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Dissecting *Wnt* signalling pathways and *Wnt*-sensitive developmental processes through transient misexpression analyses in embryos of *Xenopus laevis*

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SUMMARY

We review evidence that *Xenopus Wnts* (*Xwnts*) have activities consistent with their hypothesized roles as secreted signalling factors involved in multiple developmental processes. Transient misexpression of different Xwnts has distinct effects upon early development, and upon the formation of tissues in UV-irradiated embryos. Misexpression of Xwnts also has distinct effects on the in vitro differentiation of blastula cap explants. Cellular responses to Xwnt signals include changes in gap junctional permeability, altered responsiveness to growth factors, and possibly changes in cell adhesion. Current data suggest that a maternal Xwnt- or noggin-like activity is involved in the Nieuwkoop center activity during mesoderm induction, that Xwnt-8 participates in a pathway of differentiation as ventral mesoderm, and that Xwnt-5A is a potential modulator of morphogenetic movements.

Key words: *Wnts*, *Xenopus*, signal transduction, mesoderm, induction

INTRODUCTION

The *Wnt* genes encode a family of cysteine-rich secreted glycoproteins which are transiently expressed in embryonic development, and in a subset of adult tissues (reviewed by McMahon, 1992; Nusse and Varmus, 1992). Functionally, all members of this family represent putative developmental signalling agents, based initially on the discoveries that Wnt-1 is a secreted protein capable of transforming mammary epithelial cells, that the segment polarity gene *wingless* is the *Drosophila* ortholog of vertebrate *Wnt*-1, and the observation that ectopic expression of Wnt-1 in *Xenopus* embryos leads to a duplication of the embryonic axis. Here we review published and ongoing studies of developmental processes in *Xenopus* that may involve *Xenopus Wnts* (*Xwnts*), as well as studies seeking to elucidate signal transduction pathways employed by Xwnts, and the cellular responses to activation of these pathways.

EXPRESSION OF Xwnts

In order to interpret the consequences of misexpression of a protein, one must first know in reasonable detail when and where related proteins are normally expressed. For example, one might conclude that Wnt-1 is unimportant for formation of the neural tube, since although it is expressed both in the dorsal midline of the neural tube and anteriorly in the brain, disruption of both *Wnt*-1 alleles affects only the brain (reviewed by McMahon, 1992; Nusse and Varmus, 1992). However, as both Wnt-1 and Wnt-3A are expressed in the neural tube (reviewed by Moon, 1993), it is possible that these Wnts have partially redundant activities, and hence Wnt-3A mimics Wnt-1 activity in the neural tube of *Wnt*-1-deficient mice. Thus, cellular or developmental responses to changes in any one Xwnt activity must consider the patterns of expression of other Xwnts.

Table 1 summarizes the timing of expression and tissue localization of *Xwnts* during embryonic development (data from Moon, 1993; Ku and Melton, 1993). In general, although there is overlap in the expression of Xwnts, particularly in the developing nervous system, most Xwnts have unique domains of expression (reviewed by Moon, 1993). For example, expression of Xwnt-1, -3A, and -4 extensively overlap in the brain, but in the neural tube Xwnt-1 and -3A are found in the dorsal midline, whereas Xwnt-4 is expressed ventrally in the floor plate. In the otic vesicle, Xwnt-3A is expressed dorsally, Xwnt-4 is expressed ventrally, and Xwnt-1 is not expressed. Although most Xwnts are not detectable in development until neural

induction, Xwnt-5A (Christian et al., 1991a) and -11 (Ku and Melton, 1993) are maternally encoded, and Xwnt-8 expression commences at midblastula transition (Christian et al., 1991b). It is also important to note that all the data in Table 1 are based on the localization of *Xwnt* transcripts, as it has been difficult to generate monospecific Xwnt antisera.

