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Anterior patterning by synergistic activity of the early gastrula organizer and the anterior germ layer tissues of the mouse embryo

Patrick P. L. Tam* and Kirsten A. Steiner

Embryology Unit, Children's Medical Research Institute, Locked Bag 23, Wentworthville, NSW 2145, Australia
*Author for correspondence (e-mail: ptam@cmri.usyd.edu.au)

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SUMMARY

Fragments of the germ layer tissues isolated from the early-primitive-streak (early-streak) stage mouse embryos were tested for axis induction activity by transplantation to late-gastrula (late-streak to early-bud) stage host embryos. The posterior epiblast fragment that contains the early gastrula organizer was able to recruit the host tissues to form an ectopic axis. However, the most anterior neural gene that was expressed in the ectopic axis was *Krox20* that marks parts of the hindbrain, but markers of the mid- and forebrain (*Otx2* and *En1*) were not expressed. Anterior visceral endoderm or the anterior epiblast alone did not induce any ectopic neural tissue. However, when these two anterior germ layer tissues were transplanted together, they can induce the formation of ectopic host-derived neural tissues but these tissues rarely expressed anterior neural genes and did not show any organization of an ectopic axis. Therefore, although the anterior endoderm and epiblast together may display some inductive activity, they do not act like a classical organizer. Induction of the anterior neural genes in the ectopic axis was achieved only when a combination of the posterior epiblast fragment, anterior visceral endoderm and the anterior epiblast was transplanted to the host embryo. The formation of anterior neural structures therefore requires the synergistic interaction of the early gastrula organizer and anterior germ layer tissues.

Key words: Neural axis, Anterior patterning, Gastrula organizer, Anterior visceral endoderm, Anterior epiblast, Mouse

INTRODUCTION

In the zebrafish, amphibian and avian gastrulae, the establishment of the body plan is influenced by the activity of a group of specialized cells known as the organizer (Gilbert and Saxen, 1993; Harland and Gerhart, 1997; Shih and Fraser, 1996; Schier and Talbot, 1998; Smith and Schoenwolf, 1998). Two unique functional attributes of the organizer are its ability to exert an inductive influence on the differentiation of the tissues with which it interacts and the provision of morphogenetic cues that regulate the dimensions, the orientation and the placement of the induced tissues in the body plan. Experimentally, the organizing activity can be demonstrated by the induction of a secondary body axis in a host embryo following the transplantation of tissue fragments containing the organizer. Cells in the organizer of different vertebrates express a common set of transcription factors and other molecules that are involved with intercellular signalling, and these cells contribute to the formation of the axial mesendoderm and the midline neuroectoderm (Harland and Gerhart, 1997; Smith and Schoenwolf, 1998; Camus and Tam, 1999).

In the mouse, the node of the late-gastrula (late-streak to early-bud stage) embryo has been shown to possess organizing activity by virtue of its ability to induce axis formation following heterotopic transplantation (Beddington, 1994) and the expression of genes that are associated with the organizer of other vertebrate gastrulae (Tam and Quinlan, 1996, Tam and Behringer, 1997). In other vertebrates, cell population that displays organizer activity has been identified at the onset of gastrulation (Shih and Fraser, 1996; Zoltewicz and Gerhart, 1997; Ipzisúa Belmonte et al., 1993). However, the existence of an organizer that precedes the node in the mouse gastrula has not been demonstrated until recently by the discovery of a group of cells in the posterior epiblast of the early-streak embryo that displays cell fates, gene expression and tissue patterning activity that are characteristic of other vertebrate organizer (Tam et al., 1997; Camus and Tam, 1999). Our previous study has shown that a fragment of the posterior epiblast that is localised outside the early primitive streak can induce the formation of ectopic neural tissues, which in some cases are organized into a rudimentary axis. Ectopic neural tissue can be found in about 26% of the recipient embryos, which is comparable to the frequency of neural tissue induction by the node of the late-gastrula (Beddington, 1994; Tam et al., 1997). This group of cells may therefore be regarded as the equivalent of the organizer in the early gastrulae of other vertebrates and has been termed as the early gastrula organizer (EGO). When the node of the mouse is tested for its organizing activity by transplantation, the induced axis is typically made

up of the graft-derived tissues in the notochord and somites and host-derived neural tissues that are morphologically characteristic of the trunk neural tube but not of the brain (Beddington, 1994). The lack of anterior development in the induced axis implies that the specific head organizing activity may be absent from the node, which acts primarily as an organizer for trunk structures (Tam and Behringer, 1997; Camus and Tam, 1999; Beddington and Robsertson, 1998). Transplanted EGO, however, also seems unable to induce a full neural axis, though this has not been tested by the expression of regional markers (Tam et al., 1997). This may suggest that either the head organizing activity is found outside the EGO or the head inducing activity cannot be expressed by EGO alone when tested by heterotopic transplantation.

Recently, gene expression and experimental embryological studies have indicated that the visceral endoderm of the mouse gastrula plays an essential role in the patterning of anterior structures. The anterior visceral endoderm (AVE) is found to express genes that are also expressed in the EGO and the node (e.g. *Hnf3*β, *Gsc*; Belo et al., 1997) and those that are required for the patterning of the head or the forebrain, as revealed by mutational analysis (e.g. *nodal*, *Hesx1*, *Lim1*, *Otx2*; Varlet et al., 1997; Dattani et al., 1998; Shawlot and Behringer, 1995; Ang et al., 1996; Acampora et al., 1995; Matsuo et al., 1995). The AVE also expresses genes that are associated with head induction or axis duplication following ectopic expression in the amphibian embryos (e.g. *Gsc*, *Cerberus*; Cho et al., 1991; Bouwmeester et al., 1996; Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998). Ablation of the AVE in the mouse leads to a diminished expression of the *Hesx1* and *Otx2* gene in the forebrain (Thomas and Beddington, 1996). Furthermore, the AVE of the rabbit embryo can induce anterior neural gene activity in the chick blastoderm (Knoetgen et al., 1999). These findings therefore strongly implicate AVE as the source of anterior organizing activity that may have been separated from the early gastrula organizer in the mammalian embryo (Beddington and Robertson, 1998, 1999). An interesting finding in the zebrafish reveals that some head organizing activity may also be found in the epiblast cells in the anterior blastoderm (Houart et al., 1998), suggesting that, in addition to the AVE, the role of the epiblast in head patterning cannot be disregarded.

