Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo

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Abstract

After implantation, mouse embryos deficient for the activity of the transforming growth factor-β member Nodal fail to form both the mesoderm and the definitive endoderm. They also fail to specify the anterior visceral endoderm, a specialized signaling center which has been shown to be required for the establishment of anterior identity in the epiblast. Our study reveals that $Nodal^{-/-}$ epiblast cells nevertheless express prematurely and ectopically molecular markers specific of anterior fate. Our analysis shows that neural specification occurs and regional identities characteristic of the forebrain are established precociously in the $Nodal^{-/-}$ mutant with a sequential progression equivalent to that of wild-type embryo. When explanted and cultured in vitro, $Nodal^{-/-}$ epiblast cells readily differentiate into neurons. Genes normally transcribed in organizer-derived tissues, such as Gsc and $Foxa2$, are also expressed in $Nodal^{-/-}$ epiblast. The analysis of $Nodal^{-/-};Gsc^{-/-}$ compound mutant embryos shows that Gsc activity plays no critical role in the acquisition of forebrain characters by $Nodal$-deficient cells. This study suggests that the initial steps of neural specification and forebrain development may take place well before gastrulation in the mouse and highlights a possible role for $Nodal$, at pregastrula stages, in the inhibition of anterior and neural fate determination.
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Introduction

The transforming growth factor-β family member Nodal is one of the critical signals required for the establishment of the anterior–posterior (A–P) polarity in the mouse embryo. Embryos deficient for Nodal activity fail to specify the anterior visceral endoderm (AVE) and lack morphological and molecular evidence of mesoderm formation (Brennan et al., 2001; Conlon et al., 1994). During the early steps of gastrulation, Nodal activity in the epiblast triggers the specification of posterior cell fates and maintains molecular patterning in the adjacent extraembryonic ectoderm (Ang and Constam, 2004; Beck et al., 2002; Brennan et al., 2001). In addition, Nodal transduction within the epiblast is essential for proper formation and patterning of the AVE (Brennan et al., 2001). The AVE cells contribute to the specification of anterior identity in the adjacent epiblast cells by producing secreted antagonists such as Lefty1,

Cerberus-like (Cerl) and Dickkopf1 (Dkk1), which prevent the action of mesoderm-inducing posterior signals such as Nodal, Wnts and bone morphogenetic proteins (Bmps) (Perea-Gomez et al., 2001).

The *Squint* (*sqt*) and *cyclops* (*cyc*) genes are zebrafish orthologs of mouse *Nodal*. *One-eyed pinhead* (*oep*) is an EGF-CFC cofactor essential for Nodal signaling homologous to Cripto. Both *Sqt;cyc* double mutants and the maternal-zygotic mutation of *oep* (*Mzoep*) lack the prechordal plate, notochord and most other mesoderm and endoderm derivatives (Schier and Shen, 2000). Despite the absence of these derivatives, neural structures are formed and display evidence of A–P patterning (Feldman et al., 2000; Gritsman et al., 2000). In contrast, there are striking differences between the mouse $Nodal^{-/-}$ and $Cripto^{-/-}$ mutants. Whereas both mutants fail to form the primitive streak and mesoderm, anterior development occurs in the $Cripto^{-/-}$ embryos (Ding et al., 1998; Kimura et al., 2001; Liguori et al., 2003), but no evidence of anterior patterning has been reported so far in the $Nodal^{-/-}$ embryos (Brennan et al., 2001). Epiblast differentiation, however, may occur in *Nodal-*

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deficient embryos as indicated by the downregulation of the pluripotential marker gene *Pou5f1* (Oct3/4) (Brennan et al., 2001). The fate of *Nodal$^{-/-}$* epiblast cells therefore warrants further investigation.

In the wild-type embryo, the ectoderm germ layer derives from cells that are located in the distal and anterior regions of the epiblast and do not migrate through the primitive streak. The anterior ectoderm comprises precursor cells of the neuroectoderm and the surface ectoderm (Lawson et al., 1991; Quinlan et al., 1995). It is generally accepted that the initial step in anterior specification requires the interaction of epiblast cells with the adjacent prospective AVE cells (Beddington and Robertson, 1999; Perea-Gomez et al., 2001; Thomas and Beddington, 1996). Subsequently, during gastrulation and early somitogenesis, the specification of neuroepithelial cell fate and the acquisition of regional identity within the neural plate depend on interactions with the organizer and its derivatives, such as the axial mesendoderm (Camus et al., 2000; Hallonet et al., 2002; Kiecker and Niehrs, 2001; Robb and Tam, 2004; Tam and Steiner, 1999). The A–P patterning of the epiblast, the regionalization of the neural plate giving rise to distinct regions of the CNS, and their further subdivision repetitively require the interplay of the three major signaling pathways TGF-β, Wnt and fibroblast growth factors (FGFs) and of their respective negative regulators (Goumans and Mummery, 2000; Schier and Shen, 2000; Whitman, 2001; Wilson and Houart, 2004).

The present study investigates the fate of *Nodal*-deficient epiblast cells and demonstrates that anterior identity is established as early as 5.5 days postcoitum (dpc). A detailed qualitative and quantitative analysis of gene expression also shows that, *Nodal$^{-/-}$* epiblast cells prematurely acquire neuroepithelial characteristics and, despite the lack of AVE and mesendoderm derivatives, adopt from 6.5 dpc anterior and ventral regional identities normally specified at the early somite stages in the wild-type prospective forebrain. Explant culture assays demonstrate that *Nodal$^{-/-}$* mutant cells are competent to progress toward neuronal differentiation. Finally, *Nodal$^{-/-}$* mutants express *Gsc*, a gene normally transcribed in organizer-related tissues. However, the study of *Nodal$^{-/-}$;Gsc$^{-/-}$* compound mutant embryos shows that the acquisition of forebrain character in the *Nodal$^{-/-}$* mutant is not dependent on *Gsc* activity. The results reported here highlight a possible role for *Nodal* in preventing the emergence of anterior and neural cell fates before gastrulation and provide new insight into the mechanisms underlying neural specification and forebrain formation in the mouse.

Materials and methods

Generation of wild-type and transgenic embryos

Timed matings of Swiss outbred mice or heterozygous *Nodal$^{lacZ/+}$* mice (Collignon et al., 1996) on a Swiss background were used to collect embryos at different stages. Noon of the day of observation of a vaginal plug was defined as 0.5 dpc (day postcoitum). Embryos were staged according to the scheme described in Downs and Davies (1993). Visceral endoderm thickening provided landmarks to stage embryos at prestreak stages (Rivera-Perez et al., 2003). Measurements and pictures were taken with a Leica stereomicroscope. The

mutant *Nodal^{lacZ}* allele was genotyped as previously described (Collignon et al., 1996). *Nodal$^{-/-}$;Gsc$^{-/-}$* mutant embryos were obtained by intercrossing *Nodal$^{413d/+}$;Gsc$^{lacZ/+}$* mice. Wild-type and mutant alleles of both loci were identified as previously described (Camus et al., 2000; Conlon et al., 1994).

Explants cultures

Mutant and wild-type embryos were dissected in DMEM, 25 mM HEPES, 15% fetal calf serum (FCS). Mutant embryos were cut proximally with tungsten needles to remove the extraembryonic region and cultured in 100 μl DMEM, 15% FCS and antibiotics under mineral oil, in bacteriological 96-well plates, for 48 h. For anterior neural plate explants, a rostral segment (175 μm long and 80 μm wide) of the anterior midline tissue consisting of mesendoderm and overlying ectoderm was isolated from 7.5 dpc wild-type embryos and cultured under the same conditions.