In the remainder of this review we focus on the activities of Xwnt-5A and -8, and the developmental processes that are sensitive to their overexpression. Therefore, we shall briefly summarize their spatial patterns of expression. The localization of *Xwnt*-5A transcripts in the egg through blastula stage is unknown. During gastrulation and by neurula stage, *Xwnt*-5A transcripts are enriched in the anterior and posterior of the embryo, with no discrete boundaries of expression, and transcripts are detectable in both ectoderm and mesoderm (Moon et al., 1993a). A similar pattern of expression exists in the mouse (Gavin et al., 1990). In contrast, *Xwnt*-8 displays a striking localization in the marginal zone of the gastrula stage embryo, in cells destined to become ventral and lateral mesoderm; however, *Xwnt*-8 is excluded from the dorsal marginal zone in the region of the gastrula organizer (Christian and Moon, 1993a). As addressed below, deregulation of these patterns of expression are providing insights into the potential developmental processes which involve *Wnts*, as well as the cellular responses to *Wnt* signals.

DO DISTINCT PHENOTYPES ARISE FROM TRANSIENT OVEREXPRESSION OF Xwnts?

In order to understand the developmental processes that involve a specific Wnt, one may wish to analyze the consequences of conditional lack of function as well as conditional gain of function. At present in *Xenopus* it has not been possible to block the functions of Xwnts, but transient overexpression of Xwnts has provided a powerful tool for gaining insights into developmental processes that may involve Xwnts. This and the following sections detail experiments that have been employed to identify potential Xwnt functions.

Microinjection into fertilized eggs of synthetic RNAs encoding Xwnt-1, -3A, or -8 leads to a duplication of the embryonic axis (Fig. 1A; reviewed by Moon, 1993). Microinjection of *Xwnt*-8 DNA under the control of a promoter, which is not active until midblastula transition, allows some measure of control of the timing of expression. In the case of *Xwnt*-8, the injected plasmid is transcribed at

Table 1. Expression of *Xenopus Wnts*

Xwnt	Timing	Localization Stage	Tissue
1	Neurula onwards	Neurula:	Anterior neural fold
		Tailbud:	Dorsal midline of midbrain, extends ventrally at midbrain-hindbrain boundary, dorsal midline of posterior hindbrain and neural tube
		Adult:	Not determined
2	Neurula onwards	Not determined	
3A	Neurula onwards	Neurula:	Anterior neural fold, later extending along entire anterior-posterior axis
		Tailbud:	Dorsal midline from the forebrain-midbrain boundary, through the midbrain, and into anterior neural tube. Outside CNS in dorsal otic vesicle and tailbud
		Adult:	Not determined
4	Gastrula onwards	Neurula:	Neural folds, along entire anterior-posterior axis
		Tailbud:	Forebrain-midbrain boundary, dorsal midline of midbrain, dorsal-lateral hindbrain, posteriorally into ventral midline then floor plate of neural tube. Outside CNS in ventral otic vesicle, some ganglia
		Adult:	Skin, testes, brain
5A	Egg onwards, increase at neurula	Early embryo:	Not determined
		Tailbud:	Enriched in head and tail, expressed largely in neural and non-neural ectoderm, facial processes, less in somitic mesoderm; not restricted to specific structures
		Adult:	Not determined
6	Gastrula onwards	Tailbud:	Dorsal, punctate
		Adult:	Brain, heart
7A	Tailbud onwards	Embryo:	Brain, ventral neural tube
		Adult:	Brain
7B	Tailbud onwards	Embryo:	Not determined
		Adult:	Brain, lung
8	Mid-blastula, decline by tailbud	Gastrula:	Ventral and lateral marginal zone mesoderm, excluded from Spemann organizer field
		Neurula:	Mesoderm of ventral and lateral plate, anterior neural plate
		Adult:	Not determined
8B	Gastrula, decline by tailbud	Embryo:	Not determined
		Adult:	Brain
10	Tailbud onwards	Embryo:	Dorsal hind-brain
		Adult:	Brain
11	Egg through tadpole	Cleavage:	Vegetal hemisphere
		Blastula	Dorsal marginal zone
		Gastrula:	Ventral and lateral marginal zone
		Tadpole:	Somites, branchial arch

