Int. J. Dev. Biol. 45: 241-250 (2001)

Siamois cooperates with TGFβ signals to induce the complete function of the Spemann-Mangold Organizer

MARK J. ENGLEKA and DANIEL S. KESSLER*

Department of Cell and Developmental Biology. University of Pennsylvania School of Medicine, Philadelphia, USA

ABSTRACT In *Xenopus*, the Spemann-Mangold organizer induces and patterns the body axis. Siamois, a Wnt-responsive transcriptional activator, functions to establish and maintain the Spemann-Mangold organizer by regulating organizer gene transcription. While expression of Siamois in marginal blastomeres induces an axis consisting of both head and trunk structures, we show that expression of Siamois in animal blastomeres induces an axis that lacks head structures. Consistent with the absence of head organizer activity in Siamois-expressing animal pole tissue, Siamois did not induce animal expression of Cerberus, Frzb1 and Xlim1, genes implicated in anterior development. A dominant negative form of Siamois inhibited endogenous expression of Cerberus, Frzb1 and Xlim1, indicating that Siamois is necessary for organizer-specific expression of these head organizer genes, but is not sufficient in animal tissue. Siamois induces Cerberus, Frzb1 and Xlim1 in vegetal blastomeres and vegetal induction by Siamois is dependent on endogenous TGFβ signals. The results provide evidence that Siamois cooperates with TGFβ signals to activate the expression of organizer genes and to generate an organizer with both head- and trunk-inducing activity.

KEY WORDS: *Organizer, Xenopus, Siamois, TGFβ, Transcription.*

Introduction

Vertebrate axis formation is regulated by the activity of small groups of cells referred to as organizing centers. In *Xenopus*, the Spemann-Mangold organizer forms in the dorsal equatorial region of the blastula and acts during gastrulation to confer anteroposterior and dorsoventral pattern (Spemann and Mangold, 1924). This global patterning of the embryo results from the expression of organizer-specific genes which alter the fate of neighboring cells and regulate the differentiation of the organizer tissue into axial structures.

The Spemann-Mangold organizer forms in response to both mesoderm-inducing signals and signals that define dorsal regions of the embryo (reviewed in Kessler and Melton, 1994; Harland and Gerhart, 1997; Heasman, 1997; Moon and Kimelman, 1998). An abundance of evidence indicates that mesoderm is induced by Smad2-activating members of the TGFβ family produced by vegetal cells (reviewed in Kessler and Melton, 1994; Kimelman and Griffin, 2000). Several TGFβ ligands present in the blastula embryo, including Activin, Vg1, Derriere and the Nodal-related factors Xnr1 and Xnr2, each have the ability to induce the expression of both general and organizer-specific mesodermal markers (Asashima *et al.*, 1990; Smith *et al.*, 1990; Thomsen *et al.*, 1990; Thomsen and Melton, 1993; Jones *et al.*, 1995; Kessler and Melton, 1995; Sun *et al.*, 1999). In addition, embryos expressing one of a variety of dominant negative TGFβ receptors fail to express mesodermal genes or form differentiated mesodermal tissues (Hemmati-Brivanlou and Melton, 1992; Chang *et al.*, 1997), suggesting that mesoderm induction depends on a functional TGFβ pathway. Embryos expressing inhibitors of TGFβ signaling fail to express organizer-specific genes, suggesting a requirement for TGFβ signaling in organizer formation (Watanabe and Whitman, 1999; Agius *et al.*, 2000). Genetic studies in the mouse and zebrafish demonstrate a requirement for Nodal-related genes in mesoderm and organizer formation (Schier and Shen, 2000). Loss-of-function mutations in the mouse and the zebrafish Nodal genes result in embryos which fail to form an organizer and lack mesoderm (Conlon *et al.*, 1994; Feldman *et al.*, 1998). Likewise, inhibition of Nodal signaling in *Xenopus*, using a Nodal-specific form of Cerberus, blocks mesoderm and organizer formation (Agius *et al.*, 2000). These data suggest a critical role for Nodal-related TGFβ signals in the development of the mesodermal

Abbreviations used in this paper: Eng-Sia, Engrailed-Siamois; AC, animal cap; UV, ultraviolet; RT-PCR, reverse transcription polymerase chain reaction.

***Address correspondence to:** Daniel S. Kessler. Department of Cell & Developmental Biology. University of Pennsylvania School of Medicine, 1110 Biomedical Research Building 2/3, 421 Curie Boulevard, Philadelphia, PA 19104-6058, USA. FAX: +1-215-573-7601. e-mail: kesslerd@mail.med.upenn.edu

0214-6282/2001/$25.00

Printed in Spain
www.ijdb.ehu.es

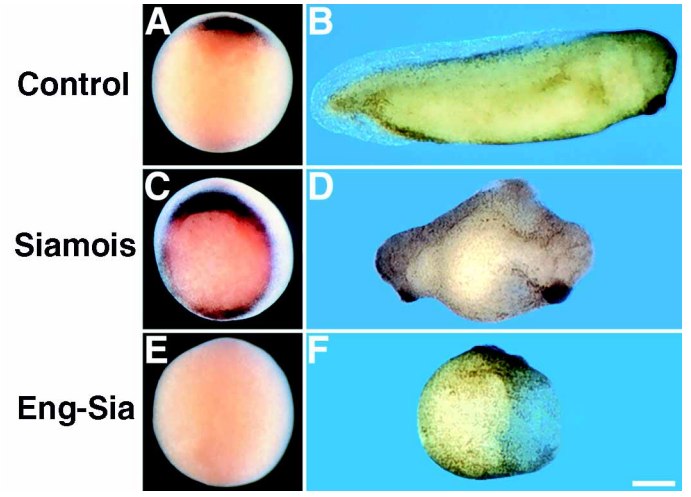

Fig. 1. Regulation of the Spemann-Mangold organizer formation by Siamois. *The expression of organizer genes in the dorsal marginal zone at the gastrula stage* **(A)** *patterns the developing embryo and regulates the formation of the body axis* **(B)**. *Misexpression of Siamois induces the ectopic expression of organizer genes* **(C)** *and generates a complete secondary axis* **(D)**. *Interference with Siamois function using an Engrailed-Siamois fusion construct inhibits organizer gene expression* **(E)** *and axial development* **(F).** *Uninjected embryos (A,B), Siamois-injected embryos (C,D) or Engrailed-Siamois-injected embryos (E,F) were analyzed by in situ hybridization for expression of the organizer gene Goosecoid (vegetal view) (A,C,E) and for axis formation (B,D,F). Scale bar, 0.45 mm. Adapted from Kessler (1997).*

lineage and the Spemann-Mangold organizer in multiple organisms including *Xenopus*.

While Nodal signaling induces mesoderm in the equatorial region of the blastula, the organizer forms in a dorsal equatorial domain in response to maternal Wnt signaling (reviewed in Harland and Gerhart, 1997; Heasman, 1997; Moon and Kimelman, 1998). Disruption of Wnt signaling by antisense ablation of the Wnt effector βcatenin or the Wnt receptor Frizzled-7 results in embryos that fail to form an organizer or undergo subsequent axial development (Heasman *et al.*, 1994; Sumanas *et al.*, 2000). Although ectopic expression of Wnt ligands or downstream signaling components can induce an ectopic axis (reviewed in Heasman, 1997; Moon and Kimelman, 1998; Sokol, 1999), an endogenous Wnt ligand required for dorsal development has not been identified. The maternal Wnt pathway is activated by cortical rotation, a movement of the cortex relative to the inner cytoplasm during the first cell cycle (Vincent *et al.*, 1986; Elinson and Rowning, 1988; Kageura, 1990; Houliston and Elinson, 1991; Marikawa *et al.*, 1997; Rowning *et al.*, 1997). Cortical rotation is a microtubule-dependent process that displaces dorsal determinants from the vegetal pole to the future dorsal domain of the marginal zone, and destabilization of microtubules by UV-irradiation inhibits rotation and disrupts dorsal development (Elinson and Kao, 1989; Gerhart *et al.*, 1989; Rowning *et al.*, 1997). The displaced dorsal determinants stimulate the nuclear localization of βcatenin in dorsal blastomeres (Schneider *et al.*, 1996; Larabell *et al.*, 1997; Rowning *et al.*, 1997), and in the nucleus, βcatenin forms a complex with TCF transcription factors and activates zygotic genes that regulate dorsal development (Behrens *et al.*, 1996; Molenaar *et al.*, 1996; Brannon *et al.*, 1997; Laurent *et al.*, 1997; McKendry *et al.*, 1997; Fan *et al.*, 1998).

