Int. J. Dev. Biol. 45: 311-320 (2001)

Role of the anterior visceral endoderm in restricting posterior signals in the mouse embryo

AITANA PEREA-GOMEZ, MURIEL RHINN[1] and SIEW-LAN ANG*

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, Illkirch Cedex, Strasbourg, France

ABSTRACT Recent genetic and embryological experiments have demonstrated that head formation in the mouse embryo is dependent on signals provided by two organising centers during gastrulation, the anterior visceral endoderm (AVE) and the anterior primitive streak (also called the Early Gastrula Organiser, EGO). However the molecular nature of the signals triggering anterior neural formation from the epiblast is not clearly understood. The analysis of mouse mutants has allowed the identification of some of the molecular players involved in the process of head formation. In this review, we describe different mutant embryos in which impairment of visceral endoderm function leads to similar defects in antero-posterior axis specification. These phenotypes are consistent with a role of the AVE in protecting anterior embryonic regions from signals that promote posterior development. We propose that a genetic cascade in the AVE, involving *HNF3β*, *Lim1*, Otx2, *Smad2* and *ActRIB*, leads to the production of secreted TGFβ antagonists that protect the anterior epiblast region from Nodal signalling.

KEY WORDS: *visceral endoderm, mouse, gastrulation, mesoderm, anterior-posterior patterning, organiser.*

Introduction

Hans Spemann and Hilde Mangold first described the existence of an organiser tissue in the Newt embryo on the basis of the ability of this structure to induce secondary axis formation when grafted ectopically (reviewed in Hamburger, 1988). Since then, embryological experiments and gene expression analyses have revealed the presence of a structure functionally equivalent to the Spemann-Mangold organiser in different vertebrate species (reviewed by Lemaire and Kodjabachian, 1996). In the mouse, the anterior primitive streak and its derivative the node, can induce secondary axis formation when grafted ectopically, and give rise to the axial mesoderm derivatives in the induced axis (Beddington, 1994; see Camus and Tam, 1999 for a review). It has been therefore proposed that the anterior primitive streak (and later the node) are the functional equivalents of the Spemann-Mangold organiser in the gastrulating mouse embryo. However, neither the mouse node nor its precursors in the anterior primitive streak (Early Gastrula Organiser, EGO) can induce ectopic formation of head structures nor ectopic expression of anterior neural markers (Beddington, 1994; Tam and Steiner, 1999). This result has led to the supposition that the mouse node/anterior primitive streak organiser is equivalent to the amphibian late gastrula trunk organiser described by Spemann (reviewed in Hamburger, 1988), and that an additional structure of the gastrulating mouse embryo might be involved in head organiser function.

Recent embryological and genetic evidence point to a role of the anterior visceral endoderm (AVE) in anterior patterning of the mouse embryo (for a review see Beddington and Robertson, 1999). The visceral endoderm (VE) germ layer surrounds the epiblast and the extraembryonic ectoderm of the early postimplantation mouse embryo (5.5 day post coitum, d.p.c.). The VE surrounding the epiblast region is progressively displaced towards the extraembryonic region where it will contribute to the formation of the visceral yolk sac. The displacement of the VE cells is thought to occur via two main mechanisms: active movement before gastrulation (Weber *et al.*, 1999; Thomas *et al.,* 1998), and replacement by definitive endoderm cells during gastrulation (Lawson and Pedersen, 1987). Besides a central role in nutrient uptake and delivery (reviewed in Bielinska *et al.*, 1999), the visceral endoderm cells play a role in the process of cavitation that allows the formation of the central amniotic cavity in the embryonic ectoderm (Coucouvanis and Martin, 1999). The patterning function of the visceral endoderm has been recently unraveled using both embryological and genetic manipulations. Ablation experiments performed at early gastrulation stages (6.5 d.p.c.) have suggested a role for the AVE cells lining the future forebrain region in controlling the formation of rostral neurectoderm structures as

Abbreviations used in this paper: AVE, anterior visceral endoderm; VE, visceral endoderm; EGO, Early Gastrula Organiser; d.p.c., day post coitum.

***Address correspondence to:** Siew-Lan Ang. Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, BP163, 67404 Illkirch Cedex, C.U. de Strasbourg, France. e-mail: siew-lan@igbmc.u-strasbg.fr

[1]Current address: Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Pfotenhauer Str. 108, 01307, Dresden, Germany.

0214-6282/2001/$25.00

Printed in Spain
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revealed by the expression of the homeobox gene *Hesx1* (Thomas and Beddington, 1996). The production of chimeras has confirmed the role of the AVE in forebrain development. This technique allows the production of embryos in which the visceral endoderm and the extraembryonic ectoderm are of a defined genotype, different from that of the epiblast cells that will give rise to all the definitive germ layers of the embryo after gastrulation is completed. The use of this technique has demonstrated essential roles of the transcription factors Otx2 (Rhinn *et al.*, 1998), HNF3β (Dufort *et al.*, 1998) and Lim1 (Shawlot *et al.,* 1999), and of the TGFβ superfamily protein Nodal (Varlet *et al.*, 1997), in the visceral endoderm for the correct development of the prospective forebrain region.

Nevertheless, recent experiments involving grafting of fragments of germ layer tissues isolated from early gastrula stage mouse embryos into ectopic locations of host mouse embryos, indicate that the AVE alone is not sufficient to induce neural development in the mouse embryo. In contrast, induction of anterior neural genes can be achieved with a graft of a combination of AVE, anterior primitive streak and anterior epiblast (Tam and Steiner, 1999). These results suggest that in the mouse gastrula, AVE signals act synergistically with signals from the anterior primitive streak and the anterior epiblast to induce anterior neural structures.

