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Otx2 is required for visceral endoderm movement and for the restriction of posterior signals in the epiblast of the mouse embryo

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SUMMARY

Genetic and embryological experiments have demonstrated an essential role for the visceral endoderm in the formation of the forebrain; however, the precise molecular and cellular mechanisms of this requirement are poorly understood. We have performed lineage tracing in combination with molecular marker studies to follow morphogenetic movements and cell fates before and during gastrulation in embryos mutant for the homeobox gene *Otx2*. Our results show, first, that *Otx2* is not required for proliferation of the visceral endoderm, but is essential for anteriorly directed morphogenetic movement. Second, molecules that are normally expressed in the anterior visceral endoderm, such as Lefty1 and Mdkk1, are not expressed in *Otx2* mutants. These secreted proteins have been reported to antagonise, respectively, the activities of Nodal and Wnt signals, which have a role in regulating primitive streak formation. The visceral endoderm defects of the *Otx2* mutants are associated with abnormal expression of primitive streak markers in the epiblast, suggesting that anterior epiblast cells acquire primitive streak characteristics. Taken together, our data support a model whereby *Otx2* functions in the anterior visceral endoderm to influence the ability of the adjacent epiblast cells to differentiate into anterior neurectoderm, indirectly, by preventing them from coming under the influence of posterior signals that regulate primitive streak formation.

Key words: Visceral endoderm, Otx2, Anterior-posterior, Lineage, Mesoderm, Forebrain, Mouse

INTRODUCTION

Before gastrulation, at 6.0 days post-coitum (d.p.c.), the egg cylinder of the mouse embryo comprises two germ layers, an outer layer, called the visceral endoderm (VE), that develops into the endoderm of the yolk sac, and an inner layer, the epiblast, that gives rise to the embryonic tissues and extra-embryonic mesoderm. Although it is difficult to identify the anterior-posterior axis of the embryo by morphology at this stage, the expression of some genes is already asymmetrically established on one side of the embryo. These genes code for transcription factors, such as Otx2 (Simeone et. al., 1992; Ang et al., 1994), Lim1 (Lhx1 – Mouse Genome Informatics; Belo et al., 1997; Perea-Gomez et al., 1999), Hex (Thomas et al., 1998) and Gsc (Belo et al., 1997; Filosa et al., 1997), and signalling molecules such as Cerberus like (Cer1) (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), Mdkk1 (Dkk1 – Mouse Genome Informatics; Pearce et al., 1999), Lefty1 (Ebaf – Mouse Genome Informatics; Oulad-Abdelghani et al., 1998) and Fgf8 (Crossley and Martin, 1995), expressed on one side of the VE. This side corresponds to the future anterior end of the embryo, hence its name, the anterior visceral endoderm (AVE) (reviewed by Beddington and Robertson, 1999; Perea-Gomez et al., 2000). The VE plays a crucial role as a trophic tissue (reviewed by Bielinska et al., 1999), and is also involved in the process of cavitation (Coucouvanis and Martin, 1999).

In recent years, genetic and embryological evidence has also supported a role for the AVE in controlling the development of anterior structures of the mouse embryo. Ablation experiments performed at early gastrulation stages (6.5 d.p.c.) have suggested a role for the AVE cells that line the future forebrain region in controlling the formation of rostral neurectoderm, as revealed by the expression of the homeobox gene *Hesx1* (Thomas and Beddington, 1996). Chimeras made from embryos and embryonic stem (ES) cells have confirmed the role of the AVE in forebrain development. This technique allows the production of embryos in which the VE and the extra-embryonic 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 the essential role of the transcription factors Otx2 (Rhinn et al., 1998), HNF3β (Foxa2 – Mouse Genome Informatics; Ang and Rossant, 1994; Filosa et al., 1997, Dufort et al., 1998) and Lim1 (Shawlot et al., 1999), and of the TGFβ superfamily protein Nodal (Varlet et al., 1997), in VE cells for the correct development of the prospective forebrain region.

Otx2 is a bicoid-class homeobox gene related to Drosophila orthodenticle. The *Otx2* gene is expressed already at the blastocyst stage (S. L. A., unpublished observations). Before the onset of gastrulation, *Otx2* is expressed in the whole epiblast and overlying VE. As gastrulation proceeds, *Otx2* expression is restricted anteriorly in the three germ layers. Later during development, *Otx2* expression is found in the forebrain and midbrain regions of the neurectoderm as well as in the rostral foregut and axial mesendoderm (Simeone et al., 1992; Ang et al., 1994). *Otx2* homozygous mutant embryos die around 10.5 d.p.c. and fail to develop anterior neural tissues rostral to rhombomere 2 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Chimera studies have demonstrated a primary function for *Otx2* in the VE for the formation of anterior neurectoderm (Rhinn et al., 1998). In addition, knock-in experiments have demonstrated that an Otx protein is necessary in the VE for the expression of *Otx2* in the epiblast (Acampora et al., 1998). However, the molecules and cellular processes regulated by *Otx2* in the VE for promoting anterior neural development are still poorly defined. To elucidate specific functions of *Otx2* in the VE and to understand the role of VE-derived signals on epiblast development, we have analysed the phenotype of *Otx2* mutants in detail by in situ hybridisation with molecular markers, and by clonal analysis of the VE using the lineage tracer horseradish peroxidase (HRP) during pre- and early gastulation stages.

Our results demonstrate two novel functions of *Otx2* in the VE. We provide the first direct demonstration that *Otx2* is required for anterior displacement of the AVE cells using lineage tracing experiments. Our data indicate that *Otx2* is not only required in the AVE, but throughout its domain of expression in the VE for displacement towards the anterior end of the embryo. *Otx2* is also required for the expression of the signalling molecules Mdkk1 and Lefty1 in the VE. Together, these VE specific defects result in abnormal patterning of the epiblast. We observe ectopic expression of primitive streak and mesoderm markers in the proximal epiblast of *Otx2* mutants. These results support a model whereby *Otx2* in the VE controls the expression and proper localisation of secreted molecules that can act as antagonists of the signals driving primitive streak formation.

MATERIALS AND METHODS

Generation and genotyping of mutant mice for in situ hybridisation

Otx2 heterozygous mutant mice on a 129/Sv × CD1 background (Ang et al., 1996) were intercrossed to obtain homozygous mutant embryos. For the genotyping of pups, DNA was extracted from tail tips as described previously (Laird et al., 1991). For genotyping of 7.5 d.p.c. or older embryos, yolk sac DNA extraction was performed according to Moens et al. (Moens et al., 1992). For 6.5 d.p.c. embryos, DNA was extracted from ectoplacental cone cultures as described (Ang and Rossant, 1994). PCR analysis of the *Otx2* locus was performed using primers and conditions described previously (Ang et al., 1996). The embryos processed for radioactive in situ hybridisation were genotyped using a 170 bp probe specific for exon2 of the *Otx2* mRNA. Staging of mutant and wild-type embryos was according to Downs and Davies (Downs and Davies, 1993).

Generation and genotyping of mutant mice for clonal analysis

Embryos were obtained by mating *Otx2lacZ* heterozygous mutant mice (Acampora et al., 1995) maintained on a C57BL6 × CBA genetic background. Tail tips from pups were genotyped according to Acampora et al. (Acampora et al., 1995). Material for genotyping cultured embryos was obtained as follows. The embryos were fixed after staining for HRP (see below) and further handled in PBS + 0.1% BSA. The portion of ectoplacental cone proximal to the VE was discarded, and the proximal part of the yolk sac with extra-embryonic ectoderm or chorion taken for genotyping. The tissue was subsequently digested in 20 μl water containing 0.6 mg/ml of Proteinase K for 4 hours at 50°C. Proteinase K was inactivated by heating for 5 minutes at 90°C. 5 μl of the supernatant were used for genotyping as previously described by Acampora et al. (Acampora et al., 1995), except that 40 cycles were carried out. A minimum of two independent tests were performed for each sample.

