Xwnt-8 modifies the character of mesoderm induced by bFGF in isolated *Xenopus* ectoderm

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In *Xenopus*, growth factors of the TGF- β , FGF and Wnt oncogene families have been proposed to play a role in generating embryonic pattern. In this paper we examine potential interactions between the bFGF and Xwnt-8 signaling pathways in the induction and dorsal–ventral patterning of mesoderm. Injection of Xwnt-8 mRNA into 2-cell *Xenopus* embryos does not induce mesoderm formation in animal cap ectoderm isolated from these embryos at the blastula stage, but alters the response of this tissue to mesoderm induction by bFGF. While animal cap explants isolated from non-injected embryos differentiate to form ventral types of mesoderm and muscle in response to bFGF, explants from Xwnt-8 injected embryos form dorsal mesodermal and neural tissues in response to the same concentration of bFGF, even if the ectoderm is isolated from the prospective ventral sides of embryos or from UV-ventralized animals. Our results support a model whereby dorso-ventral mesodermal patterning can be attained by a single mesoderm inducing agent, possibly bFGF, which is uniformly distributed across the prospective dorsal–ventral axis, and which acts in concert with a dorsally localized signal, possibly a Wnt protein, which either alters the response of ectoderm to induction or modifies the character of mesoderm after its induction.

Key words: bFGF/mesoderm induction/patterning/*Xenopus*/Xwnt-8

Introduction

During amphibian development, the basic body plan is first laid out in the mesoderm, a tissue which differentiates from marginal zone ectoderm in response to signals generated by endodermal cells of the underlying vegetal hemisphere (Nieuwkoop, 1969). Patterning of the mesoderm is believed to be accomplished predominantly by localized determinants within inducing (vegetal) cells since 180° rotation of the blastula animal hemisphere produces embryos whose dorsal–ventral polarity depends solely on the original polarity of the vegetal hemisphere (Boterenbrood and Nieuwkoop, 1973). Furthermore, isolated dorso-vegetal cells induce dorsal types of mesoderm, such as notochord, whereas ventro-vegetal cells produce ventral types of mesoderm, such as blood, when recombined with ectodermal animal cap tissue (Boterenbrood and Nieuwkoop, 1973; Dale *et al.*, 1985). The presence of two vegetal inductive centers, of dorsal and ventral specificity, would not explain the ability of dorsal mesoderm to convert ventral mesoderm to more

intermediate and dorsal types (Dale and Slack, 1987a), nor would this be sufficient to generate the actual blastula fate map (Dale and Slack, 1987b). For example, at least half of the muscle of the embryo arises from the ventral side, while ventro-vegetal blastomeres induce very little muscle when combined with isolated ectoderm (Dale and Slack, 1987a,b). Thus, it has been proposed that pattern formation in *Xenopus* requires three signals: dorsal- and ventral-type mesoderm inducing signals, which are established in the vegetal hemisphere soon after fertilization, followed by a dorsalizing signal which arises secondary to the induction of dorsal mesoderm and which ultimately gives rise to the Spemann organizer (Smith and Slack, 1983; reviewed in Smith, 1989).

The identification of peptide growth factors which can mimic the mesoderm inducing capability of the endodermal cells has led to the proposal that members of the transforming growth factor- β (TGF- β) family, such as activins, contribute the dorsal mesoderm inducing signal, and that growth factors of the fibroblast growth factor (FGF) family contribute the ventral mesoderm inducing activity of the three-signal model (Kimelman *et al.*, 1988; Smith *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1990; Amaya *et al.*, 1991; reviewed in Smith, 1989). However, these molecules alone cannot account for the dorsalizing activity which is observed *in vivo*. Specifically, while basic fibroblast growth factor can induce only ventral mesoderm and muscle, activin A and B can induce a full array of ventral and dorsal mesoderm when applied to isolated ectoderm (Green *et al.*, 1990; Sokol *et al.*, 1990; Thomsen *et al.*, 1990). Yet activin A cannot dorsalize ventral mesoderm nor can it rescue dorsal development in embryos rendered axis deficient by ultraviolet (UV) irradiation (Sokol and Melton, 1991). In contrast, endogenous inducers present in dorsal marginal zone blastomeres of early cleavage stage embryos can accomplish both of these feats (Gimlich, 1987; Kageura, 1990). Recently, selected members of a third class of developmental signaling molecules, related to and including the proto-oncogene *Wnt-1* (reviewed by McMahon, 1991), were shown to have all of the properties expected of the endogenous dorsal inducer (Sokol *et al.*, 1991). Injection of Wnt-1 or Xwnt-8 mRNA into intact cleavage stage embryos induced the formation of mesoderm in ectodermal explants isolated from these embryos. In addition, ventrally localized injections of these transcripts led to formation of a complete secondary embryo and could rescue dorsal development in UV-ventralized animals (Sokol *et al.*, 1991).