The AVE cells express transcription factors such as the homeodomain containing proteins Goosecoid (Blum *et al.*, 1992; Filosa *et al.*, 1997), Otx2 (Simeone *et al.*, 1993; Ang *et al.*, 1994) and Hex (Thomas *et al.*, 1998), the LIM-homeodomain transcription factor Lim1 (Barnes *et al.,* 1994; Shawlot and Behringer, 1995) and the winged helix protein HNF3β (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Sasaki and Hogan, 1993; Filosa *et al.*, 1997). The AVE also produces secreted molecules that might act on the adjacent epiblast, such as Cer-l (Belo *et al.*, 1997; Biben *et al.,* 1998; Shawlot *et al.*, 1998; Pearce *et al.*, 1999), mDkk-1 (Glinka *et al.*, 1998), and the TGFβ superfamily proteins Nodal and Lefty1 (Conlon *et al.*, 1994; Meno *et al.*, 1996; Varlet *et al.*, 1997; Oulad-Abdelghani *et al.*, 1998). The AVE and the anterior primitive streak organiser (EGO) share the expression of several genes such as *HNF3β*, *Lim1*, *Otx2* and *Goosecoid* further suggesting that both tissues can display organising function (Fig. 1)

The experiments mentioned above point to a role of the AVE in induction and/or patterning of the forebrain region in the mouse embryo and provide an indication of the molecular players involved in this process. However, the precise signalling events underlining the action of the AVE on the anterior epiblast, as well as the genetic cascades controlling these signals remain obscure. In this review, we will focus on the analysis of several mouse mutants in which failure of visceral endoderm function leads to similar defects in epiblast patterning during gastrulation. A comparison of the phenotypes of these mutant embryos strongly suggests that one of the main functions of the AVE is to provide signals that repress posterior fate in the anterior epiblast of the mouse gastrula, in order to allow anterior neural development in a region free of posterior signals.

Antero-posterior patterning in early mouse embryos

The anterior-posterior axis is established before gastrulation

Cell lineage studies have shown that the AVE is derived from visceral endoderm cells located at the distal tip of the embryo at 5.5 d.p.c. (reviewed in Beddington and Robertson, 1999). Distal visceral endoderm cells express the homeobox gene *Hex* at 5.5 d.p.c. and move to anterior positions to occupy the anterior midline region of the visceral endoderm between 5.5 d.p.c. and 6.0 d.p.c. (Thomas *et al.*, 1998). Although the nature of the movement of the *Hex* expressing distal visceral endoderm cells is not clearly understood, this observation indicates that the visceral endoderm is patterned along the proximodistal axis of the embryo at 5.5 d.p.c. Gene expression analysis has demonstrated that the epiblast also exhibits proximodistal asymmetry around this stage. The TGFβ molecule Nodal and the EGF/CFC family member Cripto which are initially expressed in all the epiblast at 5.5 d.p.c., become restricted to proximal epiblast at around 5.75 d.p.c. (Varlet *et al.*, 1997; Ding *et al.*, 1998). The transcription factor Brachyury (also named T, Wilkinson *et al.*, 1990) and the secreted molecule Wnt3 are expressed in the proximal epiblast at 5.75 d.p.c. (Thomas *et al.*, 1998; Liu *et al.*, 1999). The initial proximodistal asymmetry of the visceral endoderm is then transformed into an anterior-posterior asymmetry by migration of the distal *Hex* expressing visceral endoderm cells towards anterior positions (Thomas *et al.*, 1998). Simultaneously, the expression of *Brachyury, Nodal*, *Cripto* and *Wnt3* is shifted towards the posterior epiblast where the primitive streak will form at 6.5 d.p.c (Varlet *et al.*, 1997; Thomas *et al.,* 1998; Ding *et al.*, 1998; Liu *et al.*, 1999). In the primitive streak, the epiblast cells will undergo an epithelial to mesenchymal transition, delaminate and migrate as mesoderm cells that will lie between the epiblast and the endoderm germ layers (reviewed by Tam and Behringer, 1997). Fate map studies have demonstrated that during early gastrulation at 6.5 d.p.c., lateral and posterior regions of the epiblast are displaced towards the primitive streak and mainly give rise to extraembryonic mesoderm (Lawson *et al.*, 1991; reviewed by Tam and Behringer, 1997). In contrast, distal and anterior epiblast regions of the 6.5 d.p.c. embryo are displaced towards more proximal/anterior positions where they will contribute to the formation of neural structures at later stages (Lawson *et al.*, 1991; Quinlan *et al.*, 1995). Thus, from early gastrulation stages (6.5 d.p.c.), the prospective neuroectoderm of the embryo is located opposite to the epiblast region that expresses *Brachyury*, *Nodal*, *Cripto* and *Wnt3* and is in close contact to the visceral endoderm cells that express *Hex*, *Cer-l* and *Lefty1*.

Nodal related signals are involved in primitive streak formation

The signals triggering the formation of the primitive streak and mesoderm cells in the mouse embryo are not clearly identified. There is however evidence that signalling pathways mediated by TGFβ superfamily members are involved in primitive streak formation. *Nodal* homozygous mutant embryos fail to form a proper primitive streak (Conlon *et al.*, 1994), suggesting that Nodal is involved in the induction and/or maintenance of the primitive streak. Evidence from other experimental models such as *Xenopus* and zebrafish point clearly to a role of Nodal-related signalling in mesoderm formation (Osada and Wright, 1999; Feldman *et al.*, 1998; reviewed by Schier and Shen, 2000). Although clear biochemical evidence is not available, genetic analysis in mouse and gain of function analysis in *Xenopus* and zebrafish suggest that Nodal acts through ActRIB/ActRII dimers (reviewed in Whitman, 1998; Song *et al.*, 1999; Gritsman *et al.*, 1999). In agreement with a role of *Nodal* in primitive streak formation, *ActRIB* homozygous mutants and *ActRIIA;ActRIIB* double homozygous mutant embryos do not form mesoderm (Gu *et al.*, 1998; Song *et al.*, 1999). Altogether these results point to a role of TGFβ signalling, and in particular Nodal signalling, in controlling primitive streak formation in the posterior epiblast region of the mouse gastrula.