In situ hybridisation and histology

Whole-mount in situ hybridisation was performed as described previously (Conlon and Herrmann, 1993). The number of specimens used for in situ hybridisation of each gene is indicated in the figure legends as *n*=X. For section in situ hybridisation, decidua were fixed for two hours in 4% paraformaldehyde in PBS, equilibrated in 20% sucrose overnight and embedded in OCT (Tissue-Tek, Miles). In situ hybridisation on 8 μm frozen sections was performed as described by Gradwohl et al. (Gradwohl et al., 1996), and sections were counterstained with hematoxylin. The following probes were used. Hex (Thomas et al., 1998), Cer1 (Belo et al., 1997), Lefty1-3′UTR (Meno et al., 1997), Mdkk1 (Glinka et al., 1998), T (Wilkinson et al., 1990), Cripto (Ding et al., 1998), Fgf8 (Crossley and Martin, 1995), Mesp1 (Saga et al., 1996), Lefty2 (Meno et al., 1997), Wnt3 (Liu et al., 1999), Bmp4 (Winnier et al., 1995) and Nodal (Zhou et al., 1993). For histological analysis, embryos previously processed for whole-mount in situ hybridisation, were postfixed overnight in 2.5% glutaraldehyde in PBS, rinsed in PBS and embedded using the JB-4 embedding Kit (Polysciences). 5 μm sections were counterstained with safranin-O (0.01%).

Embryo culture and cell labelling

6.0 d.p.c. and 6.5 d.p.c. embryos from matings of *Otx2lacZ* heterozygotes on a mainly C57BL6 × CBA background were cultured in a 1:1 mixture of immediately centrifuged rat serum and DMEM (Dulbecco's modified minimal essential medium) in an atmosphere of 6.2% CO_2 in air as detailed previously (Lawson et al., 1986; Beddington and Lawson, 1990). Two embryos were cultured in 1 ml medium and removed as a pair from the culture tube for injection.

The procedure and equipment used for intracellular injection by iontophoresis were as described by Beddington and Lawson (Beddington and Lawson, 1990). Embryos for injection were placed in a large drop of DMEM buffered with 10 mM Hepes and containing 7.5% foetal calf serum on a single chambered tissue culture slide (Lab Tek) with the chamber removed. The embryos were viewed through an inverted microscope (Olympus, IMT-2) fitted with DIC optics according to Nomarski and an epifluorescence attachment. Images were transmitted through a CCD camera with image intensifier (Dark Star, Photonic Science), displayed on a TV monitor and recorded with a video graphic printer (Sony UP850). One visceral embryonic endoderm cell per embryo was iontophoretically injected with a

mixture of 7.4% HRP (~1000 U/mg, Boehringer) and 2.6% lysinated rhodamine dextran, M_r 10^4 ('Fluoro Ruby', Molecular Probes) in 0.05 M KCl. A 1-3 nA depolarising current was applied with a duty cycle of 500 mseconds and frequency of 1 second for 10 seconds. A similar injection was made for 5 seconds into a visceral extra-embryonic endoderm cell in the same focal plane; the resulting clone was used to establish retrospectively the angular position of the injected visceral embryonic endoderm cell with respect to the anterior-posterior axis of the embryo (Lawson et al., 1991). The proximal-distal positions of the injected cells were monitored from the rhodamine fluorescence using the image intensification system and 1% excitation of the 100W Hg lamp; the time of excitation was kept to the minimum necessary to inject and record the position of the injected cells.

Histochemistry of cultured embryos

Cultured embryos were stained with Hanker Yates' reagent for HRP (Lawson et al., 1986) for 15 minutes. They were then fixed in 4% paraformaldehyde in PBS for 2 to 24 hours and, after material was removed for genotyping, the embryos were dehydrated through an ethanol series and cleared for detailed examination in benzyl alcohol/benzyl benzoate (1:2).

RESULTS

AVE markers remain in distal positions of *Otx2* mutant embryos at the early streak stage

In *Otx2lacZ* mutant embryos, which contain an insertion of the *lacZ* coding sequence into the *Otx2* locus, the *lacZ* expression domain which is normally located anteriorly in the endoderm by 6.5 d.p.c., remained restricted to the distal part of the embryo. This suggested defects either in the genetic cascades that control the expression of the transgene, or in the displacement of *lacZ*-positive cells in the endoderm (Acampora et al, 1995). To further address these two possibilities, we first compared the expression of markers of the AVE, such as the homeobox gene *Hex* (Thomas et al., 1998) and *Cer1*, a gene related to Xenopus *Cerberus* (Belo et al., 1997; Biben et al., 1998; Shawlot et al, 1998), in *Otx2* mutants and in wild-type embryos at 6.5 d.p.c. In *Otx2* mutant embryos, *Hex* is expressed in the distal VE, in contrast to the anterior expression observed in wild-type littermates (Fig. 1B,B′ and Kimura et al., 2000). In addition, we have confirmed previous observations that *Cer1* is expressed in distal VE cells of 6.5 d.p.c. (Fig.1D,D′; Acampora et al., 1998). The expression pattern of *Hex* in *Otx2* mutants at 6.5 d.p.c. is similar to the normal expression of this gene in wild-type embryos at earlier stages, the pre-streak stage (Thomas et al., 1998). Previous studies using DiI labelling of pre-streak stage embryos have shown that AVE cells are derived from distal VE cells that migrate anteriorly in a unidirectional manner before primitive streak formation (Thomas et al., 1998). This finding raises the possibility that the abnormal expression of the AVE markers in mutant embryos is due to defects in anterior displacement of the VE, rather than to direct changes in expression of these genes.

Analysis of endoderm cell fate and cell movement in *Otx2* mutants at pre-streak and early streak stages

In order to follow the fate and morphogenetic movements of the endoderm in the mutants, we labelled single endoderm cells with HRP at pre-streak and early streak stages and traced the resulting clones after whole embryo culture. The region labelled was either within a band girdling the embryo halfway between its distal tip and the junction of epiblast and extra-embryonic ectoderm (regions A,B,C), or at the distal tip (region D).

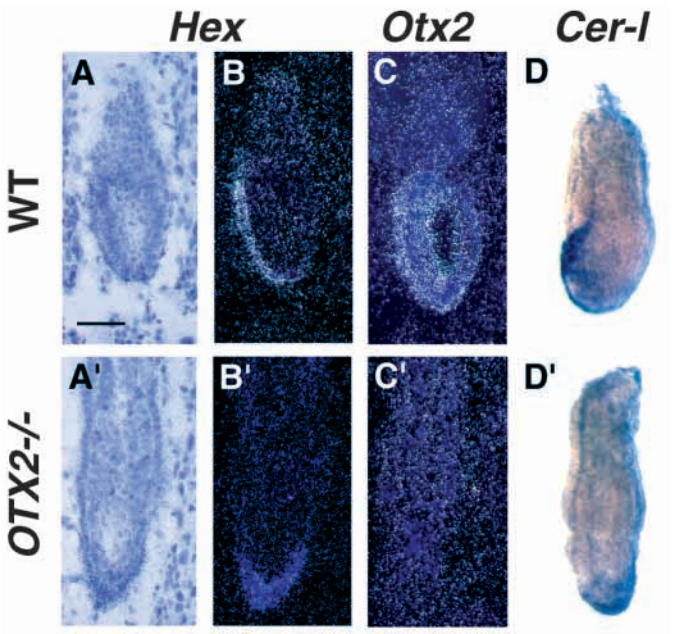

Fig. 1. AVE markers remain in distal positions in *Otx2* mutants. Radioactive in situ hybridisation on sagittal sections (A-C,A′-C′), and whole-mount in situ hybridisation (D,D′), of control wild-type or heterozygous (A-D) and *Otx2*$^{-/-}$ (A′-D′) 6.5 d.p.c. embryos. (A-C, A′-C′). (A,A′) Bright field images of B,B′. In a wild-type embryo, *Hex* transcripts are detected in the AVE (A,B). In *Otx2*$^{-/-}$ embryos, *Hex* transcripts are detected in the distal visceral endoderm (n=3) (A′,B′). (C,C′) Adjacent sections of B,B′ hybridised with *Otx2* exon 2 probe are used for identifying *Otx2* mutant (C′) and control embryos (C). (D,D′) *Cer1* is expressed in the AVE of wild-type embryos (D) and in the distal visceral endoderm of *Otx2*$^{-/-}$ mutants, (n=2) (D′). Scale bar: 100 μm. Anterior is towards the left.

Initial stages, and therefore final stages, were not evenly distributed between the different injection regions and culture times (Table 1). The majority of embryos labelled in regions A, B and C and cultured for 24 hours were initially at the early streak stage, most of those cultured for 39 hours were initially at the prestreak stage. Embryos labelled in region D and cultured for 25 hours were initially at the prestreak stage; those cultured for 31 hours were even less advanced initially and equivalent to the stage normally reached at 6.0 d.p.c. or less. No differences in cell behaviour or morphology were observed between wild type and *Otx2* heterozygous embryos in culture. These two groups were therefore pooled and are referred to as 'controls'. The *Otx2* homozygous null embryos are referred to as 'mutants'.