In this paper, we extend these studies by examining potential interactions between the Xwnt-8 and bFGF signaling pathways. We find that early, delocalized expression of Xwnt-8 in *Xenopus* embryos cannot induce differentiation of mesodermal tissues in naive ectoderm, but instead alters the response of this tissue to mesoderm induction by bFGF. Animal cap explants derived from Xwnt-8 injected embryos, but not from uninjected embryos, form dorsal mesodermal and neural tissues following applica-

tion of bFGF, even if the ectoderm is isolated from the prospective ventral sides of embryos, or from UV-ventralized animals. These data support a version of the three-signal model whereby dorso-ventral mesodermal patterning is accomplished by a single, uniformly distributed ventral mesoderm inducing factor, in combination with a dorsally localized, non-mesoderm inducing agent, which functions either to modify the initial response of ectoderm to induction or which converts newly induced ventral mesoderm to more dorsal cell types.

Results

Xwnt-8 modifies the character of mesoderm induced by bFGF

Animal cap ectoderm was isolated at stage 7–8 from embryos which had been injected with synthetic Xwnt-8 RNA at the 2-cell stage, or from uninjected control embryos, and was cultured in the presence or absence of 50 ng/ml bFGF. In the absence of bFGF, almost all ectodermal explants failed to elongate or to differentiate any recognizable structures, regardless of whether the caps were dissected from Xwnt-8 expressing embryos, or from uninjected controls (Table I, Figure 1D and G). Animal caps from uninjected control embryos responded to bFGF by elongating slightly, and by stage 40 had formed characteristic ventral-type structures with vesicles, as has been described in previous studies (Green *et al.*, 1990) (Table I and Figure 1J). In contrast, explants of animal pole tissue from embryos injected with Xwnt-8 RNA responded to bFGF by elongating dramatically, and differentiated into embryoids which in some cases demonstrated a clear dorsal–ventral and anterior–posterior polarity as well as apparent dorsal axial structures, melanocytes and occasionally, cement glands (Table I and Figure 1M).

When this same assay was repeated, but with a slightly larger piece of animal cap tissue (~35–50% of the uppermost animal hemisphere), identical results were obtained for all experimental groups with the exception of explants obtained from Xwnt-8 injected embryos which were cultured in isolation. Approximately 70% of these explants underwent an autonomous elongation in the absence of any exogenous inducing growth factor (data not shown) as described previously (Sokol *et al.*, 1991). All subsequent assays were performed using tissue comprising the uppermost 25–33% of the animal hemisphere.

To begin to analyze the types of tissues which were induced by bFGF in the presence or absence of ectopically expressed Xwnt-8, 10–20 explants from each group were immunostained, at control stage 40, using monoclonal antibodies specific for muscle (12/101, Kintner and Brockes, 1984) or for nervous tissue (2G9, Jones and Woodland, 1989). 12/101 or 2G9 immunoreactive cells were not detected in any explants cultured in the absence of bFGF, regardless of whether the caps had been dissected from embryos injected with Xwnt-8 (Figure 1E and F) or from uninjected controls (Figure 1H and I). Ten out of 10 animal caps isolated from uninjected embryos and cultured in the presence of bFGF expressed the 12/101 antigen (Figure 1K), while 10 out of 11 caps examined did not express the nerve specific 2G9 antigen (Figure 1L). These data are consistent with previous studies which demonstrated that bFGF alone induces only ventral and intermediate types of mesoderm

(Green *et al.*, 1990). Explants expressing ectopic Xwnt-8 responded to bFGF by forming muscle tissue in 100% of cases ($n = 15$, Figure 1N) and nerve tissue in 87% of cases examined ($n = 12$, Figure 1O) as evidenced by 12/101 and 2G9 staining. In many embryos, the 2G9 antibody stained a central core of nervous tissue as well as neural crest-derived melanocytes. Thus, only explants which were both derived from Xwnt-8 injected embryos and treated with bFGF consistently expressed the 2G9 antigen.

Some explants were assayed by Northern analysis for the expression of muscle-specific actin RNA (Mohun *et al.*, 1984) and for transcripts encoding XIF3, an intermediate filament gene expressed predominantly in anterior neural tissue (Sharpe *et al.*, 1989). As shown in Figure 2, explants cultured in the absence of bFGF did not express detectable levels of either cardiac actin or XIF3 RNA. In animal caps dissected from uninjected control embryos, bFGF induced very low levels of cardiac actin (not detectable on this exposure) while XIF3 transcripts were not detected. In contrast, high levels of cardiac actin RNA, as well as XIF3 transcripts, were detected in ectoderm isolated from embryos expressing ectopic Xwnt-8 and cultured in the presence of bFGF. The same filter was rehybridized with an EF1- α cDNA probe to demonstrate that all lanes contained approximately equivalent amounts of RNA (Figure 2).

The experiments described above clearly demonstrate, by immunostaining and by RNA blot analysis, that Xwnt-8 alone cannot induce naive ectoderm to differentiate into mesodermal tissues, provided that the ectoderm is obtained from the uppermost 25–33% of the animal hemisphere. Xwnt-8 is, however, capable of modifying the character of mesoderm induced by bFGF such that dorsal mesodermal tissue, as well as neural tissue, is formed.