The homeodomain protein Siamois activates the expression of organizer-specific genes and has been implicated in the transcriptional response to maternal signals that establish dorsal cell fates (reviewed in Kodjabachian and Lemaire, 1998). Siamois is expressed in dorsal blastomeres following the onset of zygotic transcription at the mid-blastula transition, and its expression precedes that of other organizer genes (Lemaire *et al.*, 1995). The Siamois promoter contains multiple TCF-binding sites that mediate Wnt-induced activation of Siamois, indicating that Siamois is a direct target of maternal Wnt signals (Brannon and Kimelman, 1996; Carnac *et al.*, 1996; Brannon *et al.*, 1997; Fagotto *et al.*, 1997; Vleminckx *et al.*, 1997; Fan *et al.*, 1998; Kessler, 1999). Ventral expression of Siamois induces ectopic expression of organizer genes and complete axial duplication (Fig. 1 C,D) (Lemaire *et al.*, 1995). In contrast to axis-inducing factors that also induce dorsal mesoderm, such as Nodal and Vg1, Siamois can induce organizer gene expression without inducing mesoderm (Lemaire *et al.*, 1995; Carnac *et al.*, 1996; Kessler, 1999). These properties of Siamois are shared by Twin, a closely related homeobox gene that is coexpressed with Siamois and exhibits similar axis-inducing activity (Laurent *et al.*, 1997).

The analysis of Siamois and Twin suggests that these genes regulate organizer formation by activating a program of organizer gene expression, rather than executing a specific organizer function. Consistent with this idea, interference with the function of endogenous Siamois has shown that transcriptional activation by Siamois is essential for organizer formation. Expression of a chimeric protein consisting of the Engrailed repressor domain fused to the Siamois homeodomain (Eng-Sia) targets this strong repressor to sites normally bound by Siamois and antagonizes endogenous Siamois activity (Fan and Sokol, 1997; Kessler, 1997). Given the high similarity of the Siamois and Twin DNA-binding domains, Eng-Sia is expected to also antagonize endogenous Twin function. Dorsal expression of Eng-Sia blocks organizer gene expression and the failure to form the Spemann-Mangold organizer results in embryos lacking dorsal axial structures (Fig.1 E,F) (Fan and Sokol, 1997; Kessler, 1997). These studies demonstrate that Siamois regulates the expression of numerous organizer genes including Goosecoid, Chordin, Noggin, Follistatin, Xnr3, Cerberus and Xlim1 (Carnac *et al.*, 1996; Fan and Sokol, 1997; Kessler, 1997, 1999). Consistent with the ability to induce organizer formation, the effect of Siamois overexpression is strikingly similar to the function of organizer tissue. Ventral expression of Siamois converts prospective ventral mesoderm into the dorsal axial tissues, notochord and muscle (Lemaire *et al.*, 1995; Carnac *et al.*, 1996). Furthermore, Siamois-expressing animal pole tissue can convert the fate of conjugated ventral mesoderm into somitic muscle, consistent with the non-autonomous dorsalizing activity of the organizer (Carnac *et al.*, 1996). This similarity between endogenous organizer function and Siamois-expressing tissues suggests that Siamois is sufficient for development of the full spectrum of organizer activities.

In this study, we have further examined the sufficiency of Siamois to induce the Spemann-Mangold organizer. We show that expression of Siamois in animal pole tissue confers only partial organizer activity, the ability to induce trunk but not head structures. Consistent with the trunk organizer activity of Siamois-expressing animal tissue, we find that Cerberus, Xlim1 and Frzb1,

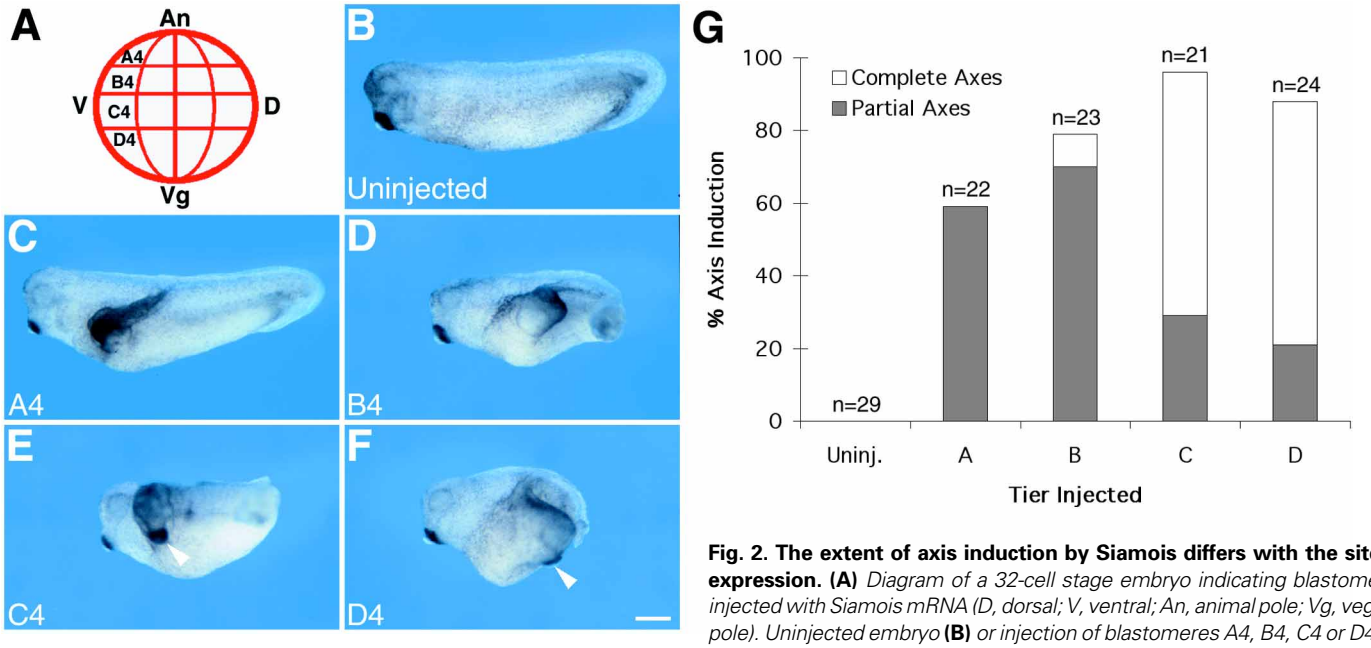

Fig. 2. The extent of axis induction by Siamois differs with the site of expression. (A) *Diagram of a 32-cell stage embryo indicating blastomeres injected with Siamois mRNA (D, dorsal; V, ventral; An, animal pole; Vg, vegetal pole). Uninjected embryo* **(B)** *or injection of blastomeres A4, B4, C4 or D4* **(C-F)** *with 20 pg of Siamois mRNA at the 32-cell stage. Injection of either tier A or B cells induced partial axes lacking head structures and injection of either tier C or D cells induced complete axes containing eyes and cement gland. The arrowhead indicates the position of ectopic cement gland.* **(G)** *Quantification of injection experiment. Gray region of bars indicate partial axes lacking head structures and white region indicates complete axes containing head structures. Scale bar, 0.5 mm.*

organizer genes implicated in anterior development, are not induced by Siamois in animal explants. In the vegetal hemisphere Siamois does induce these genes, but expression is dependent on endogenous TGFβ signals. Therefore, Siamois is sufficient for formation of trunk organizer and cooperates with vegetal TGFβ signals to generate organizer with head- and trunk-inducing activity.