β-Galactosidase staining, in situ hybridization, and histology

Detection of β-galactosidase activity, whole-mount in situ hybridization and histology were performed according to standard methods as described in Perea-Gomez et al. (2004). Embryos were sectioned at 10 μm. Antisense probes for the following genes were used: *Cerl* (Biben et al., 1998); *Dkk1* (Mukhopadhyay et al., 2001); *Dlx5* (Yang et al., 1998); *Emx2* (Suda et al., 2001); *En1* (Davis and Joyner, 1988); *Foxa2* (Filosa et al., 1997); *Foxg1* (Xuan et al., 1995); *Gbx2* (Wassarman et al., 1997); *Gsc* (Filosa et al., 1997); *Hesx1* (Thomas and Beddington, 1996); *Hoxb1* (Frohman et al., 1990); *Lefty1/2* (Oulad-Abdelghani et al., 1998); *Msx1* (Lyons et al., 1992); *Nkx2.1* (Sussel et al., 1999); *Pax6* (Walther and Gruss, 1991); *Sfrp5* (Finley et al., 2003); *Shh* (Echelard et al., 1993); *Six3* (Oliver et al., 1995); *Sox1* (Wood and Episkopou, 1999); *Vax1* (Hallonet et al., 1999).

Whole-mount immunohistochemistry and confocal microscopy

Tissue explants were stained with a neuron-specific class III β-tubulin monoclonal antibody (1: 400; T8660, Sigma), as described by Easter et al. (1993) after fixation in 2% paraformaldehyde/PBS overnight at 4°C. Biotinylated donkey anti-mouse (1:100; 715-065-151, Jackson ImmunoResearch), Alexa Fluor 568 conjugated streptavidin (1: 300; S11226, Molecular Probes) and the nuclear staining YO-PRO-1 iodide (Y3603; Molecular Probes) were used. Control experiment was always carried out without primary antibody in order to assess the specificity of the signal. Confocal sections were taken every 5 μm using a Leica SP2 AOBS scanning head and Helium Neon (543 nm) and Argon (488 nm) laser lines, and the images were prepared using ImageJ software.

Analysis of gene expression by real-time reverse transcription-PCR

mRNA quantification was done by means of the calibrator normalized relative quantification method (Bieche et al., 2001). Almost all variables influencing the final result such as RNA amount or quality, cDNA synthesis efficiency and pipetting errors were eliminated by the normalization to a reference and a calibrator. Results are expressed as normalized ratio: target/reference ratios of all samples are divided by the target/reference ratio of the calibrator. The final ratio is function of PCR efficiency and the determined crossing points. Eight serial dilutions of cDNA control were included in each run, allowing us to establish a standard curve for each primer pair. No significant differences in the final ratio were found between the two reference genes *Gapdh* and *Taf7* (only *Gapdh* normalized values are presented). In all experiments, a cDNA sample of the cephalic part anterior to the second somite of a 10- to 12-somites stage wild-type embryo (8.5 dpc) was the calibrator that we used as the basis for comparative results.

After removing the ectoplacental cone, poly A$^+$ RNA from single embryo was isolated using Dynabeads mRNA DIRECT kit (DYNAL). Random priming first strand cDNA synthesis was done at 42°C for 1 h (Roche). PCR was performed with 1/50 of the final cDNA volume and QuantiTect SYBR Green (QIAGEN) on a LightCycler (Roche). Mock reactions were carried out in the absence of reverse transcriptase for wild-type and mutant embryos (data not

shown). Primer sequences (Forward/Reverse) were designed with Oligo4.0 (National Biosciences) or obtained from Primer Bank (Wang and Seed, 2003) at http://www.pga.mgh.harvard.edu/primerbank/index.html as follow: *En1* 7106305a2; *Foxg1* 6679843a2; *Gapdh* 5′-TTC AAC AGC AAC TCC CAC TCT TC-3′/5′-CCC TGT TGC TGT AGC CGT ATT C-3′; *Gbx2* 6753952a2; *Gsc* 6754076a1; *Hesx1* 6754184a3; *Nkx2.1* 6678353a2; *Sox1* 5′-CAA GAT GGCCCAGGAAAA C-3′/5′-TCG GAC ATG ACC TTC CAC TC-3′; *Taf7* 28461141a2. The representative PCR products were analyzed on agarose gels to ascertain the specificity of the amplifications.

Results

Morphological defects in Nodal-deficient embryo and early expression of Nodal

The dimensions and the morphology of embryos homozygous for the null *Nodal*^{lacZ} allele were analyzed from 5.5 to 8.5 dpc ($n = 80$). We observed a great variability in the sizes of *Nodal*$^{-/-}$ embryos. The average length and width, however, indicate that these embryos continue to proliferate as they get older despite severe morphological defects (Fig. 1A).

Interestingly, *Nodal*$^{-/-}$ embryos were morphologically distinguishable from normal littermates at least a day earlier than previously documented. At 5.5 dpc, *Nodal*$^{-/-}$ embryos do not display the egg-cylinder shape normally seen in the wild type. Often, the boundary between the epiblast and the extraembryonic ectoderm cannot be distinguished, and the overlying visceral endoderm (VE) frequently protrudes distally (Fig. 1B).

Given that the mutant phenotype is detectable at 5.5 dpc, it is likely that Nodal activity is required before this stage. β-Galactosidase activity was analyzed in 5.0 dpc heterozygous *Nodal*$^{lacZ/+}$ embryos and detected in both the epiblast and the overlying VE ($n = 16$; Fig. 1C). This result shows that *Nodal* is expressed at least half a day before the appearance of the proximal–distal polarity (Beddington and Robertson, 1999).

Widespread and precocious expression of anterior markers in Nodal-deficient epiblast

The earliest known function of Nodal is in the specification of distal visceral endoderm (DVE) cells at 5.5 dpc (Robertson et al., 2003). This discrete population of cells expresses a specific set of markers and migrates proximally to the prospective anterior side of the embryo to constitute the AVE (Beddington and Robertson, 1999; Perea-Gomez et al., 2001; Srinivas et al., 2004; Thomas and Beddington, 1996). Consistent with an earlier study demonstrating that *Nodal*$^{-/-}$ embryos fail to establish molecular patterns within the VE (Brennan et al., 2001), no expression of the AVE markers *Cerl*, *Dkk1*, and *Sfrp5* was detectable in 6.5 dpc *Nodal*$^{-/-}$ embryos analyzed by whole-mount in situ hybridization (Table 1; data not shown).

An unexpected result was obtained when *Nodal*$^{-/-}$ embryos were tested for the expression of the anterior marker *Hesx1*. In the wild type, *Hesx1* is expressed in the AVE when gastrulation starts at 6.5 dpc and 1 day later also in the anterior proximal region of the ectoderm, which comprises the presumptive forebrain ectoderm (Thomas and Beddington, 1996). All *Nodal*$^{-/-}$ embryos examined expressed *Hesx1* (4/4 at 6.5 dpc; 3/3 at 7.5 dpc; Figs. 2A–D). Transverse sections of 6.5 and 7.5 dpc *Nodal*$^{-/-}$ embryos showed that *Hesx1* transcripts are found throughout the epiblast but not in the VE (Figs. 2A′–D′). To investigate further the possibility of an anteriorization of the
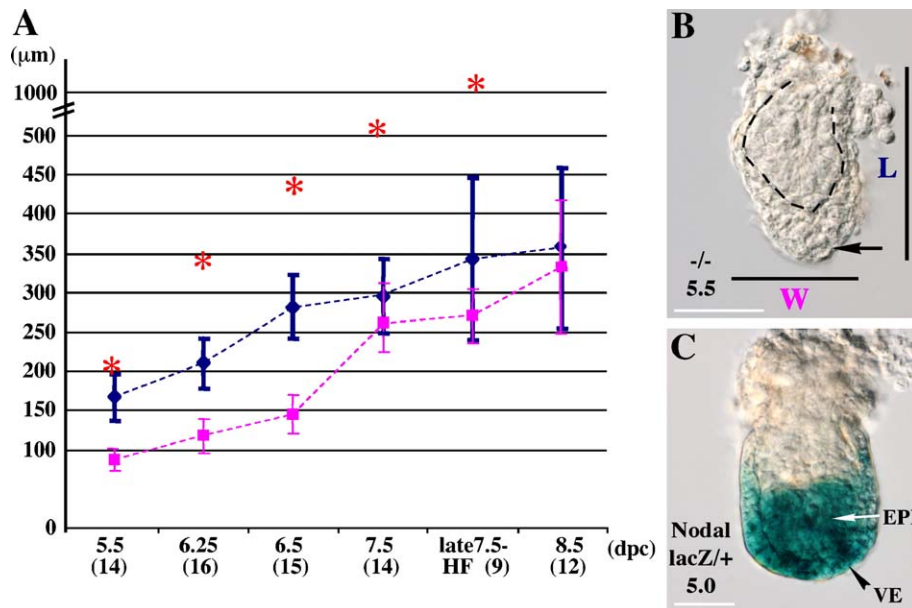