Distribution of clonal descendants

Endoderm cells at the distal tip (region D) of early prestreak stage control embryos had descendants mainly proximally, spanning the anterior midline at the embryonic/extra-embryonic junction at midstreak and late streak stages (Fig. 2). In contrast, clones obtained from region D endoderm of mutant embryos

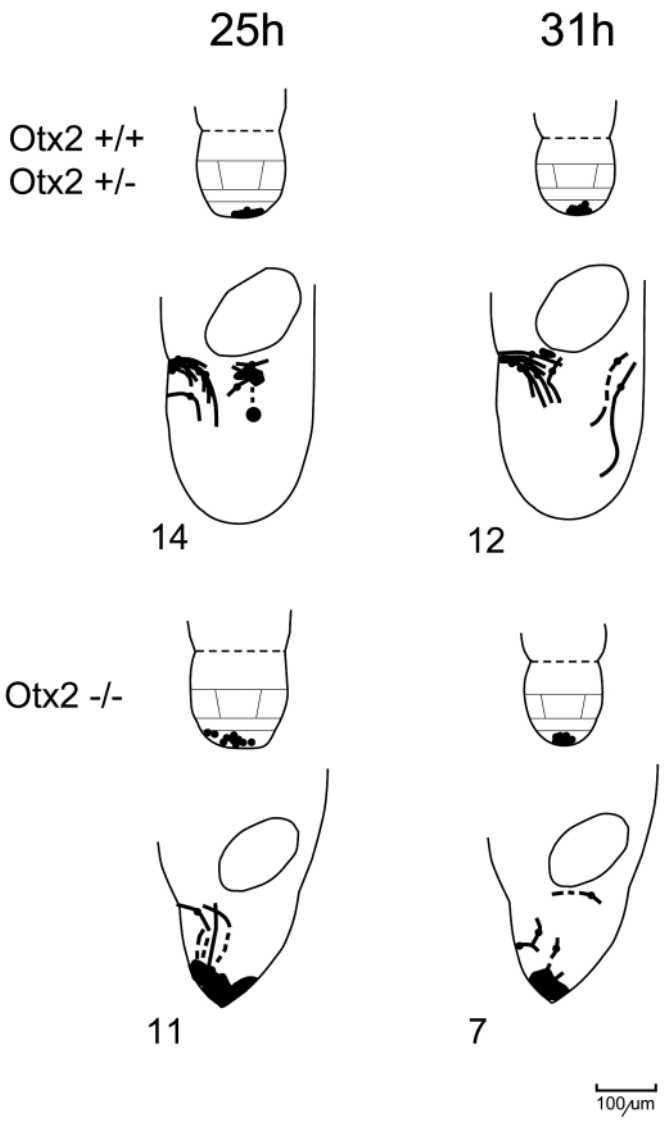

Fig. 2. Distribution of clones after labelling endoderm in region D and culturing for 25 or 31 hours. The positions of the precursor cells and of the resulting clones are projected onto embryo outlines of average dimensions (see Table 1). The upper figure of each vertical pair represents the initial stage; the lower figure of each pair represents the stage after culture. The embryonic/extra-embryonic junction is represented by a thin broken line at the initial stage and by an indentation anteriorly at the final stage; anterior is towards the left. The approximate dimensions of compact and overlapping clones are represented by black areas. Extended clones are represented by a line with a dot. Widely separated parts of a clone are joined by a thick broken line. Numbers are the clones analysed.

remained compact and mainly restricted to the distal tip after 25 and 31 hours culture. A minority extended through the anterior part of the embryo. The distribution of descendants in the mutants is statistically significantly different from the controls (P<0.001), based on whether the labelled cells were in the proximal, intermediate or distal third of the embryo (Table 2). The apparent proximal shift of mutant endoderm in embryos cultured for 31 hr (Table 2) appears to be related to a loss of distal cells (mean distal cells/clone in the mutants was 2.3 at 31 hours, compared with 8.5 at 25 hours) without a concomitant increase in labelled cells in the intermediate and proximal regions (3.6 at 31 hours, compared with 2.5 at 25 hours). This suggests that some cells in the mutant endoderm piled up at the distal tip are no longer viable after 25 hours. No obvious signs of cell death were found in histological sections (data not shown), but no TUNEL staining has been used. These results demonstrate that *Otx2* is required for the normal translocation of distal endoderm into, and beyond, the AVE.

During gastrulation, all the axial embryonic VE which includes that at the distal tip, expands along the anterior-posterior axis, mainly anteriorly (Lawson and Pedersen, 1987). We have therefore examined the behaviour of mutant endoderm located both anterior (region A) and posterior (region C) to the distal tip, as well as more laterally (region B). Region A spans the anterior midline over epiblast of which the most anterior descendants would normally give rise to forebrain (Lawson and Pedersen, 1992). At the early streak stage this endoderm would

Table 1. Initial and final stages of embryos with endoderm clones

Injection site (region)	Culture time (range)	Otx2 genotype	*n*	Initial stage*		Initial dimensions (μm): mean ± s.d.‡			Final stage*									
				PS	ES	L_1	L_2	W	ES	MS	LS	NP	HF	1,2S	3,4S	5,6S	Y	Z
A,B,C	24 hours (17.5-28.5)	+/+,+/–	30	5	25	390±83	180±38	185±34	2	1	11	12	3	1				
		–/–	15	10	5	381±89	171±26	166±35	2	0	2						9	2
	39 hours (29.5-44)	+/+,+/–	49	42	7	294±60	143±27	153±28	2	2	6	24	5	4	3	3		
		–/–	9	9	0	327±72	143±20	149±24									6	3
D	25 hours (21-28.5)	+/+,+/–	14	12	2	313±96	143±43	141±28	3	3	6	1	1					
		–/–	11	10	1	355±14	172±49	162±40	1								9	1
	31 hours (29-40)	+/+,+/–	12	12	0	228±71	124±24	130±18	3	5	2	1	1					
		–/–	7	7	0	301±92	141±26	122±10	1	1	1						4	0

*ES, early streak; HF, headfold; LS, late streak; MS, midstreak; NP, neural plate; PS, prestreak; S, somites; Y, cone ± allantoic bud; Z, elongated anterior-posterior axis ± somites.

‡L_1, length of conceptus from insertion of Reichert's membrane to distal tip; L_2, length of embryonic part; W, width of embryonic part.

Table 2. Endoderm clones initiated in region D at the prestreak stage: clones spanning the anterior midline at the embryonic/extra-embryonic junction and distribution of descendants of all clones

		Clones		Cells			
Culture time	*Otx2* genotype	*n*	Spanning midline	*n*	Proximal	Intermediate	Distal
25 hours	+/+, +/–	14	8 (57%)	106	101 (95%)	5 (5%)	0 (0%)
	–/–	11	1 (9%)	121	13 (11%)	14 (12%)	94 (77%)
31 hours	+/+, +/–	12	9 (75%)	135	123 (91%)	12 (9%)	0 (0%)
	–/–	7	0 (0%)	41	9 (22%)	16 (39%)	16 (39%)

also constitute part of the AVE (Thomas and Beddington, 1996). Region C spans the posterior midline over epiblast containing precursors of axial mesoderm and the node (Lawson et al., 1991) and having organiser properties (Tam et al., 1997, Tam and Steiner, 1999). Region B constitutes lateral endoderm linking regions A and C. All these regions would normally express *Otx2* at the prestreak stage (Acampora et al., 1995; S. L. A., unpublished observations).

In control embryos, endoderm cells from region A translocated anteriorly to the embryonic/extra-embryonic junction (Fig. 3) and, by the neural plate/headfold stage, the clones had a very extended distribution in the extra-embryonic VE stretching to the posterior of the embryo (Fig. 4). Descendants of progenitors in region C extended along and parallel to the main A-P axis (Fig. 3) and members of a clone could be widely separated by the late neural plate stage (Fig. 4). Clones initiated in region B were either aligned parallel to those from region C in the anterior half of the embryo or were located proximally (Fig. 3) and, at neural plate and headfold stages, extended posteriorly into the region where the hindgut would later invaginate (Fig. 4).