In the present study, we have examined the source of the activity for patterning anterior neural tissues in the early mouse gastrula. This is done by testing the ability of germ layer fragments isolated from the early gastrula to induce a secondary neural axis with anterior molecular characteristics after heterotopic transplantation to the late-gastrula stage host embryo. Our results suggest that the induction of the full neural axis requires the synergistic interaction of the EGO and the anterior germ layer tissues of the early gastrula.

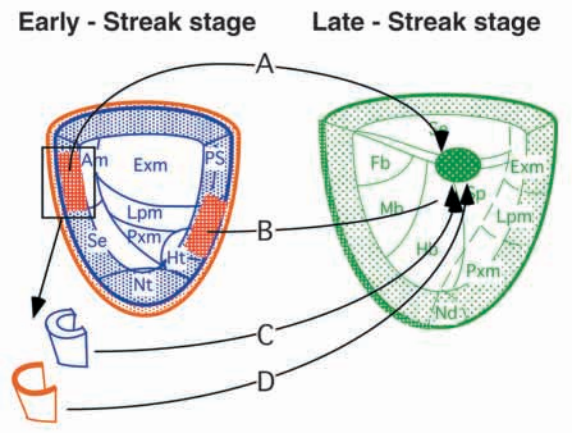


Fig. 1. The strategy of tissue transplantation. Fragments of tissues were isolated from the anterior germ layers (A) and posterior epiblast (B) of the early-streak stage embryo. The anterior germ layer fragment was dissected into (C) epiblast and (D) visceral endoderm. Various combinations of tissue fragments were grafted to the lateral region of the late-streak embryo by micro-manipulation. The prospective fate of the tissues in the epiblast of the early-streak embryo and in the ectoderm of the late-streak embryo is shown (Tam and Behringer, 1997). Exm, extraembryonic mesoderm; Lpm, lateral plate mesoderm; Pxm, paraxial mesoderm; Nt, neural tube; Ht, heart mesoderm; Se, surface ectoderm; Am, amnion; Fb, forebrain, Mb, midbrain; Hb, hindbrain; Sp, spinal cord; Nd, node; PS, primitive streak.

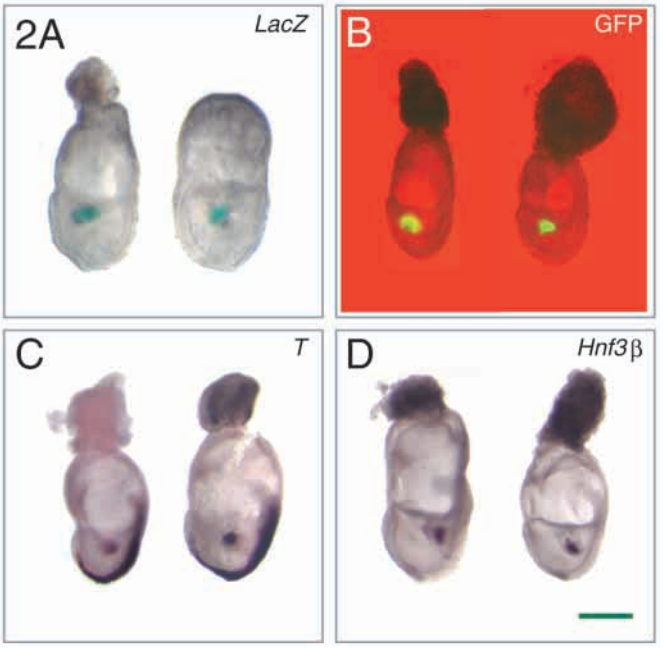


Fig. 2. Recipient embryos containing the same combined grafts of anterior visceral endoderm (AVE), anterior epiblast (EPI) and posterior epiblast (EGO) in the lateral region of the host embryo. The recipient embryos were examined at 5-6 hours after tissue transplantation. The grafted tissues show reporter activity encoded by (A) the *lacZ* and (B) the EGFP transgene (Hadjantonakis et al., 1998). (C) *Brachyury* activity is expressed widely in the graft but (D) *Hnf3*β activity is restricted to the posterior part of the grafted tissue (which is most likely to be derived from the EGO that was grafted usually posterior to the EPI and AVE). *Brachyury (T)* activity is also found in (C) the primitive streak and anterior midline mesendoderm and *Hnf3*β is expressed in (D) the node of the host embryo. The *lacZ* activity was revealed by X-gal staining, GFP by fluorescence microscopy (using Leica MZFLIII microscope with GFP-2 filter set at 480 nm absorbance) and gene expression by whole mount in situ hybridization. Anterior is to the left. Bar, 200 μm.