Fig. 1. Growth and morphology of the mutant embryos and *Nodal* expression at 5.0 dpc. (A) Graph showing average length (L) in dark blue and width (W) in pink of the mutant from 5.5 to 8.5 dpc and the length of the wild-type embryo (red star; data for pregastrula embryos are from Perea-Gomez et al. (2004) and Rivera-Perez et al. (2003). L, length of the egg cylinder: from the base of the ectoplacental cone to the distal tip of the embryonic region; W, width of the embryonic region. The number of mutants analyzed is indicated between brackets. Standard errors are shown as bars on each dot. The difference between L and W values decreases: mutants become rounded with time. (B) Morphology of 5.5 dpc *Nodal*$^{-/-}$ embryo. The arrow points to an abnormal accumulation of VE. The black dotted line demarcates the epiblast layer. (C) β-Galactosidase activity in 5.0 dpc *Nodal*$^{lacZ/+}$ embryo. EPI, epiblast; VE, visceral endoderm. Scale bars are 50 μm.

Table 1
Summary of the whole-mount in situ hybridization experiments on Nodal^{−/−} mutants

Gene	No of mutants [a]		
	5.5 dpc	6.5 dpc	7.5 dpc
Cerl	0/7	0/7	
Dkk1	0/5	0/3	
Lefty1/2 [b]	0/5	–	
Sfrp5	0/5	0/3	
Gsc		7/7	4/4
Foxa2		3/3	3/3
Shh		0/5	5/6
Hesx1	2/2 [c]	4/4	3/3
Dlx5		2/2	3/3
Sox1		8/8	3/3
Msx1		0/5	0/5
Six3		3/3	3/3
Foxg1		0/4	6/6
Nkx2.1		0/10	9/9
Vax1		0/2	4/6
Emx2		0/4	0/4
Pax6		–	0/7
En1		0/6	0/6
Gbx2		0/3	0/2
Hoxb1		0/3	0/2

[a] Number of positive embryos out of the total number of mutant embryos analyzed.
[b] The probe used recognizes Lefty1 and Lefty2 mRNA.
[c] Expression detected within the epiblast layer but not in the VE.

mutant epiblast, the expression of Dlx5 was analyzed. Dlx5 is one of the earliest known markers for the most rostral ectoderm, before the formation of an overt neural plate at 7.5 dpc. Subsequently, its expression demarcates the anterior neural ridge (ANR), which defines the rostral boundary of the neural plate at 8.0 dpc, and extends laterally, marking presumptive neural crest precursors (Yang et al., 1998). Widespread expression of Dlx5 was found in the epiblast of Nodal^{−/−} embryos at 6.5 dpc (2/2) and at 7.5 dpc (3/3) (Figs. 2E–G′). The precocious and widespread expression of anterior ectoderm markers in the mutant epiblast led us to examine the patterning of the VE a day earlier. The AVE could have been specified at 5.5 dpc but subsequently not maintained at 6.5 dpc. Similar to the findings at 6.5 dpc, no expression of AVE markers was found in the Nodal^{−/−} mutant at 5.5 dpc (Table 1; data not shown). All together, these results indicate that, in the Nodal^{−/−} mutant, epiblast cells precociously adopt an anterior ectoderm character despite the absence of the AVE.

Nodal-deficient epiblast cells prematurely acquire a neural character

A detailed molecular analysis was performed to address the question whether Nodal^{−/−} epiblast cells can acquire neural identity. Sox1 is the earliest known specific marker of the neuroectoderm, the onset of its expression correlates with the formation of the neural plate at late headfold stages (7.75 dpc; Pevny et al., 1998; Wood and Episkopou, 1999). Sox1 was detected in 6.5 dpc Nodal^{−/−} embryos (8/8), a developmental stage during which this early neural marker is not normally

expressed, and was maintained at 7.5 dpc (3/3) (Figs. 2H–J). Histological analysis of 6.5 and 7.5 dpc Nodal^{−/−} embryos showed that most epiblast cells express Sox1 (Figs. 2I′, J′). These findings indicate that Nodal^{−/−} epiblast cells are prematurely committed to a neuroepithelial cell fate.

Some mutant epiblast cells do not express Sox1 (Fig. 2J′), yet the non-neural ectoderm marker Dlx5 is detectable at this stage. In wild-type embryos, Dlx5-expressing ectoderm gives rise to various cell types, including epidermis, sensory placodes, and neural crest cells (Knecht and Bronner-Fraser, 2002; Osumi-Yamashita et al., 1997; Yang et al., 1998). However, in Nodal^{−/−} embryos, no expression of Msx1, a marker of the cephalic neural crest at early somite stages (Lyons et al., 1992), was detected at 6.5 or 7.5 dpc (Table 1; Figs. 2K–M). Therefore, although non-neural cells are present in the mutants, we found no evidence of neural crest cell differentiation.

Nodal-deficient ectoderm layer principally consists of presumptive anterior and ventral forebrain cells

Expression analysis of regional markers of fore-, mid- or hindbrain territories was performed to investigate whether the Sox1-expressing cells found in Nodal^{−/−} embryos could acquire A–P regional identities. We found that the anterior neural plate marker gene Six3, which demarcates the prospective forebrain at 7.75 dpc (Oliver et al., 1995), was strongly expressed throughout Nodal^{−/−} epiblast as early as 6.5 dpc (3/3; Figs. 3A, B). In contrast, no expression was detectable for the midbrain marker En1 (0/6 at 6.5 dpc, data not shown; 0/6 at 7.5 dpc; Figs. 3D, E), the rostral hindbrain marker Gbx2 and the posterior neuroectoderm marker Hoxb1 (Table 1; data not shown). We conclude that the Nodal^{−/−} epiblast prematurely adopts a neuroectoderm fate but of forebrain character only.

