Control of early anterior–posterior patterning in the mouse embryo by TGF- β signalling

Elizabeth J. Robertson^{*}, Dominic P. Norris[†], Jane Brennan[¶]
and Elizabeth K. Bikoff

Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

Prior to gastrulation the mouse embryo exists as a symmetrical cylinder consisting of three tissue layers. Positioning of the future anterior–posterior axis of the embryo occurs through coordinated cell movements that rotate a pre-existing proximal–distal (P–D) axis. Overt axis formation becomes evident when a discrete population of proximal epiblast cells become induced to form mesoderm, initiating primitive streak formation and marking the posterior side of the embryo. Over the next 12–24 h the primitive streak gradually elongates along the posterior side of the epiblast to reach the distal tip. The most anterior streak cells comprise the ‘organizer’ region and include the precursors of the so-called ‘axial mesendoderm’, namely the anterior definitive endoderm and prechordal plate mesoderm, as well as those cells that give rise to the morphologically patent node. Signalling pathways controlled by the transforming growth factor- β ligand *nodal* are involved in orchestrating the process of axis formation. Embryos lacking *nodal* activity arrest development before gastrulation, reflecting an essential role for *nodal* in establishing P–D polarity by generating and maintaining the molecular pattern within the epiblast, extraembryonic ectoderm and the visceral endoderm. Using a genetic strategy to manipulate temporal and spatial domains of *nodal* expression reveals that the nodal pathway is also instrumental in controlling both the morphogenetic movements required for orientation of the final axis and for specification of the axial mesendoderm progenitors.

Keywords: mouse embryo; axis formation; nodal; transforming growth factor- β signalling

1. INTRODUCTION

By the time the mouse embryo implants into the uterine epithelium *ca.* 4 days after fertilization, it comprises three tissue layers. The founder tissue of the embryo proper, the epiblast, forms a radially symmetric cup of epithelial cells that grows in close apposition to the ExE and the VE. This simple cylindrical structure exhibits a distinct molecular pattern along its P–D axis (reviewed in Beddington & Robertson 1999; Lu *et al.* 2001). The A–P axis of the embryo is positioned later via coordinated cell movements that rotate this pre-existing P–D axis (Thomas & Beddington 1996). Mesoderm cells are induced to form at a single site on the prospective posterior side of the epiblast marking the primitive streak, while cells situated diametrically opposite to the primitive streak are fated to give rise to neurectoderm in response to signals provided by the AVE, a specialized population of VE cells.

The activities of secreted growth factors belonging to the TGF- β superfamily are critical for establishing the

body axes of the vertebrate embryo (reviewed in Whitman 2001). In particular, nodal homologues have been shown to contribute at distinct stages, both during the initial specification and subsequent elaboration of the A–P axis. *nodal* genes also act in a conserved molecular pathway to determine the L–R axis, the process by which distinct and invariant handedness is conferred upon the embryonic body plan. Thus, in all vertebrates examined, expression of a *nodal* family member exclusively in left LPM precedes the development of overt differences between the left and right sides of the embryo (reviewed in Whitman 2001).

In the post-implantation mouse embryo, sites of *nodal* functional activity closely correlate with highly dynamic expression domains. Thus, our previous studies have shown that *nodal* expression in the posterior epiblast is required for primitive streak formation (Varlet *et al.* 1997), and for inducing the anterior organizing centre or AVE, essential for patterning the most anterior regions of the CNS (reviewed in Beddington & Robertson 1999; Lu *et al.* 2001). Collectively, these experiments suggest that *nodal* plays a key role during the establishment of the initial A–P pattern in the pre-gastrulation stage embryo. Based on its node-specific expression pattern at later stages (Zhou *et al.* 1993; Conlon *et al.* 1994; Collignon *et al.* 1996) *nodal* has also been implicated in the formation and/or function of the mouse node, the organizing centre responsible for patterning the midline and posterior regions of the embryo (Beddington & Robertson 1999).

^{*} Author and address for correspondence: Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA (ejrobert@fas.harvard.edu).

[†] Present address: Mouse Genome Centre, Medical Research Council, Harwell, Oxfordshire OX11 0RD, UK.

[¶] Present address: Centre for Stem Cell Research, University of Edinburgh, West Mains Road, Edinburgh EH9 3JQ, UK.