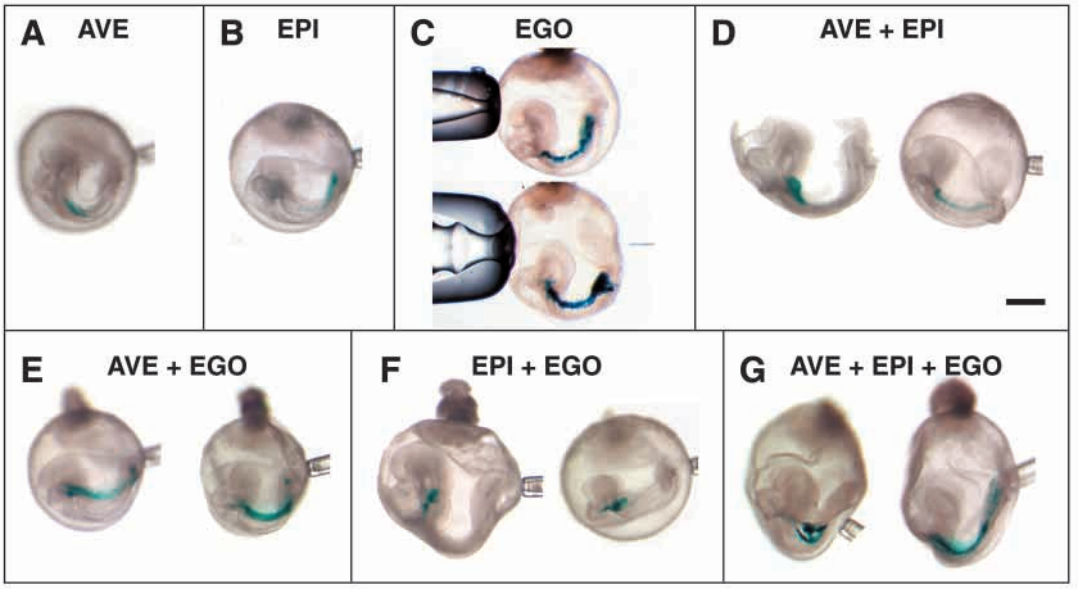
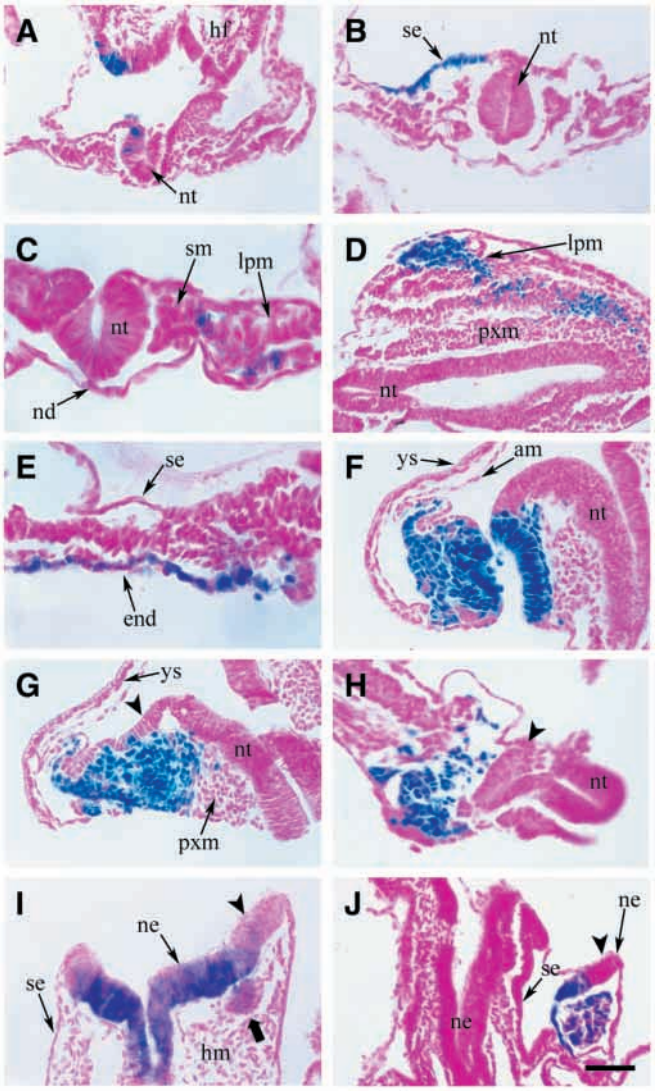


Fig. 3. The incorporation of germ layer grafts in the host embryo after 24 hours of in vitro development. Both local incorporation and widespread colonization of graft-derived *lacZ*-expressing tissues (blue colour) are found in all seven types of transplantation, but grafts containing the early gastrula organizer tend to show more extensive colonization. The implant received by each of the embryos is indicated. Anterior visceral endoderm (AVE), anterior epiblast (EPI), early gastrula organizer (EGO). Bar, 250 μm.



MATERIALS AND METHODS

Experimental strategy

Early- and late-gastrula stage gastrula embryos were explanted from pregnant mice at 6.5 days and 7.5 days post coitum respectively. The early-primitive-streak (early-streak) stage embryos were obtained from transgenic mice that express a *HMG-nls-lacZ* reporter (Tam and Tan, 1992) or an EGFP transgene (Hadjantonakis et al., 1998). Tissue fragments isolated from the transgenic early-streak embryo were used as donor tissues for transplantation into non-transgenic ARC strain late-gastrula (late-streak to early-bud; Downs and Davies, 1993) stage embryo. Since the transgene is expressed ubiquitously in all embryonic tissue lineages, it therefore allows the unequivocal tracking of the distribution and differentiation of the graft-derived cells in the host tissues. It is not possible in the present study to test whether the grafted tissue can elicit de novo induction of neural tissues because the grafts were placed either within or close to the germ layer tissues