Interestingly, we observed an apparent reduction in Six3 expression between 6.5 and 7.5 dpc, suggesting that the prospective anterior neuroepithelial tissue in the Nodal^{−/−} mutants continues to differentiate during the next 24 h of development (3/3; Figs. 3B, C). This would be in accordance with the acquisition of early regional identity observed in the wild-type anterior neural plate at early somite stages (Kobayashi et al., 2002). 6.5 and 7.5 dpc Nodal^{−/−} embryos were tested for the expression of genes whose activities direct the development of distinct forebrain regions. Foxg1 is first detected at 3-somites stage in the ANR and subsequently in the anterior telencephalic neuroectoderm at 8-somites stage (Xuan et al., 1995). Nkx2.1 is expressed at 3-somites stage in the ventral part of the forebrain (Sussel et al., 1999). The expression of Vax1 is detectable at 8.0 dpc and is restricted to the most rostral level of the medial neural plate, including the ANR and adjacent ectoderm (Hallonet et al., 1999). None of these markers could be detected at 6.5 dpc in Nodal^{−/−} embryos (0/4; 0/10 and 0/2 for Foxg1, Nkx2.1, and Vax1, respectively; Figs. 3F, G, I, J and data not shown), but at 7.5 dpc, the anterior neuroectoderm marker Foxg1 and the ventral diencephalon marker Nkx2.1 were found strongly expressed in discrete patches in Nodal^{−/−} embryos (6/6 and 9/9, respectively; Figs. 3H, H′, K, K′). Similarly, Vax1
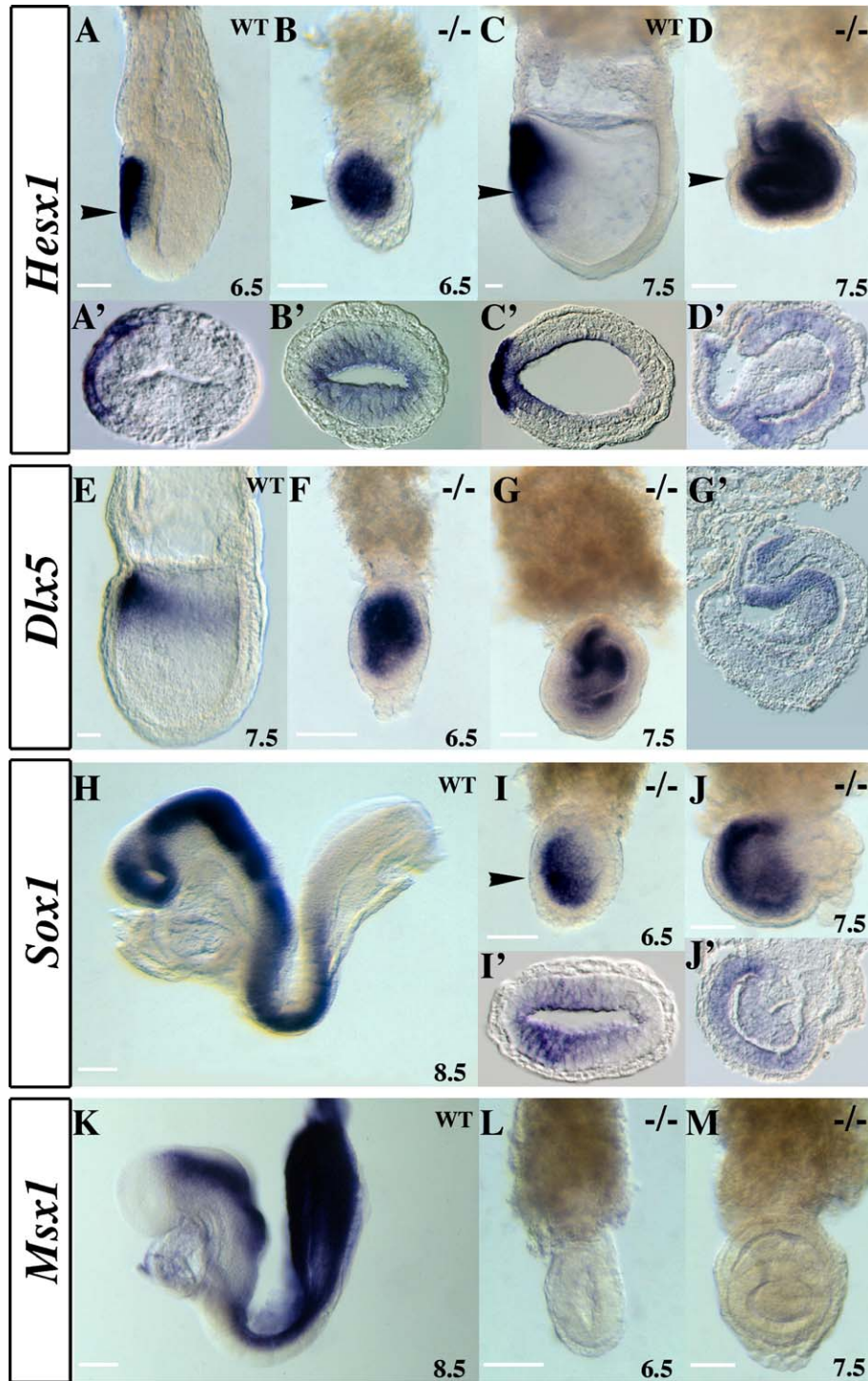
Fig. 2. Anterior patterning and widespread expression of the *Sox1* neuroectoderm marker in *Nodal*$^{-/-}$ mutants. (A–D′) *Hesx1* expression in wild-type embryos, (A) at 6.5 and (C) at 7.5 dpc; in *Nodal*$^{-/-}$ mutants, (B) at 6.5 and (D) at 7.5 dpc. (A′–D′) Corresponding transverse sections. Panels B′ and D′ show that expression is absent from the VE in the mutant. (E–G′) *Dlx5* expression in wild-type at 7.5 dpc (E); in *Nodal*$^{-/-}$ (F) at 6.5 and (G) at 7.5 dpc. (G′) Corresponding sagittal section. Note that the expected asymmetric expression of *Hesx1* and *Dlx5* is lost in the mutants, transcripts are found instead throughout the epiblast. (H–J′) *Sox1* expression in wild-type at 8.5 dpc (H); in *Nodal*$^{-/-}$ (I) at 6.5 and (J) at 7.5 dpc. Corresponding transverse (I′) and sagittal (J′) sections. (K–M) *Msx1* expression in wild-type at 8.5 dpc (K); in *Nodal*$^{-/-}$ (L) at 6.5 and (M) at 7.5 dpc. (D′, G′, and J′) The continuous growth and folding of the epithelium result in the formation of distorted cavities. Arrowheads in panels (A), (B), (C), (D), and (I) show levels of transverse sections. Scale bars are 100 μm.

transcripts were observed in discrete regions of the epiblast in 4 out of 6 *Nodal*$^{-/-}$ embryos examined at 7.5 dpc (data not shown). In contrast to anterior and ventral regional markers, the dorsal forebrain markers *Emx2* and *Pax6* were not expressed