Xwnt-8 acts upon the ectoderm, but requires an endodermal signal to modify mesoderm induction

To determine whether the enhanced response of ectodermal cells to bFGF requires that Xwnt-8 polypeptides act upon the responding cells directly, Xwnt-8 RNA was injected into two blastomeres at the extreme animal or vegetal pole of 32- to 64-cell embryos. In all cases, two adjacent cells were injected, one on the prospective dorsal side, and one on the prospective ventral side of the embryo. Although Xwnt-8

Table I. Inductive effects of bFGF and Xwnt-8 on blastula ectoderm

Xwnt-8	bFGF	Elongation at stage 18				Tissues induced at stage 40							
		0	+	+++	<i>n</i>	Epi	Ves	Ax	Mel	Eyes	Cg	<i>n</i>	
–	–	98	2	0	64	100	0	0	0	0	0	10	
+	–	88	12	0	57	95	5	0	0	0	0	21	
–	+	1	99	0	75	2	91	7	16	0	0	44	
+	+	0	4	96	82	0	4	96	77	0	31	26	

Animal caps excised at stage 7–8 from Xwnt-8 injected or control embryos were cultured in the presence (+) or absence (–) of bFGF as indicated, and were scored for degree of elongation at control stage 18 (0, no elongation; +, slight elongation characteristic of ventral mesodermal explants; ++, extensive elongation characteristic of dorsal mesodermal explants). Some explants were cultured to control stage 40 and classified according to types of tissue induced. Epi, atypical epidermal differentiation; Ves, vesicles characteristic of ventral mesoderm; Ax, axial, muscle containing elongated structures; Mel, neural crest-derived melanocytes; Cg, cement gland. All numbers are expressed as percentages except n which refers to total sample size.

is believed to be secreted, evidence suggests that Wnt proteins remain tightly adherent to the cell surface or extracellular matrix following secretion and act in an autocrine or paracrine fashion over a distance of only a few cells (Bradley and Brown, 1990; Papkoff and Schryver, 1990). Thus it is reasonable to assume that the vegetally introduced Xwnt-8 protein will not directly contact cells of the extreme animal pole, or vice versa. If Xwnt-8 is required within, or at the cell-surface of, ectodermal cells then only ectoderm isolated from embryos receiving injections into animal pole blastomeres should show extensive elongation and differentiation in response to bFGF. In contrast, if the enhanced response of ectodermal cells occurs via a secondary signal initiated in, and transmitted by, the inducing cells,

then ectoderm derived from vegetally injected embryos might demonstrate enhanced induction by bFGF. Embryos were allowed to develop to the mid-blastula stage at which time ectodermal caps were isolated from some of the injected embryos, and cultured in the presence or absence of bFGF. In the absence of bFGF, all but one explant failed to elongate or differentiate into non-ectodermal structures (Table II). Explants which were isolated from embryos vegetally injected with Xwnt-8, and cultured in the presence of bFGF, differentiated into characteristic ventral-type structures with vesicles in 89% of cases. In contrast, 91% of explants from embryos expressing ectopic Xwnt-8 in animal pole cells, and treated with bFGF, elongated dramatically and differentiated in a fashion identical to that observed in ectoderm isolated

Fig. 1. Early, delocalized expression of Xwnt-8 in *Xenopus* animal cap cells modifies the character of mesoderm induced by bFGF. Whole stage 40 embryos (A–C), or ectodermal explants isolated from uninjected embryos (G–I) or embryos injected with Xwnt-8 RNA at the 2-cell stage (D–F and M–O) and cultured in the absence (D–I) or presence (J–O) of bFGF to an equivalent stage are shown. In the first column, intact unstained embryos or explants are shown. Columns two and three illustrate embryos or explants stained with the muscle-specific monoclonal antibody (mAb) 12/101 or the nerve-specific mAb 2G9 as indicated. Arrows denote specific staining.

Fig. 2. Induction by bFGF of neural- and muscle-specific RNAs in ectoderm from embryos injected with Xwnt-8 mRNA. Explants were isolated at stage 7–8 from embryos injected with Xwnt-8 RNA at the 2-cell stage or from uninjected control embryos, and were cultured in the presence or absence of bFGF as indicated above each lane. RNA prepared from whole embryos (W) or from explants at stage 26 was subjected to gel blot analysis. The filter was successively hybridized to a ^{32} P-labeled XIF3 riboprobe and to cardiac actin and EF1- α cDNA probes as indicated to the left of each panel. Asterisk to the right of the second panel indicates the position of muscle-specific actin transcripts, while the upper two bands in this panel represent cytoskeletal actin RNAs.

Table II. Differential induction by bFGF of ectoderm isolated from embryos expressing Xwnt-8 in vegetal or animal pole blastomeres

Xwnt-8	bFGF	Elongation at stage 18				Tissues induced at stage 40						
		0	+	++	+++	n	Epi	Ves	Ax	Mel	Eyes	Cg
Veg	–	95	5	0	20	95	5	0	0	0	0	20
Veg	+	0	93	8	12	0	89	11	11	0	0	9
Ani	+	0	9	91	11	0	9	91	64	0	9	11

Xwnt-8 mRNA was injected into two animals (Ani) or vegetal (Veg) pole blastomeres of 32- to 64-cell embryos. Animal caps excised from these embryos at stage 7–8 were cultured in the presence (+) or absence (–) of bFGF and were scored for degree of elongation as well as for type of tissue induced at control stage 18 or 40 as indicated. Numbers expressed as percentages except for *n* which denotes sample size. Abbreviations as in Table I.

from embryos injected with Xwnt-8 at the 2-cell stage (Table II). This supports the hypothesis that Xwnt-8 acts directly upon the ectodermal cells to modify their response to bFGF.