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Figure 1. Distinct patterning defects exhibited by *nodal*-deficient epiblast tissue. Whole-mount *in situ* hybridization analysis of early gastrulation (a) wild-type or (b) *nodal*. Expression of *cripto* and *wnt3* is restricted to the proximal-posterior region of the wild-type embryo. *nodal* mutants lack both *cripto* and *wnt3* expression. The pluripotential marker *oct-4* (*pou5f1*) is abundantly expressed in wild-type but drastically downregulated in *nodal* mutants. In comparison with the ExE of wild-type, the *bmp4* expression domain is smaller and less robust in *nodal* mutant embryos.

In keeping with this idea, loss of *nodal* expression in the node has recently been shown to prevent the normal establishment of the L–R axis (Brennan *et al.* 2002).

Transgenic approaches have been used to map *cis*-regulatory elements responsible for spatially and temporally restricted *Nodal* expression patterns (Adachi *et al.* 1999; Norris & Robertson 1999; Brennan *et al.* 2001). An intronic enhancer, termed the ASE, controls early expression in the epiblast and VE, and also at later stages on the left side of the embryo. The minimal ASE enhancer region contains two *FoxH1* binding sites (Saijoh *et al.* 2000). Interestingly, *FoxH1*-dependent *cis*-acting regulatory elements have been mapped within the *Nodal* locus of all vertebrates examined (reviewed in Whitman 2001). *FoxH1* (formerly known as FAST1/2) binds as part of a complex to the activin-responsive element of target genes (Chen *et al.* 1996, 1997), suggesting that the ASE acts in an autoregulatory fashion in response to *nodal* signals. In support of this conclusion, we previously showed that transgenes carrying the ASE fail to be activated in either *nodal* null (Brennan *et al.* 2001) or *FoxH1* mutant embryos (Norris *et al.* 2002).

We review the role played by *nodal* signalling during early axis patterning. Our studies show that *nodal* signalling in the epiblast is required for the establishment of initial molecular polarity of the embryo. *nodal* acts to promote posterior cell fates in the epiblast and to maintain the molecular pattern in the adjacent ExE (Brennan *et al.* 2001). Moreover, *nodal* signals from the epiblast also pattern the VE by activating the pathway required for specification of anterior identity in overlying epiblast cells. To evaluate the role of the *FoxH1* feedback enhancer in reg-

ulating *nodal* activity we have used a genetic strategy to eliminate the enhancer from the mouse germline. These studies show that *nodal* activity is not required in the VE for establishment of the AVE. However, the enhancer plays an important role in regulating dose-dependent *nodal* signals, since lowering *nodal* activity in the embryo results in a failure either to rotate the initial P–D axis of the embryo or to correctly specify mesendoderm precursors. Thus, *nodal* signalling in the epiblast both guides the morphogenetic movements that establish the final A–P axis of the embryo, and contributes to the specification of derivatives of the anterior streak.

2. RESULTS AND DISCUSSION

(a) *Nodal* signalling in the epiblast controls P–D molecular patterning of the early embryo

Nodal is expressed throughout the epiblast and the overlying VE at early post-implantation stages (Varlet *et al.* 1997). As development proceeds, a P–D gradient of *nodal* expression formed within the epiblast rapidly resolves to the future posterior side where the primitive streak will form. Embryos lacking *nodal* activity arrest shortly before gastrulation and fail to establish a primitive streak (Zhou *et al.* 1993; Conlon *et al.* 1994). To understand the patterning defects that accompany this block to development we examined the expression of a panel of marker genes that are normally expressed in a restricted fashion in the three cell lineages of the early embryo. As shown in figure 1, *wnt3* is initially expressed radially in the proximal epiblast and rapidly becomes confined to the posterior side of the embryo, consistent with its essential roles during

Figure 2. *nodal*-deficient VE lacks anterior identity. β gal and whole-mount *in situ* hybridization analysis of (a) wild-type and (b) *nodal* mutant embryos at early gastrulation. Lateral views with anterior to the left and posterior to the right. The *hex-lacZ* transgene activated in AVE of wild-type embryos is not expressed in *nodal* mutants. Similarly, *lefty1* marks AVE of wild-type embryos but is not expressed in *nodal* mutants. *Lim-1* (*lhx1*), normally present throughout the VE prior to gastrulation, is also absent from *nodal* mutants. As a control for the presence of VE, both wild-type and *nodal* mutant embryos express H19 in the outermost VE cells.