Results

Siamois induces a partial axis when expressed in animal blastomeres

Marginal zone expression of native and dominant negative forms of Siamois has previously suggested that Siamois was both necessary and sufficient for formation of the Spemann-Mangold organizer (Lemaire *et al.*, 1995; Carnac *et al.*, 1996; Fan and Sokol, 1997; Kessler, 1997). To further examine the ability of Siamois to induce organizer formation, Siamois was misexpressed along the animal-vegetal axis. Siamois mRNA was injected into a single ventral blastomere of each tier of the 32-cell stage embryo (Fig. 2A) and resulting ectopic axial structures were scored at the tailbud stage. Expression in vegetal tiers (C4 or D4) resulted in induction of complete axes containing head and trunk structures (Fig. 2 E,F), consistent with previous studies (Lemaire *et al.*, 1995; Kessler, 1997). In contrast, when expressed in animal tiers (A4 or B4), Siamois induced partial axes consisting of trunk structures only (Fig. 2 C,D). While expression in tier C or D resulted in complete axis formation in nearly 70% of injected embryos, induction of complete axis formation was never observed with tier A injection and was observed at low frequency (9%) with tier B injection (Fig. 2G). Therefore, the extent of axis induction by Siamois differs with the position of injection, and in animal regions Siamois is insufficient for complete axis formation.

Animal explants expressing Siamois have trunk organizer activity

Given the ability of Siamois to induce most, if not all organizer genes, and the apparent requirement for Siamois function in organizer formation, the failure of Siamois to induce complete axial duplication when expressed in animal blastomeres was unexpected. One possible explanation for this result is that animal expression of Siamois induces organizer tissue in a position too distant from the tissues that must be patterned to generate a complete axis. To further examine the organizer activity of Siamois-expressing tissue we utilized the Einsteck assay (Geinitz, 1925;

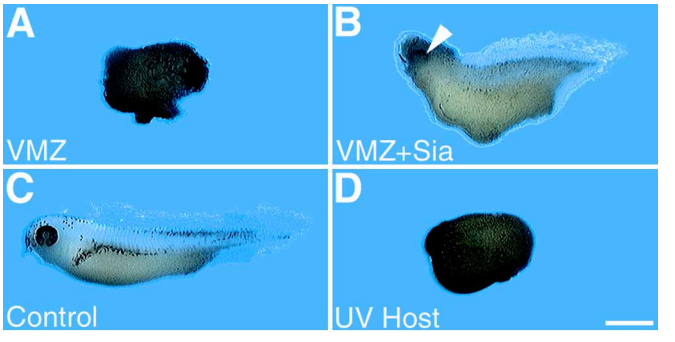

Fig. 3. Siamois induces head and trunk organizer activity in ventral marginal zone explants. *At the four-cell stage, 30 pg of Siamois mRNA was injected into the marginal zone of each blastomere. Ventral marginal tissue from uninjected embryos* **(A)** *or Siamois-injected embryos* **(B)** *was explanted at the early gastrula stage and transplanted into the blastocoel of UV-irradiated hosts. Representative non-irradiated* **(C)** *and UV-irradiated* **(D)** *embryos are shown. A Siamois-injected ventral marginal zone completely rescues axial development including head and trunk structures. The arrowhead indicates the position of differentiated eye. Scale bar, 1.0 mm.*

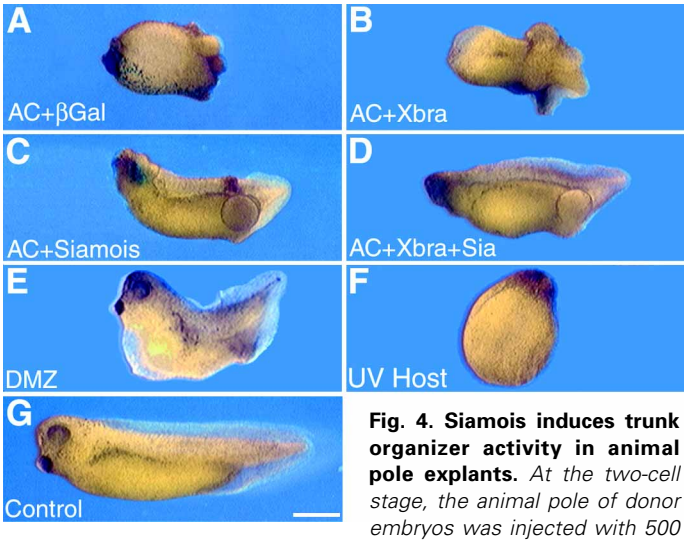

Fig. 4. Siamois induces trunk organizer activity in animal pole explants. *At the two-cell stage, the animal pole of donor embryos was injected with 500 pg of β–galactosidase mRNA* **(A-D)** *in combination with 1 ng of Brachyury mRNA (Xbra)* **(B)**, *100 pg of Siamois mRNA* **(C)** *or both Brachyury and Siamois mRNAs* **(D)**. *Animal pole explants (AC) were transplanted into the blastocoel of UV-irradiated hosts at the late blastula stage and evaluated for rescue of axial structures at the tailbud stage. Transplantation of dorsal marginal zone (DMZ)* **(E)**, *which rescues a complete axis, serves as a positive control. Representative UV-irradiated* **(F)** *and non-manipulated (Control)* **(G)** *embryos are shown. Compared to β–galactosidase alone, the Siamois-expressing animal explant rescues a partial axis, indicated by elongation and the presence of dorsal fin, that lacks head structures. A Brachyury-expressing explant does not rescue axial structures, and Brachyury does not alter the extent of axial rescue when coexpressed with Siamois. See Table 1 for quantification. Scale bar, 1.0 mm.*

Marx, 1925; Slack and Isaacs, 1994). In this assay, tissue with putative axis-inducing activity is transplanted into the blastocoel of an UV-irradiated embryo and the rescue of axial development is assessed in the ventralized host. As a positive control for the Einsteck assay, Siamois mRNA was injected into the ventral marginal zone at the four-cell stage and at the gastrula stage, ventral marginal zone explants were prepared and transplanted into UV-irradiated hosts. Consistent with the ability of Siamois to induce complete axis formation when expressed in the marginal zone of untreated or UV-irradiated embryos, transplantation of a Siamois-expressing ventral marginal zone rescued complete axis formation, including head structures (Fig. 3B). An uninjected ventral marginal zone did not rescue axis formation (Fig. 3A). Therefore, when expressed in ventral mesoderm Siamois is sufficient for induction of the Spemann-Mangold organizer, and the activity of the tissue can be demonstrated using the Einsteck assay.

To test the organizer function of Siamois-expressing animal tissue, Siamois mRNA was injected into the animal pole of both blastomeres at the two-cell stage and at the blastula stage, animal explants were prepared and transplanted into UV-irradiated hosts. In contrast to Siamois-expressing ventral marginal zone, Siamois-expressing animal explants rescued a partial axis consisting of trunk structures that underwent elongation and developed a dorsal fin (Fig. 4C). Differentiation of somitic muscle and notochord was observed with transplantation of Siamois-expressing animal explants (data not shown). Siamois-expressing animal explants did not rescue formation of anterior structures such as eyes or cement gland. Consistent with the results of Siamois injection into animal blastomeres of the intact embryo, Siamois-expressing animal tissue transplants induce formation of trunk structures containing dorsal mesodermal tissues, but not head structures (Table 1). The results confirm that Siamois can impart trunk organizer activity upon animal tissue, but indicate that Siamois cannot confer head organizer function on this tissue.

One significant difference between the ventral marginal zone and the animal pole is the mesodermal specification of ventral marginal cells. To determine whether this mesodermal state influences the ability of Siamois to induce organizer, we coexpressed Siamois and Brachyury in animal tissue and transplanted this tissue into UV-irradiated hosts. In animal explants, Brachyury induces mesodermal fate, but not organizer formation (Cunliffe and Smith, 1992). Animal tissue expressing Siamois and Brachyury induced trunk formation, identical to the activity of Siamois alone, while tissue expressing Brachyury alone did not rescue axis formation (Fig. 4 B,D and Table 1). The results suggest that the inability of Siamois to induce head organizer function in animal tissue is not a consequence of the non-mesodermal state of the animal tissue.