approximately the same time as the endogenous gene (Fig. 2). When the plasmid is targeted to the dorsal marginal zone in which *Xwnt*-8 is not normally expressed, embryos develop with head malformations, and lack a notochord (Fig. 1B; Christian and Moon, 1993a). Importantly, targeting the plasmid to the region of the marginal zone, which normally expresses *Xwnt*-8, has no effect upon development. The difference in phenotype when compared to the RNA injection, which has been reviewed elsewhere (Christian and Moon, 1993b), supports the idea that the phenotype from *Xwnt*-8 RNA arises by the Xwnt acting before midblastula transition, to promote formation of a Nieuwkoop center. The phenotype arising from injection of *Xwnt*-8 plasmid in the dorsal marginal zone may involve Xwnt-8 acting to alter cellular responses to dorsalizing signals from the gastrula organizer. The differences observed in the response to pre-MBT versus post-MBT expression of the same *Xwnt*-8 signal may in part reflect the limited period when embryos can form or respond to Nieuwkoop center signals (Fig. 2).

In contrast to the two distinct *Xwnt*-8 phenotypes, injection of *Xwnt*-5A RNA leads to a complex phenotype involving shortened anterior-posterior axis, and a variety of abnormalities including dorsal-ward shifting of the cement gland and an abnormal notochord (Fig. 1C; Moon et al., 1993a). Overexpression of Xwnt-5A from microinjected plasmid constructs yields a similar though less pronounced phenotype as injection of the RNA, suggesting that the abnormal phenotype arises by the activity of Xwnt-5A after midblastula transition (Moon et al., 1993a).

What can be inferred from these phenotypes? In the case of *Xwnt*-8, the distinct phenotypes arising from ectopic expression before or after midblastula transition resemble abnormalities that might be predicted by altering the process of formation of the Nieuwkoop center, dorsal mesoderm, and the gastrula organizer, in a specific manner. In the case of *Xwnt*-5A, the phenotype suggests a perturbation of the movements of gastrulation. Although none of these phenotypes are an answer in terms of defining Xwnt activity or function - they allow one to move backwards in time with the goal of determining which processes were perturbed to give rise to this phenotype, and to determine how cells respond to Xwnt signals in a normal time frame of minutes to hours after activation of a hypothetical receptor-mediated