Some embryos which received animal pole or vegetal pole injections of Xwnt-8 RNA were allowed to develop intact and were scored for external phenotype at stage 26. All embryos receiving vegetally directed Xwnt-8 RNA developed with some degree of dorsal–anterior duplication ($n = 48$), as has previously been described (Christian *et al.*, 1991; Sokol *et al.*, 1991). In contrast, of 50 embryos expressing Xwnt-8 in extreme animal pole cells, 48 appeared completely normal and the remaining two developed a short secondary dorsal axis. Thus, injection of Xwnt-8 into blastomeres most distal to the vegetal cells, which release the endogenous mesoderm inducing signal(s) (Nieuwkoop, 1969), has no dorsalizing effect. In contrast, when Xwnt-8 is delivered to vegetal cells, the dorsal character of mesoderm which is induced is enhanced, consistent with the hypothesis

that Xwnt-8 acts upon the marginal zone ectoderm in concert with a vegetally derived signaling agent. These experiments involving intact embryos receiving vegetal or animal pole injections of Xwnt-8, or ectoderm isolated from such embryos, provide further evidence that Xwnt-8 alone is not capable of redirecting the fate of naive ectoderm. However, in combination with bFGF (in isolated ectodermal caps), or with an endogenous vegetally derived signal (in intact animals), Xwnt-8 can redirect the fate of newly induced mesoderm along a more dorsal route.

Xwnt-8 overcomes the differential response of prospective dorsal and ventral ectoderm to bFGF

Recent studies have demonstrated that prospective dorsal and ventral regions of *Xenopus* blastula animal cap tissue respond differently to identical concentrations of the two mesoderm inducing growth factors activin A and bFGF (Ruiz i Altaba and Jessell, 1991; Sokol and Melton, 1991). Specifically, the dorsal halves of ectodermal explants differentiate into anterior dorsal structures in response to activin A, and into posterior dorsal structures in response to bFGF. The ventral halves of these explants respond to an identical concentration of either growth factor by forming mesenchyme and muscle. Thus, ventral ectoderm is normally incompetent to form extreme dorsal mesodermal structures in response to these peptide growth factors. The ability of Xwnt-8 to redirect the fate of mesoderm along a more dorsal route, both in the intact animal and in isolated animal pole tissue, raises the possibility that a Wnt protein may play some role in establishing this differential response of dorsal and ventral ectodermal cells to mesoderm inducing growth factors. To test this possibility, dorsal and ventral ectodermal cells were dissected from embryos at stage 7–8 and cultured in the presence or absence of bFGF. Half of these caps were from embryos in which Xwnt-8 had been injected into both blastomeres at the 2-cell stage while half were from uninjected controls. Dorsal and ventral caps from uninjected embryos developed into solid masses of epidermal cells when cultured in the absence of bFGF, as judged by external appearance and histological examination (Table III). Dorsal (Figure 3A) and ventral (Figure 3G) caps derived from uninjected embryos responded to bFGF by elongating slightly and forming vesicular structures (Figure 3B and H, Table III). Histological sections of these caps revealed the presence of muscle, mesenchyme, mesothelium, and often a structure resembling the proctodeum (Figure 3C and I, Table III), all of which are characteristic of explants induced by bFGF (Green *et al.*, 1990). Notochord or neural tissue was not detected in any of the dorsal or ventral explants which were examined, consistent with the inability of bFGF to induce these structures in whole explants (Green *et al.*, 1990). Differences in the response of prospective dorsal and ventral halves to bFGF included a greater degree of elongation of dorsal caps (compare Figure 3A and G) and the infrequent appearance of melanocytes in dorsal, but not ventral explants (Table III). These differences are similar to, but more subtle than, those reported by Ruiz i Altaba and Jessell (1991). This discrepancy might be due to differences in the amount of animal pole tissue which was explanted (our caps were restricted to 25–30% of the animal pole) or to differences in the developmental stage at which caps were obtained (stage 7–8 in our experiments, versus stage 10 in theirs). The latter explanation seems less likely since dorsal–ventral patterning

Table III. Xwnt-8 alters the response of dorsal and ventral ectoderm to induction by bFGF

	Elongation at stage 18				Tissues induced at stage 40							
	0	+	+++	<i>n</i>	Epi	NT	Not	Mel	Cg	Mus	Mt	<i>n</i>
Dorsal half	100	0	0	8	100	0	0	0	0	0	0	8
+bFGF	0	100	0	21	0	0	0	25	0	88	63	15
+bFGF +Xwnt-8	0	21	79	28	0	88	88	81	31	94	37	16
Ventral half	100	0	0	7	100	0	0	0	0	0	0	5
+bFGF	0	100	0	20	0	0	0	0	0	87	38	15
+bFGF +Xwnt-8	0	24	76	29	0	77	71	82	18	94	29	17

Stage 7–8 animal caps were isolated from Xwnt-8 injected or uninjected embryos as described in text, cut into dorsal and ventral halves and incubated in the presence or absence of bFGF. Explants were scored for elongation at control stage 18 (abbreviations as in Table I), and analyzed histologically at control stage 40. Abbreviations: Epi, atypical epidermis; NT, neural tissue; Not, notochord; Mel, neural crest-derived melanocytes; Cg, cement gland; Mus, muscle; Mt, mesothelium. Numbers expressed as percentages except for *n* which denotes sample size.