Siamois activates a subset of organizer genes in animal tissue

Given the failure of Siamois-expressing animal tissue to provide head organizer function, the ability of Siamois to activate organizer genes implicated in anterior development, including Cerberus, Xlim1, and Frzb1, was examined. In *Xenopus*, Cerberus induces ectopic head formation (Bouwmeester *et al.*, 1996), Frzb1 enhances head formation and inhibits trunk formation (Leyns *et al.*, 1997), and an activated form of Xlim1 induces anterior neural markers (Taira *et al.*, 1992) . Siamois or Activin mRNA was injected into the animal pole at the two-cell stage and animal explants prepared at the midblastula stage were harvested for RT-PCR analysis at the gastrula stage. Although Siamois strongly induced the expression of Goosecoid, activation of Cerberus, Xlim1 or Frzb1 was not observed (Fig. 5, lane 2). Activin was sufficient for the induction of all of these organizer genes (Fig. 5, lane 3). Therefore, in animal tissue Siamois was not sufficient, even at doses as high as 3 ng (data not shown), for the activation of several genes implicated in head organizer function. This inability of

TABLE 1

AXIAL RESCUE BY SIAMOIS-EXPRESSING ANIMAL POLE EXPLANTS

Donor Tissue	N	Head Structures	Trunk Structures
None	36	0	2
β–galactosidase Animal Cap	25	0	1
Xbra Animal Cap	18	0	0
Siamois Animal Cap	24	0	11
Xbra+Siamois Animal Cap	17	0	11
Dorsal Marginal Zone	47	18	37
Ventral Marginal Zone	44	0	3

Embryos were injected with the indicated RNA and explants were transplanted into the blastocoel cavity of a UV-irradiated host at the blastula stage. Axial rescue was scored at stage 35 by morphology and immunohistochemistry. Head structures were scored by the presence of eyes and cement gland, and trunk structures were scored by trunk elongation, notochord differentiation and the presence of dorsal fin.

Siamois to induce head organizer genes may account for the absence of head organizer function in Siamois-expressing animal tissue. These results are consistent with previous work suggesting that the Wnt pathway and Siamois cannot induce Cerberus or Xlim1 in animal tissue (Carnac *et al.*, 1996; Darras *et al.*, 1997).

To examine the role of Siamois in the endogenous expression of Cerberus, Xlim1 and Frzb1, Eng-Sia was used to inhibit the function of endogenous Siamois. Eng-Sia or native Siamois was injected into the marginal zone of both blastomeres at the two-cell stage, and intact embryos were harvested at the gastrula stage for RT-PCR analysis. Siamois injection resulted in a slight upregulation of the organizer genes, an inhibition of Xwnt8, and no effect on Brachyury (Fig. 6, lane 3). Eng-Sia inhibited the expression of each organizer gene examined, including Cerberus, Frzb1, Xlim1 and Goosecoid, but did not effect Xwnt8 or Brachyury expression (Fig. 6, lane 4). Therefore, Siamois is necessary for the endogenous expression of these head organizer genes in the marginal zone, but is not sufficient for their expression in animal tissue. The observed dependence of Cerberus and Xlim1 on Siamois function is consistent with previous results (Darras *et al.*, 1997; Fan and Sokol, 1997; Kessler, 1997).

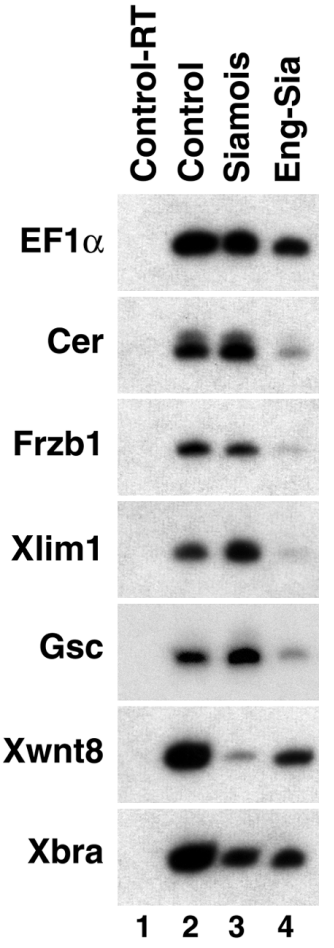

Fig. 6. Siamois regulates the endogenous expression of Cerberus, Frzb1 and Xlim1. *At the two-cell stage, 30 pg of Siamois or Eng-Sia mRNA was injected into the marginal region of both blastomeres. Embryos were harvested for RT-PCR at the gastrula stage. Siamois* **(lane 3)** *inhibited the expression of Xwnt8, slightly increased the expression of the organizer genes Cerberus (Cer), Frzb1, Goosecoid (Gsc) and Xlim1, and had no effect on Brachyury (Xbra) expression. Eng-Sia* **(lane 4)** *inhibited the expression of each organizer gene and had no effect on Xwnt8 and Brachyury. EF1α serves as a control for RNA recovery and loading. Uninjected embryos, Control,* **(lane 2)** *serve as a positive control and an identical reaction prepared without reverse transcriptase, Control-RT,* **(lane 1)** *controls for PCR contamination.*

Cooperation of Siamois and TGFβ signals in activation of organizer gene expression

The failure of Cerberus, Xlim1 and Frzb1 to respond to Siamois in animal tissue may be due to the absence of a positive signal that cooperates with Siamois, or the presence of a negative signal that blocks the response to Siamois. To determine if Siamois can induce the expression of these genes in any region outside of the marginal zone, Siamois was expressed in vegetal tissue. At the one-cell stage the vegetal pole was injected with Siamois mRNA and vegetal explants prepared at the late blastula stage were harvested for RT-PCR at the early gastrula stage. In contrast to the response of animal tissue, vegetal expression of Siamois induced expression of Cerberus, Xlim1 and Frzb1 (Fig. 7A, lane 2). Therefore, vegetal cells are competent to express head organizer genes in response to Siamois.

One difference between animal and vegetal tissues that may account for the differential response to Siamois is the presence of active TGFβ signaling in vegetal tissues (Watabe *et al.*, 1995; Faure *et al.*, 2000). To determine if vegetal activation of head organizer genes by Siamois was influenced by endogenous TGFβ signaling, Siamois was coexpressed with a truncated Activin type II receptor (Δ1XAR1) that blocks signaling by Nodal-related factors, as well as other TGFβ ligands (Hemmati-Brivanlou and Melton, 1992; Dyson and Gurdon, 1997). The induction of Cerberus, Xlim1 and Frzb1 by Siamois was reduced to near basal levels by Δ1XAR1 (Fig. 7A, lane 3), indicating that endogenous TGFβ signals are required for induction of these genes by Siamois in vegetal tissue. Vegetal induction of Goosecoid was only slightly inhibited by Δ1XAR1, consistent with the ability of Siamois to activate Goosecoid in animal tissue lacking TGFβ signals. As a positive control for Δ1XAR1 function, *Xenopus* Nodal-related-1 (Xnr1) was expressed in animal explants alone or with Δ1XAR1 and induction of each organizer gene by Xnr1 was severely reduced by Δ1XAR1 (Fig. 7B, lanes 8-9). The results indicate that Siamois cooperates with vegetal TGFβ signals to activate expression of the head organizer genes Cerberus, Xlim1 and Frzb1. The dependence on TGFβ signals for activation of this subset of organizer genes may account for the inability of Siamois to induce these genes and head organizer function in animal tissue. Furthermore, the results support the idea that Siamois-mediated Wnt signals and TGFβ signals collaborate to direct formation of the Spemann-Mangold organizer.