(Table 1; Figs. 3L–N and data not shown). Together, these results show that a subset of 6.5 dpc *Six3*-expressing mutant cells differentiate further at 7.5 dpc and acquire anterior and ventral forebrain characters.
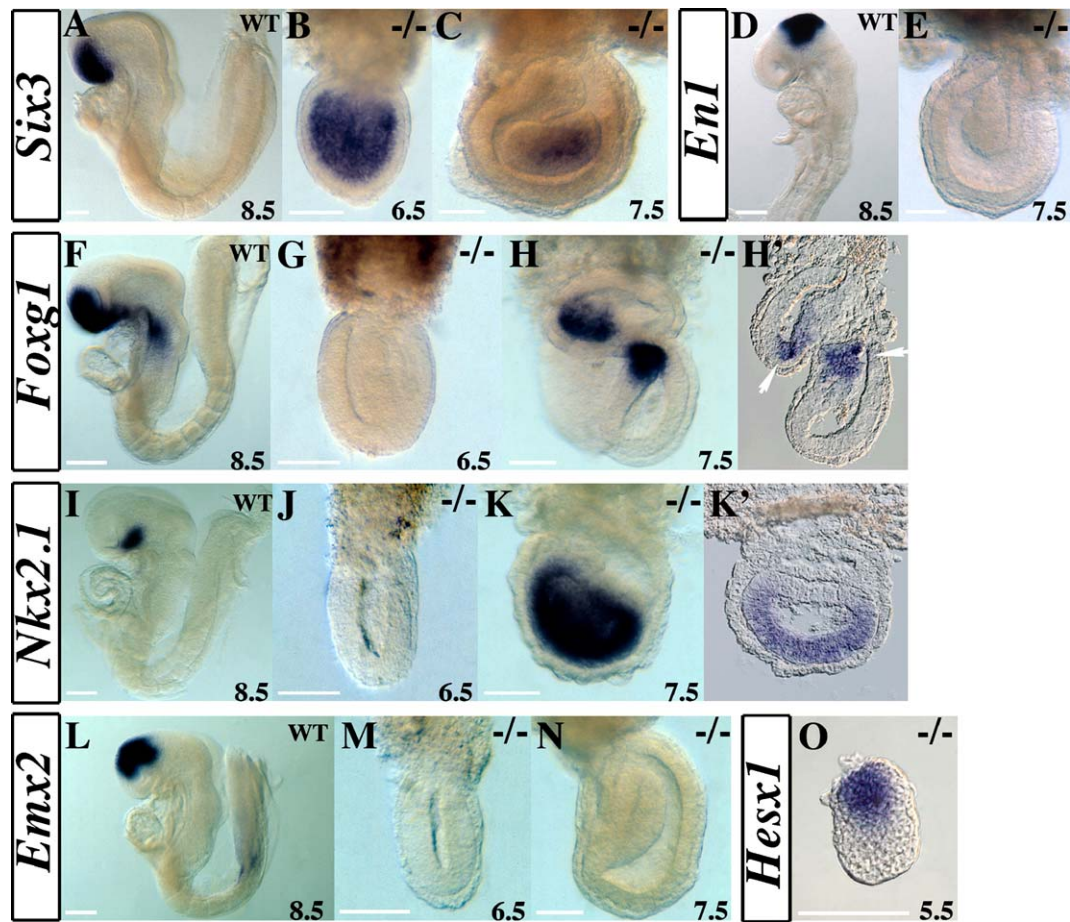
Fig. 3. *Nodal*-deficient ectoderm layer acquires prematurely anterior and ventral forebrain characters. (A–C) *Six3* expression in wild-type at 8.5 dpc (A); in *Nodal*$^{-/-}$ (B) at 6.5 and (C) at 7.5 dpc. (D and E) *En1* expression in wild-type at 8.5 dpc (D); in *Nodal*$^{-/-}$ at 7.5 dpc (E). (F–H′) *Foxg1* expression in wild-type at 8.5 dpc (F); in *Nodal*$^{-/-}$ (G) at 6.5 and (H) at 7.5 dpc. (H′) Corresponding parasagittal section showing *Foxg1* expressed in two distinct domains (arrows). (I–K′) *Nkx2.1* expression in wild-type at 8.5 dpc (I); in *Nodal*$^{-/-}$ (J) at 6.5 and (K) at 7.5 dpc. (K′) Corresponding sagittal section. (L–N) *Emx2* expression in wild-type at 8.5 dpc (L); in *Nodal*$^{-/-}$ (M) at 6.5 and (N) at 7.5 dpc. (O) *Hesx1* expression in 5.5 dpc *Nodal*$^{-/-}$ epiblast. Scale bars are 100 μm.

Gsc gene activity plays no critical role in the acquisition of forebrain characters by Nodal-deficient cells

Nodal$^{-/-}$ embryos fail to form a primitive streak or a node and lack morphological and molecular evidence of mesoderm formation. Interestingly, a broad expression of *Gsc*, *Foxa2*, and *Shh*, three genes associated with patterning activity in organizing tissues, was seen in the epiblast layer of *Nodal*$^{-/-}$ embryos. *Gsc* and *Foxa2* expressions were detected from 6.5 dpc (7/7 and 4/4 for *Gsc*, 3/3 and 3/3 for *Foxa2* at 6.5 and 7.5 dpc, respectively; Figs. 4A–D and data not shown; see also Brennan et al., 2001 for *Foxa2*). *Shh* expression was detected a day later than that of *Foxa2* in the *Nodal*$^{-/-}$ embryos (0/5 and 5/6 at 6.5 and 7.5 dpc, respectively; Figs. 4E–G). This delay is consistent with the timing of activation that occurs in normal development. In embryonic structures where they are both expressed, *Foxa2* expression precedes that of *Shh*.

Experiments performed in other vertebrates species have emphasized the role of *Gsc* in the specification of anterior neural tissues (Blum et al., 1992; Izpisua-Belmonte et al., 1993; Latinkic and Smith, 1999; Yao and Kessler, 2001). However, loss-of-function analysis in the mouse has shown that the lack

of *Gsc* activity does not prevent the formation of a patterned neural tube (Rivera-Perez et al., 1995; Yamada et al., 1995). Nevertheless, a functional interaction between *Gsc* and *Foxa2* is required for the patterning of the neural tube (Filosa et al., 1997). Moreover, heterospecific transplantation experiments suggest that *Gsc* activity plays a role in the ability of the mouse node to ectopically induce *Sox2* and *Sox3* neural-specific markers in the chick host embryo (Zhu et al., 1999). The role of *Gsc* in the development of forebrain tissues in the *Nodal*-deficient embryo was addressed by analyzing the patterning of *Nodal*$^{-/-}$;*Gsc*$^{-/-}$ compound mutants using real-time RT-PCR. Embryos were collected at 6.5 and 7.5 dpc, and no obvious morphological difference could be observed between mutant embryos of distinct genotypes: *Nodal*$^{-/-}$;*Gsc*$^{+/+}$ ($n = 3$), *Nodal*$^{-/-}$;*Gsc*$^{+/-}$ ($n = 3$), and *Nodal*$^{-/-}$;*Gsc*$^{-/-}$ ($n = 9$) (data not shown). Moreover, the comparison of the normalized values obtained, at 6.5 and 7.5 dpc, for *Sox1*, *Hesx1*, *Nkx2.1*, and *Foxg1* transcripts, revealed no significant difference between embryos of distinct genotypes (Fig. 4H; data not shown at 6.5 dpc). The quantitative expression study of *Nodal*$^{-/-}$;*Gsc*$^{-/-}$ compound mutants revealed that anterior and ventral neuroectoderm is formed despite the absence of *Gsc* activity. We
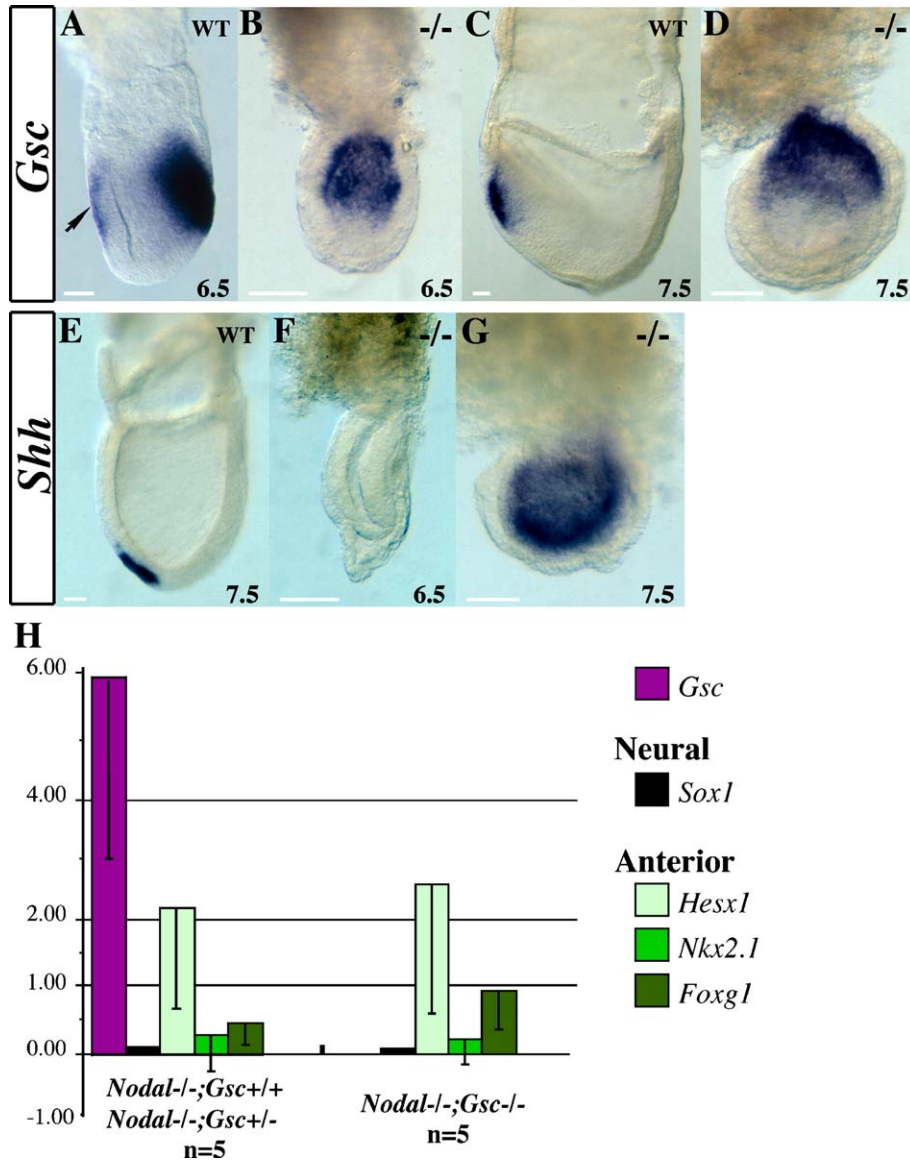
Fig. 4. The acquisition of forebrain characters is not dependent on *Gsc* activity. (A–D) *Gsc* expression in wild-type, (A) at 6.5 and (C) at 7.5 dpc; in *Nodal*$^{-/-}$ (B) at 6.5 and (D) at 7.5 dpc. *Gsc* is expressed in both the VE (arrow) and in the organizer region (A) and in the prechordal plate and ventral neuroectoderm (C) in the wild-type. Note the absence of expression in the mutant VE layer (B). (E–G) *Shh* expression in wild-type at 7.5 dpc (E); in *Nodal*$^{-/-}$ (F) at 6.5 and (G) at 7.5 dpc. *Shh* is expressed in the nascent mesendoderm in the wild-type (E). Scale bars are 100 μm. (H) Relative quantification of anterior and neural gene expressions in *Nodal;Gsc* compound mutants at 7.5 dpc. Representation of the mean values. Two groups of a total of 5 individual embryos of distinct genotypes. The comparison of the normalized values for all genes analyzed revealed no significant difference between embryos of distinct genotypes, except for *Gsc* in *Nodal*$^{-/-}$;*Gsc*$^{-/-}$ (statistical analysis was performed by means of a Wilcoxon test on JMP software, SAS Institute Inc., Cary, NC). The standard error is shown as a bar within each column.

