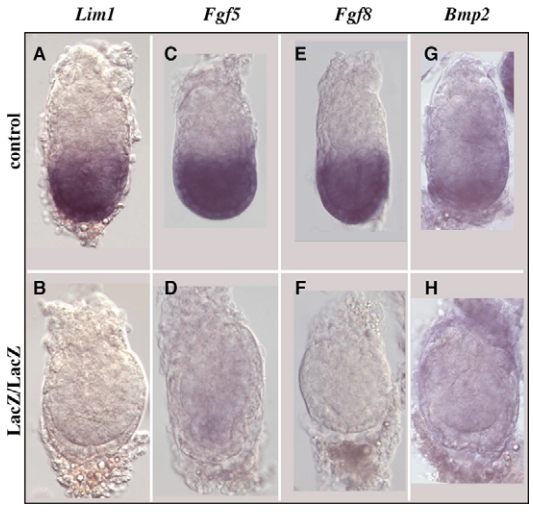

Fig. 3. Early Nodal signaling before DVE formation is essential to specify EmVE. In pre-DVE control embryos (**A**,**C**,**E**,**G**), which have not yet upregulated HexP-GFP expression (E5.25, see Materials and methods for accurate staging of embryos), *Lim1, Fgf5, Fgf8* and *Bmp2* are specifically expressed in the EmVE. By contrast, all of these markers fail to be induced in Nodal mutants (**B**,**D**,**F**,**H**). Note that Fgf5 and Fgf8 also show expression in the epiblast of wild-type embryos, which is downregulated in Nodal$^{lacZ/lacZ}$ null mutants (C,E,D,F).

Nodal is required before E5.5 to maintain pluripotency in the epiblast

Previous work suggests that Nodal processing is also essential for the epiblast to maintain expression of Oct4 during gastrulation (Beck et al., 2002). To assess whether Nodal already influences pluripotency during implantation, we monitored the expression of *Oct4* and other markers of pluripotent cells in early Nodal mutants and control littermates before E5.5. Whereas *Fgf4* and *Sox2* were normally expressed, *Oct4*, *Nanog*, *Foxd3* and *Cripto* expression levels were already reduced or undetectable at E5.0 (Fig. 6A), suggesting that pluripotency is severely compromised or lost by that stage.

Self-renewing pluripotent mouse ES cells depend on the presence of Bmp4 in the culture medium (Chambers et al., 2003), raising the question of whether Nodal mutants cannot maintain *Oct4* expression simply because they downregulate *Bmp4* (Brennan et al., 2001). Contrary to this idea, we found that *Bmp4* remained normally expressed in Nodal mutants at E5.25 (Fig. 6B). Likewise, the proprotein convertases Furin and Pace4 were still present (Fig. 2P, Fig. 6B), suggesting that markers of pluripotency are not simply lost at this early stage due to a lack of ExE. Therefore, Nodal either directly induces *Oct4* in the epiblast, or maintains pluripotent cells indirectly by specifying the EmVE. To distinguish between these possibilities, the effect of Nodal on the expression of *Oct4* was analyzed in cultured embryo explants. Epiblast explants isolated at E5.75 without VE failed to maintain *Nodal* expression, due to the absence of Nodal proprotein convertases and the resulting inhibition of auto-induction (Guzman-Ayala et al., 2004). Likewise, epiblast explants cultured without VE and ExE also downregulated *Oct4* (Fig. 6C). However, *Oct4* expression could be restored in the absence of VE by adding recombinant Nodal. This effect was specific, as it was blocked by the Nodal receptor antagonist SB-431542 (Fig. 6C). Treatment of epiblast explants with recombinant Nodal also restored expresson of *Cripto* (Beck et al., 2002) and *Fgf4* (Guzman-Ayala et al., 2004). It follows that Nodal signaling within the epiblast is sufficient to stimulate *Oct4* expression and several other markers of pluripotency independently of the VE.