Fig. 4. (A-F) Examples showing the colonization of host tissues by transplanted tissues. Graft-derived cells express *lacZ* activity and were stained blue by X-gal histochemistry. Examples are shown of cell colonization in (A) the neuroepithelium of the head fold and neural tube, (B) surface ectoderm, (C) somite and lateral plate mesoderm, (D) presomitic mesoderm and somatopleure of the lateral plate mesoderm and (E) the endoderm of the open midgut. F shows an example of self-differentiation of the graft to epithelial and mesenchymal tissues, which has produced a hiatus in the lateral region of the host embryo. (G-J) The formation of ectopic neural tissues in the host embryo (arrowheads). (G) Thickening of the surface ectoderm to columnar/pseudostratified epithelium. (H) Duplicated neural tube. (I) An expanded domain of *Otx2* expression in the neural plate of the head fold (arrowhead) overlying the graft-derived tissues (arrow, tissue stained by magenta-gal). (J) Ectopic neural plate. (A,C,E) EPI graft, (B) AVE+EPI graft, (D,F,G,H) AVE+EPI+EGO graft, (I) EGO graft and (J) EPI+EGO graft. (A-H and J) X-gal and nuclear fast red staining, (I) in situ hybridization revealed by NBT/BCIP staining and tissues counterstaining with nuclear fast red. am, amnion; hf, head fold; lpm, lateral plate mesoderm; nd, notochord; ne, neuroepithelium, nt, neural tube; pxm, paraxial mesoderm; se, surface ectoderm; sm, somite; ys, yolk sac. Bar, 50 μm.

that are normally allocated to the formation of prospective neural structures (Fig. 1). In view of this caveat, the response of the host tissues to the transplanted tissue was assessed specifically for the formation of ectopic neural tissues and the induction of region-specific neural gene activity. The outcome of the axis induction experiment could potentially be affected by the competence of the germ layer of the host embryo to tissues from the early gastrula. However, previous studies have shown that axis induction can be achieved at a reasonable rate by EGO and node (Beddington, 1994; Tam et al., 1997), and the expression of neural genes such as *Sox2*, *En1*, *Hesx1* and *Otx2* can be induced by transplanting axial mesendoderm to the lateral region of the late-gastrula stage host embryo (A. Camus and P. P. L. T., unpublished). A similar experimental approach was therefore undertaken for the present study.

Isolation of embryonic tissues for transplantation

Donor transgenic early-streak embryos were explanted from the decidua and the Reichert's membrane was removed. Different tissue fragments were isolated from the early-streak embryo (Fig. 1) for transplantation by dissecting with finely drawn glass needles. A tissue fragment contained within a zone of 50-100 μm from the border of the epiblast and the extraembryonic ectoderm was dissected from the posterior region of the embryo. The adherent visceral endoderm was removed. This epiblast fragment has been shown to display axis induction activity after heterotopic transplantation to a late-streak host embryo (Tam et al., 1997). This fragment was trimmed down to about 40×40 μm containing approximately 40 cells. Another tissue fragment of similar size was dissected from the anterior region of early-streak embryo. This fragment, which contained the AVE and the epiblast (EPI), was either used as such for transplantation or the AVE and EPI were separated from each other by micro-dissection with glass needles and grafted separately. The EGO, AVE and EPI fragments were transplanted either separately (EGO, AVE or EPI) or in various combinations (AVE+EPI, AVE+EGO, AVE+EPI or AVE+EPI+EGO; Fig. 1; see Tables 1 and 2) to the late-gastrula host embryo (late-streak to early bud stages, Downs and Davies, 1993).

Transplantation to recipient embryos

The tissue fragments were transplanted to the lateral region of the late-gastrula host embryo that contains the precursor tissues of the surface ectoderm, the lateral mesoderm and the neuroectoderm of the hindbrain and the spinal cord (Fig. 1). During transplantation, an incision (70-80 μm, about one-third the anterior-posterior length of the host embryo) was made using electrolytically polished alloy metal needles. The host embryo was then held by suction onto a holding micropipette and the donor tissue fragments, held by an injection micropipette operated with a Leica micromanipulator, were lodged in the incision (Fig. 2A,B), so that the transplanted tissues were in direct contact with all three germ layers of the host embryo. When a combination of tissue fragments was grafted, the epiblast tissue (EGO or EPI) was transplanted first followed by the endoderm (AVE). The host embryo was then cultured in the DME medium supplemented with 75%(v/v) rat serum in a rotating embryo culture apparatus for 24-26 hours (Sturm and Tam, 1993).

Some embryos that received transplants of AVE+EPI+EGO were examined for the expression of *T* and *Hnf3β* genes at 5-6 hours after transplantation by whole-mount in situ hybridization (for protocol, see later section) to reveal whether the grafted tissues displayed organizer and primitive streak activity.

Analysis of embryonic development

At the end of in vitro culture, host embryos were removed from the culture medium, washed three times with PBS and fixed with 4% paraformaldehyde. The embryos were then stained with X-gal (Progen) or Magenta-gal (Biosynth AG) reagent to reveal the β-gal activity of the graft-derived cells. The stained embryos were allocated randomly either for histology, to examine the developmental fate of the grafted cells, or for whole-mount in situ hybridization, to analyze gene expression (Wilkinson and Nieto, 1993, modified by Davidson et al., 1999). The expression of five neural genes, *Otx2* (fore- and mid-brain), *En1* (mid-brain), *Krox20* (rhombomere 3 and 4), *Hoxb1* (rhombomere 4 and the spinal cord) and *Sox2* (the pan-neural marker), was examined to assess the extent of axis induction by the transplanted tissues. The expression of engrailed protein was studied in embryos receiving AVE+EPI+EGO grafts by immunostaining using the αEnhb-1 antibody (Davies et al., 1991).