conclude that the acquisition of anterior and neural characters in *Nodal*$^{-/-}$ mutants does not require *Gsc* activity.

Differentiation in Nodal$^{-/-}$ *mutants recapitulates the sequential events of specification and refinement of anterior neuroectoderm*

In order to establish with precision the primary molecular defect and the time course of marker expressions, we examined individual *Nodal*$^{-/-}$ embryos using quantitative real-time RT-PCR. We performed a relative quantification of gene expression on single embryos from 5.5 dpc to 7.5 dpc (2 to 4 embryos were analyzed per day of gestation). For all

analyzed transcripts, normalized values showed a variability between embryos recovered on the same day of gestation, but they confirmed the data obtained by in situ hybridization: anterior and neural gene markers were consistently detected, whereas no *En1* or *Gbx2* transcript was found from 5.5 dpc to 7.5 dpc (Fig. 5A; data not shown). In addition, this analysis reveals that anterior identity appears to be specified first, with the *Hesx1* transcript being detectable in *Nodal*$^{-/-}$ embryos as early as 5.5 dpc (data not shown). In situ hybridization was performed on 5.5 dpc *Nodal*$^{-/-}$ embryos with the *Hesx1* probe and showed that transcripts are expressed by cells of the epiblast layer exclusively (*n* = 2; Fig. 3O). *Sox1* transcripts were detected at 6.25 dpc, as assessed by the
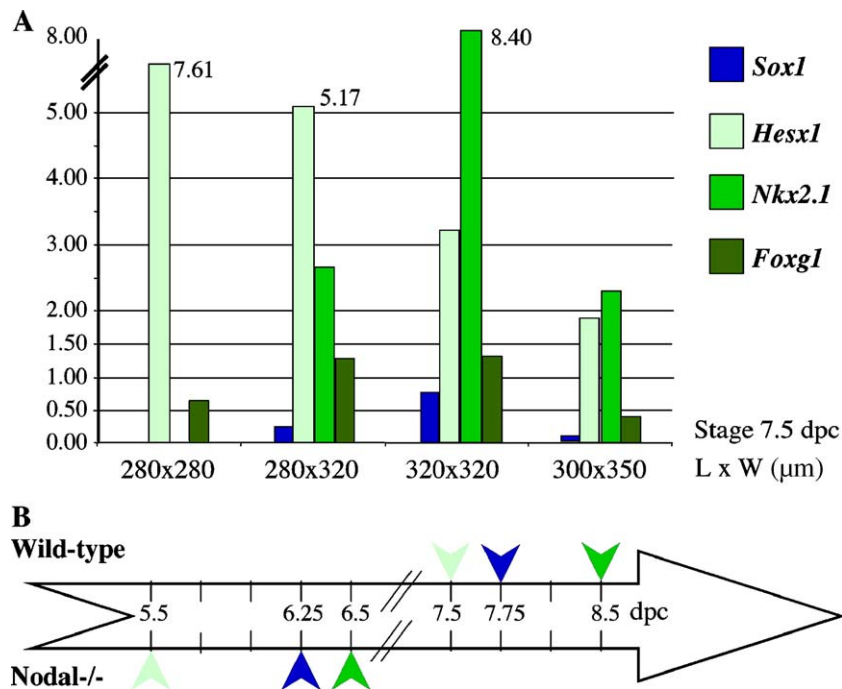
Fig. 5. Anterior and ventral regional cell fates are specified prematurely in $Nodal^{-/-}$ mutants with a progression similar to that of the wild-type embryo. (A) Relative quantification of gene expression in four individual 7.5 dpc $Nodal^{-/-}$ embryos by real-time RT-PCR. Size increases from left to right. L, egg cylinder length; W, embryonic width. (B) Summary of the time course analysis of molecular defects in the $Nodal^{-/-}$ mutant (real-time RT-PCR data) in parallel with the wild-type embryo (real-time RT-PCR and whole-mount in situ hybridization data) from 5.5 to 8.5 dpc. Colored arrowheads correspond to gene markers as in (A) and point to the developmental time of onset of expression.

size of the mutants (Fig. 1A) and by the developmental stage of the littermates. Therefore, Sox1 expression demonstrates that, at 6.25 dpc, the $Nodal^{-/-}$ epiblast adopts a neural fate. Finally, a low level of Nkx2.1 transcripts could be detected as early as 6.5 dpc (data not shown). At 7.5 dpc, the abundance of Nkx2.1 transcripts confirms the presumptive forebrain character of $Nodal^{-/-}$ epiblast-derived cells. Foxg1 and Nkx2.1 transcripts appear to be regulated independently in a given mutant embryo, as revealed by the variations in the normalized values (Fig. 5A). Fig. 5B summarizes and compares the chronology of the molecular defects seen in the $Nodal^{-/-}$ mutants and the anterior patterning of the wild-type embryo. Altogether, these results demonstrate that anterior and neural identities are specified and further regionalization of forebrain tissues takes place precociously in $Nodal^{-/-}$ mutants, albeit with a sequential and temporal progression similar to that of wild-type embryos.