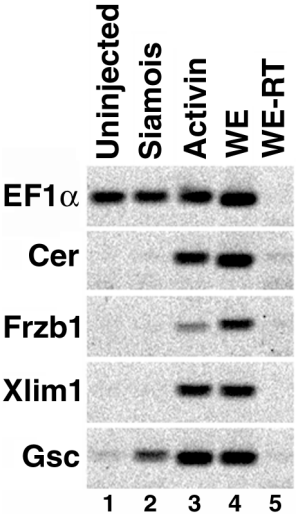

Fig. 5. Siamois does not induce the head organizer genes, Cerberus, Frzb1 and Xlim1, in animal pole explants. *At the two-cell stage, 100 pg of Siamois mRNA or 10 pg of Activin mRNA was injected into the animal pole. Animal pole tissue was explanted at the midblastula stage, cultured to the gastrula stage and harvested for RT-PCR analysis. Siamois induces the expression of Goosecoid (Gsc), but does not activate the expression of Cerberus (Cer), Frzb1 or Xlim1* **(lane 2).** *Activin induces the expression of each organizer gene* **(lane 3)** *and none of the genes is expressed in uninjected explants* **(lane 1)**. *EF1α serves as a control for RNA recovery and loading. Whole embryos, WE,* **(lane 4)** *serve as a positive control and an identical reaction prepared without reverse transcriptase, WE-RT,* **(lane 5)** *controls for PCR contamination.*

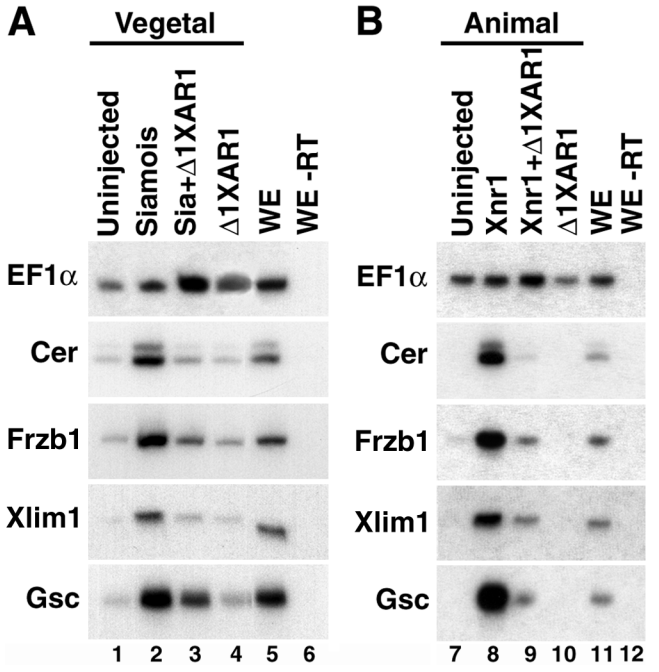

Fig. 7. Siamois induction of Cerberus, Frzb1 and Xlim1 in vegetal pole explants is dependent on endogenous TGFβ signals. (A) *At the one-cell stage, the vegetal pole was injected with 3 ng of mRNA encoding a truncated Activin type II receptor (Δ1XAR1) and at the two-cell stage, 30 pg of Siamois mRNA was injected into the vegetal pole. Vegetal tissue was explanted at the late blastula stage and harvested at the gastrula stage for RT-PCR. Siamois (lane 2) induced the vegetal expression of Cerberus (Cer), Frzb1 and Xlim1, and this induced expression was inhibited by coexpression of Δ1XAR1 (lane 3). Goosecoid (Gsc) was also induced by Siamois, but expression was only weakly effected by coexpression of Δ1XAR1. The organizer genes were expressed at a low basal level in uninjected explants (lane1) and Δ1XAR1-expressing explants (lane 4).* **(B)** *At the one-cell stage, 3 ng of Δ1XAR1 mRNA was injected into the animal pole and at the two-cell stage, 300 pg of Xnr1 mRNA was injected into the animal pole. Animal pole tissue was explanted at the late blastula stage and harvested at the gastrula stage for RT-PCR. Xnr1 induced the animal pole expression of each gene (lane 8) and this expression was inhibited by Δ1XAR1 (lane 9). These genes were not expressed in uninjected explants (lane7) and Δ1XAR1-expressing explants (lane 10). EF1α serves as a control for RNA recovery and loading. Whole embryos (WE, lanes 5 and 11) serve as a positive control and an identical reaction prepared without reverse transcriptase (WE-RT, lanes 6 and 12) controls for PCR contamination.*

Discussion

In recent years, a large amount of work has been directed at defining the molecular events that lead to the formation of the Spemann-Mangold organizer. Much has been learned, including the demonstration that TGFβ signals and Wnt signals are essential for the formation of this important organizing center (Kessler and Melton, 1994; Harland and Gerhart, 1997; Heasman, 1997; Moon and Kimelman, 1998). Characterization of the Wnt-responsive genes, Siamois and Twin, has identified these genes as zygotic transcriptional effectors of maternal events that initiate dorsal development (reviewed in Kodjabachian and Lemaire, 1998). Despite these advances, the respective role each of these pathways plays in organizer formation is still not fully understood.

In this paper we examined the ability of Siamois to induce organizer function in animal tissue, in the absence of mesoderm-inducing TGFβ signals or induced mesodermal tissues. The results show that although Siamois is sufficient to induce a subset of organizer functions in an animal explant, it is insufficient in the context of this tissue to induce the full organizer activity required to induce a complete axis. In addition, we show that Siamois fails to induce the expression of a subset of organizer genes, including Cerberus, Frzb1 and Xlim1, in the animal pole. This inability to induce a full complement of organizer genes may account for the absence of head organizer activity in Siamois-expressing animal explants. A dominant negative form of Siamois inhibited endogenous expression of Cerberus, Xlim1 and Frzb1, indicating that Siamois is necessary for organizer-specific expression of these head organizer genes, but is not sufficient in animal tissue. Furthermore, we show that Siamois can induce Cerberus, Frzb1 and Xlim1 in more vegetal regions, and this response is dependent on endogenous TGFβ signals. We propose that the combined action of the Wnt-responsive transcription factors, Siamois and Twin, and vegetal TGFβ signals is required for full organizer function.

Siamois induces a complete secondary axis when expressed in ventral mesoderm and activates many organizer genes in animal tissue (Lemaire *et al.*, 1995; Carnac *et al.*, 1996; Kessler, 1999). Therefore, it was surprising to find that Siamois induces only a partial axis when expressed in animal pole tissue. Given this failure to induce complete axial development, the activity of Siamois in animal tissue may reflect the true activity of Siamois in the absence of additional inducers. Alternatively, animal tissue may fail to incorporate into the proper position in the gastrula to effectively pattern neighboring tissues. This appears not to be the case because we were able to detect donor cells that successfully incorporated into the rescued axis (data not shown). Another possibility is that animal tissue may be unable to fully respond to organizer inducers or support organizer function. For example, animal tissue may fail to secrete the dorsalizing signals necessary for axial development. However, animal explants expressing Siamois have been shown to dorsalize conjugated ventral mesoderm, indicating that animal tissue is indeed capable of secreting dorsalizing signals (Carnac *et al.*, 1996). Furthermore, several lines of evidence suggest that animal tissue can support a greater degree of organizer function than that observed with Siamois-expressing explants. Transplants of Activin-induced animal explants have previously been shown to induce complete axes when transplanted into normal embryos (Ruiz i Altaba and Melton, 1989). Likewise, Cerberus-expressing animal explants can induce ectopic heads in an Einsteck assay (Bouwmeester *et al.*, 1996). Taken together, these data suggest that the animal explant is capable of forming a complete organizer in response to appropriate signals and can release the dorsalizing signals required for complete axial development.

Our results suggest that Siamois is insufficient to provide the appropriate signals to induce head organizer. Because this contrasts with the ability of Siamois to induce a complete organizer when expressed in the marginal zone, additional factors present in more vegetal regions of the embryo may cooperate with Siamois to generate full organizer function. The ability of Siamois to induce a complete axis when expressed in the ventral marginal zone suggests that cooperating factors would not be restricted to dorsal tissues and instead, would be present throughout the vegetal hemisphere. The cooperating factors appear not to be components

of prospective mesoderm because inducing a general mesodermal character in animal tissue with Brachyury does not alter the extent of axial rescue. Instead, induction of head structures by Siamois may require cooperation with the vegetal secreted signals that induce mesoderm.