RESULTS

Graft-derived cells display diverse fate in the host embryo

A previous study on the organizing activity of the posterior epiblast of the early mouse gastrula has revealed that 5-6 hours after heterotopic transplantation, the graft tissues express *T*, *Lim1* and *Hnf3β* activity (Tam et al., 1997). In the present study, the combined graft of EGO, AVE and EPI showed strong expression of *T* (51%, 18 of 35 transplants examined; Fig. 2C) and *Hnf3β* activity (34%, 15 of 44 transplants; Fig. 2D). These findings show that the grafted tissues of EGO and AVE+EPI+EGO may have either acquired or maintained the molecular property of the gastrula organizer and the primitive streak. The anterior epiblast (EPI) fragments show weak in situ hybridization for *T* and *Lim1* (Tam et al., 1997) but no *Hnf3β* activity. The AVE was not examined for gene expression because of the poor rate of recovery of grafts in the host embryos.

Recipient embryos with grafts of germ layer tissues developed to the early-somite-stage (average 7.6 pairs of somite, range: 3-10 somites) after 24-26 hours of culture. In most embryos, graft-derived tissues were incorporated into the flank of the host embryo frequently at the hindbrain and upper trunk levels (Fig. 3A-G), which is consistent with the initial location of the graft in the body plan of the late-gastrula embryo. There were examples of localised incorporation of the graft into the hindbrain or the flank of the host embryo, as well as extensive contribution of the grafted tissues along the length of the host body axis in all seven types of transplantation (Fig. 3A-G). In some embryos, the grafted tissues failed to mix with the host tissues and differentiated to discrete epithelial structures and clumps of mesenchymal cells (Figs 3D,F, 4F). The localised integration and self-differentiation of the graft tissues (Figs 3G, 4F) sometimes disrupted the morphology of the host embryo.

The host tissues that were most frequently colonized by the graft-derived cells were the paraxial mesoderm and the neural tube (Fig. 4A,C,D; Table 1). Graft-derived cells were also found in the surface ectoderm, lateral plate mesoderm and the endoderm (Fig. 4B,C,E). However, without further analysis for expression tissue-specific markers, it is not possible to determine if the graft-derived cells were incorporated passively in the host tissues or have acquired the phenotypic property of the tissues they colonized. Grafts of AVE showed the lowest rate of incorporation and the least extensive contribution to the host tissues (Fig. 3A; Table 1). Some AVE-derived cells were found in the neural tube and mesoderm of the host embryo. Extensive colonization of the host tissues along the anterior-posterior length of the body was more frequently found with grafts containing the anterior epiblast tissues or the early

Table 1. Tissue colonization by cells derived from transplants of the anterior visceral endoderm (AVE), anterior epiblast (EPI) and early gastrula organizer (EGO) of the early-streak stage mouse gastrula

Graft	No. Embryo showing colonization of tissues								Self differ	Extended tisssue colonization*
	No. analyzed	NT	SE	Pxm	Lpm	End	HT/AA	Exm		
AVE	13	2			4	3		3	2	1/12
EPI	22	9	2	7	1	1		4	6	6/6
EGO	23	9	7	13	2	1			5	8/10
AVE+EPI	12	5	4	9	1	2			5	5/15
AVE+EGO	9	4		9	3	5	2		2	7/9
EPI+EGO	12	1		4	3	3	5	2	6	6/12
AVE+EPI+EGO	12	7	4	10	5	8	2	1	4	38/82

*The fraction represents the number of embryos showing extended tissue colonization/ total number examined.

Abbreviation: NT, neural tube; SE, surface ectoderm; Pxm, paraxial mesoderm; Lpm, lateral plate mesoderm; End, endoderm; HT/AA, heart and associated vessels such as aortic arches; Exm, extraembryonic mesoderm; Self differ, self differentiation to epithelial or cystic structures.

Table 2. The induction of neural tissue specific genes in the host tissues by transplants containing anterior visceral endoderm (AVE), anterior epiblast (EPI) and early gastrula organizer (EGO) of the early-primitive-streak stage mouse gastrula

Graft	Neural tissues induced % (N)	*Otx2* induction	*En1* induction	*Krox20* induction	*Sox2/Hoxb1* induction
AVE	0% (13)	0/13†			
EPI	0% (22)	0/4			
EGO	24% (46)	1/14	0/5	5/9	
AVE+EPI	8% (12)	1/22	1/5	2/7	
AVE+EGO	11% (9)	0/5	0/4		
EPI+EGO	42% (12)	0/5			
AVE+EPI+EGO	75% (12)*	12/47	5/26	3/10	14/28

N=number of embryos examined histologically for the presence of ectopically induced neural tissues.

†The fraction represents the number of embryos expressing the gene in the induced tissues/ total number of embryos analyzed. Blank= not analyzed.

*Significant difference between AVE+EPI+EGO and all other groups except EPI+EGO by χ^2 test at $P<0.01$.

gastrula organizer (e.g., Fig. 3C,E,G). Grafts of EPI, EGO or a combination of these two tissues displayed more widespread colonization of the ectodermal (neural plate and surface ectoderm, Fig. 4A,B), mesodermal (paraxial and lateral plate mesoderm, Fig. 4C,D) and endodermal tissues (Fig. 4E; Table 1). The most extensive contribution to host tissues was made by transplants that contained all three types of tissue fragment (AVE+EPI+EGO; Table 1).