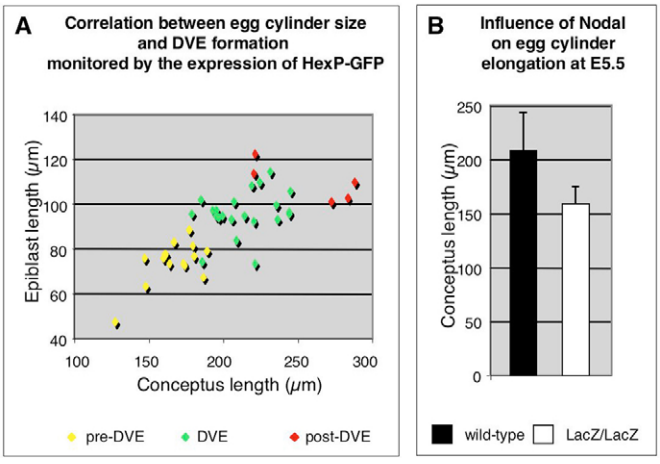

Fig. 4. The egg cylinder of Nodal mutants does not reach the size that is required for DVE formation. (**A**) In normal embryos, DVE formation monitored by HexP-GFP expression occurs only when the apex of the egg cylinder has reached a certain distance from the ExE (epiblast length). (**B**) While the length of the epiblast is difficult to measure in Nodal mutants due to the lack of a sharp epiblast/ExE boundary, a reduction in overall size (conceptus length) is readily apparent. Note that Nodal mutants at E5.5 do not reach the conceptus length that appears to be required for the DVE to be induced.

During the course of these experiments, we observed that epiblasts isolated before E5.5 together with VE maintained expression of *Oct4* for at least 30 hours. In addition, such explants also maintained significant expression levels of *Nodal* and *Furin* (Fig. 6D, top panels). However, in the presence of SB-431542 or the Furin inhibitor dec-RVKR-CMK, both *Oct4* and *Nodal* were downregulated (Fig. 6D). We conclude that endogenous Nodal and Furin activities are necessary to maintain *Nodal* and *Oct4* expression in embryo explants. However, once Nodal has been processed, its activity in the epiblast no longer depends on the VE (Fig. 6C).

DISCUSSION

The earliest known function of Nodal and its proprotein convertases Furin and Pace4 in the mouse embryo is to establish the DVE as a signaling center (Brennan et al., 2001; Beck et al., 2002), which defines the position of the future AP body axis