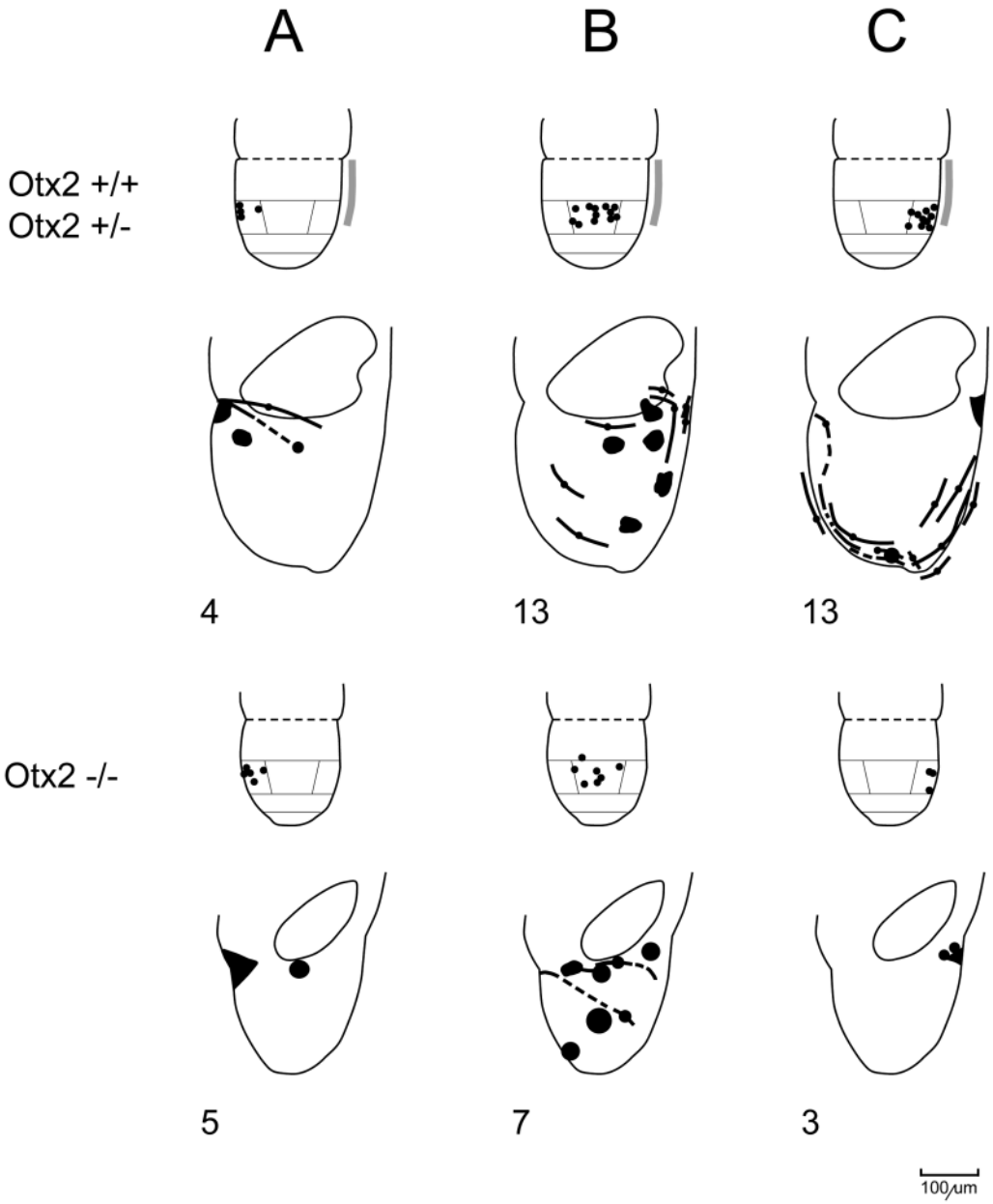

Fig. 3. Distribution of clones after labelling endoderm in regions A, B and C and culturing for 25 hours. The grey bar in the top row of figures indicates the length of the primitive streak at the early streak stage. For clarity, some extended clones along the A-P axis have been displaced outside the embryo outline. The upper figure of each vertical pair represents the initial stage; the lower figure of each pair represents the stage after culture. The embryonic/extra-embryonic junction is represented by a thin broken line at the initial stage and by an indentation anteriorly at the final stage; anterior is towards the left. The approximate dimensions of compact and overlapping clones are represented by black areas. Extended clones are represented by a line with a dot. Widely separated parts of a clone are joined by a thick broken line. Numbers are the clones analysed.

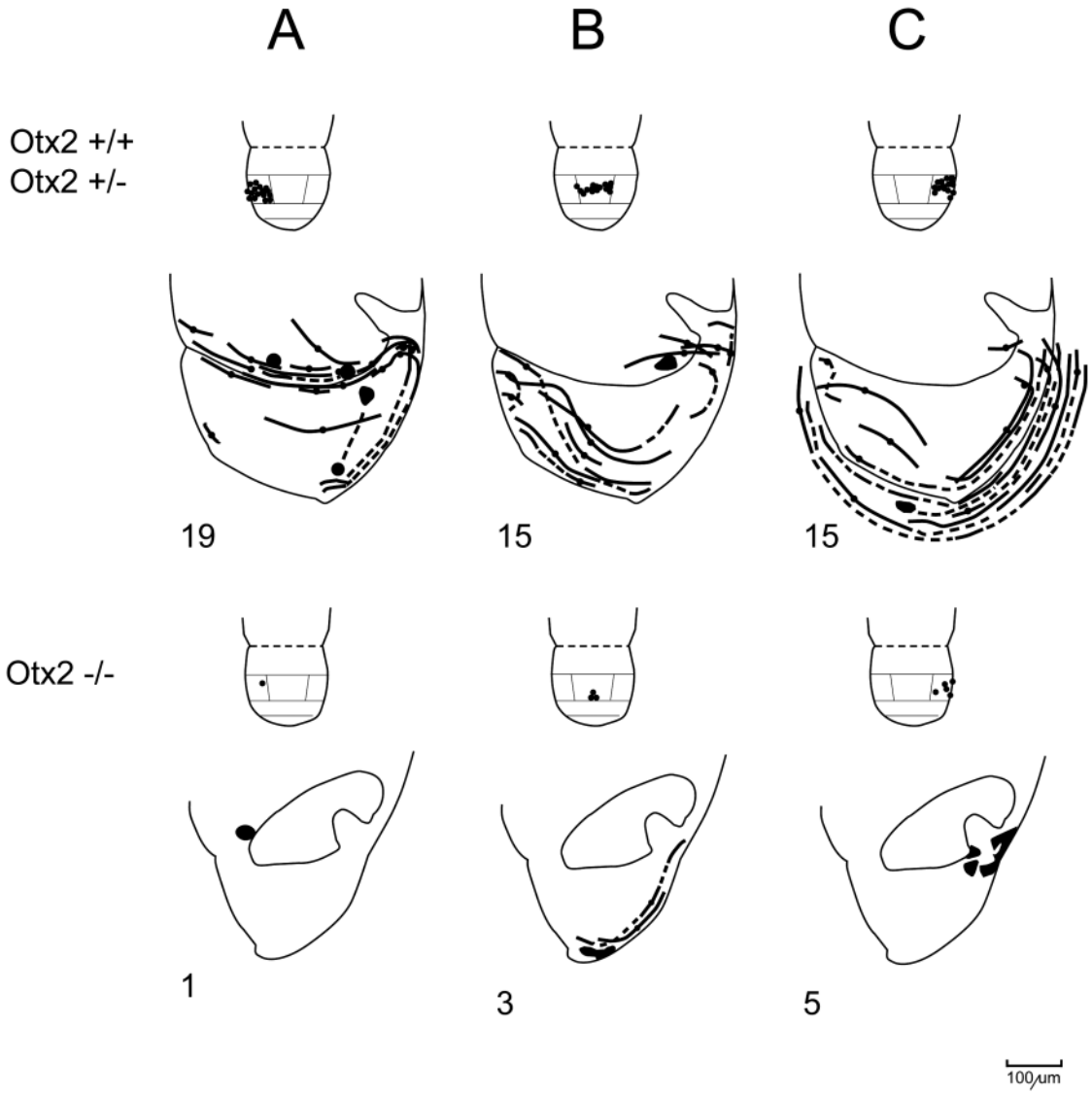

Fig. 4. Distribution of clones after labelling endoderm in regions A,B and C and culturing for 39 hours. The upper figure of each vertical pair represents the initial stage; the lower figure of each pair represents the stage after culture. The embryonic/extra-embryonic junction is represented by a thin broken line at the initial stage and by an indentation anteriorly at the final stage; anterior is towards the left. The approximate dimensions of compact and overlapping clones are represented by black areas. Extended clones are represented by a line with a dot. Widely separated parts of a clone are joined by a thick broken line. Numbers are the clones analysed.