Lessons from the mutants: AVE signals are necessary to prevent posterior development in the anterior epiblast

HNF3β;Lim1 double mutants

HNF3β and *Lim1* code for two transcription factors of the winged helix and Lim homeodomain families respectively (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Sasaki and Hogan, 1993; Barnes *et al.*, 1994; Shawlot and Behringer, 1995). These two genes are coexpressed in the visceral endoderm surrounding the epiblast before gastrulation (5.5 d.p.c.). At 6.5 d.p.c., *HNF3β* and *Lim1* are coexpressed in the anterior primitive streak and in the AVE. In addition, *Lim1* is expressed in the mesoderm wings exiting the primitive streak, and *HNF3β* is expressed in the visceral endoderm surrounding the extraembryonic region (Perea-Gomez *et al.*, 1999). Both genes are coexpressed in the node at 7.5 d.p.c. but only *HNF3β* expression is maintained in the axial mesoderm derivatives, as well as in the gut and in the ventral neural tube at 8.5 d.p.c. Homozygous *HNF3β* mutant embryos die at 10.5 d.p.c. and present dorsoventral patterning defects along the neural tube (Ang and Rossant, 1994; Weinstein *et al.*, 1994) due to the absence of node and axial mesoderm structures. Chimera experiments have further demonstrated that *HNF3β* functions cell autonomously in the formation of the node and axial mesendoderm. In addition *HNF3β* function is required in the VE for elongation of the primitive streak (Dufort *et al.,* 1998). *Lim1* mutant embryos die at 11.5 d.p.c. and show a headless phenotype with truncations of the rostral neuroectoderm up to rhombomere 3 in the hindbrain (Shawlot and Behringer, 1995). Chimera analysis has demonstrated that *Lim1* function is required both in the AVE and in the axial mesendoderm precursors for the normal induction and patterning of the rostral CNS (Shawlot *et al.*, 1999).

We recently reported the analysis of double homozygous mutant embryos for *HNF3β* and *Lim1* (*HNF3β*$^{-/-}$;*Lim1*$^{-/-}$ embryos, referred to as *HNF3β;Lim1* embryos, Perea-Gomez *et al.*, 1999). These double mutant embryos have a more severe phenotype than each of the single mutants and show profound disturbance of axial patterning from very early stages of development. In particular at the onset of gastrulation (6.5 d.p.c.), *HNF3β;Lim1* embryos have a disorganised epiblast in which cells have lost epithelial appearance and show mesenchymal characteristics. Primitive streak markers, such as *Brachyury* and *Fgf8* (Wilkinson *et al.*, 1990; Crossley and Martin, 1995), are ectopically expressed in *HNF3β;Lim1* embryos in all the epiblast including anterior and distal regions. In addition, the bHLH transcription factor Mesp1 (Saga *et al.*, 1996), and the TGFβ -related molecule Lefty2 (Meno *et al.*, 1997), expressed in two lateral bands of mesoderm cells on either side of the primitive streak in wildtype embryos, are ectopically expressed in the anterior and distal epiblast regions of *HNF3β;Lim1* embryos at 6.5 d.p.c. (Perea-Gomez *et al.*, 1999). These observations suggest that the primitive streak is abnormally expanded in *HNF3β;Lim1* embryos and that ectopic mesoderm formation occurs in anterior regions of the mutant embryos. In agreement with this interpretation, *Otx2* expression which normally marks epiblast cells outside the primitive streak region at 6.5 dpc (Ang *et al.*, 1994), is absent in *HNF3β;Lim1* embryos.

We have generated chimeras where the extraembryonic tissues (VE and extraembryonic ectoderm) are made of *HNF3β;Lim1* double mutant cells, whereas the epiblast is made of wild-type cells (Perea-Gomez *et al.*, 1999). In these chimeric embryos the *HNF3β;Lim1* phenotype is not rescued despite the extensive colonisation of the epiblast by wild-type cells. Thus, the expansion of the primitive streak and the absence of anterior epiblast development can be attributed to the lack of *HNF3β* and *Lim1* function in the extraembryonic tissues. As *HNF3β* and *Lim1* are not expressed in the extraembryonic ectoderm, the *HNF3β;Lim1* phenotype is due to a primary defect in the visceral endoderm germ layer.

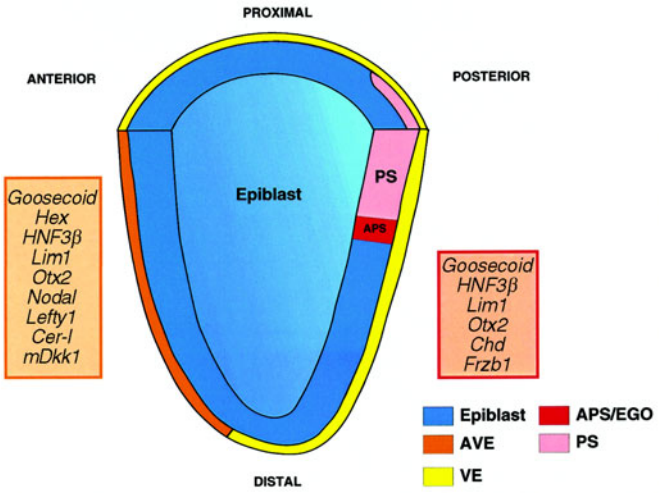

Fig. 1. Organizing centers in the early mouse gastrula express an overlapping set of genes. *Schematic representation of the distal region of a 6.5 d.p.c. mouse embryo. The extraembryonic region is not shown. The AVE (orange) overlies the prospective anterior neural region of the mouse embryo. In the proximal posterior epiblast, the primitive streak (PS, pink) is the site of mesoderm formation (pink lateral band). The anterior primitive streak (APS) or early gastrula organiser (EGO, red), expresses characteristic organiser genes some of which are also expressed in the AVE. References for the expression patterns can be found in text except for Frzb-1 (Leyns et al., 1997) and Chordin (Rhinn et al., 1998; Bachiller et al., 2000).*

Altogether these results suggest that in the absence of *HNF3β* and *Lim1* function in the AVE, anterior epiblast fates and notably *Otx2* expression are not maintained, whereas the posterior epiblast region is expanded as revealed by widespread expression of both primitive streak and mesoderm markers (Fig. 2A).