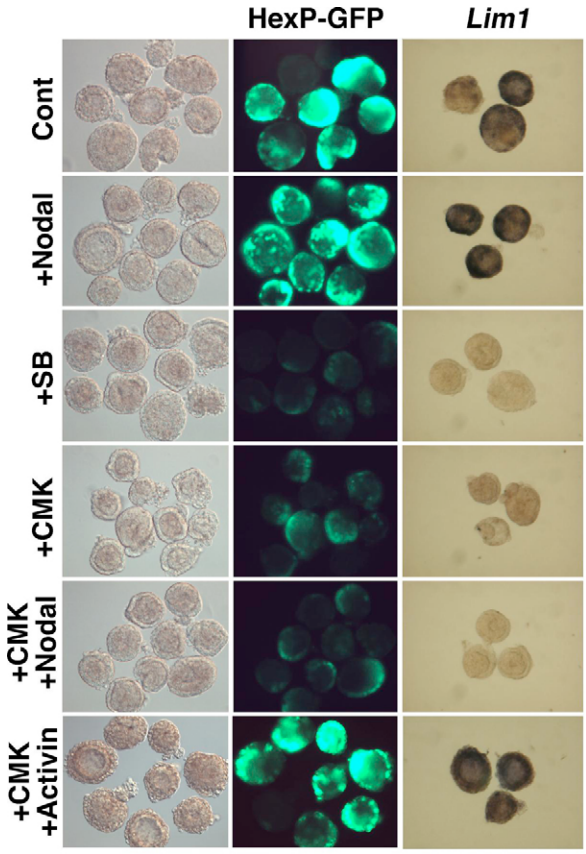

Fig. 5. Even in the absence of dominant inhibitory signals from ExE, induction of DVE depends on Nodal and proprotein convertase activities. As expected (Rodriguez et al., 2005), embryo explants stripped of ExE at the DVE stage (E5.5) induce *Lim1* and HexP-GFP throughout the EmVE endoderm (top row). Purified recombinant Nodal (50 μg/ml) does not prevent ectopic induction of DVE markers (second row). By contrast, blocking endogenous Nodal activity through inhibition of its signaling receptors (+SB) or proprotein convertases (+CMK) prevents ectopic induction of both HexP-GFP and *Lim1*. Processed Nodal (50 μg/ml) administred to the outer (apical) membrane of the visceral endoderm is not sufficient to ectopically induce HexP-GFP and *Lim1*, whereas processed Activin (50 μg/ml) is (bottom rows). Note that the thickness of the VE is significantly increased upon inhibition of Nodal signaling compared with untreated controls or explants treated with Activin. SB, SB-431542 (10 μmol/l); CMK, dec-RVKR-CMK (25 μmol/l).

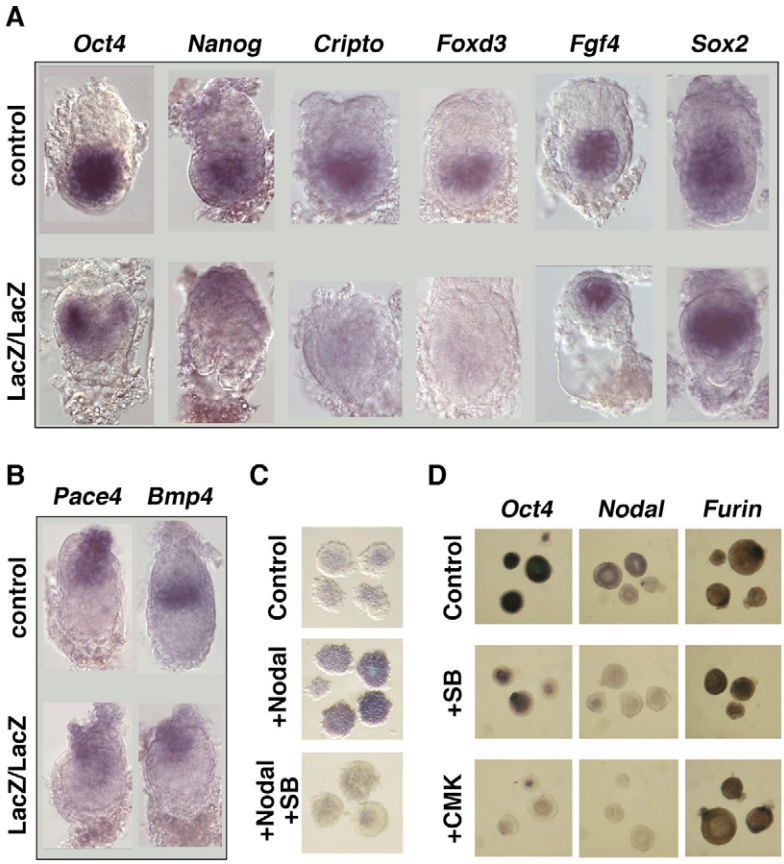

Fig. 6. Nodal mutants prematurely downregulate several determinants of pluripotency during implantation. (**A**) Whereas expression of *Fgf4* and *Sox2* is still unaffected, *Oct4*, *Nanog, Foxd3* and *Cripto* are already downregulated or silenced in Nodal mutants between E5.0 and 5.5. (**B**) By contrast, molecular markers of the ExE, such as *Pace4* and *Bmp4*, are not affected at this stage. (**C**) Epiblast explants of post-DVE embryos (E5.75) stripped of ExE and VE fail to maintain expression of Oct4/Pou5f1, unless they are cultured in the presence of recombinant Nodal. Administration of SB-431542 blocked the effect of exogenous Nodal protein, confirming that it is specific, even though in this experiment we used unpurified protein produced in human 293T cells (Beck et al., 2002). (**D**) Earlier explants isolated from DVE- and pre-DVE-stage embryos without VE did not survived well under the conditions examined (not shown). However, in the presence of VE, they maintained expression of *Oct4*, *Nodal*, as well as *Furin* (top row). By contrast, *Oct4* and *Nodal* were downregulated in explants cultured with inhibitors of endogenous Nodal (SB) or Furin (CMK) activities. Furin remained expressed in the VE, suggesting that these treatments are not toxic.