Fig. 3. Dorsal and ventral ectoderm from Xwnt-8 injected embryos differentiates identically, forming dorsal mesoderm and neural tissue in response to bFGF. Dorsal (A–F) or ventral (G–L) halves of ectodermal caps cut from embryos injected with Xwnt-8 RNA at the 2-cell stage (D–F, J–L) or from uninjected embryos (A–C, G–I) were cultured in the presence of bFGF until sibling embryos reached stage 18 or 40 as indicated at the head of each column. Columns one and two show intact explants, column three represents histological sections. Abbreviations: p, proctodeum; m, muscle; mes, mesothelium; n, neural tissue; no, notochord; mel, neural crest-derived melanocytes.

of the ectoderm has been shown to be established at least as early as stage 6.5 (Sokol and Melton, 1991).

The response to bFGF of dorsal and ventral caps derived from Xwnt-8 injected embryos was indistinguishable. Caps from dorsal (Figure 3D) or ventral (Figure 3J) ectodermal halves elongated dramatically resembling explants of normal dorsal marginal zone tissue (Symes and Smith, 1987), and

differentiated into embryoids, which externally appeared to have formed dorsal axial structures, and in some cases cement glands (Figure 2E and K, Table III). Histological examination demonstrated the presence of abundant neural tissue, notochord and segmented muscle (Figure 3F and L, Table III). Thus, Xwnt-8 not only modifies the overall response of ectoderm to the mesoderm inducing growth

factor, bFGF, but changes the competence of ventral ectoderm, enabling it to differentiate into dorsal mesoderm tissues.

Xwnt-8 rescues the ability of ectoderm from UV-ventralized embryos to form dorsal structures

In *Xenopus*, UV irradiation of the egg during the first half of the cell cycle produces embryos in which dorsal mesoderm is re-routed along ventral pathways and dorsal axial structures are lacking (Gerhart *et al.*, 1989). We isolated ectoderm from UV-treated embryos which had been injected with Xwnt-8 at the 2-cell stage, and from uninjected controls, and cultured the tissue in the presence of bFGF. Explants in each group differentiated identically to those derived from non-irradiated explants; ectoderm from uninjected controls developed vesicles characteristic of dorsal inductions and ectoderm from Xwnt-8 injected embryos elongated and formed dorsal axial structures (Table IV). Thus, Xwnt-8, along with a ventral mesoderm inducing agent, can rescue the dorsal axial development of embryos whose endogenous dorsal center has been destroyed by UV irradiation.

Discussion

Our results show that expression of Xwnt-8 within prospective ectodermal cells of early *Xenopus* embryos has no effect on the fate of ectoderm cultured in isolation, but alters the response of this tissue to mesoderm induction by bFGF. Whereas naive ectoderm differentiates into ventral types of mesoderm upon exposure to bFGF, an identical concentration of this growth factor produces dorsal mesoderm and, secondarily, neural tissue in ectoderm exposed to Xwnt-8 during early cleavage stages.

Previous studies have shown that ectopic expression of Xwnt-8 or Wnt-1 in intact cleavage stage embryos induces multiple axis formation (McMahon and Moon, 1989; Christian *et al.*, 1991) and rescues dorsal development in embryos ventralized by UV irradiation, suggesting that these signaling molecules can induce the formation of a new Spemann organizer (Sokol *et al.*, 1991). Our results complement these studies and provide new information regarding early embryonic events which may ultimately lead to induction of the organizer.

In *Xenopus*, the dorso-ventral axis is established as a result of a pre-cleavage cortical cytoplasmic rotation which is believed to activate locally dorsal determinants (Gerhart *et al.*, 1989). The dorsalizing activity induced by this cytoplasmic reorganization is segregated to dorsal vegetal blastomeres by the end of the 8-cell stage (Kageura and Yamana, 1984; Cardellini, 1988) and appears to become progressively restricted to the dorsal marginal zone as development proceeds (Smith and Slack, 1983; Gimlich and Gerhart, 1984; Gimlich, 1986; Kageura, 1990). During the blastula stage, signals generated by vegetal endodermal cells induce the overlying ectoderm to form mesoderm (Nieuwkoop, 1969), the dorso-ventral character of which is imposed upon the responding (ectodermal) tissue by the inducing (endodermal) cells (Boterenbrood and Nieuwkoop, 1973). The current model of mesoderm induction proposes that induction and patterning of this germ layer are achieved by three signals: (i) a ventro-vegetal signal which induces ventral mesoderm, (ii) a dorso-vegetal signal, activated by the pre-cleavage cytoplasmic reorganization, which induces