Otx2 mutants

The *Otx2* gene codes for a transcription factor containing a bicoid class homeodomain (reviewed by Simeone, 1998). *Otx2* is expressed in the epiblast and in the visceral endoderm of the 5.5 d.p.c. mouse embryo, at pregastrulation stages (Simeone *et al.*, 1993; Ang *et al.*, 1994). During gastrulation, *Otx2* expression in the epiblast and in the visceral endoderm becomes restricted to anterior regions (Ang *et al.*, 1994) and is maintained in the anterior epiblast that will give rise to the forebrain and midbrain regions at headfold stages (7.5 d.p.c.). *Otx2* homozygous mutant embryos die around 10.5 d.p.c. and show an anterior truncation rostral to rhombomere 2 (reviewed by Simeone, 1998). Chimera analysis has demonstrated that the expression of *Otx2* in the visceral endoderm is essential for the induction of the anterior neural plate (Rhinn *et al.*, 1998). However *Otx2* is also required in the neural plate for the correct regionalisation of the forebrain and midbrain

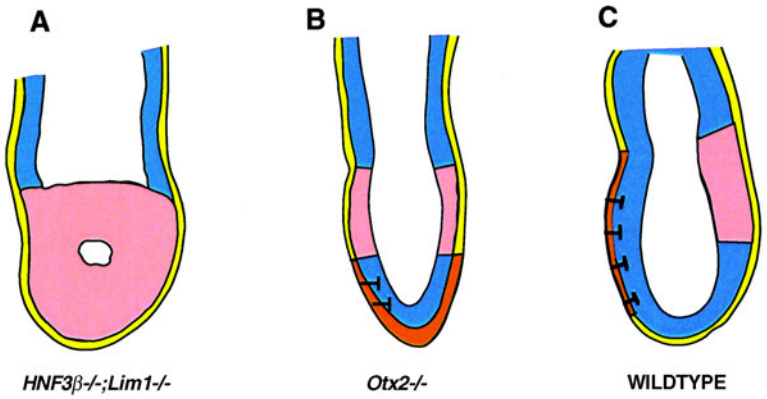

Fig. 2. Visceral endoderm defects lead to expansion of posterior epiblast tissues in *HNF3β;Lim1* and *Otx2*$^{-/-}$ mutants. *Schematic representation of sagittal sections through mutant and wildtype embryos at 6.5 d.p.c.* **(A)** *In HNF3β;Lim1 mutants, the anterior epiblast is absent and the whole embryonic region is composed of cells with primitive streak or mesoderm characteristics (pink). This defect is phenocopied by +/+<-> HNF3β;Lim1 chimeras, where the visceral endoderm is HNF3β;Lim1 mutant.* **(B)** *In Otx2*$^{-/-}$ *mutants, due to the abnormal location of the AVE (orange) the inhibitory interactions occur distally and not anteriorly. This defect can be rescued in embryos expressing hOtx1in the VE.* **(C)** *In a wildtype embryo, the primitive streak (pink) appears in the proximal-posterior epiblast. Analysis of the phenotypes of mutant embryos suggests that in wildtype embryos, the AVE (orange) provides signals (black bars) that antagonise posterior development in anterior regions (blue) of the mouse gastrula.*

regions. In particular *Otx2* is required cell autonomously for the expression of the homeodomain transcription factor Hesx1 in the forebrain and the secreted molecule Wnt1 in the midbrain (Rhinn *et al*., 1999). In addition, knock-in experiments have demonstrated that *Otx2* expression is required in the visceral endoderm for its own expression in the epiblast (Acampora *et al*., 1998). Thus, *Otx2* plays a clear role in the AVE at least in the induction and/or maintenance of *Otx2* expression in the epiblast. Recent observations have shown that two genes normally expressed in the posterior epiblast, *Fgf8* and *Brachyury*, are ectopically expressed in the anterior epiblast of *Otx2* mutants (Perea-Gomez *et al.,* 2000, submitted; Fig. 2B). Thus, some aspects of the phenotype in *Otx2* homozygous mutant embryos, are reminiscent of the one observed in *HNF3β;Lim1* embryos. In addition, the abnormal expression of *Brachyury* observed in *Otx2* mutants is rescued in embryos expressing the related gene *hOtx1* in the visceral endoderm but lacking *Otx2* expression in the epiblast (Acampora *et al*., 1998), suggesting that *Otx2* function is required in the visceral endoderm for correct *Brachyury* expression in the posterior epiblast. *Otx2* is not expressed in the AVE in *HNF3β;Lim1* embryos. Thus a part of the phenotype of these embryos could be due to the absence of *Otx2* transcripts in the AVE. However, the phenotype of *HNF3β;Lim1* embryos is more severe than the one of *Otx2* mutants (Fig. 2), suggesting that *HNF3β* and *Lim1* might have other targets in the visceral endoderm besides *Otx2*. The report of a direct protein-protein interaction of Otx2 with Hnf3β and Lim1, reinforces the hypothesis that these transcription factors act in a common pathway in the visceral endoderm cells where they are coexpressed (Nakano *et al*., 2000), and that regulatory interactions can take place between Otx2, Hnf3β and Lim1 in controlling the transcription of target genes.