Table 3. Clone doubling time* of endoderm: mean time in hours ± s.d. (n)

		Region				
Culture time	Genotype	A	B	C	D	A-D
18-28.5 hours	Control	11.3±5.3 (4)	9.0±1.7 (13)	10.5±2.6 (13)	9.8±2.8 (14)	9.9±2.8 (44)
	Mutant	10.0±2.8 (5)	11.8±4.3 (6)	11.1±6.0 (2)	8.6±2.3 (11)	9.9±3.3 (24)
29-44 hours	Control	12.2±2.5 (19)	13.4±2.7 (14)	11.9±3.8 (15)	9.2±1.1 (13)‡	11.7±3.0 (61)
	Mutant	9.8 (1)	13.1±3.5 (4)	13.0±2.7 (5)	12.5±2.5 (7)‡	12.6±2.7 (17)

*Clone doubling time (G) was calculated assuming exponential growth: $G = (\ln 2 \times T)/(\ln N_t - \ln N_0)$, where T = hours after labelling, N_t = labelled cells after time T, N_0 = initially labelled cells (this is usually 1, but a sister cell connected by a cytoplasmic bridge may also be labelled).

‡Difference between control and mutant is statistically significant: t=3.33, df=18, $P<0.005$.

Endoderm clones from region A of mutant embryos were located at the anterior embryonic/extra-embryonic junction after 24 hours culture, as in the controls, except that they remained compact without extension towards the posterior of the embryo (Fig. 3). The single clone obtained after 39 hours culture showed little change in position, unlike the controls (Fig. 4). Clones from region C in the mutants behaved differently from the controls: all clones were compact and located at the posterior

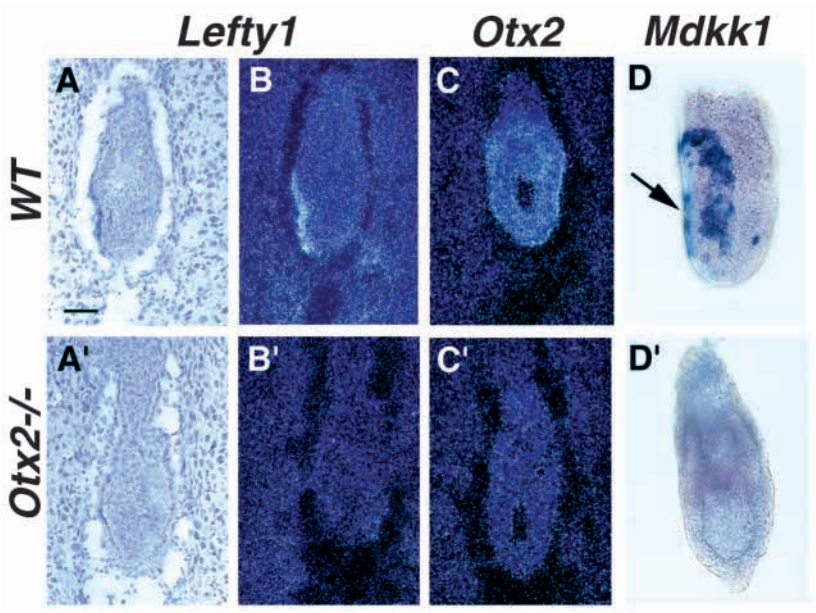

Fig. 5. Expression of VE signalling molecules in *Otx2* mutants. Radioactive in situ hybridisation on sagittal sections (A-C,A′-C′), and whole-mount in situ hybridisation (D,D′), of wild-type (A-D) and *Otx2*$^{-/-}$ (A′-D′) 6.5 d.p.c. embryos. (A,A′) Bright field images of B,B′. (C,C′) adjacent sections of B,B′ hybridised with *Otx2* exon 2 probe. (A-C, A′-C′) *Lefty1* transcripts are present in the AVE of wild-type embryos (A-B), but are not detected in *Otx2*$^{-/-}$ embryos (*n*=3) (A′-B′). (D,D′) D shows a slightly tilted view of the left side of a 6.5 d.p.c. wild-type embryo. *Mdkk1* is expressed in VE cells in an arc of cells spanning the anterior midline, including the most proximal region of the AVE. The arrow indicates the position of *Mdkk1*-positive cells on the right side of the embryo. In *Otx2*$^{-/-}$ embryos, *Mdkk1* transcripts are not detected in the VE (*n*=5) (D′). Scale bar: 100 μm. Anterior is towards the left.

embryonic/extra-embryonic junction, after both 24 and 39 hours; there was no extension along the streak or anterior to it (Figs 3, 4). Clones from region B remained initially compact and located over the lateral, mainly proximal, sides of the epiblast cup (Fig. 3), becoming aligned parallel with the primitive streak by 39 hours, but, unlike the controls, making no contribution to endoderm in the anterior half of the embryo (Fig. 4).

The compact clones in the mutants, and the eventual loss of viability in distal endoderm that failed to translocate anteriorly, suggested that *Otx2* might be required for normal proliferation in the endoderm. However, when clone doubling times were compared, no significant difference was found for any injection region, except in region D after 29-44 hours culture (Table 3). In addition, there was no indication of bias in the number of injected cells that resulted in clones: 92% and 90% injected cells gave rise to labelled clones after one day in controls and mutants respectively; the corresponding proportions for the longer culture period were 76% and 72% (data not shown). Therefore the limited proximal displacement of endoderm from regions A and C, and the failure of distal endoderm to translocate anteriorly in the mutants can not be attributed to reduced cell proliferation or viability, but instead support the conclusion that *Otx2* is required for morphogenetic movement throughout the embryonic VE.

Otx2 regulates the expression of signalling molecules *Lefty1* and *Mdkk1* in the VE

In addition to a role in regulating cell movements, we also investigated whether *Otx2* is required for the expression of signalling molecules in the VE. Besides Cer1, two other secreted molecules, Lefty1 and Mdkk1 are expressed in the VE. Mouse Lefty1 and Lefty2 belong to a novel subclass of TGFβ superfamily molecules (Meno et al., 1997; Oulad-Abdelghani et al., 1998), whose members include zebrafish antivin (Thisse and Thisse, 1999) and the human endometrial bleeding associated factor, Ebaf (Kothapalli et al., 1997). *Lefty1* is expressed specifically in the AVE at the early streak stage (Fig. 5B). This expression is transient and disappears by the mid-to late streak stage (Oulad-Abdelghani et al., 1998). At early somite stages, *Lefty1* is also expressed asymmetrically in the left side of the prospective floor plate and weakly in the left half of the lateral plate mesoderm (Meno et al., 1997). *Lefty1* expression is missing in the AVE of *Otx2* mutant embryos at the pre- to mid-streak stage, indicating that *Otx2* is required for initiating *Lefty1* expression (Fig. 5B′ and data not shown).

Mdkk1 is a cysteine rich protein, that functions as a potent antagonist of Wnt signalling in *Xenopus* gain-of-function experiments (Glinka et al., 1998). At the early to mid-streak stage, *Mdkk1* is expressed in VE cells that form an arc on either side of the anterior midline which includes the most anterior part of the AVE (Fig. 5D). At the late streak stage, expression is observed in the endoderm covering the anterior third of the embryo (data not shown). When expression of *Mdkk1* was examined from the pre-streak to the late streak stage, no expression was ever detected in the *Otx2* mutants (Fig. 5D′ and data not shown). These results therefore show that, as for *Lefty1*, *Otx2* is also required for the onset of *Mdkk1* expression.

Abnormal expression of posterior epiblast markers in anterior regions of *Otx2* mutant embryos

Gain-of-function experiments performed in *Xenopus* and zebrafish embryos have demonstrated a role for Cer1, Lefty1 and Mdkk1 as Wnt and/or Nodal extracellular antagonists (Belo et al., 1997; Biben et al., 1998; Glinka et al., 1998; Pearce et al., 1999; Thisse and Thisse, 1999; Belo et al., 2000). Genetic evidence in mouse embryos indicate that Nodal and Wnt signalling are involved in primitive streak formation (Liu et al., 1999; reviewed by Schier and Shen, 2000). The lack of expression of *Lefty1* and *Mdkk1* in the VE and the abnormal distal expression of *Cer1* at early streak stages in *Otx2* mutants prompted us to analyse primitive streak formation and epiblast patterning in these mutants.