dorsal mesoderm in a restricted region of the embryo, and (iii) a signal emitted by the newly induced dorsal mesoderm, or organizer tissue, which converts adjacent ventral mesoderm to more intermediate and dorsal types during the late blastula to gastrula stages (Smith and Slack, 1983). While the molecular nature of these signalling agents is unknown, the identification of peptide growth factors which can mimic the action of endogenous inducers has led to the suggestion that the ventro-vegetal signal may consist of bFGF or a low concentration of activin, while the dorso-vegetal signal could include a higher concentration of activin, or of a different, possibly non-mesoderm inducing, member of the TGF- β family acting synergistically with bFGF (reviewed by Smith, 1989; Slack *et al.*, 1989). Our results support a version of the three-signal model in which the dorsalizing activity which becomes localized following fertilization is an agent which lacks intrinsic mesoderm inducing activity itself, but which can alter the initial response of ectodermal cells to a uniform ventral mesodermalizing signal. Thus, induction of dorsal mesoderm (step two of the model) would require both this dorsal determinant and a general mesoderm inducing agent. While dorsal patterning could not be accomplished by either factor alone, an interaction between the two would generate the embryonic dorsal center, as defined by Kageura (1990) which ultimately leads to formation of the Spemann organizer (Smith and Slack, 1983). This scenario is consistent with recent studies demonstrating that animal hemisphere ectoderm acquires a dorso-ventral pre-pattern by the early blastula stage, which is revealed by the action of mesoderm inducing growth factors (Ruiz i Altaba and Jessell, 1991; Sokol and Melton, 1991). Our results support this model, and demonstrate that Xwnt-8 can substitute for the locally activated dorsal determinant, and bFGF for the ventral mesodermalizing component of the model.

Xwnt-8 can induce the formation of dorsal axial structures in isolated ectoderm, but only in the presence of the ventral mesodermalizing agent, bFGF. It has previously been concluded that Xwnt-8 has the ability to induce mesoderm formation directly, based on the observation that ectodermal explants from Xwnt-8 injected embryos elongate autonomously and express muscle-specific actin transcripts (Sokol *et al.*, 1991). This apparent discrepancy is most likely due to the amount of animal cap tissue excised, since in our hands explants comprising ~50% of the animal hemisphere elongate in the absence of exogenous growth factor if derived from Xwnt-8 injected, but not from uninjected embryos. Slightly smaller explants of Xwnt-8 injected embryos differentiate only in the presence of bFGF, demonstrating

Table IV. Basic FGF induces formation of dorsal axial structures in ectodermal explants from Xwnt-8 injected, UV-irradiated embryos

Xwnt-8	bFGF	Elongation at stage 18				Tissues induced at stage 40						
		0	+	+++	n	Epi	Ves	Ax	Mel	Eyes	Cg	n
−	+	0	100	0	14	7	86	7	7	0	0	14
+	+	0	22	78	18	0	23	77	47	0	12	17

Eggs were UV-irradiated prior to first cleavage. Animal caps were excised at stage 7–8 from Xwnt-8 injected or control embryos cultured in the presence of bFGFa and scored for degree of elongation and for tissue differentiation at control stages 18 and 40 as indicated. Abbreviations as in Table 1. Numbers expressed as percentages except for n which denotes sample size.

that this ectoderm is still competent to respond to mesoderm inducing signals, but that Xwnt-8 alone is insufficient to accomplish this induction. An alternate interpretation of the data, that Xwnt-8 is a weak inducer whose action is potentiated by bFGF, is possible but seems unlikely given our inability to detect mesodermal differentiation by Northern blot analysis, by immunostaining or by histological examination of explants derived from Xwnt-8 injected embryos cultured in the absence of bFGF. The observation that dorsal duplications can be produced by injection of Xwnt-8 into extreme vegetal pole cells, but not animal pole cells, of intact 32- to 64-cell embryos further supports the contention that Xwnt-8 has no direct mesoderm inducing activity, but requires a vegetally derived inducing signal for activity. While these results might also be explained by postulating that translation of Xwnt-8 is blocked in animal pole cells, but not in vegetal pole cells, this is unlikely for several reasons. First, while injections of Xwnt-8 into animal pole blastomeres of 32- to 64-cell embryos did not produce dorsal duplications, explants dissected from random members of this same group of embryos showed an enhanced response to bFGF demonstrating that Xwnt-8 RNA is active when introduced into animal pole cells. In addition, injection of Wnt-1 transcripts into animal pole cells of *Xenopus* blastula produces an identical enhanced response of animal cap ectoderm to bFGF (D.J.Olson and R.T.Moon, unpublished data) and a myc-epitope tagged version of this RNA has been shown to be translated throughout the *Xenopus* embryo (McMahon and Moon, 1989; D.J.Olson and R.T. Moon, unpublished data). Thus, our data are consistent with a model whereby a vegetal mesoderm inducing signal interacts with a graded (dorsal to ventral) dorsalizing signal, and the local concentration of both agents determines the threshold for mesoderm induction, as well as the character of mesoderm to be induced. The vegetal inducing factor most likely does not penetrate throughout the entire animal hemisphere, and thus its scope of action is limited to a band of marginal zone ectoderm. This would explain why a small cap of ectoderm from the animal pole of an Xwnt-8 injected embryo does not differentiate in the absence of exogenous bFGF, while a slightly larger cap from an Xwnt-8 injected embryo, which contains endogenous mesoderm inducing agents, possibly bFGF, can differentiate autonomously.