mesoderm formation (Liu *et al.* 1999). Surprisingly, *nodal*-deficient embryos lack posterior markers including *wnt3* (figure 1), *eomes*, *T* and *fgf8* (data not shown). Similarly *nodal* mutants fail to express *cripto* (figure 1), a marker normally expressed throughout the epiblast of wild-type embryos before regressing to the posterior side (figure 1), and *lefty2* a TGF- β family member initially expressed at gastrulation (not shown). The striking absence of any posterior markers is not simply caused by a generalized block to differentiation, because *nodal* mutants rapidly downregulate expression of the pluripotential marker gene *oct-4* (figure 1). We next asked whether these epiblast patterning defects are associated with abnormal development of the ExE by examining the expression of two distal markers *bmp4* (figure 1) and *eomes*. Expression of both genes is transiently detected in *nodal* mutants (figure 1 and data not shown). Thus, continued *nodal* signalling in the epiblast is also required for maintaining the molecular pattern in the adjacent ExE.

Considerable data argue that signals from the distal-most VE cells, fated to form the AVE, normally antagonize signals received by the proximal epiblast and thus act to maintain anterior identity (reviewed in Beddington & Robertson 1999; Lu *et al.* 2001). The discrete population of VE overlying the distal end of the epiblast selectively expresses *hex*, *lefty1* and *cer1*, whereas *nodal*, *otx2*, *lhx1* (*lim1*) and *hmf3 β* (*Foxa2*) are broadly expressed throughout this tissue layer. We used a *lacZ* transgene regulated by *hex* enhancer sequences active in the VE, as well as a panel of gene markers, to assess the extent of VE patterning. Overall, we observe that *nodal* mutants fail to

establish a molecular pattern within the VE (figure 2 and data not shown). As a control for the integrity of the VE these mutant embryos were shown to express abundant levels of H19 (figure 2).

(b) *Transcriptional control of nodal in the early embryo*

We and others (Adachi *et al.* 1999; Norris & Robertson 1999) have previously identified two discrete regulatory elements in the *nodal* locus, one lying 5' to exon 1 and the other within the first intron (ASE), that collectively direct *nodal* expression during early development (figure 3). The 5' enhancer is expressed initially in the proximal epiblast and concomitant with the onset of gastrulation activity becomes restricted to epiblast cells on the posterior side of the embryo. Expression from this enhancer is not detectable in the overlying VE cells. By contrast, in wild-type embryos at early post-implantation stages, the ASE transgene is active throughout the epiblast and VE, but expression becomes localized to the prospective posterior prior to the initiation of gastrulation (figure 1b; Norris & Robertson 1999). Sequences lying within the *nodal* intronic enhancer are recognized by the forkhead DNA-binding protein FoxH1, which associates with activated smad2/3/4 complexes formed in response to activin/nodal signals (Chen *et al.* 1996, 1997). Thus, the intronic enhancer could potentially regulate aspects of *nodal* expression via an autoregulatory mechanism. In keeping with this notion, deletion of the 100 bp core region, containing the two FoxH1 binding sites, causes striking changes to the expression of a *Nodal*^{LacZ} reporter

Figure 3. The *nodal* locus contains two physically distinct *cis*-acting regulatory elements active in the early post-implantation embryo. LacZ expression patterns in transgenic embryos harbouring either the 5' enhancer (denoted by green circle) or intronic ASE (denoted by red circle). Embryonic stages shown are (from left to right) 5.5, 6.5 and 6.75 dpc, with anterior to the left. The 5' enhancer is expressed in the proximal epiblast at day 5.5, and becomes restricted to the prospective posterior side of the epiblast by day 6.5. Transverse sectioning (red line) shows that transgene activity is confined to the epiblast. By contrast, the ASE is robustly expressed throughout the epiblast and VE at 5.5 dpc, becoming restricted to the posterior side of the embryo coincident with the onset of gastrulation.

allele (Norris & Robertson 1999). Thus, LacZ activity was restricted to the proximal epiblast, absent from the VE and severely attenuated in the LPM of *Nodal*^{Δ100, lacZ/+} embryos. Moreover, our previous experiments have shown that expression of the ASE is strictly dependent on *nodal* activity since the transgene is not expressed in *nodal* null embryos (Brennan *et al.* 2001). In sum, it seems likely that transcriptional activation of the *nodal* locus may occur via a two-step process. Transcription is initiated by activation of an early enhancer, perhaps the 5' enhancer or another as yet unidentified *cis*-regulatory element. Expression then spreads throughout the epiblast via activation of the autoregulatory intronic enhancer, consequently allowing activation of *nodal* transcription in the overlying VE.