Brachyury (*T*) codes for a transcription factor expressed in the primitive streak that might be a direct target of the Wnt /β-catenin pathway (Wilkinson et al., 1990; Yamaguchi et al., 1999; Arnold et al., 2000). In *Otx2* mutants, *T* expression is found in a proximal ring in both anterior and posterior regions

(Fig. 6A′ and Kimura et al., 2000), in contrast to its normal expression in the posterior primitive streak region (Fig. 6A). Similarly, the expression of *Cripto* (*D16Mit11* – Mouse Genome Informatics) a gene coding for an EGF-CFC protein that functions as a permissive cofactor of Nodal signalling (Ding et al., 1998; Gritsman et al., 1999), is abnormally distributed in the anterior proximal epiblast of *Otx2* mutants (Fig. 6B′) whereas it is restricted posteriorly in wild-type littermates (Fig. 6B). These results suggest that anterior epiblast may acquire primitive streak characteristics in *Otx2* mutants. The expression patterns of *Cripto* and *T* resemble earlier stages of expression of these genes in wild-type embryos at the prestreak stage (Ding et al., 1998; Thomas et al., 1998), suggesting that the expression of these genes is abnormally maintained in anterior regions of *Otx2* mutants after the onset of gastrulation. Alternatively, it is possible that *Otx2* mutants may be developmentally delayed and thus show earlier patterns of expression of *T* and *Cripto*.

To distinguish between these two possibilities, we analysed the expression of other primitive streak markers, whose expression is initiated in the posterior epiblast later than *T* and *Cripto*. The gene for fibroblast growth factor 8 (*Fgf8*) is expressed just prior to streak formation in a patch of epiblast cells on the proximal prospective posterior side of the embryo, as well as in the VE (Crossley and Martin, 1995; Sun et al., 1999; Fig. 6C,C′). In *Otx2* mutants, *Fgf8* is expressed in a proximal ring, encompassing both anterior and posterior regions of the embryo. The T-box gene eomesodermin (*Eomes* – Mouse Genome Informatics) is expressed in the extra-embryonic ectoderm and in the posterior epiblast at 5.75 d.p.c. During gastrulation, *Eomes* is expressed in the extra-embryonic ectoderm, in the primitive streak and nascent mesoderm cells (Russ et al., 2000). In *Otx2* mutants, *Eomes* is expressed in the entire proximal epiblast at early streak stages (n=2, data not shown). Thus, we conclude that the anterior expression of posterior epiblast markers in *Otx2* mutants is due to abnormal regulation of primitive streak formation, and not to developmental delay of the mutant embryos.

We next asked whether the ectopic expression of primitive streak markers in the anterior epiblast of *Otx2* mutants resulted in abnormal mesoderm formation. To this aim, we analysed the expression of *Lefty2* and the bHLH gene *Mesp1*. In wild-type late-streak stage embryos, *Lefty2* (*Leftb* – Mouse Genome Informatics) is expressed in the medial regions of the primitive streak, and *Mesp1* is expressed in the mesodermal wings (Meno et al., 1999; Saga et al., 1996; see Fig. 6E,F). In *Otx2* mutants, *Lefty2* and *Mesp1* are expressed in a ring near the embryonic/extra-embryonic junction both in epiblast and mesoderm cells (Fig. 6E′,F′), suggesting that mesoderm is formed ectopically in anterior proximal regions of the mutant embryos.

Genetic and promoter studies indicate that Nodal might trigger an autoregulatory loop in the epiblast, so that Nodal itself could be a downstream target of Nodal signalling (Norris and Robertson, 1999; Saijoh et al., 2000). *Nodal* shows a very dynamic expression pattern from pre-streak to mid-streak stages. It is first broadly expressed in the epiblast and subsequently becomes restricted to the posterior primitive streak and mesoderm wings (Varlet et al., 1997). In *Otx2* mutant embryos, *Nodal* transcripts are detected in anterior regions, whereas they are already lost in anterior regions of wild-type littermates (Fig. 7F,F′). This result suggests that there is persistent expression of *Nodal* in anterior regions of *Otx2* mutants at early gastrulation stages.

Wnt3 is another gene required for primitive streak formation (Liu et al., 1999). *Wnt3*, like *T*, is initially expressed in the entire proximal region, and progressively becomes restricted to the posterior primitive streak region (Fig. 7B). Analogous to the expression of the other primitive streak markers, *Wnt3* expression also was found in a broad proximal ring in mutant embryos at the early streak stage (Fig. 7B′ and Kimura et al., 2000).

The analysis of the phenotypes of *Bmp4* and *Bmpr1* mutant embryos has suggested that BMP signalling could be involved in primitive streak and mesoderm formation in the mouse embryo (Winnier et al., 1995; Mishina et al., 1995). We asked whether increased *Bmp4* expression could explain the abnormal expression of primitive streak markers in *Otx2* mutant embryos. Our results indicate that *Bmp4* expression is not affected in the extra-embryonic ectoderm of *Otx2* mutants when compared with wild-type littermates (Fig. 7D,D′).

In summary, we have observed abnormal persistent expression of *Nodal* and *Wnt3* in the proximal epiblast of *Otx2* mutant embryos during gastrulation. These results suggest that Nodal and Wnt3 signalling could be involved in driving ectopic primitive streak formation in the entire proximal epiblast of *Otx2* mutants.

DISCUSSION

Our results from clonal and marker analyses demonstrate two novel functions of *Otx2* in the VE. Previous studies, based on the analysis of gene expression in *Otx2* mutants, have suggested that the expression of AVE markers in distal rather than anterior positions is due to defects in cell movement rather than to abnormal gene regulation (Acampora et al., 1995; Kimura et al., 2000). In this paper, we have provided the first direct demonstration of defects in VE cell movements by performing lineage tracing experiments in *Otx2* mutant embryos. Our data show that *Otx2* is not only required in the AVE but throughout its expression domain in the VE for displacement towards the anterior end of the embryo. In addition, *Otx2* regulates the expression of secreted molecules, such as Lefty1 and Mdkk1, that influence the developmental fate of the epiblast.

Inability of *Otx2*$^{-/-}$ VE cells to disperse and to translocate anteriorly

We have used clonal analysis to determine if the abnormal expression of AVE markers in distal regions of mutant embryos is due to defects in migration of these cells. The four regions of endoderm examined in the distal half of the prestreak stage embryo make up much of the embryonic VE at neural plate/headfold stages, as well as contributing to the adjacent extra-embryonic VE. The clonal distributions in the controls exemplify the dynamic behaviour of the endoderm, showing mainly anterior extension and displacement along and parallel with the elongating AP axis. Clones translocated to the embryonic/extra-embryonic junction then continue to expand posteriorly. These extensive displacements have been linked to the associated behaviour of the underlying epiblast and axial