Support for this idea comes from the examination of Siamois regulation of several organizer genes implicated in anterior development. Our results show that while Cerberus, Frzb1 and Xlim1 are induced by Siamois in vegetal cells, these genes are not induced in animal tissue. The induction of Cerberus expression by Siamois in vegetal, but not animal cells, is consistent with previous work (Darras *et al.*, 1997) showing that Cerberus is not induced in animal tissue by vegetal cortical cytoplasm known to contain Wnt/Siamois-like dorsalizing activity. This identifies a class of organizer genes that differ from others, including Goosecoid, Chordin and Noggin, which can be induced by Siamois in animal tissue. These data suggest that additional factors present in the vegetal pole are required for the expression of Cerberus, Frzb1 and Xlim1. Previous work indicates that TGFβ signals are active in vegetal regions, but not in the animal pole (Faure *et al.*, 2000). We show that the ability of Siamois to induce Cerberus, Frzb1 and Xlim1 in the vegetal pole is sensitive to the level of TGFβ signaling. This suggests that expression of the head organizer genes depends on cooperation of Siamois with vegetal TGFβ signals. Although genes like Goosecoid and Chordin also require TGFβ signaling for their endogenous expression (Agius *et al.*, 2000), this requirement can be circumvented in animal tissue by overexpression of Siamois. The requirement for TGFβ signaling for the expression of Cerberus, Frzb1 and Xlim1 is more absolute since overexpression of Siamois in animal tissue is not sufficient for their expression. Whether this difference reflects a qualitative difference in how Cerberus, Frzb1 and Xlim1 are regulated, or simply reflects a greater requirement for TGFβ signaling, is yet to be determined. It will be interesting to learn if additional genes involved in anterior development are regulated similarly to Cerberus, Frzb1 and Xlim1.

The absence of Cerberus, Frzb1 and Xlim1 in Siamois-expressing animal explants may account for the inability of this tissue to induce anterior structures when transplanted into a ventralized host. In addition, the requirement for TGFβ signaling for the expression of Cerberus, Frzb1 and Xlim1 in vegetal cells provides evidence that Siamois may cooperate with TGFβ signals to induce the expression of genes which regulate anterior development. Cooperation between the Wnt and TGFβ pathways has been proposed as a patterning mechanism that localizes the organizer to the dorsal marginal zone (Christian *et al.*, 1992; Kimelman *et al.*, 1992; Christian and Moon, 1993; Watabe *et al.*, 1995; Crease *et al.*, 1998; Moon and Kimelman, 1998; Kessler, 1999). Post-translational activation of Smad2 (Faure *et al.*, 2000) and transcriptional activation of an Activin-responsive promoter (Watabe *et al.*, 1995) has demonstrated that high levels of Smad2-activating TGFβ signals are present throughout the vegetal hemisphere, while little or no signaling occurs in the animal pole. βcatenin, a Wnt pathway effector, localizes to cell nuclei in a broad dorsal domain that extends to the animal pole (Schneider *et al.*, 1996; Larabell *et al.*, 1997). This localization of βcatenin suggests that maternal Wnt signaling extends beyond the marginal zone blastomeres that form the organizer. However, the regions of active signaling for these two pathways overlap in the dorsal marginal zone of the blastula embryo, and this overlap region corresponds well to the position of the Spemann-Mangold organizer. Several lines of evidence suggest that the Wnt and TGFβ pathways cooperate to regulate organizer gene expression. The organizer gene Goosecoid has been shown to be regulated by both TGFβ-responsive and Wnt/Siamois-responsive promoter elements (Watabe *et al.*, 1995; Fan and Sokol, 1997; Kessler, 1997; Laurent *et al.*, 1997) and can be synergistically activated by Activin and Siamois (DSK, unpublished). In addition, Smad4 and βcatenin form a protein complex that activates transcription of Twin (Nishita *et al.*, 2000). Our description of the influence of endogenous TGFβ signals on Siamois activity provides functional evidence for cooperation between these factors in the formation of the organizer.

Rather than being a single homogenous population of cells, the organizer exhibits regional differences in gene expression and inducing properties soon after its formation. The organizer appears to consist of two domains, a vegetally positioned head-inducing region (head organizer) and an animally positioned trunk-inducing region (trunk organizer) (Spemann, 1931; Zoltewicz and Gerhart, 1997). It is interesting to speculate that the differences we observe in the response of animal versus marginal cells to Siamois provide a potential mechanism for the early patterning of the organizer. It is possible that within the marginal zone, cells positioned closer to the animal pole or vegetal pole respond differently to Siamois, resulting in formation of trunk organizer and head organizer, respectively. One prediction of this mechanism is that head organizer genes, like Cerberus, Frzb1 and Xlim1, will be expressed in a more vegetal domain of the organizer. Recent observations in the zebrafish suggest a requirement for higher levels of TGFβ signaling for the formation of anterior axial structures than for posterior structures (Schier and Shen, 2000; Thisse *et al.*, 2000). Inhibition of TGFβ signaling with Antivin blocks prechordal plate formation at low doses and posterior mesodermal structures at higher doses, suggesting that development of anterior structures are dependent on higher levels of TGFβ signals than are posterior structures (Schier and Shen, 2000). These results suggest that the cooperation of TGFβ signals with transcriptional regulators, such as Siamois, may induce organizer formation, and regulate the anteroposterior patterning of the organizer at the early gastrula stage.

We have characterized the sufficiency of Siamois to induce the Spemann-Mangold organizer. Although the ability of Siamois to induce a complete organizer when expressed in marginal blastomeres is well-established (Kodjabachian and Lemaire, 1998), this work reveals that when expressed in animal blastomeres, Siamois is sufficient to induce only partial organizer function. This work suggests that Siamois cooperates with TGFβ signals present in the vegetal pole of the embryo to activate the complement of organizer genes necessary to generate a complete Spemann-Mangold organizer.

Numerous studies in the mouse, chick and zebrafish have demonstrated the importance of TGFβ and Wnt signaling in regulating vertebrate axis formation. Wnt3 and Nodal function are required in the mouse for gastrulation and node formation (Liu *et al.*, 1999; Conlon *et al.*, 1994), Wnt1 and Vg1 can each induce ectopic organizer gene expression in the chick (Joubin and Stern, 1999), and in the zebrafish, βcatenin localizes to the nuclei of dorsal blastomeres and Nodal function is required for organizer formation (Schneider *et al.*, 1996; Feldman *et al.*, 1998). Although the Wnt and TGFβ pathways are implicated in axis formation in the mouse, chick and zebrafish, the mechanisms of cooperation between these pathways have not been well defined. Wnt and TGFβ signals may have separate roles in dorsal-ventral patterning

and germ layer formation or may cooperate to generate the organizer. A number of observations suggest some degree of cooperation in axis formation. Wnt1 and Vg1 can act synergistically to activate the expression of organizer genes in the chick (Joubin and Stern, 1999). A genetic interaction has been observed between Bozozok, a putative Wnt-responsive transcription factor, and Squint, a Nodal-related gene, in zebrafish axis formation, indicating that these genes cooperate to regulate organizer formation (Sirotkin *et al.*, 2000). Furthermore, the presence of conserved Wnt/Siamois-response and TGFβ-response elements in the promoters of the *Xenopus*, zebrafish and mouse Goosecoid genes, points to an interaction of the two pathways in regulating organizer gene transcription (Watabe *et al.*, 1995; McKendry *et al.*, 1998). The analysis of additional organizer gene promoters has identified potential Wnt/Siamois- and TGFβ-response elements (MJE and DSK, unpublished), suggesting that cooperative activation of transcription may be a general mechanism for the coordinate regulation of organizer gene expression. Further study will be necessary to determine if conserved mechanisms integrate the signaling outputs of the Wnt and TGFβ pathways and regulate formation of the vertebrate organizer.

Materials and Methods

Embryos and Microinjection

Embryos were collected, fertilized, injected and cultured as previously described (Yao and Kessler, 1999), and embryonic stage was determined according to Nieuwkoop and Faber (1967). Dorsal and ventral blastomeres were identified by pigmentation differences (Klein, 1987). Explants were prepared using a hair knife or a Gastromaster microsurgery instrument (Xenotek Engineering). For Einsteck transplants, explants were inserted into a slit in the animal pole of early blastula hosts and cultured in 0.5X MMR. Host embryos were prepared by UV-irradiating the vegetal pole for 65 seconds at 35 minutes post-fertilization. Capped, *in vitro* transcribed RNA was synthesized using a Message Machine kit (Ambion) programmed with linearized DNA template, and 5-10 nl of RNA solution was injected. Templates for *in vitro* transcription were pCS2-Siamois, pCS2-Engrailed-Siamois (Kessler, 1997), pSP64T-Brachyury (Cunliffe and Smith, 1992), pSP64T-ActivinβB (Sokol *et al.*, 1991), pSP64T-Δ1XAR1 (Hemmati-Brivanlou and Melton, 1992) and pCS2-Xnr1 (Sampath *et al.*, 1997).