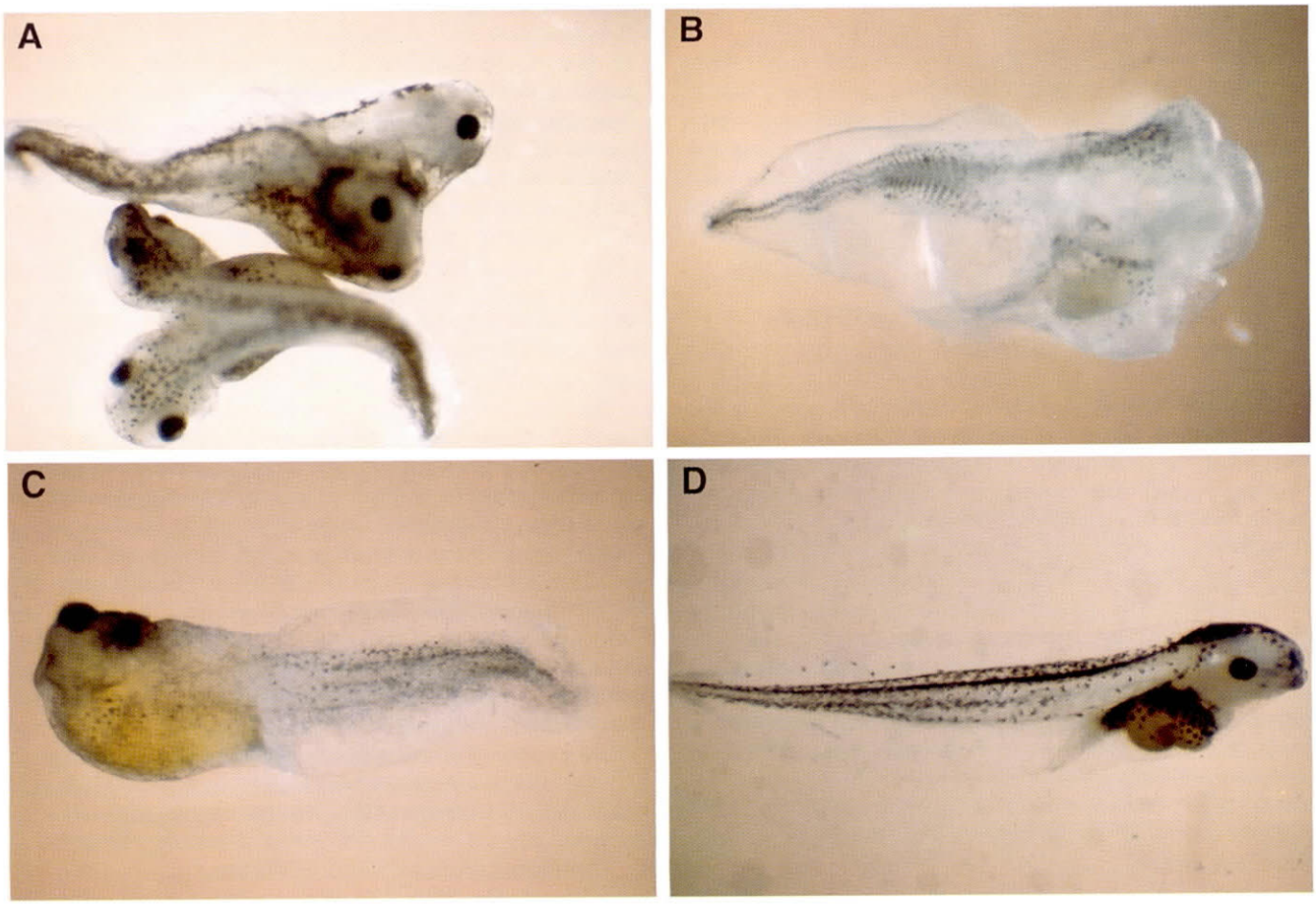


Fig. 1. Phenotypes arising by overexpression of Xwnts. (A) Injection of *Xwnt*-8 RNA into the marginal zone or vegetal hemisphere of ventral blastomeres leads to duplication of the embryonic axis. (B) Injection of *Xwnt*-8 under the control of a cytoskeletal actin promoter into the dorsal marginal zone leads to expression after midblastula transition, and to embryos lacking head structures and notochord. (C) Injection of *Xwnt*-5A RNA or expression constructs into the dorsal marginal zone leads to shortened anteroposterior axis and head defects. (D) Control tadpole. Reprinted, with permission, from *BioEssays*.