(c) Testing the *in vivo* role of the autoregulatory feedback enhancer

To examine ASE function *in vivo*, we had previously generated a deletion allele in which a 600 bp region containing the two FoxH1 binding sites was selectively removed from the wild-type locus (Norris *et al.* 2002). To assess the effect of removing the ASE we examined the temporal and spatial distribution of *nodal* transcripts in *Nodal*^{Δ600/-} and *Nodal*^{Δ600/Δ600} compared to wild-type (figure 4). In *Nodal*^{Δ600/-} weak levels of *nodal* transcripts are detectable throughout the proximal epiblast, but absent from the VE. In *Nodal*^{Δ600/Δ600} embryos the level of *nodal* is enhanced and shows the same spatial distribution as in wild-type embryos, being localized to the proximal posterior side of the embryo, but absent from the VE. We

conclude that ASE activity maintains widespread expression in a broader domain of the epiblast and activates *nodal* transcription in the VE.

To determine the phenotypic consequences of modulating the level of *nodal* expression in the epiblast we next examined *Nodal*^{Δ600/-} and *Nodal*^{Δ600/Δ600} embryos. At day 6.5, the majority of *Nodal*^{Δ600/-} embryos (60%) are indistinguishable from wild-type. However, the remaining 40% develop abnormally and display a characteristic morphology (figure 5). In these overtly abnormal *Nodal*^{Δ600/-} embryos gastrulation is initiated and mesoderm induced radially around the proximal epiblast. These embryos also have a thickened patch of VE characteristic of the AVE located at or just lateral to the distal tip of the epiblast, suggesting that the AVE forms normally but fails to rotate as is the case for *Otx2* and *Cripto* mutants (Ding *et al.* 1998; Perea-Gomez *et al.* 2001). These embryos also display a marked constriction at the embryonic/extra-embryonic boundary (figure 5). To further assess tissue disturbances, we analysed a panel of molecular markers. In the overtly abnormal *Nodal*^{Δ600/-} embryos, posterior epiblast markers such as *Cripto* (Ding *et al.* 1998), *T* (Thomas *et al.* 1998) and *Wnt3* (Liu *et al.* 1999) are ectopically expressed throughout proximal regions of the epiblast (figure 5). Collectively, these results demonstrate that mesoderm induction occurs throughout the proximal epiblast in *Nodal*^{Δ600/-} embryos. *hmf3β* (Foxa2) is initially expressed throughout the VE, but by 6.5 dpc localizes to the AVE and the anterior primitive streak (figure 5; Perea-Gomez *et al.* 1999). In mutant *Nodal*^{Δ600/-} embryos *FoxA2*

Figure 4. Disturbed *Nodal* expression patterns in *Nodal*^{Δ600/-} and *Nodal*^{Δ600/Δ600} embryos. Whole-mount *in situ* hybridization analysis of *nodal* mRNA in wild-type, *Nodal*^{Δ600/-} and *Nodal*^{Δ600/Δ600} embryos reveals altered spatial distribution of transcripts and lowered levels of expression resulting from deletion of the ASE. In contrast to *Nodal*^{Δ600/-} embryos where low levels of *nodal* are confined proximally and the P-D axis fails to rotate, increasing *nodal* expression levels in *Nodal*^{Δ600/Δ600} embryos at 6.5 dpc allow establishment of a normal A-P axis by 7.5 dpc.

transcripts are found at the proximal and distal-most extent of the embryonic region, suggesting the proximal distal axis is formed correctly but fails to rotate. Additional AVE markers including *Hex* (Thomas *et al.* 1998) and *Lhx1* (Perea-Gomez *et al.* 1999) gave similar results (not shown). Expression of the extraembryonic markers *Eomesodermin* (*Eomeso*) and *Bmp4* is unperturbed in *Nodal*^{Δ600/-} embryos (not shown). In sum, we observe a distinctive perturbation of the A-P axis position in a high proportion of *Nodal*^{Δ600/-} embryos.