Further evidence that Xwnt-8 mimics the endogenous dorsal determinant is provided by the observation that Xwnt-8 can overcome the pre-existent dorsal-ventral bias in isolated ectoderm such that prospective ventral, as well as dorsal, ectoderm can form axial mesoderm in response to bFGF, and in a secondary induction can form neural structures. In addition, if the endogenous dorsal center is destroyed by UV irradiation, it can be restored by injection of Xwnt-8, such that isolated ectoderm is again competent to form dorsal structures in response to mesoderm inducing growth factors. In the intact embryo, injection of Xwnt-8 alone can rescue dorsal axial development in UV-ventralized embryos, suggesting that the endogenous mesoderm inducing signals are intact in these embryos (Sokol *et al.*, 1991).

Are Xwnt-8 and bFGF the endogenous ectodermal patterning and mesodermalizing signals?

Our results suggest that Xwnt-8 can mimic or replace the endogenous dorsal determinant which is locally activated by

cortico-cytoplasmic rotation. However, Xwnt-8 does not meet all of the criteria for such an agent. Xwnt-8 transcripts are first detected after the mid-blastula transition (Christian *et al.*, 1991), whereas the endogenous dorsalizing signal is active soon after fertilization. Most likely, ectopically expressed Xwnt-8 is activating a receptor for a maternally expressed member of the Wnt family, which normally functions as the endogenous dorsal determinant (McMahon and Moon, 1989; Christian *et al.*, 1991; Sokol *et al.*, 1991). Alternatively, Xwnt-8 may be activating its own, or a heterologous receptor, at an inappropriate time in development, setting in motion a signal transduction process which is normally employed by a non-Wnt-related endogenous dorsalizing agent. The molecular mechanism by which Xwnt-8 modifies mesodermal patterning is unknown, but the observation that lithium mimics many of its actions provides one clue as to how its effects may be mediated. Lithium, like Xwnt-8 or Wnt-1 (Sokol *et al.*, 1991), respecifies ventral mesoderm such that it differentiates into the Spemann organizer (Kao and Elinson, 1988), and can induce a secondary axis on the ventral side of the embryo, or rescue dorsal development in an embryo ventralized by UV irradiation (Kao *et al.*, 1986). Embryos respond rapidly to administration of Wnt-1, Xwnt-8 or lithium, as evidenced by the ability of each to enhance ventral gap junctional communication at the 32-cell stage (Nagajski *et al.*, 1989; Olson *et al.*, 1991). Finally, lithium enhances the response of ectoderm to bFGF in that explants elongate extensively and produce more muscle tissue, but, unlike Xwnt-8 treated explants, do not form notochord or neural tissue (Slack *et al.*, 1988). Evidence suggests that these effects of lithium are mediated by an inhibition of the polyphosphoinositide cycle (Busa and Gimlich, 1989). Whether the endogenous dorsalizing agent utilizes a similar pathway remains to be determined.

That Xwnt-8 can dorsalize ventral mesoderm is surprising, given that endogenous Xwnt-8 transcripts are largely restricted to the ventral marginal zone of mid-blastula stage embryos (unpublished data) and to the ventral mesoderm of early gastrulae (Christian *et al.*, 1991). Endogenous Xwnt-8 has no apparent dorsalizing effect in unperturbed embryos, in that notochord does not normally form at the ventral site of endogenous Xwnt-8 expression. In addition, while bFGF induces expression of Xwnt-8 transcripts in isolated animal cap ectoderm, these caps differentiate only ventral mesoderm (Christian *et al.*, 1991). This suggests that ectopically expressed Xwnt-8 may affect the primary response of ectoderm to mesoderm induction, rather than having a later, dorsalizing effect on ventral mesoderm, although the present studies do not absolutely distinguish between these possibilities. How then can we reconcile the different response of cells in the early embryo to signals produced by endogenous versus injected Xwnt-8? It is possible that ectopically expressed Xwnt-8 polypeptides produce a short lived primary signal when introduced into cleavage stage embryos and this leads to a rapid, heritable change within cells of early blastulae. By the mid-blastula stage, when Xwnt-8 transcripts are first detected, endogenous Xwnt-8 may produce the same primary signal in the ventral marginal zone cells where it is present, but this same signal might generate a completely different secondary response. Alternatively, injected and endogenous Xwnt-8 may be acting at distinct receptors coupled to unique signal transduction pathways, in cleavage stage versus

mid-blastula stage embryos respectively, and thus produce vastly different responses.

The proposition that bFGF is the endogenous mesodermalizing agent is a more attractive possibility. *Xenopus* oocytes and early embryos contain a store of bFGF which is present at high enough concentrations to account for induction of ventral, but not dorsal mesoderm (Kimelman *et al.*, 1988; Slack and Isaacs, 1989). FGF receptors are also present in a fairly uniform distribution in the early embryo (Gillespie *et al.*, 1989; Musci *et al.*, 1990). In addition, the range of embryonic stages over which ectoderm is competent to respond to bFGF, as well as the minimum time of contact required for stable induction by bFGF are consistent with that determined for the endogenous vegetal inducer (Jones and Woodland, 1987; Slack *et al.*, 1988, 1989). Thus, bFGF may be the endogenous agent which sends a uniform signal to dorsal and ventral ectoderm, with polarity arising due to the synergistic action of a dorsally localized, non-mesoderm inducing agent.