(Thomas et al., 1998; Yamamoto et al., 2004). In addition, Nodal signaling has recently been implicated in maintaining the pluripotency of cultured human ES cells (James et al., 2005). The present study reveals two important novel functions for mouse Nodal that pre-date DVE formation. First, we have shown that Nodal, Furin and Pace4 are already expressed during implantation, and that early Nodal signaling is essential to specify EmVE and confine the expression of Furin to the extra-embryonic region. As Furin and Pace4 potentiate Nodal activity, this conversion of PrE into EmVE is likely to be important to spatially regulate Nodal processing during gastrulation. In addition, differentiation of the EmVE, and adhesion of the epiblast are necessary to enlarge the egg cylinder (Fassler and Meyer, 1995; Coffinier et al., 1999; Smyth et al., 1999; Miner et al., 2004) and thereby distance its apex from the source of dominant signals in the ExE that prohibit DVE formation (Rodriguez et al., 2005). Furthermore, our embryo explant experiments show that Nodal and Furin activities are essential even in the absence of ExE to induce DVE markers. In the simplest model, Furin expression in the VE of such explants is necessary to activate proNodal, even though its loss in whole embryos is partially compensated by ExE-derived Pace4 (Roebroek et al., 1998; Beck et al., 2002). Thus, we conclude that besides stimulating egg cylinder growth, processed Nodal also provides an instructive signal to specify DVE. A second novel function for early Nodal signaling uncovered before E5.5 is to maintain molecular markers of pluripotency in the epiblast, including *Oct4*, *Nanog*, *Foxd3* and *Cripto*. Together, these results provide insights into the early function of Nodal that are essential to define how inductive tissue interactions after implantation control the fate of undifferentiated progenitor cells in the mammalian embryo.

The finding that several components of the Nodal pathway are already expressed during implantation raises the question of whether Nodal at this early stage mainly acts as an anti-differentiation signal, or whether it also provides important polarity cues anticipating future asymmetries within the embryo. In particular, the presence of Lefty1 and Hex may be indicative of a vestigial DVE, as these markers at E5.5 are confined to the distal tip of the egg cylinder (Oulad-Abdelghani et al., 1998; Thomas et al., 1998). While our results do not exclude a role for Lefty1 in the blastocyst, several observations argue against the idea that the DVE is already induced at E4.5. First of all, targeted inactivation of Lefty1 does not disrupt AP asymmetry (Meno et al., 1998), because the DVE at later stages expresses the unrelated Nodal antagonist Cer1, which is sufficient to repress posterior cell fates in the adjacent epiblast (Perea-Gomez et al., 2002). It follows that Lefty1 cannot be used as a reliable marker to decide whether a functional DVE is present or not. However, *Cer1* mRNA expression is undetectable before E5.5, even though in-situ hybridization reveals a very robust signal once the DVE is induced. Together, these observations argue against the idea that a functional DVE is established before E5.5. Finally, and most importantly, Lefty1 and Hex mRNA are not continuously expressed between E4.5-5.5, but instead are transiently downregulated between E5.0 and 5.25. This intriguing on/down/on pattern strongly suggests that the VE is not competent to form DVE before E5.5.

Previous work has shown that dominant inhibitory signals from the ExE are sufficient to prevent induction of HexP-GFP and necessary at E5.5 to confine DVE formation to the apex of the egg cylinder (Rodriguez et al., 2005). As the ExE arises during the time window between E5.0 and 5.25 when *Lefty1* and *Hex* are downregulated, this tissue is likely to be responsible for repressing DVE formation before E5.5. Therefore, we propose that DVE