Induction of ectopic neural tissue by the early gastrula organizer is enhanced by the presence of anterior germ layer tissues

Results of this study show that neither the AVE nor the EPI induced any formation of ectopic neural tissue in the host tissue, but a combination of AVE and EPI has induced ectopic neural tissues in 1 of the 12 (8%) embryos analyzed (Table 2). The posterior epiblast fragment containing the EGO induced the formation of ectopic neural tissues in about 24% of embryos, which is similar to the frequency previously reported for similar transplantation (Tam et al., 1997). The combination of the AVE with EGO did not change the incidence of ectopic neural tissues produced by combining AVE with EPI (11% versus 8%). However, the frequency of ectopic neural tissue formation was increased by the combining the EGO and the EPI in the graft (Table 2; Fig. 4J). The highest incidence (75%) of induced ectopic neural structures was found in embryos receiving combined grafts of all three tissue fragments. (AVE+EPI+EGO, Table 2; Fig. 4G,H). In the host embryos, the ectopic neural tissues were identified by the ectoderm acquiring a tall columnar to pseudostratified epithelial architecture, which was found either to be contiguous with the neural tube (Fig. 4G) or in the flank of the embryo (Fig. 4J), as the diverticulation of the neural tube (Fig. 4H) or as an expansion of the neural plate (Fig. 4I). Most of the ectopic neural tissues were recruited primarily from the host tissues (e.g. Fig. 4G,H) but there were examples of mixed contribution by host and graft-derived cells (e.g. Fig. 4J).

The combination of AVE, EPI and EGO promotes the expression of anterior neural genes in the ectopic axis

Previous analysis of gene expression in the ectopic neural tissues formed after EGO transplantation showed that they expressed the pan-neural marker *NCAM* (Tam et al., 1997), but region-specific neural markers have not been examined to reveal the anterior-posterior extent of the neural axis. The inference of the absence of anterior neural tube structure was based solely on the lack of head fold formation in the ectopic axis. This observation has been confirmed in the present study which showed the absence of *Otx2* (except one, see Fig. 4I) and En expression in the ectopic tissues formed in the embryo receiving the EGO graft (Table 2). However, *Krox20*-expressing tissues were present in the neural tube structure induced by the EGO graft (Fig. 5A-C), suggesting that the ectopic neural axis has extended to at least the level of the upper hindbrain. A survey of *Otx2* and *En1* expression in host embryos that received other types of grafts (Table 2) revealed that AVE, EPI and EPI+EGO could not induce any *Otx2* or *En1* expression. In view of this finding, the expression of more posterior neural markers was not examined for these grafts.