nodal mRNA expression, as assessed by whole-mount *in situ* hybridization, is greatly reduced in the absence of the feedback loop (figure 4). Nonetheless, the low levels of *nodal* expression in the epiblast of *Nodal*^{Δ600/-} embryos are sufficient for establishment of a correct P-D pattern, and promote expression of genes such as *Wnt3* and *Eomeso* required for mesoderm formation. *Nodal*^{Δ600/-} embryos also maintain expression of *Bmp4* in the adjacent extraembryonic ectoderm, and markers of the AVE such as *Hex* are induced in the overlying VE. In a high percentage of *Nodal*^{Δ600/-} embryos the P-D axis is imprecisely converted to an A-P pattern, a phenotype also seen in embryos homozygous for a hypomorphic *Nodal* allele (Lowe *et al.* 2001). Since the A-P axis is rescued in *Nodal*^{Δ600/Δ600}

embryos we conclude that this defect reflects decreased levels of *Nodal* signalling in the epiblast. Thus, *Nodal* transcription is initiated via upstream regulatory elements, probably the proximal epiblast enhancer. In *Nodal*^{Δ600/-} embryos, *nodal* activity is sufficient to induce mesoderm formation, but insufficient to promote cell movements that position the A-P axis.

(d) Failure to specify anterior mesendoderm populations in *Nodal*^{Δ600/-} embryos

While only 40% of *Nodal*^{Δ600/-} embryos exhibit overt morphological defects at early gastrulation stages, by 7.5 dpc all *Nodal*^{Δ600/-} embryos are distinctly abnormal. The class of embryos displaying a visible constriction at the embryonic/extraembryonic boundary at 6.5 dpc develop external to the VYS. The majority of mutant embryos develop within the VYS, exhibit a normal midline, somites and yolk sac morphology, but have defective cardiac looping (data not shown). By 8.5 dpc *Nodal*^{Δ600/-} embryos begin to display a marked reduction in anterior neural tissues. This defect is not associated with failure to correctly specify the AVE, node or notochord. Rather, these developmental abnormalities seem to result from defective formation of the ADE and PCP (collectively the

Figure 5. Defective P-D to A-P rotation in *Nodal*^{Δ600/-} embryos. Primitive streak and AVE marker analysis in 6.5 dpc embryos. Wild-type (wt) controls are shown in (a); *Nodal*^{Δ600/-} mutants in (b). Cartoons indicate primitive streak/mesoderm markers and AVE markers. Anterior is to the left. *Cripto*, *T* (*Brachyury*) and *wnt3* normally localized to the posterior epiblast are expressed throughout the proximal epiblast *Nodal*^{Δ600/-} mutant embryos. *hnf3β* (*FoxA2*) is expressed in the anterior primitive streak and the AVE. In *Nodal*^{Δ600/-} mutant embryos *Hnf3β* is expressed in distal VE and epiblast. Note that in the majority of *Nodal*^{Δ600/-} embryos a distinctive constriction is visible between the embryonic and extraembryonic regions.

anterior mesendoderm), tissues arising from the anterior streak that are known to be responsible for patterning the CNS (Martinez Barbera *et al.* 2000). In keeping with this conclusion, *Hex* expression, a marker of definitive endoderm, is not detected in *Nodal*^{Δ600/-} embryos from gastrulation stages onwards. Expression of *Shh*, a marker of PCP, is punctate and highly downregulated even in grossly normal *Nodal*^{Δ600/-} embryos. The anterior *Shh* domain normally extends into the ventral forebrain by 8.5 dpc, but in *Nodal*^{Δ600/-} mutant embryos, expression is truncated at the hindbrain. The most severely affected *Nodal*^{Δ600/-} embryos also display decreased midline tissue and deficiencies in definitive endoderm formation.