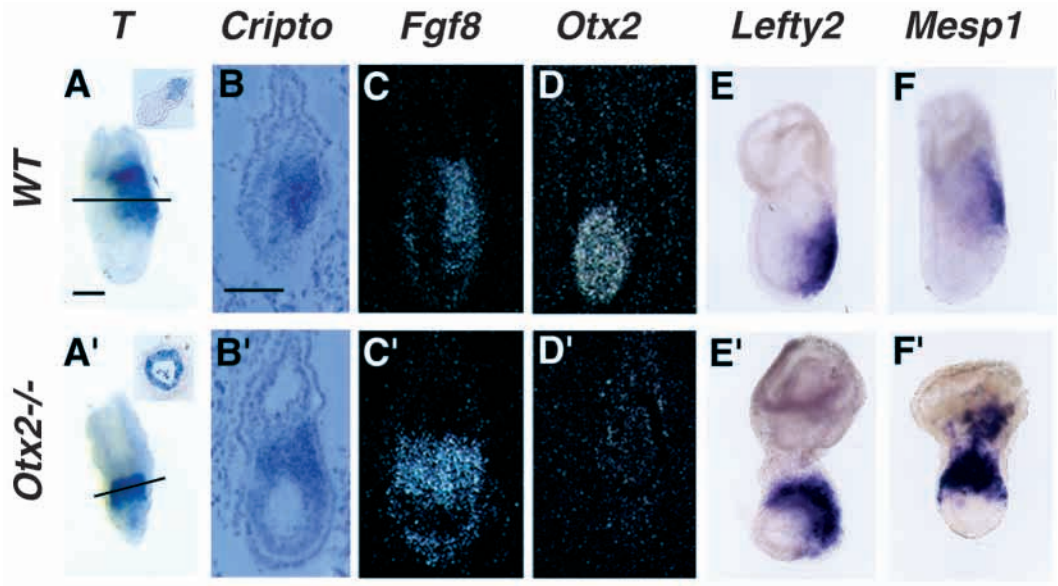

Fig. 6. Posterior epiblast and mesoderm markers are expressed in anterior regions of *Otx2* mutants. Radioactive in situ hybridisation on sagittal sections (B-D,B′-D′), and whole-mount in situ hybridisation (A,A′,E,F,E′,F′), of wild-type (A-F) and *Otx2*$^{-/-}$ (A′-F′) 6.5 d.p.c. (A-D,A′-D′,F,F′) and 7.25 d.p.c. (E,E′) embryos. (B,B′) Bright field images. (D,D′) Adjacent sections of C,C′, hybridised with *Otx2* exon 2 probe. (A-D,A′-D′) *T*, *Cripto* and *Fgf8* are expressed in posterior epiblast and nascent mesoderm wings of wild-type embryos at 6.25 d.p.c. (A, B and C, respectively). By contrast, in *Otx2*$^{-/-}$ embryos, *T* (*n*=3), *Cripto* (*n*=2) and *Fgf8* (*n*=1) are expressed in a ring of proximal epiblast and mesoderm cells (A′,B′ and C′, respectively). The insets in A and A′ show transverse sections of the corresponding embryos at the level indicated by the black bars. (E,E′) *Lefty2* is expressed in mesoderm cells exiting the middle part of the primitive streak in wild-type embryos (E); in *Otx2*$^{-/-}$ embryos *Lefty2* is expressed in proximal mesoderm cells (*n*=1) (E′). (F,F′) *Mesp1* is expressed in mesoderm cells exiting the posterior part of the primitive streak in wild-type embryos (F); in *Otx2*$^{-/-}$ embryos, *Mesp1* is expressed in proximal mesoderm cells in a wider region than in wild-type embryos (*n*=1) (F′). Scale bars: in A, 100 μm for A,A′,E,E′,F,F′; in B, 100 μm for B,B′,C,C′,D,D′. Anterior is towards the left.

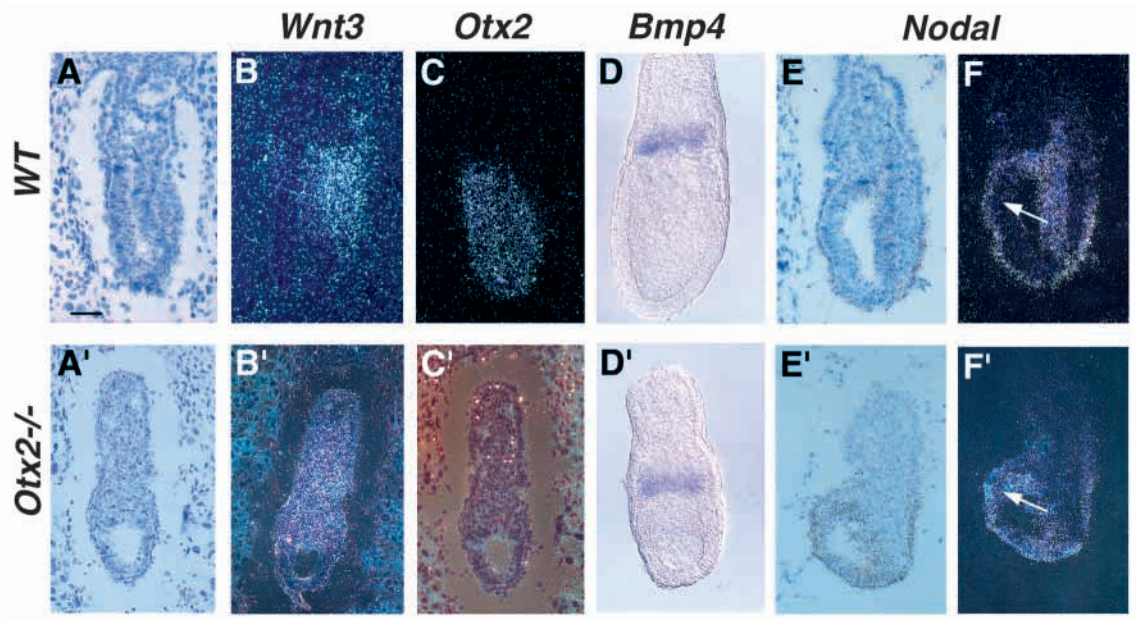

Fig. 7. Abnormal expression of signalling molecules in the anterior epiblast. Radioactive in situ hybridisation on sagittal sections (A-C,A′-C′ and E,F,E′,F′) and whole-mount in situ hybridisation (D,D′) of wild-type (A-F) and *Otx2*$^{-/-}$ (A′-F′) 6.5 d.p.c. (A-D, A′-D′) and 6.75 d.p.c. (E,F,E′F′) embryos. (A,A′ and E,E′) Bright field images of C,C′ and F,F′, respectively. (C,C′) Adjacent sections of B,B′, hybridised with *Otx2* exon 2 probe. (A-C,A′-C′) *Wnt3* is expressed in the posterior epiblast and mesoderm cells of wild-type embryos (B). In *Otx2*$^{-/-}$ embryos, *Wnt3* is expressed in the entire proximal epiblast and mesoderm cells (*n*=3) (B′). (D,D′) *Bmp4* transcripts are found in distal extra-embryonic ectoderm cells at comparable levels both in wild-type (D) and *Otx2* mutant embryos (D′). (E-F,E′-F′) *Nodal* transcripts are restricted to posterior epiblast in wild-type embryos (F). In *Otx2*$^{-/-}$ embryos, *Nodal* transcripts are found in the entire proximal epiblast (*n*=2) (F′). White arrows show anterior epiblast. Scale bar: 50 μm. Anterior is towards the left.

lengthening during gastrulation (Lawson and Pedersen, 1987), but are already under way at 5.5 to 5.75 d.p.c. when distal endoderm undergoes an anterior, asymmetrical displacement, as shown by tracing experiments with DiI (Thomas et al., 1998; Weber et al., 1999); the displacements are likely to be the continuation of a polonaise movement initiated at a still earlier stage in the development of the VE (Weber et al., 1999). Using clonal analysis we have shown that *Otx2* is necessary for the

anteriorly directed axial expansion of the embryonic VE. Clones generated in the embryonic VE in the mutants remained compact, and distal endoderm clones strikingly failed to extend anteriorly. This failure in anterior translocation of distal endoderm in the absence of *Otx2* could explain the failure of *Cer1* and *Hex* expression patterns to shift anteriorly into the AVE.

Alteration in the behaviour of endoderm clones in the mutants was not limited to the most distal cells. Clones generated more proximally, in regions A and C, shifted proximally to the embryonic/extra-embryonic junction, but remained compact and in their original angular relationship to the AP axis. This behaviour is similar to that of normal extra-embryonic VE, which does not express *Otx2*: clones of extra-embryonic VE generated near the embryonic/extra-embryonic junction at 6.0 d.p.c. and later are compact and become displaced proximally, but retain their original position relative to the AP axis. An explanation for the location of A and C region endoderm clones at the embryonic/extra-embryonic junction in the mutants can be found in the pattern of insertion of epiblast derived cells into definitive endoderm in these embryos. Region D epiblast gives rise to definitive endoderm cells covering the truncated anterior half of the mutant embryo, while descendants of region C epiblast abundantly colonise the endoderm covering the primitive streak (K. A. L. and S. M., unpublished observations). Thus, the insertion of definitive endoderm cells could passively displace the VE cells from regions A and C to more proximal positions in the mutants, whereas in controls the two populations partly overlap. As extensive translocation appears to be blocked throughout mutant embryonic VE, the anterior movement of endoderm along the AP axis may require *Otx2* from the onset of expression and involve cells throughout the expression domain in the VE.

The changes in VE cell movements observed in *Otx2* mutants suggest that the target genes controlled by the transcriptional activity of *Otx2* include genes involved in cell adhesion, cell shape and cell motility. Interestingly, we have previously shown that *Otx2* regulates the expression of cell adhesion molecules, specifically R-cadherin and EphrinA2 in the midbrain (Rhinn et al., 1999). Whether expression of similar types of cell adhesion molecules are affected in the VE of *Otx2* mutants remains to be determined. Changes in adhesiveness of *Otx2* mutant cells could also explain why the cells of a mutant VE clone tend to remain compact (Figs 2-4), and also why *Otx2* mutant cells form compact aggregates that segregate from the wild-type cells in the midbrain of chimaeric embryos (Rhinn et al., 1999). It is noteworthy that in zebrafish embryos, overexpression of *Otx1* can promote cell aggregation, which also suggests a role for Otx proteins in the control of cell interactions through regulation of the expression of cell adhesion molecules (Bellipanni et al., 2000). It may seem paradoxical that loss- and gain-of-function of *Otx* genes result in similar phenotypes, but this could be explained by the finding that variations in amount of the same adhesion molecule can lead to differences in cellular adhesiveness (Steinberg and Takeichi, 1994).