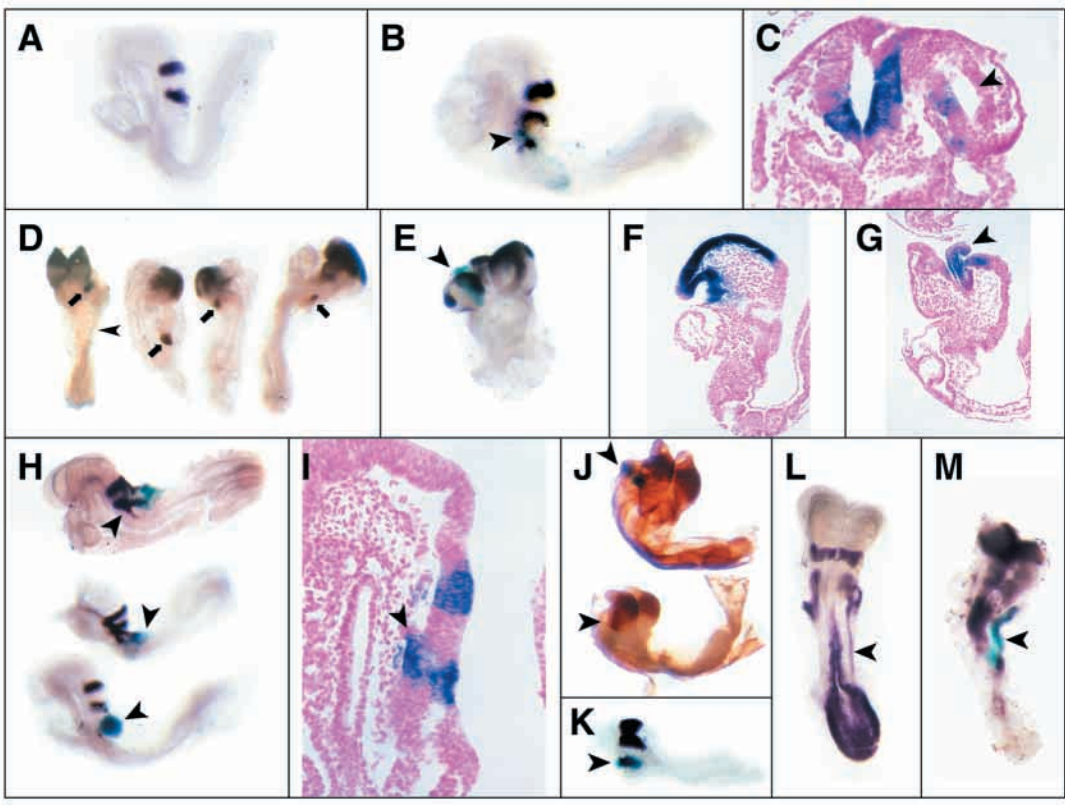


Fig. 5. The induction of neural gene expression by EGO (A-C) and AVE+EPI+EGO grafts (D-G). (A) Normal *Krox20* expression in control early-somite stage embryo, (B) host embryo with EGO graft showing an extra domain of *Krox20* expression (arrowhead) adjacent to the graft and (C) the expression of *Krox20* in the duplicated neural tube (arrowhead) next to the host hindbrain. (D-G) *Otx2* expression in embryos with AVE+EPI+EGO grafts. (D) Expression of *Otx2* in the ectopic neural tissues (arrows) in four recipient embryos. In the left-most embryo, an apparently complete secondary body axis has been induced (arrowhead), the posterior part of the ectopic axis fuses with the host axis. (E) The extra head fold on the right hand side of the graft (blue tissue indicated by the arrowhead) shows ectopic *Otx2* expression. (F) The head fold of control early-somite embryo showing the expression of *Otx2* in the forebrain and midbrain and (G) expression of *Otx2* in the extra neural tissues (arrowhead) in between the head folds of the host embryo. (H,I) Induction of *Krox20*-expressing tissues by the AVE+EPI+EGO grafts in (H) the lower hindbrain (arrowheads) of whole-mount embryos and (I) in the duplicated neural epithelium (arrowhead) in parasagittal section of the hindbrain. (J) Engrailed expression in the ectopic tissues induced by the AVE+EPI+EGO grafts lateral to the head folds (expression of the EN protein is revealed by immunostaining as dark brown patches marked by arrowheads) and (K) *En1* expression in the neural tissue (arrowhead) induced by the AVE+EPI graft revealed by in situ hybridization. (L) Expression of *Hoxb1* and (M) *Sox2* in the ectopic axis (arrowhead) induced by the AVE+EPI+EGO graft. The graft-derived tissues are stained bright blue, and the in situ hybridization signal is dark purple in whole mount (A,B,E,H,K-M) and purplish blue on sections (C,F,G,I).

Combined AVE+EGO grafts also failed to induce *Otx2* and *En1* expression. In contrast, AVE+EPI grafts can induce expression of *En1* (Fig. 5K), *Otx2* and *Krox20* (data not shown, Table 2), suggesting that these two anterior germ layer tissues together can impart some anterior characteristics to the ectopic neural tissues. When the AVE and EPI were combined with the EGO, strong induction of the *Otx2* gene (Fig. 5D-G), and expression of the engrailed protein (Fig. 5J,K) was observed in the ectopic neural tissues. More posterior genes such as *Krox20* (Fig. 5H,I) and *Hoxb1* (Fig. 5L) and the pan-neural *Sox2* gene (Fig. 5M) were also expressed in the ectopic neural tissue induced by the AVE+EPI+EGO graft (Table 2). These findings strongly suggest a complete neural axis may be induced by the synergistic activity of the EGO and the anterior germ layer tissues.

DISCUSSION

Anterior patterning requires the interaction of early gastrula organizer with anterior germ layer tissues

Results of this study have shown that neither AVE nor EPI alone induces any expression of anterior neural genes in the host tissues. However, when AVE and EPI are grafted together, ectopic neural tissues can be induced but only at a low frequency. In the induced tissue, expression of *Otx2, En1* and *Krox20* genes can be detected, suggesting the AVE and EPI together may elicit the differentiation of anterior neural tissues. Despite this, the combined AVE+EPI graft does not induce the formation of ectopic neural axis in the host. The AVE and the EPI, either separately or together, therefore do not possess the classical organizing activity that recruits the host tissues to the formation of a secondary neural axis. The present study and a previous one (Tam et al., 1997) show that the ability to organize the host tissue into an ectopic body axis is specifically associated with the posterior epiblast fragment that contains the early gastrula organizer. However, the analysis of region-specific molecular markers has shown that the induced neural axis is a partial one that lacks anterior neural characteristics, as previously inferred based on the morphology of EGO- and node-induced neural tube (Beddington, 1994; Tam et al., 1997). The combination of EGO with AVE does not increase the frequency of axis induction, but EGO with EPI has raised the frequency to 42%. Nevertheless, neither combination has induced the expression of the anterior neural genes (*Oxt2* and *En*) in the ectopic axis. Expression of anterior neural genes is induced only when the EGO, the anterior visceral endoderm and the anterior epiblast adjacent to the AVE are transplanted

together to the recipient embryo. Our results therefore provide compelling evidence for the requirement of the synergistic interaction between the EGO-containing posterior epiblast and the anterior germ layer tissues for the organization of the full neural axis.

The role of anterior germ layer tissues in anterior patterning

The AVE has been implicated to play a crucial role in the patterning of anterior neural structures. An increasing number of genes have been found to be expressed by the visceral endoderm of the early mouse gastrula and some of these are expressed specifically in the anterior endoderm. These genes included those, such as *Hnf3β* and *Gsc*, that are also expressed in the gastrula organizer, and others that are expressed in both the AVE and other germ layer derivative during gastrulation and organogenesis including *Hesx1*, *Lim1*, *Otx2*, *Mrg1*, *Cerrl* and *nodal* (Ang et al., 1996; Shawlot and Behringer, 1995; Hermesz et al., 1996; Thomas and Beddington, 1996; Belo et al., 1997; Varlet et al., 1997; Biben et al., 1998, Dunwoodie et al., 1998; Shawlot et al., 1998; Thomas et al., 1998; Pearce et al., 1999; reviewed by Bouwmeester and Leyns, 1997; Beddington and Robertson, 1998, 1999). For two of these genes (*Lim1* and *Otx2*), the loss-of-function mutation has led to the deficiency or absence of anterior structures (Shawlot and Behringer, 1995; Ang et al., 1996; Acampora et al., 1995; Matsuo et al., 1995). Whether this is the result of the loss of the anterior patterning activity specifically from the AVE is not fully understood. Chimeric studies of the *Otx2* mutant embryo reveal that the gene activity in the visceral endoderm is necessary for anterior patterning but is not sufficient to overcome the defects due to *Otx2* deficiency in the epiblast-derived tissues (Rhinn et al., 1998; Suda et al., 1999). Mutation of the *Hesx1* gene, which is specifically expressed in the AVE at early gastrulation, or the ablation of the *Hesx1*-expressing endoderm does not affect the global patterning of anterior structures but leads to defective differentiation of the forebrain (Dattani et al., 1998). In mutant embryos that display head deficiency (such as $Otx2^{-/-}$ and $Lim1^{-/-}$), the expression domain of the AVE genes (e.g. *Cerr1* and *Hex*) is displaced to ectopic sites (Biben et al., 1998; Shawlot and Behringer, 1998). This finding suggests that not only the gene activity but also the correct positioning of this AVE population may be critical for anterior patterning. The role of *Cerr1* in anterior patterning is also consistent with its effect in head induction in *Xenopus* embryos when the gene is expressed ectopically (Bouwmeester et al., 1996). The consequence of the mutation of the orthologs of *Cerberus* and other related genes (Pearce et al., 1999) in the mouse is not fully elucidated. However, the phenotype of the $Cripto^{-/-}$ (Ding et al., 1998) and $Hnf3\beta^{-/-}$ mutant embryo (Dufort et al., 1998) has suggested that some anterior patterning may be achieved even in the absence of the gastrula organizer, therefore raising the possibility that the activity of tissues outside the organizer, such as AVE and EPI, may be sufficient to bring about a limited degree of axis patterning.