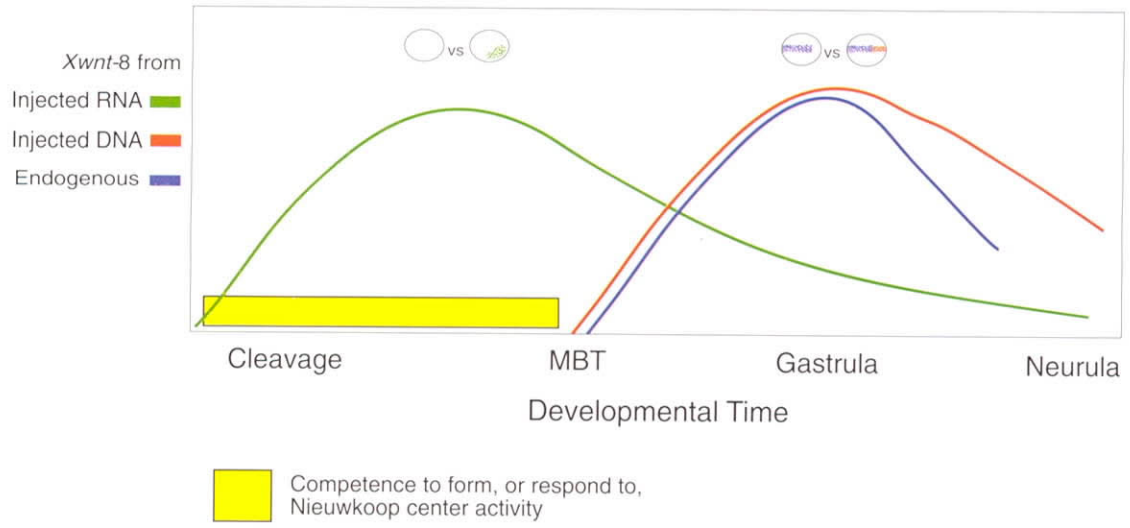


Fig. 2. Ectopic expression of Xwnt-8 from injected RNA or plasmid constructs. Expression of Xwnt-8 from synthetic RNA leads to expression in the cleavage stage embryo in the absence of endogenous Xwnt-8 expression, and overlapping the period when the embryo is competent to form, and to respond to, Nieuwkoop center activity. Expression of Xwnt-8 from expression constructs leads to expression at midblastula transition (MBT), at the same time as the endogenous gene.

signalling pathway. In the absence of an established Xwnt receptor and signal transduction pathway, this experimental scheme allows one to investigate signalling pathways which might be employed by a Xwnt receptor, and to investigate cellular targets of receptor activation, as well as how cells integrate this information to influence developmental processes.

ARE ALL EMBRYONIC CELLS COMPETENT TO RESPOND TO Xwnt SIGNALS?

The *Xenopus* embryo has distinct domains in terms of the localization of signalling agents, and the fate of cells. Regarding the localization of signalling agents, mesoderm inducing signals likely arise from the vegetal hemisphere and within the marginal zone, which is fated to give rise to mesoderm (reviewed by Kimelman et al., 1992). Regarding the fate of cells, by the 32-cell stage specific blastomeres have a predictable probability of contributing to certain structures, based on fate maps of the embryo. Thus, by injecting *Xwnt* RNA or plasmid into specific regions of the embryo, one can deduce whether specific embryonic structures are sensitive to a given Xwnt. Lack of response might be an indication of the absence of some component in the signal transduction pathway downstream of the putative signalling agent, or the presence of inhibitory activities. Lack of a response might also indicate that the signalling pathway is already operative in these cells. Positive responses to injection of *Xwnt* RNAs, monitored by the phenotype, would indicate the likely presence of a functional signalling pathway, and help one interpret whether abnormal phenotypes make sense given the fate of the injected cells, and whether the phenotypes are cell autonomous.

In these experiments one can include a marker RNA, such as β-galactosidase RNA (Smith and Harland, 1992), colloidal gold (Niehrs and De Robertis, 1991), or fluorescent dextrans to confirm that the injected blastomeres give rise to the predicted structures. These types of experiments have established that blastomeres fated to give rise to ventral structures are sensitive to injection of *Xwnt*-1, -3A, and -8 RNA, resulting in the duplication of the embryonic axis (Fig. 3). In contrast, injection of the marginal zone of dorsal blastomeres, or the animal poles of blastomeres, has no or negligible effect on development (reviewed by Christian and Moon, 1993b), presumably because the signalling pathway is already operative in dorsal cells. Surprisingly, expression of the same Xwnt-8 signal after midblastula transition, from the plasmid transcribed at the same time as the endogenous gene, reveals a reversal in blastomere sensitivity measured by phenotypic effects (Fig. 3). That is, targeting the plasmid to ventral blastomeres where the endogenous gene is expressed now has no effect on development, whereas targeting to the dorsal side where *Xwnt*-8 is not normally expressed produces head abnormalities and lack of notochord (Christian and Moon, 1993a). In considering these results, it is important to keep in mind that although targeting *Xwnt*-8 plasmids to the ventral marginal zone has no effect on development, the ventral marginal zone is the location where the endogenous *Xwnt*-8 gene is expressed, and these cells are already responding to this signal, hence additional signal may not alter the pathway of differentiation of these cells.

These results indicate that both the incidence of abnormal phenotypes (Fig. 1), and the blastomere sensitivity (Fig. 3), differ for the same Xwnt-8 signal, depending upon the time of expression (Fig. 2). In terms of sensitivity to Xwnt-5A overexpression, targeting *Xwnt*-5A RNA or plasmid expression constructs to the ventral side of 4- or 32-cell stage embryos produces few overt effects, and primarily the

dorsal blastomeres are sensitive (Figs 1, 3; Moon et al., 1993a).