Nodal-deficient cells are competent to progress towards neuronal differentiation

Following neural induction, neurogenesis takes place in specific areas along the A–P and dorso-ventral axis of the neural plate around 9.5 dpc (Bally-Cuif and Hammerschmidt, 2003). We therefore investigated whether neuronal differentiation could occur in $Nodal^{-/-}$ embryos. Past 8.5 dpc, $Nodal^{-/-}$ embryos start to degenerate (Conlon et al., 1994). Therefore, we performed tissue explant cultures to prolong the survival of the mutant tissues in vitro (Liguori et al.,

2003). The anterior neural plate of a 7.5 dpc wild-type embryo was used as control explant (Fig. 6A). After 48 h of culture in vitro, analysis was performed by whole-mount immunohistochemistry with an anti-β-tubulin subunit III antibody that specifically marks neurons and axons (Easter et al., 1993). Three out of 4 control explants displayed a faint β-tubulin subunit III staining. A few fusiform cells without process were immunopositive (Fig. 6B) likely indicators of the beginning of neurogenesis in control explants after 48 h of culture. In 7.5 dpc $Nodal^{-/-}$ explants cultured under the same conditions (Fig. 6C), a dense network of immunopositive cells was detected (in 4 out of 5 explants). Numerous axonal projections had grown into the space between the neuroepithelial-like inner layers and the thickened VE (Figs. 6D, E). These results demonstrate that $Nodal^{-/-}$ epiblast-derived cells are committed to a neural fate and are competent to differentiate into neurons.

Discussion

Anterior patterning takes place in Nodal-deficient mouse embryo

Previous studies in zebrafish reported that severe disruptions of mesendodermal derivatives in the absence of Nodal signaling do not prevent the formation of an A–P patterned neural tube (Schier and Shen, 2000). Our finding that anterior neural patterning occurs in $Nodal^{-/-}$ embryos partly reconciles the discrepancies between mouse and zebrafish studies
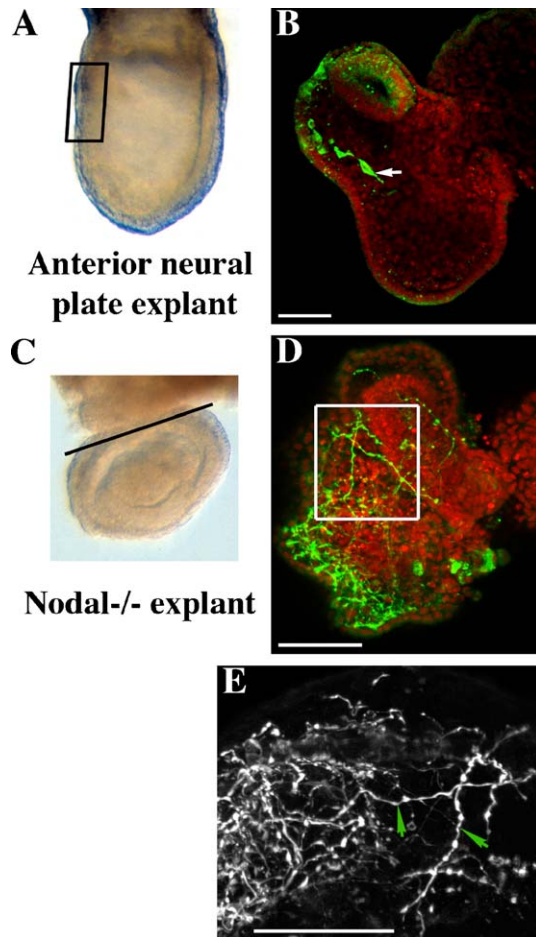
Fig. 6. In vitro assay of neuronal differentiation. (A and B) Anterior neural plate dissected from a 7.5 dpc wild-type embryo cultured for 48 h. (B) β-Tubulin subunit III antibody seen in green and nuclear staining in red. Fusiform cell without process (white arrow). (C–E) 7.5 dpc $Nodal^{-/-}$ explants cultured for 48 h to assess neuronal differentiation using β-tubulin subunit III antibody seen in green and nuclear staining in red (D). (E) Higher magnification of the boxed region in panel D (rotated 90°) showing β-tubulin subunit III antibody only. Note the numerous mature neurones forming a complex network of axonal projections (green arrows). Using ImageJ software, we calculated the fluorescence intensity per μm³ for the β-tubulin subunit III signal from whole explants. The values were 5.74 and 28.54 for the wild-type (B) and $Nodal^{-/-}$ (D) explants respectively. This quantification shows that $Nodal^{-/-}$ explant displays 5 times more fluorescence than the wild-type explant. Scale bars are 100 μm.

on Nodal signaling. The comparison of the identity of neural tissues generated by mutants in both species, however, reveals important differences suggesting that *Nodal* plays a distinct role in the mouse. Diagnostic markers identifying fore-, mid- and anterior hindbrain territories are detectable in both *Mzoep* and *Sqt;Cyc* zebrafish mutants and in the $Cripto^{-/-}$ mouse embryos (Ding et al., 1998; Kimura et al., 2001; Liguori et al., 2003). In contrast, our study indicates that $Nodal^{-/-}$ derived epiblast cells do not generate tissues of midbrain or hindbrain types. The detailed qualitative and quantitative molecular analysis shows that the expression profile observed from 5.5 to 7.5 dpc in $Nodal^{-/-}$ embryos is reminiscent of the distinct steps of prospective anterior and ventral forebrain development characterized in the wild-type embryo. These differences might be explained by the existence of Cripto-

independent Nodal signaling or by the presence of other EGF-CFC genes, such as *Cryptic* (Schier and Shen, 2000; Whitman, 2001), which could allow partial Nodal signaling in $Cripto^{-/-}$ embryos. Interestingly, the topological relationship between the different A–P neural marker domains persists in the $Cripto^{-/-}$ mutants (Liguori et al., 2003). Cells with caudal-most neural identity are found adjacent to the extraembryonic ectoderm where residual posterior signals are predominant (Ding et al., 1998). In the $Nodal^{-/-}$ mutants, *Foxg1*- or *Nkx2.1*-expressing cells were indifferently located in the distal or the proximal part of the embryos, and the levels of transcription of *Foxg1* and *Nkx2.1* revealed striking variations between mutant embryos at a given stage. These results indicate that the intrinsic competence and/or the various instructive cues that trigger the development of the anterior neural tissues likely vary from one $Nodal^{-/-}$ embryo to another. The origin of this heterogeneity remains unknown but deserves to be investigated in future studies.

In contrast to other vertebrate models, in which mutations that either disrupt the Nodal signaling pathway or lead to the absence of mesendoderm derivatives result in the lack of ventral neuroectoderm (Kiecker and Niehrs, 2001; Klingensmith et al., 1999; Schier and Shen, 2000), our study reveals that mouse embryos lacking Nodal signaling form tissue of a ventral forebrain character. It has been shown that the morphogenetic activities of the organizer and the organizer-derived anterior axial mesendoderm (the prechordal plate) are essential for the specification and maintenance of anterior neural cell fates in the mouse embryo (Camus et al., 2000; Hallonet et al., 2002; Tam and Steiner, 1999). A broad expression of *Gsc* and *Foxa2*, two genes associated with patterning activity in organizing tissues (Camus and Tam, 1999), was seen in the epiblast layer of $Nodal^{-/-}$ embryos. After gastrulation, *Gsc* and *Foxa2* genes are also transcribed in the ventral part of the forebrain of the wild-type embryo (Filosa et al., 1997; Wilson and Rubenstein, 2000). Thus, the presence of these marker genes in $Nodal^{-/-}$ embryos may be indicative of the ventral character of the developing neuroectoderm rather than of any residual signaling activities characteristic of the early organizer or the mesendoderm precursors. Our study of $Nodal^{-/-};Gsc^{-/-}$ compound mutants revealed that the acquisition of anterior and neural characters in $Nodal^{-/-}$ mutants does not require *Gsc* activity. Due to the difficulty in obtaining fertile $Nodal^{+/-};Foxa2^{+/-}$ females (A.C. and J.C., unpublished results), we were not able to examine the consequences of the loss of *Foxa2* activity in the $Nodal^{-/-}$ context, leaving this question unresolved. Several reports have suggested that *Shh* activity is necessary for the ventral patterning throughout the nervous system, including the telencephalon (Wilson and Rubenstein, 2000). A more recent study on mouse embryonic stem cells has shown that the addition of Wnt and Nodal antagonists to a neural differentiation assay efficiently increases the generation of the telencephalic precursors that acquire a ventral diencephalic character in response to Shh treatment (Watanabe et al., 2005). It is likely that *Shh* activity directs neuroectoderm cells towards a ventral diencephalic fate in the Nodal-deficient embryonic context.