Smad2 mutants

Smad proteins are intracellular transducers of TGFβ superfamily signalling pathways. In particular Smad2 is activated upon phosphorylation by type I TGFβ and activin receptors. Activated Smad2 forms a complex with Smad4, translocates to the nucleus and binds to regulatory sequences of target genes in association with transcription factors such as Fast1 (reviewed by Whitman, 1998). In the mouse embryo, *Smad2* is ubiquitously expressed during gastrulation (Waldrip *et al*., 1998). Five different mutations in the *Smad2* gene have been reported. Three of these mutations lead to a very severe phenotype as homozygous mutant embryos do not form mesoderm and show a high degree of disorganisation in the egg cylinder (Nomura and Li, 1998; Weinstein *et al*., 1998). The two other mutations, S*mad2*^{Robm} and *Smad2*^{dex2}, lead to a less severe phenotype (Waldrip *et al.*, 1998; Heyer *et al*., 1999). In *Smad2*^{Robm} homozygous mutant embryos (*Smad2*^{Robm} embryos), *Otx2* is not expressed in the epiblast, whereas *Brachyury*, *Nodal* and *Fgf8* expression is abnormally expanded in most of the epiblast. As a result of this abnormal epiblast patterning, *Smad2*^{Robm} embryos form normal extraembryonic structures with extraembryonic endoderm, ectoderm and mesoderm, but show complete absence of embryonic derivatives at head fold stages (7.5 d.p.c.). Diploid chimeras in which the extraembryonic tissues are formed of *Smad2*^{Robm} cells and the epiblast is largely contributed by wild type cells, phenocopy *Smad2*^{Robm} embryos, indicating that *Smad2* function is required in the extraembryonic tissues (Waldrip *et al*., 1998). The reverse experiment has been performed and demonstrates that wild type extraembryonic cells rescue the early phenotype of *Smad2*^{Dex2} embryos, however *Smad2* function is also essential in the epiblast derivatives for the correct morphogenesis of the forebrain, and for the proper embryo turning and looping of the cardiac tube (Heyer *et al*., 1999). The phenotypes of *Smad2*^{Robm} embryos and *HNF3β;Lim1* embryos during gastrulation are very similar. Although chimera analysis does not allow to distinguish between a requirement for *Smad2* in the visceral endoderm versus the extraembryonic ectoderm, it is tempting to speculate that *Smad2* function, like *HNF3β* and *Lim1,* is required in the visceral endoderm. In agreement with this hypothesis, *Lim1* and *HNF3β* transcripts are not detected in *Smad2*^{Robm} embryos at 6.5 d.p.c. (Waldrip *et al*., 1998), suggesting that *Smad2, HNF3β* and *Lim1* act in the same pathway in the visceral endoderm cells and that *Smad2* could act upstream of *HNF3β* and *Lim1* .

ActRIB mutants

ActRIB is a type 1 transmembrane serine/threonine kinase receptor that can mediate activin signal upon phosphorylation by type 2 activin receptors A or B (ActRIIA/B) bound to activin dimers (reviewed by Whitman, 1998). *ActRIB* is expressed in the epiblast and in the extraembryonic ectoderm prior to gastrulation, and is detected in the visceral endoderm from 6.5 d.p.c. onwards (Gu *et al*., 1998). Targeted mutation of *ActRIB* leads to embryonic lethality (Gu e*t al.*, 1998). *ActRIB* homozygous mutant embryos show a severe disorganisation of epiblast and extraembryonic ectoderm and do not form mesoderm. Diploid chimeras generated by injec-

tion of wild-type cells into *ActRIB* mutant morulae (*+/+<->ActRIB*$^{-/-}$ chimeras), have demonstrated that *ActRIB* function is essential in the extraembryonic tissues for correct patterning of embryonic tissues (Gu *et al.*, 1998). Indeed in these chimeric embryos, like in *Smad2*^{Robm} mutants (Waldrip *et al.*, 1998), no embryonic ectoderm or mesoderm derivatives are formed and only extraembryonic tissues are present, indicating an essential function of *ActRIB* in the visceral endoderm or the extraembryonic ectoderm in controlling the development of embryonic derivatives. *+/+<->ActRIB*$^{-/-}$ chimeras were analysed at 7.5 d.p.c., thus it is not known whether these defects result from abnormal epiblast patterning at earlier stages. However the similarity of the phenotype of these embryos and *Smad2*^{Robm} embryos at 7.5 d.p.c. suggest that as for *Smad2*^{Robm} mutants, epiblast patterning might be affected in *+/+<->ActRIB*$^{-/-}$ chimeras at 6.5 d.p.c. *Smad2* is a transducer for activin and TGFβ signalling pathways (reviewed by Whitman, 1998). Thus *ActRIB* could be upstream of *Smad2*, *HNF3β, Lim1* and *Otx2* in the VE pathway that leads to restriction of posterior development. This hypothesis is tempting in light of the data obtained in *Xenopus* and zebrafish embryos, demonstrating that the homologues of *HNF3β* and *Lim1* are inducible by the TGFβ family members Nodal and activin (Toyama *et al.*, 1995; Rebbert and Dawid, 1997; Howell and Hill, 1997; Feldman *et al.*, 1998). Alternatively, *ActRIB* might function in the extraembryonic ectoderm where *Smad2* is also expressed, to indirectly regulate the patterning function of the VE.

In summary the analysis of the phenotypes of the mutants and chimeric embryos described above suggest that *ActRIB*, *Smad2*, *HNF3β*, *Lim1* and *Otx2*, are required in the VE for patterning of the epiblast before gastrulation. In *HNF3β*$^{-/-}$*;Lim1*$^{-/-}$, *Otx2*$^{-/-}$ and *Smad2*$^{Robm-/-}$ embryos, posterior epiblast markers are abnormally expressed in the anterior epiblast region. One possible explanation for these mutant phenotypes is that during normal development, the AVE secretes inhibitory molecules that antagonise the activity of posterior signals involved in specifying posterior epiblast fates. According to this hypothesis, the expression of these inhibitory signals might be either downregulated or absent in the AVE of the mutant embryos described above, thus leading to the expression of posterior epiblast markers anteriorly. The similarity of the phenotypes of the various mutants also suggests that a common pathway involving *ActRIB*, *Smad2*, *HNF3β*, *Lim1* and *Otx2* might function to positively regulate the expression of the inhibitory signals in the AVE.