A valuable complement to scoring sensitive blastomeres based on phenotype is to identify transcripts that change in abundance and/or localization in response to Xwnt signals. Whole-mount in situ hybridization is a useful assay to monitor these changes relative to control-injected embryos. Fig. 3 illustrates the blastomeres of the 4-cell stage embryo, which are sensitive to different Xwnt signals, and shows the effects of these Xwnt signals on expression of *goosecoid*, a gastrula organizer marker (Christian and Moon, 1993a; Moon et al., 1993a). Importantly, monitoring changes in gene expression in response to ectopic expression of a putative signaling agent allows one to visualize cellular responses prior to overt changes in the embryonic phenotype. Thus, one can move closer to identifying rapid cellular responses to ectopic signals, and one can use the changes in gene expression as an end point to dissect signalling pathways.

DO *Xwnts* DIVERT THE DIFFERENTIATION OF BLASTULA CAPS AWAY FROM EPIDERMIS?

Explants of the animal hemisphere of the blastula embryo (animal caps) differentiate in vitro as atypical epidermis, assuming that the explant is not contaminated with marginal zone cells, which induce mesoderm. This provides a useful cellular context in which to ask whether exogenous putative mesoderm-inducing agents will indeed divert the differentiation of the cap into dorsal or ventral mesoderm (reviewed by Kimelman et al., 1992). *Xwnts* have been tested in this assay, in the presence or absence of exogenous mesoderm-inducing factors such as basic fibroblast growth factor (bFGF) or activin.

This assay has revealed that expression of Xwnt-8 or Xwnt-5A early in development from injected RNA, does not divert the differentiation of the blastula caps into either dorsal or ventral mesoderm, and hence they are not acting as mesoderm inducing growth factors (Christian et al., 1992; Moon et al., 1993a). Xwnt-8 expressed after midblastula transition, from injected plasmid, does lead to formation of ventral mesoderm in these blastula caps, consistent with its hypothesized activity as being in a pathway giving rise to ventral mesoderm (Christian and Moon, 1993a,b). We do not know why *Xwnt*-8 RNA does not also promote ventral mesoderm formation in these blastula caps. It could be due to the lack of persistence of protein from the injected RNA, it could be that the competence modifying activity of Xwnt-8 expressed from RNA (discussed below) precludes subsequent formation of ventral mesoderm, or it could be that early expression of Xwnt-8 persistently downregulates hypothetical endogenous Xwnt-8 receptors in the blastula cap, rendering them unavailable after midblastula transition to participate in ventral mesoderm formation.

Xwnts CAN ACT AS COMPETENCE MODIFYING AGENTS

Although neither Xwnt-8 nor Xwnt-5A expressed from injected RNAs directly induce mesoderm in blastula caps, both alter the sensitivity of blastula caps to mesoderm-inducing growth factors, but in dramatically distinct ways (Fig. 4). Xwnt-8 expressed from RNA dorsalizes the response of the blastula cap to either bFGF (Christian et al., 1992) or to low concentrations of activin (Sokol and Melton, 1992). Specifically, bFGF added alone to animal caps induces ventral mesoderm and no notochord, whereas bFGF plus Xwnt-8 produces dorsal mesoderm, including a notochord. These data provide the key basis for proposals that the localized Nieuwkoop center activity of the blastula may be a *Xwnt*-like activity which works by altering the competence of cells to respond to mesoderm inducing growth factors (Kimelman et al., 1992; Moon and Christian, 1992; Christian and Moon, 1993b; Sive, 1993). As *noggin*, the prospective dorsalizing signal of the gastrula organizer