Another factor contributing to cell movements is the change in cell shape and polarity. Just prior to gastrulation, the distal VE of wild-type embryos undergoes changes in cell shape from columnar to squamous epithelium. This cellular differentiation event does not occur in *Otx2* mutants (Figs 1A′, 6B′, 7A′), as well as in other embryos with likely defects in the migration of VE such as those carrying mutations in *Cripto* (Ding et al., 1998) or the winged-helix gene *HNF3β* (Ang and Rossant, 1994; Dufort et al., 1998).

Finally, it is noteworthy that in *Xenopus* embryos, *Otx2* plays a role in excluding anterior neuroepithelial cells from convergent extension movements by controlling the expression of *XclpH3* (Morgan et al., 1999). XclpH3 is related to calponin, a protein that can prevent the movement of myosin filaments. Our results indicate that the activity of *Otx2* in the VE stimulates rather than inhibits cell motility. These apparently conflicting results might be explained by the different experimental conditions. Whether the mammalian calponin homologues are also targets of *Otx2* remains to be determined.

VE secretes antagonists to restrict primitive streak formation in the epiblast

The data reported in this paper using molecular markers revealed alterations in the expression pattern of genes both in the VE and in the epiblast. The abnormal expression of posterior primitive streak markers in anterior regions of mutant embryos indicate that A-P patterning of the epiblast is severely affected in *Otx2* mutants. In addition, *Otx2* mutants also show loss or abnormal expression of signalling molecules in the VE.

The molecules involved in primitive streak formation in the mouse remain to be identified. However, the phenotypes of mice carrying mutations in *Nodal* and *Wnt3* have strongly suggested roles for these genes in primitive streak formation and/or maintenance (Conlon et al., 1994; Liu et al., 1999). Moreover, gain- and loss-of-function studies in zebrafish demonstrate a role for *Nodal*-related genes in mesoderm and endoderm formation (reviewed in Schier and Shen, 2000). In chick embryos, Wnt and TGFβ signals seem also involved in primitive streak formation (reviewed by Bachvarova, 1999). In this paper, we have demonstrated that the expression of *Nodal* and *Wnt3* persists in the entire proximal epiblast of *Otx2* mutants at 6.5 d.p.c.; in contrast to the restricted posterior expression of these genes in wild-type embryos at the same stage. We, thus, speculate that the ectopic expression of primitive streak and mesoderm markers observed in *Otx2* mutants is a consequence of abnormal persistent Nodal/Wnt signalling in the entire proximal epiblast of *Otx2* mutant embryos. Previous studies have shown that the abnormal proximal expansion of *T* in *Otx2* mutants is rescued by substituting human OTX1 for Otx2 activity in the VE (Acampora et al., 1998). Together with this finding, our results strongly suggest that the defects in epiblast patterning are primarily due to a requirement for Otx2 activity in the VE.

We have also found novel changes in gene expression in the AVE of *Otx2* mutant embryos. Both *Mdkk1* and *Lefty1* are not expressed in *Otx2* mutants. Our results on the analysis of *Lefty1* expression differ from those of a recent report (Kimura et al., 2000), where *Lefty1* expression was found at the distal tip of *Otx2* mutants at mid- to late streak stage. One possible explanation is that the onset of expression of *Lefty1* may be delayed in *Otx2* mutants, so that no expression is found at early to mid-streak stages (our results). Alternatively, the expression

of *Lefty1* observed by Kimura et al., may correspond to a later domain of expression of *Lefty1* in definitive endoderm cells (Constam and Robertson, 2000).

Altogether, these results are consistent with a model in which the AVE provides secreted molecules such as Cer1, Lefty1 and Mdkk1 that can act as antagonists of primitive streak inducing signals like Nodal and Wnt3. This mechanism allows the existence of a zone of anterior proximal epiblast that is not under the influence of primitive streak promoting/derived signals. In the case of *Otx2* mutants, *Mdkk1* and *Lefty1* are not expressed and *Cer1* expression is mislocalised in the distal tip of the embryo. These VE defects result in expansion of the territory expressing primitive streak markers in the proximal epiblast during gastrulation. In line with this interpretation, proximal anterior epiblast cells display a significant bias towards a mesodermal fate in *Otx2*$^{-/-}$ gastrulating embryos (K. A. L. and S. M., unpublished observations).

Based on this model, an important function of the AVE is to maintain a region in the anterior epiblast, free from the influence of signals involved in mesoderm and endoderm formation derived from the posterior epiblast. The anterior proximal epiblast initially 'protected' by AVE factors, can subsequently respond to signals coming from the anterior part of the primitive streak and/or its descendants to initiate anterior neural development (Shawlot et al., 1999; Martinez-Barbera et al., 2000; Foley et al., 2000; Kimura et al., 2000). Our model provides new insights into the molecular nature of the signalling between the AVE and the epiblast, and elaborates on our previous model based on the analysis of the phenotype of *HNF3β*$^{-/-}$*;Lim1*$^{-/-}$ double mutants (Perea-Gomez et al., 1999; Perea-Gomez et al., 2000).

Lefty1 and *Cer1* mutants have recently been reported. None of these single mutants shows a phenotype in epiblast patterning during gastrulation (Meno et al., 1998; Simpson et al., 1999; Belo et al., 2000; Shawlot et al., 2000; Stanley et al., 2000). In contrast, *Otx2* mutants exhibit defects in epiblast patterning that are most likely due to loss of expression of multiple secreted TGFβ and/or Wnt antagonists. Taken together, these results suggest that *Cer1*, *Lefty1* and *Mdkk1* might act cooperatively, and that the presence of one biochemically active Nodal and/or Wnt antagonist might be sufficient for correct epiblast patterning. Thus, understanding the function of *Lefty1*, *Cerl* and *Mdkk1* in the AVE awaits the production of compound mutant embryos.

Additional functions of the AVE in anterior neural development

The AVE also regulates anterior migration of node descendants to generate the axial mesendoderm. We and others have previously reported that the anterior axial mesendoderm fails to form in *Otx2* mutants (Acampora et al., 1995; Ang et al., 1996; Kimura et al., 2000). However, formation of these cells can be rescued if Otx1 or Otx2 proteins are expressed specifically in the VE and not in embryonic tissues (Acampora et al., 1998; Kimura et al., 2000). Thus, *Otx2* is required in the VE for anterior migration of axial mesendoderm cells. Since anterior axial mesendoderm is also required for the maintenance and growth of the forebrain (Filosa et al., 1997; Shawlot et al., 1999; Camus et al., 2000), AVE could also indirectly influence forebrain development through its effects on axial mesendoderm.

In addition, recent embryological and dye-labelling experiments in chick show that the hypoblast, equivalent to the mouse AVE, regulates cell movements in the epiblast before primitive streak formation (Foley et al., 2000). This anterior displacement of the epiblast results in the positioning of the prospective forebrain territory away from primitive streak-derived posteriorising signals. Whether the AVE has a similar function in coordinating epiblast movements in mouse embryos requires lineage tracing experiments of the epiblast before gastrulation. The progressive restriction of *T* expression from a proximal ring of expression to posterior regions that occur normally in wild-type embryos from 5.5 d.p.c. to 6.5 d.p.c. may suggest that anterior to posterior cell movements occur prior to gastrulation. Whether the persistent expression of *T* in *Otx2* mutants is in part due to defects in epiblast cell movements remains to be determined.

Anterior endoderm, containing mostly hypoblast cells, has also been implicated in the specification of prechordal mesoderm identity in chick embryos, by repressing notochord characteristics (Vesque et al., 2000). It is not known whether this function of the hypoblast is conserved in the AVE of mouse embryos.

We have previously shown that expression of *Otx2* in the epiblast depends on signals coming from underlying mesendoderm tissues (Ang et al., 1994), using germ layer explant assays. In addition, the transcriptional activity of the *Otx2* promoter in the epiblast is also dependent on Otx activity in the VE (Acampora et al., 1998). Altogether, these results suggest that *Otx2* is required in the VE and/or axial mesendoderm for activation and/or maintenance of its own expression in the epiblast. What is the signal from the VE that regulates *Otx2* expression in the epiblast? Recent transplantation experiments suggest that Fgf8 transiently induces the expression of *Otx2* in the epiblast in avian embryos (Streit et al., 2000). However, in *Fgf8* null embryos, *Otx2* expression is still initiated albeit in a broader domain than in normal embryos (Sun et al., 1999). Thus, *Fgf8* is not required to initiate *Otx2* expression in the epiblast of mouse embryos. It will be important to determine in the future the nature of the VE signals regulating *Otx2* expression in the epiblast. The identification of other downstream targets of *Otx2* in the VE via other approaches, such as subtractive hybridisation (Shimono and Behringer, 1999) will also further our understanding of the role of the VE in regulating cell movements and patterning of the epiblast.

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