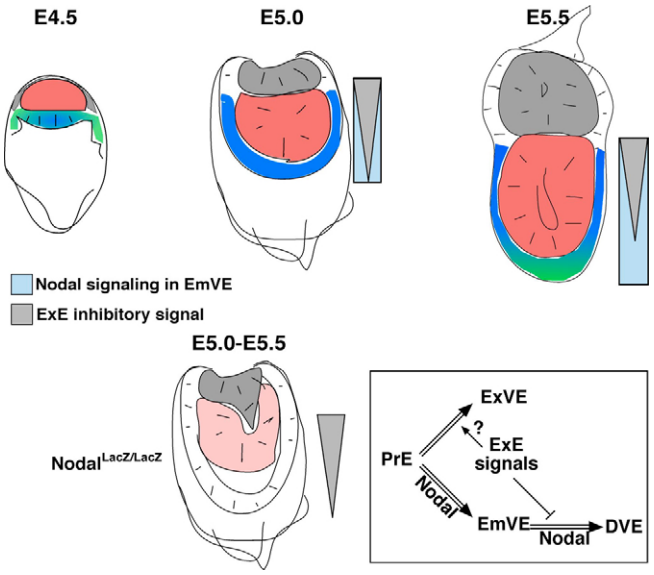

Fig. 7. Role of early Nodal signaling in overcoming dominant inhibitory signals from the ExE. Nodal initially maintains pluripotent cells in the epiblast (dark red) and must overcome dominant inhibitory signals from the ExE before it can specify DVE. Between E4.5 and 5.0, Nodal signaling (blue) specifies the VE abutting the epiblast to become EmVE and provide an adhesive substratum for the epiblast. Paracrine signals from the ExE (gray) pattern the VE in the extra-embryonic region (ExVE) (Dziadek, 1978), and prevent the conversion of EmVE into DVE (Rodriguez et al., 2005). Therefore, to establish the competence for DVE formation, we propose that EmVE first must promote elongation of the egg cylinder to distance its apex from the ExE. In addition, after overcoming the non-permissive stage, sustained Nodal activity potentiated by the proprotein convertases Furin and Pace4 (Beck et al., 2002) is also essential as an instructive signal, which actively induces DVE-specific genes. In Nodal$^{lacZ/lacZ}$ null mutants, the entire VE assumes an extra-embryonic character (ExVE) and subsequently fails to form DVE. In addition, the epiblast prematurely downregulates determinants of pluripotency (light red). Whether this is a consequence or a cause of the absence of the EmVE remains to be further investigated.

cannot form until the apex of the egg cylinder has reached a certain distance from the ExE (Fig. 7). According to this model, Nodal signaling in the VE is required before E5.5 to impose an embryonic identity on the VE that is in contact with the epiblast. In response to Nodal, we have found that the EmVE broadly expresses Fgf8 and Fgf5 and provides an adhesive substratum that is essential for normal growth of the epiblast (Smyth et al., 1999). It is plausible that the resulting growth of the egg cylinder sufficiently removes the ExE for distalmost cells to become competent to form DVE. Moreover, our explant experiments show that, even in the absence of ExE, the DVE does not arise by default. Instead, Nodal is essential both to overcome the inhibitory influence of the ExE *and* as an instructive signal to induce DVE-specific genes.