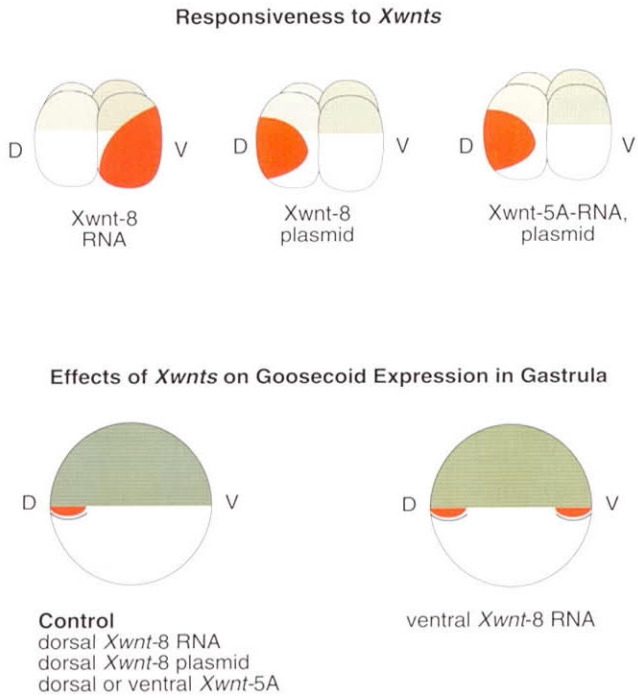


Fig. 3. Sensitivity of cells to *Xwnt* signals. The upper panel depicts responsiveness to *Xwnts* in terms of altered phenotype. Injection of synthetic *Xwnt* RNAs into dorsal (D) or ventral (V) blastomeres at the 4-cell stage reveal that ventral blastomeres are more sensitive (red area) to *Xwnt*-8, whereas dorsal blastomeres are more sensitive (red area) to *Xwnt*-5A, in terms of development with an abnormal phenotype. Dorsal blastomeres are also more sensitive to overexpression of Xwnt-8 after midblastula transition, achieved through injection of plasmid constructs. In the lower panel, prior to overt effects of *Xwnts* upon the embryonic phenotype, one can observe that *Xwnts* affect gene expression in the marginal zone. Monitoring expression of *goosecoid* by whole-mount in situ hybridization, ventral injection of *Xwnt*-1, -8, and likely -3A positively regulate *goosecoid* expression, (shown in red with a curved black line under to represent a dorsal lip at the gastrula stage), by mimicking the Nieuwkoop center activity. In contrast, *Xwnt*-5A RNA or plasmid injected on either the dorsal or ventral side has no effects on goosecoid expression. Similarly, targeting *Xwnt*-8 RNA or DNA to the dorsal side does not affect expression of *goosecoid*, presumably because there is an endogenous Nieuwkoop center activity already present.

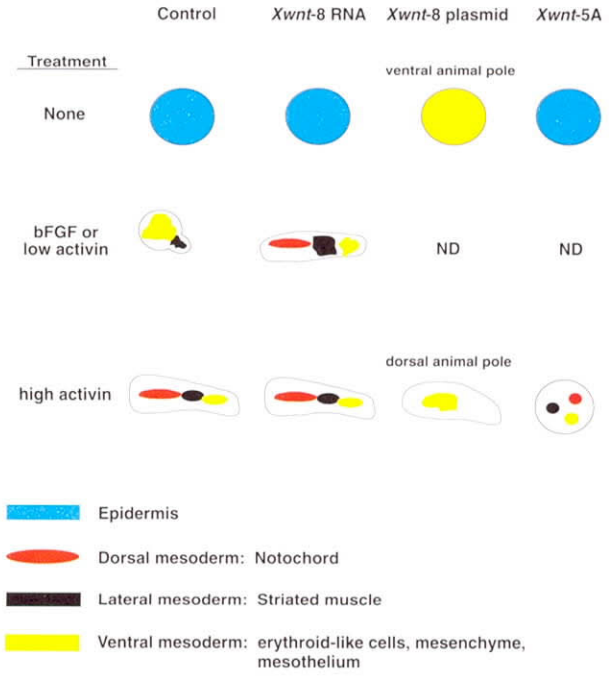


Fig. 4. Effects of *Xwnts* on the differentiation of blastula caps. Xwnt-8 and -5A expressed by injection of RNA do not induce mesoderm, whereas Xwnt-8 expressed from plasmid constructs promotes formation of ventral mesoderm. In the presence of bFGF or low concentrations of activin, *Xwnt*-8 RNA alters the differentiation from ventral mesoderm, to differentiation as dorsal mesoderm. In the presence of higher concentrations of activin, Xwnt-5A does not alter the type of mesoderm induced in response to activin, but it does block the morphogenetic movements associated with mesoderm induction. Xwnt-8 expressed from dorsal animal pole injection of an expression construct reduces the incidence of notochord formation in response to high activin (see Christian and Moon, 1993a for discussion). (ND denotes not determined.)