Despite the aforementioned findings that implicate the AVE in anterior patterning, results of our study on the patterning activity of transplanted germ layer tissues show that the AVE alone is not sufficient to induce neural development in the mouse embryo. This is in contrast with the ability of the rabbit AVE to induce neural gene (*GANF* and *cSox3*) expression in the extraembryonic tissues of the chick blastoderm (Knoetgen et al., 1999). In the chick embryo, anterior hypoblast alone does not induce neural gene expression but prechordal mesendoderm does (Knoetgen et al., 1999). However, when the hypoblast of the pre-incubated embryo is placed together with the Hensen's node beneath the anterior blastoderm, a miniature axis with the anterior pole directed towards the hypoblast is induced (Callebaut et al., 1999). The anterior-posterior extent of the induced axis is not known since no marker expression was studied. This may suggest that in the mouse, anterior induction may require more than the activity of the endoderm. In the zebrafish, the patterning of the forebrain has been found to depend on a small population of epiblast cells in the prospective head region (Houart et al., 1998). However, while the mouse EPI alone does not induce any neural gene activity (this study), the zebrafish epiblast population can induce forebrain-specific *dlx1* and *emx1* gene expression (Houart et al., 1998). The different outcome of induction by germ layer tissues in different vertebrates might be due to the species-specific variation in the localization of patterning activity in the germ layers of the early gastrula or to the stage-related (pre-incubated stage X and HH3 mid-gastrula in the chick and quail, 70-75% epiboly mid-gastrula in the zebrafish versus late gastrula in the mouse) competence of the host tissues to respond to inductive signals (Ang and Rossant, 1993; Koshida et al., 1998; Knotegen et al., 1999).

Anterior patterning in the mouse involves successive steps of tissue interaction brought about by morphogenetic tissue movement

In the pre-gastrulation mouse embryo, the *Hex* gene is expressed initially in the visceral endoderm in the distal part of the egg cylinder (Thomas et al., 1998). During pre-gastrulation development of the mouse embryo, the expression domain of *Hex* genes in the visceral endoderm shifts from the apex to the anterior side of the egg cylinder (Thomas et al., 1998). This shift in expression domain is apparently brought about by an anterior displacement of the visceral endoderm, which continues till late gastrulation (Lawson et al., 1986; Lawson and Pedersen, 1987; Tam and Beddington, 1992). This results initially in the translocation of apical endoderm to the anterior region of the early gastrula and subsequently the displacement of the AVE to the yolk sac. Shortly before the onset of gastrulation, the anterior visceral endoderm also starts to express the *Cerrl* and the *Hesx1* gene (Thomas and Beddington, 1996; Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998; Pearce et al., 1999). The mouse *Cerrl* and the *Xenopus Cerberus* genes are known to encode factors that display anti-Wnt and anti-BMP activity (Biben et al., 1998; Piccolo et al., 1999). In *Xenopus*, it has been postulated that the anti-BMP activity is important for the maintenance and patterning of the anterior endoderm (Zorn et al., 1999) and perhaps is critical for antagonizing the ventralizing effect of the BMP factors (Piccolo et al., 1999).

The AVE of the mouse embryo during its ontogeny has been juxtaposed successively with the apical and the anterior epiblast which are fated to form the neuroectoderm of the fore- and midbrain (Lawson et al., 1991; Quinlan et al., 1995; Thomas and Beddington, 1996). Although definitive evidence of inductive interaction between the visceral endoderm and the epiblast is lacking, the transition of *Hesx1* expression from the

AVE to the anterior epiblast (Thomas and Beddington, 1996) suggests that some cross-talk may have taken place between the germ layers. This interaction may be critical for the establishment of the anterior potency. It may enable the descendants of the anterior epiblast that remains in the anterior ectoderm (Lawson et al., 1991) to act synergistically with the tissues derived from early gastrula organizer to initiate head development. During gastrulation, cellular descendants of the early gastrula organizer are allocated, as a result of morphogenetic movement, to the midline mesendoderm, the floor plate and the cranial mesenchyme that are associated with anterior neural plate (Lawson et al., 1991; Parameswaran and Tam, 1995; Tam et al., 1997; Tam and Behringer, 1997). The interaction of the mesendodermal derivatives of the EGO that are brought into contact with the descendants of the anterior epiblast may thus lead to the morphogenesis of anterior neural structures. The ability to induce anterior neural gene expression in the host tissues by the combination of AVE, EPI and EGO therefore emulates, in an experimental setting, the normal outcome of the interactions of these tissues during anterior patterning. The EGO of the mouse may possess both the head and trunk organizing activity. The head organizing activity, however, is ineffective in the absence of the synergistic action of the epiblast that has been primed for head formation by the anterior visceral endoderm. Therefore, anterior patterning is brought about by successive inductive interaction between the anterior germ layers and the gastrula organizer that is enabled by morphogenetic tissue movement during gastrulation.

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