Using embryo explant cultures, we could show that Nodal signaling is also necessary and sufficient to maintain the expression of *Oct4* in the epiblast independently of its role in the VE. This observation is consistent with the fact that *Oct4* expression at E6.5 is drastically reduced in mutant embryos lacking Nodal or its proprotein convertases Furin and Pace4 (Brennan et al., 2001; Beck et al., 2002). Therefore, we asked whether Oct4 or other known markers of pluripotent cells already depend on Nodal during implantation. Indeed, analysis of Nodal mutants revealed that expression of *Oct4* and *Foxd3* was already downregulated before E5.5, whereas that of *Sox2* and *Fgf4* was unaffected at that stage. However, *Fgf4* expression is lost by E6.5 (Guzman-Ayala et al., 2004), as expected if Oct4 protein is no longer present in sufficient amounts to synergize with Sox2 and directly activate the Fgf4 promoter (Yuan et al., 1995; Nichols et al., 1998). Foxd3 is essential in the ICM to derive ES cell lines, and to sustain a normal epiblast after implantation (Hanna et al., 2002). In particular, while loss of Foxd3 apparently does not affect DVE formation, it abolishes the expression of *Nodal* and its targets in the epiblast during gastrulation, including Cripto (Hanna et al., 2002). Foxd3 thus emerges as a key mediator of Nodal activities in the embryonic lineage. However, the direct target genes of Foxd3 required in ES cells or during gastrulation are unknown. One candidate is Foxa2 (previously Hnf3β), which is induced in the primitive streak but absent in ES cells, possibly because a physical interaction with Oct4 can mask the DNA-binding domain of Foxd3 (Guo et al., 2002). Finally, progenitor cells in the ICM also depend on the homeobox transcription factor Nanog (Mitsui et al., 2003). Nanog is highly expressed in early blastocysts, but downregulated during implantation (E4.5) when the ICM loses its potential to generate self-renewing ES cells (Chambers et al., 2003). During egg cylinder formation (E5.0-5.5), we detected low levels *Nanog* mRNA in the epiblast of normal embryos, but not in Nodal mutants. Even though the functional significance of Nanog at this stage is unknown, this result together with the observed downregulation of Foxd3 and Oct4 strongly suggests that pluripotent cells are prematurely lost in Nodal mutants. Recently, Nodal signaling has also been implicated in inhibiting the differentiation of cultured human ES cells (James et al., 2005). By contrast, mouse ES cells can be derived and cultured in the absence of Nodal and its signal transduction machinery (Varlet et al., 1997; Gu et al., 1998; Sirard et al., 1998; Tremblay et al., 2000). It follows that Nodal activity in the mouse embryo is required only after implantation to sustain pluripotent cells. We have not analyzed the fate of cells that prematurely downregulate markers of pluripotency, as Nodal-deficient epiblasts do not adhere well to VE and become disorganized, possibly as a secondary consequence of their exposure to ectopic ExVE. Nodal mutants also ectopically express *Furin* in the epiblast, which may contribute to premature or aberrant differentiation. Consistent with this idea, forced expression of Furin in wild-type mouse ES cells blocks self-renewal and triggers rapid differentiation (D.B.C., unpublished). It will be interesting to determine in future experiments whether or not this effect of ectopic Furin expression relies on Nodal processing, and to characterize the resulting cell types.

Recently, the TGFβ family member Gdf3 has been shown to play an important role in embryonic stem cells and in peri-gastrulation mouse embryos. By E6.5, Gdf3 null mutants share some defects of *Nodal*$^{-/-}$ embryos (Chen et al., 2006). However, it is controversial whether Gdf3 primarily acts as a Nodal/activin family member (Chen et al., 2006) or rather as a Bmp inhibitor (Levine and Brivanlou, 2006). To address this issue, it will be necessary to characterize in future studies the expression of Nodal target genes in the EmVE and epiblast of Gdf3 null mutants between E5.0 and 5.5.

Besides its role in the VE and epiblast, Nodal is also required to maintain trophoblast stem cells in ExE during gastrulation (Guzman-Ayala et al., 2004). However, between E5.0 and 5.5, we did not observe impaired expression of ExE markers in Nodal$^{lacZ/lacZ}$ null mutant embryos. In particular, *Bmp4*, *Furin* and *Pace4* and the trophoblast stem cell marker *Cdx2* were unaffected, and the differentiation marker *Mash2* (*Ascl2* – Mouse Genome Informatics) was not precociously upregulated (unpublished data). These results suggest that the ExE does not depend on Nodal until after E5.5.

Overall, this study demonstrates that Nodal signaling in the mouse embryo is already required during implantation to expand a population of pluripotent progenitor cells in the epiblast, and to render visceral endoderm competent to form DVE, before its known role during gastrulation and axis specification. Secondly, it uncovers a novel role for Furin in potentiating Nodal signaling locally within the primitive endoderm and embryonic visceral endoderm during a limited time window, a function that has been masked in Furin mutants (Roebroek et al., 1998) due to the partially redundant activity of the related Nodal convertase Pace4 (Beck et al., 2002). Together, these results suggest a model in which proNodal matures locally in the embryonic hemisphere during implantation, before the expression of Furin and Pace4 becomes restricted to the extra-embryonic region.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/13/2497/DC1

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