Cer-l and Lefty1 are TGFβ antagonists expressed in the AVE

Good candidates to mediate the action of the AVE on the adjacent distal and anterior epiblast are the secreted molecules Cer-l and Lefty1. Cer-l belongs to the DAN family of secreted factors, related to the *Xenopus* protein Cerberus (Bouwmeester *et al.*, 1996; Belo *et al.*, 1997; Biben *et al.,* 1998; Shawlot *et al.*, 1998; Pearce *et al.*, 1999). Injection of *Cerberus* mRNA in *Xenopus* embryos leads to ectopic head induction and to suppression of trunk-tail mesoderm formation (Bouwmeester *et al.*, 1996). Biochemical studies have demonstrated that *Xenopus* Cerberus inhibits the signalling activities of *Xenopus* BMP4, *Xenopus* Nodal-related1 (Xnr-1) and *Xenopus* Wnt8, through direct binding to these molecules (Piccolo *et al.*, 1999). These results led to the proposal that head formation in *Xenopus* requires the simultaneous inhibition of Nodal, BMP and Wnt signalling (Glinka *et al.*, 1997; Piccolo *et al.*, 1999). *Cer-l* mRNA is unable to mimic the head inducing activity of *Xenopus Cerberus* when injected ventrally in *Xenopus* embryos. However Cer-l is able to inhibit BMP activity in *Xenopus* and mammalian assays (Belo *et al.*, 1997; Biben *et al.*, 1998; Pearce *et al.*, 1999), as well as Nodal and X-Wnt8 activities in *Xenopus* assays (Belo *et al.*, 1999).

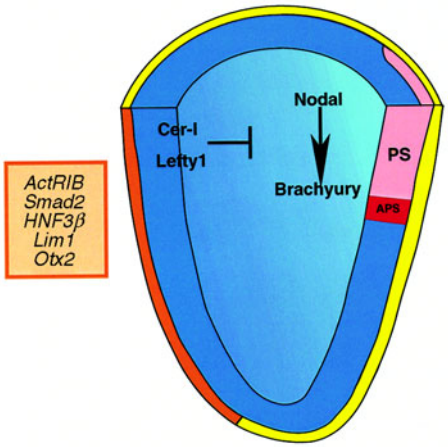

Fig. 3. A possible model for the action of the AVE in restricting posterior signals during mouse gastrulation. *The AVE produces the TGFβ antagonists, Cer-l and Lefty1. These secreted molecules in turn act on the adjacent anterior epiblast to antagonise Nodal signalling and prevent the appearance of primitive streak cells (expressing Brachyury) in anterior epiblast regions. Genetic studies suggest that the expression of Cer-l and Lefty1 in the AVE is controlled by a molecular cascade involving HNF3β, Lim1, Otx2, ActRIB and Smad2.*

Lefty1 belongs to a new subclass of the TGFβ superfamily, comprising also mouse Lefty2 (Meno *et al.,* 1996; Meno *et al.*, 1997; Oulad-Abdelghani *et al.*, 1998;), human LeftyA/EBAF and Lefty-B (Kothapalli *et al.*, 1997; Kosaki *et al.*, 1999), zebrafish Antivin/Lefty1 and Lefty2 (Thisse and Thisse, 1999; Bisgrove *et al.*, 1999), Xenopus Antivin (Cheng *et al.*, 2000), and chick Antivin/Lefty1 (Ishimaru *et al.*, 2000). Lefty proteins display distinct features when compared to other TGFβ superfamily proteins. In particular, they lack a cysteine residue required for dimerisation through disulfide linkage of TGFβ precursors. In addition, structural predictions by computer analysis indicate that zebrafish antivin lacks an α-helix normally required for dimerisation of TGFβ precursors (Thisse and Thisse, 1999). Overexpression of zebrafish *antivin*, or mouse *Lefty1* in zebrafish embryos leads to a depletion of mesendoderm tissue in a dose dependent manner (Thisse and Thisse, 1999; Bisgrove *et al.*, 1999). This effect can be overcome by coexpression of *activin* or *Cyclops*, a zebrafish *Nodal*-related gene. Antivin and Lefty could accordingly function as inhibitors of activin and/or Nodal signalling, possibly by competing for binding to activin and/or Nodal receptors. Supporting evidence for such a model has come from the analysis of mouse *Lefty2* homozygous mutant embryos. *Lefty2* is expressed in mesoderm cells exiting the mid-distal region of the primitive streak at 6.75 d.p.c., and in the lateral plate mesoderm during early somitic stages (Meno *et al.*, 1997). *Lefty2* homozygous mutant embryos show an expanded primitive streak and excess mesoderm production (Meno *et al.*, 1999). Based on the biochemical properties of Lefty proteins, it has

been proposed that *Lefty2* acts to restrict the extent of Nodal signalling arising in the primitive streak region. In agreement with this interpretation, the *Lefty2* mutant phenotype is partially suppressed by heterozygosity for *Nodal* (Meno *et al.*, 1999), further supporting that Lefty proteins function by antagonising Nodal-like signals. Thus, both biochemical and genetic evidence support the hypothesis that Cer-l and Lefty1 could function as Nodal antagonists.