The depletion of posteriorizing signals promotes forebrain specification in absence of a functional AVE

Previous expression analysis of early gene markers in the *Nodal*⁻/⁻ embryos led to the conclusion that Nodal activity is required to maintain posterior signals, such as *Bmp4* and *Wnt3* and for proper formation and patterning of the AVE (Brennan et al., 2001). Our study reveals that anterior epiblast fates are specified, and subsequent steps leading to forebrain tissue formation take place in *Nodal*-deficient embryos, despite the absence of a functional AVE. The AVE has often been proposed to be the "head inducer", playing a central role in the induction of the forebrain in mammals (Beddington and Robertson, 1999). To our knowledge, this is the first mutation in the mouse where anterior neuroectoderm cell fates are established in the absence of AVE signals. Transplantations, germ layer explant assays, and previous genetic studies have indicated that the AVE alone cannot induce the anterior neuroectoderm but imparts an anterior fate on the adjacent epiblast cells by preventing the action of mesoderm-inducing posterior signals, such as Nodal, Bmp, and Wnt (Kimura et al., 2000; Perea-Gomez et al., 2002; Tam and Steiner, 1999). In the *Nodal*⁻/⁻ mutant context, where posterior signals fail to be maintained, the lack of AVE does not prevent the emergence of anterior cell fates.

Moreover, our cell fate analysis of the *Nodal*-deficient epiblast shows that the neuroectoderm formed lacks dorsal forebrain identity and that the non-neural ectoderm cells fail to differentiate into placodal tissues or neural crest cells (absence of *Emx2*, *Pax6* and *Msx1*) (Gunhaga et al., 2003; McLarren et al., 2003; Theil et al., 2002; Tribulo et al., 2003). These findings are further evidence that *Nodal*-deficient tissues retain none or an insignificant level of Bmp and Wnt signaling after 6.5 dpc.

The analysis of the *Nodal*⁻/⁻ embryo provides a paradigm to study the mechanism underlying forebrain specification in the mouse. Genetic analyses have established that an excess of signaling of both Wnt and Bmp leads to forebrain truncations in the mouse (Anderson et al., 2002; del Barco Barrantes et al., 2003; Lagutin et al., 2003; Mukhopadhyay et al., 2001). The transcriptional co-repressor Drap1 is critical for the attenuation of the positive feedback loop of the Nodal signaling pathway. Drap1-deficient embryos display enlarged axial mesendoderm, but no neuroectoderm is formed, suggesting that inhibition of Nodal is also essential for anterior neural development (Iratni et al., 2002). In *Xenopus*, upon overexpression of full-length *Cer*, a multifunctional antagonist of Nodal, Bmp, and Wnt signaling, neural tissue of a forebrain character, very similar to that found in the *Nodal*⁻/⁻ mouse mutant, is formed exclusively (Agius et al., 2000; Bouwmeester et al., 1996; De Robertis and Kuroda, 2004; Piccolo et al., 1999). Altogether, this striking phenotypic resemblance, the genetic data, and the finding that posterior signals such as Bmp and Wnt are not maintained in *Nodal*-deficient embryos argue that the specification of the anterior-most neural precursors requires the combined inhibition of the Bmp, Wnt, and Nodal signaling pathways in the mouse.

What is the nature of the signal that mediates neural tissue formation in the Nodal⁻/⁻ *mutant?*

The latest studies in chick and *Xenopus* place the initial steps of neural tissue specification before gastrulation, thus before the organizer is formed (Delaune et al., 2005; Kuroda et al., 2004; Stern, 2005). Although considerable progress has been made, our understanding of the molecular and cellular mechanisms that govern the emergence of neural cells is still the subject of constructive debate (De Robertis and Kuroda, 2004; Munoz-Sanjuan and Brivanlou, 2002; Stern, 2005; Wilson and Edlund, 2001). In the mouse, fate mapping by clonal analysis at the early gastrula stage demonstrated that, although a regionalization of cell fate exists within the epiblast, this embryonic tissue is pluripotent. The progeny of a single cell can contribute to any somatic tissue lineage before the completion of gastrulation (Lawson et al., 1991; Tam and Behringer, 1997). It is not known when the initial step in neural specification occurs in the mouse embryo. Moreover, the nature of the signals required for the epiblast cells to become neuroectoderm remains uncertain.

This study shows that, in the absence of Nodal signaling, epiblast cells adopt an anterior identity and express *Hesx1* prematurely, as early as 5.5 dpc, whereas this expression begins in the wild-type rostral ectoderm only after gastrulation has been completed. Shortly after, at 6.25 dpc, *Nodal*⁻/⁻ epiblast cells display molecular and cellular characteristics that are indicative of their precocious commitment to neural fate and have the competence to differentiate in vitro into mature neurons. A possible explanation for the precocious neural phenotype characterized in the mutant embryos is that the inactivation of *Nodal* leads to early deficits that leave the epiblast cells with no instructive or permissive signals other than those directing them towards neural differentiation, and that neural progenitor cells are initially of anterior character (Foley et al., 2000). An alternative explanation is that like in other chordates, the first step toward neural differentiation is initiated well before gastrulation in the mouse and that *Nodal* plays an active role in preventing epiblast cells from further differentiation down the neural lineage by transiently blocking neural commitment.

Consistent with the later hypothesis, recent studies suggested a role for several components of the TGF-β/activin/Nodal pathway in the maintenance of the undifferentiated state in human embryonic stem cells (Besser, 2004; James et al., 2005; Vallier et al., 2004). Discrepancies between mouse and zebrafish Nodal pathway mutants could be explained if one considers that *Nodal* acts as a key modulator of cell fate decision within the pluripotent epiblast of the mouse pregastrula. In the zebrafish embryo, the *Sqt* and *Cyc* expression domains are restricted to the dorsal marginal cells that are fated to form endoderm and axial mesoderm (Feldman et al., 2000), whereas in the mouse, *Nodal* is broadly expressed in the whole epiblast well before gastrulation starts. From 5.5 dpc, the dynamics of expression of *Cripto* and Nodal antagonists, together with the extraembryonic ectoderm-

localized expression of the proprotein convertases Spc1/4 required for the maturation of Nodal, contribute to the establishment of a proximal–distal gradient of Nodal activity within the epiblast (Ang and Constam, 2004; Robertson et al., 2003). Previous studies have provided evidence that high and sustained Nodal activity in the proximal epiblast promotes endoderm and mesoderm fates (Lowe et al., 2001; Norris et al., 2002; Vincent et al., 2003). Depending on the concentration but also on the duration of the exposure to Nodal signal, a given epiblast cell will adopt a particular cell fate. Interestingly, clonal analysis indicates that a majority of the distal epiblast cells, labeled near the midline at the prestreak stage, gives rise to descendants that contribute to the midline of the anterior neuroectoderm at early somite stages (K.A. Lawson, personal communication). According to the proximal–distal gradient of *Nodal* activity, these distal epiblast cells would be the less exposed to *Nodal* signal. Based on the data presented here, we propose that moderate and transient Nodal activity would prevent the early commitment of the epiblast cells to anterior and neural cell fates. In this model, Nodal signal would play a central role in maintaining epiblast cells in a competent state to sustain normal growth and patterning of the embryo during gastrulation. The blockage in neural commitment would be released toward the end of gastrulation when the mesoderm and definitive endoderm layers are fully formed and can impart a complete A–P polarity to the emerging neuroectoderm cells. Such a mechanism mediated directly or indirectly by *Nodal* renders compatible the co-existence of an early step in anterior neural specification and the remarkable plasticity of the epiblast cells characteristic of the mouse embryo. The model discussed above is speculative, further work will be required to establish whether Nodal regulates anterior neural differentiation during early postimplantation development. The unique characteristics of the *Nodal*-deficient embryo make it appropriate for future investigations into the mechanisms of neural specification and forebrain development in the mouse.

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