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from explants and embryos using a RNAqueous kit (Ambion) and cDNA synthesis and PCR were performed as described (Wilson and Melton, 1994). Radiolabelled PCR products were resolved on 5% native polyacrylamide gels. For unlabelled PCR products, Vistra Green (Amersham) was added (1:10000) to each sample, resolved on 2% agarose gels, and fluorescent amplification products were detected using a Storm 850 fluorimager (Molecular Dynamics). Primers and PCR conditions for EF1α, Cerberus, Frzb1, Xlim1, Goosecoid, Xwnt8 and Xbra were as described (Taira *et al.*, 1992; Wilson and Melton, 1994; Bouwmeester *et al.*, 1996; Leyns *et al.*, 1997).

Acknowledgements

We thank Ali Hemmati-Brivanlou, Harv Isaacs, Patrick Lemaire, Jerry Thomsen and Chris Wright for providing plasmids. This work was supported by grants from the NIH (R01-HD35159 and T32-HD07516), the American Digestive Health Foundation and the Pew Scholars Program.

References

AGIUS, E., OELGESCHLAGER, M., WESSELY, O., KEMP, C. and DE ROBERTIS, E.M. (2000). Endodermal Nodal-related signals and mesoderm induction in *Xenopus*. *Development* 127: 1173-1183.

ASASHIMA, M., NAKANO, H., SHIMADA, K., KINOSHITA, K., ISHII, K., SHIBAI, H. and UENO, N. (1990). Mesodermal induction in early amphibian embryos by activin A (erythroid differentiation factor). *Roux's Arch. Dev. Biol.* 198: 330-335.

BEHRENS, J., VON KRIES, J.P., KUHL, M., BRUHN, L., WEDLICH, D., GROSSCHEDL, R. and BIRCHMEIER, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 382: 638-642.

BOUWMEESTER, T., KIM, S.-H., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* 382: 595-601.

BRANNON, M., GOMPERTS, M., SUMOY, L., MOON, R.T. and KIMELMAN, D. (1997). A β-catenin/XTcf-3 complex binds to the *siamois* promoter to regulate axis specification in *Xenopus*. *Genes Dev.* 11: 2359-2370.

BRANNON, M. and KIMELMAN, D. (1996). Activation of Siamois by the Wnt pathway. *Dev. Biol.* 180: 344-347.

CARNAC, G., KODJABACHIAN, L., GURDON, J.B. and LEMAIRE, P. (1996). The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. *Development* 122: 3055-3065.

CHANG, C., WILSON, P.A., MATHEWS, L.S. and HEMMATI-BRIVANLOU, A. (1997). A *Xenopus* type I activin receptor mediates mesodermal but not neural specification during embryogenesis. *Development* 124: 827-837.

CHRISTIAN, J.L. and MOON, R.T. (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes Dev* 7: 13-28.

CHRISTIAN, J.L., OLSON, D.J. and MOON, R.T. (1992). Xwnt-8 modifies the character of mesoderm induced by bFGF in isolated *Xenopus* ectoderm. *EMBO J.* 11: 33-41.

CONLON, F.L., LYONS, K.M., TAKAESU, N., BARTH, K.S., KISPERT, A., HERRMANN, B. and ROBERTSON, E.J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* 120: 1919-1928.

CREASE, D.J., DYSON, S. and GURDON, J.B. (1998). Cooperation between the activin and Wnt pathways in the spatial control of organizer gene expression. *Proc. Natl. Acad. Sci. USA* 95: 4398-4403.

CUNLIFFE, V. and SMITH, J.C. (1992). Ectopic mesoderm formation in *Xenopus* embryos caused by widespread expression of a Brachyury homologue. *Nature* 358: 427-430.

DARRAS, S., MARIKAWA, Y., ELINSON, R.P. and LEMAIRE, P. (1997). Animal and vegetal pole cells of early *Xenopus* embryos respond differently to maternal dorsal determinants: implications for the patterning of the organiser. *Development* 124: 4275-4286.

DYSON, S. and GURDON, J.B. (1997). Activin signaling has a necessary function in *Xenopus* early development. *Curr. Biol.* 7: 81-84.

ELINSON, R.P. and ROWNING, B. (1988). A transient array of parallel microtubules in frog eggs: potential tracks for a cytoplasmic rotation that specifies the dorso-ventral axis. *Dev. Biol.* 128: 185-197.

ELINSON, E.P. and KAO, K.R. (1989). The location of dorsal information in Frog early development. *Dev. Growth Differ.* 31: 423-430.

FAGOTTO, F., GUGER, K. and GUMBINER, B.M. (1997). Induction of the primary dorsalizing center in *Xenopus* by the Wnt/GSK/beta-catenin signaling pathway, but not by Vg1, Activin or Noggin. *Development* 124: 453-460.

FAN, M.J., GRUNING, W., WALZ, G. and SOKOL, S.Y. (1998). Wnt signaling and transcriptional control of Siamois in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* 95: 5626-5631.

FAN, M.J. and SOKOL, S.Y. (1997). A role for siamois in Spemann organizer formation. *Development* 124: 2581-2589.

FAURE, S., LEE, M.A., KELLER, T., TEN DIJKE, P. and WHITMAN, M. (2000). Endogenous patterns of TGFβ superfamily signaling during early *Xenopus* development. *Development* 127: 2917-2931.

FELDMAN, B., GATES, M.A., EGAN, E.S., DOUGAN, S.T., RENNEBECK, G., SIROTKIN, H.I., SCHIER, A.F. and TALBOT, W.S. (1998). Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature* 395: 181-185.

GEINITZ, B. (1925). Embryonale Transplantation zwischen Urodelen und Anuren. *Wilhelm Roux Arch. Entwicklungsmech. Org.* 106: 357-408.

GERHART, J., DANILCHIK, M., DONIACH, T., ROBERTS, S., ROWNING, B. and STEWART, R. (1989). Cortical rotation of the *Xenopus* egg: consequences for the

anteroposterior pattern of embryonic dorsal development. *Development* 107 (Suppl.): 37-51.

HARLAND, R. and GERHART, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* 13: 611-667.

HEASMAN, J. (1997). Patterning the *Xenopus* blastula. *Development* 124: 4179-4191.

HEASMAN, J., CRAWFORD, A., GOLDSTONE, K., GARNER-HAMRICK, P., GUMBINER, B., MCCREA, P., KINTNER, C., NORO, C.Y. and WYLIE, C. (1994). Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* 79: 791-803.

HEMMATI-BRIVANLOU, A. and MELTON, D.A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* 359: 609-614.

HOULISTON, E. and ELINSON, R.P. (1991). Patterns of microtubule polymerization relating to cortical rotation in *Xenopus laevis* eggs. *Development* 112: 107-118.

JONES, C.M., KUEHN, M.R., HOGAN, B.L., SMITH, J.C. and WRIGHT, C.V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* 121: 3651-3662.

JOUBIN, K. and STERN, C.D. (1999). Molecular interactions continuously define the organizer during the cell movements of gastrulation. *Cell* 98: 559-571.

KAGEURA, H. (1990). Spatial distribution of the capacity to initiate a secondary embryo in the 32-cell embryo of *Xenopus laevis. Dev. Biol.* 142: 432-438.

KESSLER, D.S. (1997). Siamois is required for formation of Spemann's organizer. *Proc. Natl. Acad. Sci. USA* 94: 13017-13022.

KESSLER, D.S. (1999). Maternal signaling pathways and the regulation of cell fate. In *Cell lineage and fate determination* (Ed. S. A. Moody). Academic Press, San Diego. pp. 323-340.

KESSLER, D.S. and MELTON, D.A. (1994). Vertebrate embryonic induction: mesodermal and neural patterning. *Science* 266: 596-604.

KESSLER, D.S. and MELTON, D.A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* 121: 2155-2164.

KIMELMAN, D., CHRISTIAN, J.L. and MOON, R.T. (1992). Synergistic principles of development: overlapping patterning systems in *Xenopus* mesoderm induction. *Development* 116: 1-9.

KIMELMAN, D. and GRIFFIN, K.J. (2000). Vertebrate mesendoderm induction and patterning. *Curr. Opin. Genet. Dev.* 10: 350-356.