The expression of Nodal in the early mouse embryo has been followed using a *Nodal-LacZ* line, in which the reporter gene *LacZ* is cloned downstream of the regulatory sequences of the endogenous *Nodal* locus via the knock-in approach (Varlet *et al.*, 1997). *Nodal* is expressed in the whole epiblast and visceral endoderm at 5.5 d.p.c., before the onset of gastrulation. Between 5.5 d.p.c. and 6.5 d.p.c., *Nodal* expression disappears first from the distal tip of the embryo and shortly thereafter from the anterior epiblast, and is maintained in posterior epiblast regions where the primitive streak arises around day 6.5 d.p.c. (Varlet *et al.*, 1997). There is increasing evidence that Nodal triggers a positive autoregulatory loop so that Nodal signalling is necessary for maintenance of *Nodal* expression. In zebrafish, the maintenance of expression of *Cyclops* and *Squint*, two nodal-related genes, is dependent on Nodal signalling (Meno *et al.*, 1999). In *Lefty2* homozygous mouse mutant embryos, the increase in Nodal signalling leads to upregulation of *Nodal* expression (Meno *et al.*, 1999). Recent analysis of the regulatory elements driving the expression of *Nodal* in the mouse embryo have demonstrated that the expression of *Nodal* in the epiblast from 5.5 d.p.c. to 6.5 d.p.c., and in the left lateral plate mesoderm at 8.0 d.p.c., is driven by an intronic enhancer that can respond to Nodal in a luciferase reporter assay (Saijoh *et al.*, 2000). Thus, Nodal could be involved in maintaining its own expression in the epiblast and in the lateral plate mesoderm.

We hypothesize that Cer-l and Lefty1 proteins secreted from the distal and anterior visceral endoderm cells, diffuse to the adjacent epiblast tissue and inhibit Nodal signalling. Inhibition of Nodal signalling might block the autoregulatory loop triggered by Nodal, resulting in downregulation of *Nodal* transcripts in the distal and anterior epiblast, and in restriction of *Nodal* expression to proximal and posterior epiblast regions (Fig. 3). This mechanism would allow the development of distal and anterior epiblast in a region free of Nodal signalling, and the formation of the primitive streak under the influence of Nodal activity in proximal and posterior epiblast regions.

Mutations in *Lefty1* and *Cer-l* have been reported. None of these mutants show a phenotype in epiblast patterning during gastrulation. *Lefty1* mutants display left-right positional defects in visceral organs (Meno *et al.*, 1998), whereas *Cer-l* mutants do not show any obvious phenotype during gastrulation (Simpson *et al.*, 1999; Belo *et al.,* 2000; Shawlot *et al.,* 2000; Stanley *et al.,* 2000). These results suggest that *Cer-l* and *Lefty1* might act cooperatively, and that the presence of one biochemically active Nodal antagonist might be sufficient for correct epiblast patterning. Such a phenomenon of redundancy between coexpressed unrelated proteins with similar biochemical activities has been recently exemplified by the case of the BMP antagonists Noggin and Chordin. *Chordin*$^{-/-}$*;Noggin*$^{-/-}$ compound mutant embryos show defects in forebrain development, whereas none of the single mutants show defects in anterior-posterior patterning (Bachiller *et al.,* 2000). Thus, understanding the function of *Lefty1* and *Cer-l* in the AVE awaits the production of compound mutant embryos.

Consistent with a role of *Lefty1* and *Cer-l* in restricting the extent of primitive streak in the mouse embryo, the expression of these two genes is altered in the mutants described in this review. In *HNF3β;Lim1* embryos, *Lefty1* expression is not detected, and *Cer-l* expression is greatly diminished (Perea-Gomez *et al.*, 1999). *Cer-l* is expressed in the distal visceral endoderm of *Otx2* mutants (Biben *et al.*, 1998) whereas *Lefty1* expression is absent (our unpublished results). It is thus tempting to correlate the less severe phenotype of *Otx2* mutants when compared to *HNF3β;Lim1* embryos with the persistent expression of *Cer-l* in the distal endoderm of *Otx2* mutants. In *Smad2*^{Robm} mutant embryos, *Cer-l* is not expressed in the visceral endoderm at 6.5 d.p.c. (Waldrip *et al.*, 1998), but the expression of *Lefty1* in these mutant embryos is not known. Thus *Cer-l* expression seems to be downstream of Smad2*,* HNF3β and Lim1 activities, whereas *Lefty1* expression might be controlled by HNF3β, Lim1 and Otx2.

Altogether, the results presented above point to a role of the AVE in limiting the effect of posterior signals on anterior epiblast, through the action of Nodal antagonists such as Cer-l and Lefty1 whose expression might be downstream of *ActRIB*, *Smad2*, *HNF3β*, *Lim1* and *Otx2* (Fig. 3).

Visceral endoderm inhibits *Brachyury* expression in epiblast explants

In order to test the action of the VE on epiblast patterning, we have used an embryological approach by culturing explants of different germ layers of the early mouse gastrula. In intact prestreak or early streak stage embryos, the distal epiblast region does not express *Brachyury*. When explanted and cultured for 1 day in medium containing 15% serum, distal epiblast explants dissected away from the overlying visceral endoderm express widespread *Brachyury*, presumably as a result of mesoderm induction from serum factors in the medium (Fig. 4A). However when both distal visceral endoderm and distal epiblast are cultured, these explants fail to express *Brachyury* after 1 day culture, suggesting that the presence of visceral endoderm can prevent the action of mesoderm inducers present in the medium on the epiblast tissue (Fig. 4B). The absence of *Brachyury* expression is not due to a lost of epiblast tissue as revealed by widespread *Otx2* expression in these explants (our unpublished results). These results indicate that distal visceral endoderm can provide signals that antagonise *Brachyury* induction in distal epiblast explants, suggesting that such an antagonising activity could also exist in the embryo.