By contrast, similar analysis of embryos homozygous for the Δ600 allele shows that none displays these morphological abnormalities (figure 5 and data not shown). As shown in figure 4, in comparison with *Nodal*^{Δ600/-} embryos, *Nodal* expression within the epiblast is elevated roughly twofold in *Nodal*^{Δ600/Δ600} homozygous mutants. This increased dosage is sufficient to rescue ADE and PCP formation and hence axis patterning is normal in these embryos. Interestingly, in these embryos *nodal* mRNA is expressed at significantly reduced levels in the epiblast and is undetectable in the VE. We conclude that *nodal* expression within the VE is not essential for either specification or positioning of the AVE. Rather, these processes depend on the strength of nodal signals within the epiblast.

3. CONCLUSION: DOSE-DEPENDENT ROLES FOR NODAL SIGNALLING IN PATTERNING THE A-P AXIS

In sum, the current experiments demonstrate that nodal signals from the epiblast dictate molecular identity within all three tissues along the P-D axis. It remains to be determined how the directed anterior movement of distal VE cells and accompanying posteriorward movement of

proximal epiblast cells subsequently give rise to the definitive embryonic A-P axis. Recent data indicate that the *nodal* cofactor *cripto* and the transcription factor *otx2* are involved, as loss of either molecule causes the AVE to remain distal (Ding *et al.* 1998; Kimura *et al.* 2000). However, *cripto* mutant embryos express posterior markers such as *brachyury* and *fgf8* and form extraembryonic mesoderm. Thus *nodal* can act via *cripto*-independent pathways to promote posterior fates, but *cripto* is essential for *nodal* functions in the epiblast leading to displacement of the AVE. We suggest that *cripto* may modulate the strength or possibly the duration of *nodal* signalling and thus contribute to the complex regulatory mechanisms that control *nodal* activity in discrete tissue sites of the early embryo. In keeping with this conclusion we have shown that selectively lowering *nodal* activity in the epiblast via deletion of the ASE results in a similar phenotype.

The highly dynamic *nodal* expression patterns in the early embryo appear to be governed by the interplay of a minimum of two distinct *cis*-regulatory regions. Recent *in vitro* studies suggest that the range and intensity of *nodal* signals are, in part, controlled by the FoxH1-dependent autoregulatory feedback loop (reviewed in Whitman 2001). Our studies underscore this conclusion. We find *in vivo* that acting in an autocrine fashion, the ASE maintains and amplifies *nodal* transcription and thereby increases the amount of ligand produced locally. The ASE also functions in a paracrine fashion to expand expression domains. Specifically, the ASE controls *nodal*-dependent target gene expression in the VE. The *FoxH1*-dependent autoregulatory enhancer thus establishes a gradient of *nodal* signals that is critical for mesoderm induction, endoderm specification and A-P axis patterning during early mouse development.

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Discussion

R. L. Gardner (*Mammalian Development Laboratory, Department of Zoology, University of Oxford, Oxford, UK*). According to unpublished observations of Rosa Beddington, cells in the visceral endoderm at the distal tip of the 5.5 day *post coitum* egg cylinder which give rise to the AVE are distinctive in being more columnar than their neighbours (cited in Thomas *et al.* 1998). Moreover, another group claim that cells in this region are double-layered rather than single-layered (Kimura *et al.* 2001). This prompts the obvious question whether the cells that form the AVE might originate from the epiblast rather than the visceral endoderm. Can this possibility, which would obviate the need to postulate that AVE activity is transferred from visceral to definitive endoderm as the former is displaced extraembryonically, be discounted?

E. J. Robertson. Our recent experiments indicate that the AVE is exclusively of extraembryonic origin. We have performed an *in vivo* marking experiment using a Cre recombinase transgene that is expressed exclusively in the epiblast (activated as early as the blastocyst stage: see Hayashi *et al.* (2002)). When crossed to the R26R reporter strain (Soriano 1999) to mark the entire epiblast, we never see blue cells emerging into in the superficial VE. This argues that the AVE derives from the VE.

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GLOSSARY

ADE: anterior definitive endoderm
ASE: asymmetric element
A-P: anterior-posterior
AVE: anterior visceral endoderm
 β gal: β -galactosidase
CNS: central nervous system
dpc: days *post coitum*

ExE: extraembryonic ectoderm
LPM: lateral plate mesoderm
L-R: left-right
PCP: prechordal plate
P-D: proximal-distal
TGF- β : transforming growth factor- β
VE: visceral endoderm
VYS: visceral yolk sac