KLEIN, S.L. (1987). The first cleavage furrow demarcates the dorsal-ventral axis in *Xenopus* embryos. *Dev. Biol.* 120: 299-304.

KODJABACHIAN, L. and LEMAIRE, P. (1998). Embryonic induction: is the Nieuwkoop centre a useful concept? *Curr. Biol.* 8: R918-921.

LARABELL, C.A., TORRES, M., ROWNING, B.A., YOST, C., MILLER, J.R., WU, M., KIMELMAN, D. and MOON, R.T. (1997). Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* 136: 1123-1136.

LAURENT, M.N., BLITZ, I.L., HASHIMOTO, C., ROTHBACHER, U. and CHO, K.W. (1997). The *Xenopus* homeobox gene twin mediates Wnt induction of goosecoid in establishment of Spemann's organizer. *Development* 124: 4905-4916.

LEMAIRE, P., GARRETT, N. and GURDON, J.B. (1995). Expression cloning of Siamois, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* 81: 85-94.

LEYNS, L., BOUWMEESTER, T., KIM, S.H., PICCOLO, S. and DE ROBERTIS, E.M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* 88: 747-756.

LIU, P., WAKAMIYA, M., SHEA, M.J., ALBRECHT, U., BEHRINGER, R.R. and BRADLEY, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* 22: 361-365.

MARIKAWA, Y., LI, Y. and ELINSON, R.P. (1997). Dorsal determinants in the *Xenopus* egg are firmly associated with the vegetal cortex and behave like activators of the Wnt pathway. *Dev. Biol.* 191: 69-79.

MARX, A. (1925). Experimentelle Untersuchungen zur Frage der Determination der Medullarplatte. *Wilhelm Roux Arch. Entwicklungsmech. Org.* 105: 20-44.

MCKENDRY, R., HSU, S.C., HARLAND, R.M. and GROSSCHEDL, R. (1997). LEF-1/TCF proteins mediate Wnt-inducible transcription from the *Xenopus* nodal-related-3 promoter. *Dev. Biol.* 192: 420-431.

MCKENDRY, R., HARLAND, R.M. and STACHEL, S.E. (1998). Activin-induced factors maintain goosecoid transcription through a paired homeodomain binding site. *Dev. Biol.* 204: 172-186.

MOLENAAR, M., VAN DE WETERING, M., OOSTERWEGEL, M., PETERSON-MADURO, J., GODSAVE, S., KORINEK, V., ROOSE, J., DESTREE, O. and CLEVERS, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell* 86: 391-399.

MOON, R.T. and KIMELMAN, D. (1998). From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus. Bioessays* 20: 536-545.

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal Table of Xenopus laevis (Daudin).* North Holland Publishing Company, Amsterdam.

NISHITA, M., HASHIMOTO, M.K., OGATA, S., LAURENT, M.N., UENO, N., SHIBUYA, H. and CHO, K.W. (2000). Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature* 403: 781-785.

ROWNING, B.A., WELLS, J., WU, M., GERHART, J.C., MOON, R.T. and LARABELL, C.A. (1997). Microtubule-mediated transport of organelles and localization of beta-catenin to the future dorsal side of *Xenopus* eggs. *Proc. Natl. Acad. Sci. USA* 94: 1224-1229.

RUIZ I ALTABA, A. and MELTON, D.A. (1989). Interaction between peptide growth factors and homeobox genes in the establishment of antero-posterior polarity in frog embryos. *Nature* 341: 33-38.

SAMPATH, K., CHENG, A.M., FRISCH, A. and WRIGHT, C.V. (1997). Functional differences among *Xenopus* nodal-related genes in left-right axis determination. *Development* 124: 3293-3302.

SCHIER, A.F. and SHEN, M.M. (2000). Nodal signalling in vertebrate development. *Nature* 403: 385-389.

SCHNEIDER, S., STEINBEISSER, H., WARGA, R.M. and HAUSEN, P. (1996). Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech. Dev.* 57: 191-198.

SIROTKIN, H.I., DOUGAN, S.T., SCHIER, A.F. and TALBOT, W.S. (2000). *bozozok* and *squint* act in parallel to specify dorsal mesoderm and anterior neuroectoderm in zebrafish. *Development* 127: 2583-2592.

SLACK, J.M. and ISAACS, H.V. (1994). The Einsteck-method: position and structure of projections formed by implants of a ventral character. *Dev. Biol.* 161: 313-317.

SMITH, J.C., PRICE, B.M.J., VAN NIMMEN, K. and HUYLEBROECK, D. (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homolog of activin A. *Nature* 345: 729-731.

SOKOL, S., CHRISTIAN, J.L., MOON, R.T. and MELTON, D.A. (1991). Injected wnt RNA induces a complete body axis in *Xenopus* embryos. *Cell* 67: 741-752.

SOKOL, S.Y. (1999). Wnt signaling and dorso-ventral axis specification in vertebrates. *Curr. Opin. Genet. Dev.* 9: 405-410.

SPEMANN, H. (1931). Uber den Anteil von Imlantat und Wirtskeim an der Orientierung and Beschaffenheit der induzierten Embryonalanlage. *Roux Arch. Entwicklungsmech.* 123: 389-517.

SPEMANN, H. and MANGOLD, H. (1924). Uber Induktion von Embryonanlagen durch Implantation artfremder Organisatoren. *Arch. mikr. Anat. EntwMech.* 100: 599-638.

SUMANAS, S., STREGE, P., HEASMAN, J. and EKKER, S.C. (2000). The putative wnt receptor *Xenopus* frizzled-7 functions upstream of beta-catenin in vertebrate dorsoventral mesoderm patterning. *Development* 127: 1981-1990.

SUN, B.I., BUSH, S.M., COLLINS-RACIE, L.A., LAVALLIE, E.R., DIBLASIO-SMITH, E.A., WOLFMAN, N.M., MCCOY, J.M. and SIVE, H.L. (1999). *derriere*: a TGF-beta family member required for posterior development in *Xenopus. Development* 126: 1467-1482.

TAIRA, M., JAMRICH, M., GOOD, P.J. and DAWID, I.B. (1992). The LIM domain-containing homeobox gene *Xlim*-1 is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev.* 6: 356-366.

THISSE, B., WRIGHT, C.V. and THISSE, C. (2000). Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature* 403: 425-428.

THOMSEN, G., WOOLF, T., WHITMAN, M., SOKOL, S., VAUGHAN, J., VALE, W. and MELTON, D.A. (1990). Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. *Cell* 63: 485-493.

THOMSEN, G.H. and MELTON, D.A. (1993). Processed Vg1 protein is an axial mesoderm inducer in *Xenopus. Cell* 74: 433-441.

VINCENT, J.-P., OSTER, G.F. and GERHART, J.C. (1986). Kinematics of gray crescent formation in *Xenopus* eggs. The displacement of cortical cytoplasm relative to the egg surface. *Dev. Biol.* 113: 484-500.

VLEMINCKX, K., WONG, E., GUGER, K., RUBINFELD, B., POLAKIS, P. and GUMBINER, B.M. (1997). Adenomatous polyposis coli tumor suppressor protein has signaling activity in *Xenopus laevis* embryos resulting in the induction of an ectopic dorsoanterior axis. *J. Cell Biol.* 136: 411-420.

WATABE, T., KIM, S., CANDIA, A., ROTHBACHER, U., HASHIMOTO, C., INOUE, K. and CHO, K.W. (1995). Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between *Xenopus* and mouse. *Genes Dev.* 9: 3038-3050.

WATANABE, M. and WHITMAN, M. (1999). FAST-1 is a key maternal effector of mesoderm inducers in the early *Xenopus* embryo. *Development* 126: 5621-5634.

WILSON, P.A. and MELTON, D.A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Curr. Biol.* 4: 676-686.

YAO, J. and KESSLER, D.S. (1999). Mesoderm induction in *Xenopus*: oocyte expression system and animal cap assay. In *Developmental Biology Protocols, Vol. III* (Eds. R. S. Tuan and C. W. Lo). Methods in Molecular Biology, Vol. 137. Humana Press, Totowa. pp. 169-178.

ZOLTEWICZ, J.S. and GERHART, J.C. (1997). The Spemann organizer of *Xenopus* is patterned along its anteroposterior axis at the earliest gastrula stage. *Dev. Biol.* 192: 482-491.