Conclusions

Over the last few years, the study of the visceral endoderm germ layer has gained interest. Evidence from embryological and genetic experiments have accumulated and point to an essential role of the AVE in the development of forebrain tissues in the mouse embryo. It appears however, that the action of the AVE requires synergistic function of other regions of the early mouse embryo and in particular of the anterior primitive streak (or EGO) and of the anterior epiblast (Tam and Steiner, 1999). Thus, the AVE seems necessary but not sufficient for anterior neural development in the mouse embryo. In contrast, pre-streak stage rabbit AVE can induce ectopic anterior neural development when grafted into the embryonic or extraembryonic ectoderm of a chick embryo (Knoetgen

et al., 1999), indicating that a mammalian AVE can initiate anterior neural development. Whether the different activities of the AVE of mouse and rabbit embryos reflect real differences between the two species remains an open question as the assays used to address the potentialities of the AVE of these two species are not the same.

Nevertheless the requirement of signals derived from the AVE for proper anterior development has been clearly established in the mouse embryo. In this review, we have attempted to place some of the molecular components of the AVE into a pathway involved in anterior neural tissue formation. We have compared several mouse mutants in which visceral endoderm function impairment leads to similar phenotypic features during gastrulation: expansion of proximal-posterior epiblast tissue and reduction or absence of distal-anterior epiblast derivatives. We propose that in wildtype embryos, distal and anterior visceral endoderm are involved in restricting the extent of the epiblast region contributing to primitive streak formation. A molecular cascade involving ActRIB, Smad2, HNF3β, Lim1 and Otx2 might act to regulate the expression of two secreted Nodal antagonists, Cer-l and Lefty1, in the AVE (Fig. 3). These molecules might in turn act on the adjacent epiblast tissue by antagonising Nodal signalling and restricting the site of primitive streak formation to the proximal-posterior epiblast. In this way, AVE-derived signals would allow distal and anterior regions of the epiblast to develop into anterior neural structures in an environment free of posterior signals.

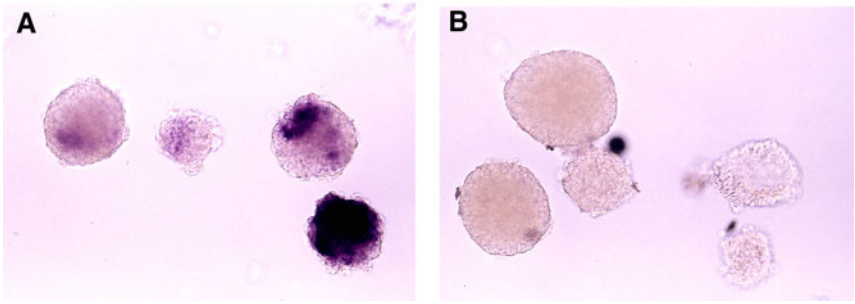

Fig. 4. Distal visceral endoderm prevents *Brachyury* expression in distal epiblast explants. (A) *6.25 d.p.c. distal epiblast explants cultured for 1 day in medium containing 15% serum express Brachyury.* **(B)** *Explants of 6.25 d.p.c. distal VE and distal epiblast fail to turn on Brachyury after 1 day culture in 15% serum m*edium.

In addition to Nodal, other signalling molecules are involved in development of posterior epiblast in the mouse embryo. In particular, the Wnt signalling pathway has been shown to participate in primitive streak formation. *Wnt3* homozygous mutant embryos do not form a primitive streak and lack mesoderm tissues (Liu *et al.*, 1999). Misexpression of chick *Wnt8* induces an additional streak in the mouse embryo (Pöpperl et al., 1997). Moreover, a mutation in *axin,* coding for an intracellular inhibitor of Wnt signalling pathway, results in embryos with duplicated primitive streaks (Zeng *et al.*, 1997). Finally, promoter analyses have demonstrated that *Brachyury* is a target of the Wnt/ß-catenin pathway (Yamaguchi *et al.*, 1999; Arnold *et al.*, 2000). Taken together these results indicate a role for Wnt signalling in primitive streak formation in the mouse embryo. Thus, according to the model we propose, in addition to Nodal inhibitors, the AVE could also provide a source of molecules that antagonise Wnt signalling. Accordingly, the extracellular Wnt antagonist mDkk-1 is expressed in the visceral endoderm at early stages of development (Glinka *et al.*, 1998; Pearce *et al.*, 1999). In addition, Cer-l might be a Wnt antagonist as well as a Nodal antagonist, as it has been shown to inhibit Wnt signalling mediated by Xenopus Wnt8 (Belo *et al.,* 1999). Thus, it is possible that AVE-derived signals also act on the epiblast tissue to antagonise Wnt signalling involved in primitive streak formation.

Another possible player in primitive streak formation is the TGFβ molecule BMP4. BMP4 is expressed in the extraembryonic ectoderm abutting the epiblast from 5.5 d.p.c. onwards. Homozygous mutant embryos for BMP4 or its type I receptor (type I BMP-2/4 receptor), show defects in primitive streak formation and mesoderm generation (Winnier *et al.,* 1995; Mishina *et al.,* 1995). These defects might be due to a reduction in epiblast proliferation in the mutant embryos, however it is not possible to exclude a direct role for BMP4 in primitive streak formation. Thus, Cer-l secreted from the AVE could also function to inhibit BMP4 function in the anterior epiblast.

In summary, we propose that as for Xenopus embryos (Piccolo *et al.*, 1999, reviewed in Niehrs, 1999), the formation of anterior neural structures in the mouse embryo requires the production of inhibitors of the TGFβ and Wnt pathways that restrict the extent of the epiblast region influenced by posterior signals. This model of AVE action by «posterior inhibition» has to be challenged by the analysis of mutant embryos for the secreted TGFβ and Wnt antagonists expressed in the AVE.

Note added in proof:

Since this article was submitted, a role for the visceral endoderm in suppressing posterior signals was also suggested by Kimura *et al., Dev. Biol.* 225: 304-321.

Acknowledgements

We are grateful to Marc Hallonet for critical reading of the manuscript. Work in our laboratory is supported by research grants from the Human Frontier Science Program and the European Community Biotech Programme, and by funds from the Institute National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer, and the Hôpital Universitaire de Strasbourg.

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