Our results do not support a role for bFGF in anterior patterning since explants challenged with bFGF and Xwnt-8 did not develop eyes. Despite this, some anterior neural tissue was induced as evidenced by the presence of XIF3 transcripts and what appeared to be brain tissue in histological sections of these explants. This inability to achieve complete anterior inductions cannot be reconciled by proposing that a second, dorsally localized anterior mesoderm inducing factor exists in the intact embryo but is missing in our assays, since ventral injection of Xwnt-8 RNA alone into intact embryos produces secondary embryos complete with head and eyes (Sokol *et al.*, 1991). More likely a second inducing signal, which is uniformly distributed within marginal zone cells, is either locally activated only within cells of the late-blastula organizer, or can induce anterior tissues only in ectoderm which is pre-patterned as anterior by the dorsal determinant whose action is mimicked by Xwnt-8. Activin B, which is expressed following the mid-blastula transition (Thomsen *et al.*, 1990), and which can induce anterior structures in dorsal, but not ventral ectoderm (Sokol and Melton, 1991) is a good candidate for such an agent. This proposal is consistent with the demonstration that dorsal ectoderm is pre-patterned as anterior by the early blastula stage (Sokol and Melton, 1991), and with studies which suggest that activin and bFGF specify anterior and posterior mesodermal cell fates respectively (Ruiz i Altaba and Melton, 1989; Cho and De Robertis, 1990; Amaya, 1991).

In our experiments, while all explants challenged with both bFGF and Xwnt-8 differentiate dorsal mesoderm as well as neural tissue, these tissues are not always arranged in a normal spatial pattern with respect to each other. The endogenous dorsal organizer, as well as the dorsal center which precedes it (Kageura, 1990), exhibit many of the properties of a morphogenetic gradient field (reviewed by De Robertis *et al.*, 1991). Thus, the fact that Xwnt-8 was not delivered to the explants as a point source may explain the disorganized pattern. Regardless of this, our results support a model whereby dorsal-ventral mesodermal patterning can be attained by a single mesoderm inducing agent, possibly bFGF, acting in concert with a dorsally localized signal, possibly a Wnt protein, which either modifies the response of ectoderm to induction or modifies the character of mesoderm following its induction. While

Xwnt-8 does not meet the criteria for the endogenous dorsally localized signal, it provides a valuable tool with which to test hypotheses regarding the molecules and mechanisms used to generate embryonic pattern in *Xenopus*. The true identity of signaling molecules which play these roles *in vivo* remains to be discovered.

Materials and methods

Embryo culture and manipulations

Xenopus laevis eggs were obtained and embryos cultured in modified Barth solution (MBS) as described by Moon and Christian (1989). All embryonic stages are according to Nieuwkoop and Faber (1967).

Ultraviolet irradiation of fertilized eggs was as described by Christian *et al.* (1991). In all experiments, some embryos were allowed to develop to stage 28, at which time they were assigned a score of dorso-anterior index (DAI) as described by Kao and Elinson (1988). Only experiments in which the average DAI for UV-irradiated embryos was <0.5 were included in the results.

To determine dorsal-ventral polarity of animal caps, the prospective dorsal midline was marked with 1% Nile blue at the 8-cell stage. At this time, prospective dorsal blastomeres are smaller and more lightly pigmented than their ventral counterparts (Nieuwkoop and Faber, (1967). When control embryos were allowed to develop until the dorsal lip of the blastopore was visible, the Nile blue mark was found to be within 15° of the future dorsal midline in 95–98% of cases.

Mesoderm induction assays

Blastula animal caps were obtained at stage 7–8 and cultured as described by Christian *et al.* (1991). In all experiments, the tissue used consisted of the uppermost 25–33% of the animal cap unless otherwise stated. Recombinant *Xenopus* bFGF was used at a concentration of 50 ng/ml. Induction was scored by the degree of convergent extension of explants observed at control stage 18, by external evidence of mesodermal tissues, and/or by identification of various mesodermal cell types in histological sections of explants fixed at control stage 40.

Histology

Animal caps were fixed for 2 h in 4% paraformaldehyde/0.25% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4, and then washed overnight in PBS. Fixed explants were embedded in paraffin and 7 µm thick sections were cut and stained with hematoxylin-eosin. Differentiated cell types were identified by morphology as described by Green *et al.* (1990).

Immunolabeling of whole embryos and explants

Whole mount immunocytochemical analysis using the muscle specific monoclonal antibody 12/101 (Kintner and Brockes, 1984) or the nerve specific monoclonal antibody 2G9 (Jones and Woodland, 1989) was as described by Dent *et al.* (1989).

RNA analysis

RNA was extracted from embryos or explants and analyzed by Northern hybridization to ³²P-labeled cDNA or cRNA probes as described by Christian *et al.* (1991).

In vitro transcription and microinjection of RNA

Capped synthetic RNA was generated by *in vitro* transcription of pXwnt-8 (Christian *et al.*, 1991). Approximately 5–10 pg of RNA was injected into the animal hemisphere of each blastomere of embryos at the 2-cell stage (or 32- to 64-cell stage if indicated) as described by Moon and Christian (1989). In all experiments, some embryos were allowed to develop to stage 28 at which time >90% of Xwnt-8 injected embryos showed some degree of dorso-anterior duplication as has been described (Christian *et al.*, 1991) while this phenotype was never observed in control uninjected embryos.

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