(Smith et al., 1993), also possesses this activity, it is unclear at present which maternal factor(s) normally function in the Nieuwkoop center (reviewed by Christian and Moon, 1993b). Interestingly, Xwnt-8 expressed from plasmids may also alter cellular competence to respond to *noggin* (Christian and Moon, 1993a).

In contrast, *Xwnt*-5A RNA does not alter the balance between dorsal and ventral mesodermal types induced in response to activin. Instead, this *Xwnt* blocks the elongation of the blastula cap typically associated with mesoderm induction (Moon et al., 1993a). Thus, *Xwnt*-5A affects cellular movements in this assay, but has no effect on differentiation.

DO *Xwnts* ALTER THE DIFFERENTIATION OF TISSUES IN UV-IRRADIATED EMBRYOS?

Cortical rotation, a slight displacement of the cytoplasm relative to the cortex, is required for activation of the Nieuwkoop center activity, which establishes the position of the gastrula organizer. UV irradiation of the vegetal hemisphere of fertilized *Xenopus* eggs early in the first mitotic cycle prevents normal cortical rotation. Thus, the embryo develops without an organizer, and develops predominantly ventral characteristics. This provides a valuable background into which investigators can introduce cells, cytoplasm, factors, or RNAs to assay for their ability to rescue dorsal mesodermal or neural structures. *Xwnt*-8 RNA can fully rescue normal development in UV-irradiated embryos and can do so even when the RNA is directed to vegetal blastomeres not giving rise to the dorsal axial structures, indicating that the injected cells have acquired Nieuwkoop center signalling activity (Smith and Harland, 1991). In contrast, *Xwnt*-5A does not rescue dorsal mesoderm or neural structures in this assay, further arguing that it is not involved in Nieuwkoop center or gastrula organizer activity (Moon et al., 1993a). Finally, *Xwnt*-11 rescues neural structures and somitic mesoderm, but not notochord, in UV-irradiated embryos, indicating that it is not sufficient to mimic Nieuwkoop center activity (Ku and Melton, 1993).

FROM PHENOTYPES TO MECHANISMS - INQUIRIES INTO CELLULAR RESPONSES TO Xwnt SIGNALS PREDICT Wnt SIGNALLING PATHWAYS

The types of experiments outlined above are generally useful for investigating the potential activities of factors and, in the case of *Xwnts*, contribute to our hypotheses on the functions of *Xwnts* during development. To pursue the mechanisms through which *Xwnts* may affect development clearly requires analyses performed soon after the controlled expression of a Xwnt signal, and requires the ability to analyze specific cellular responses.

We have investigated whether *Xwnts* have the capacity to modulate gap junctional permeability. We found that those *Xwnts* that cause a duplication of the embryonic axis (e.g., *Xwnt*-1 and -8) enhance gap junctional permeability on the ventral side of 32-cell *Xenopus* embryos, as monitored by transfer between cells of the dye Lucifer yellow (Olson et al., 1991; Olson and Moon, 1992; Fig. 5). In contrast, *Xwnt*-5A has no ability to modulate gap junctions. These data demonstrate: (1) that cells can respond within hours of receiving a Xwnt signal, even in the absence of transcription, which does not begin until midblastula stage, and (2) that some Xwnts are likely to activate different cell physiological responses. As reviewed elsewhere (Moon et al., 1993b), it is plausible but untested that the changes in gap junctional communication are an indirect consequence of changes in cell adhesion in response to Xwnt-8. Whether gap junctional permeability is normally modulated in response to endogenous Xwnt signals is not addressed by these ectopic expression studies.

How can the data on gap junctions point towards a signalling pathway? LiCl treatment of embryos also enhances gap junctional permeability in this assay (reviewed by Olson et al., 1991) and, depending on the time of administration, produces embryonic phenotypes resembling those obtained by injection of *Xwnt*-8 RNA or plasmids (reviewed by Christian and Moon, 1993b). As LiCl is thought to act via

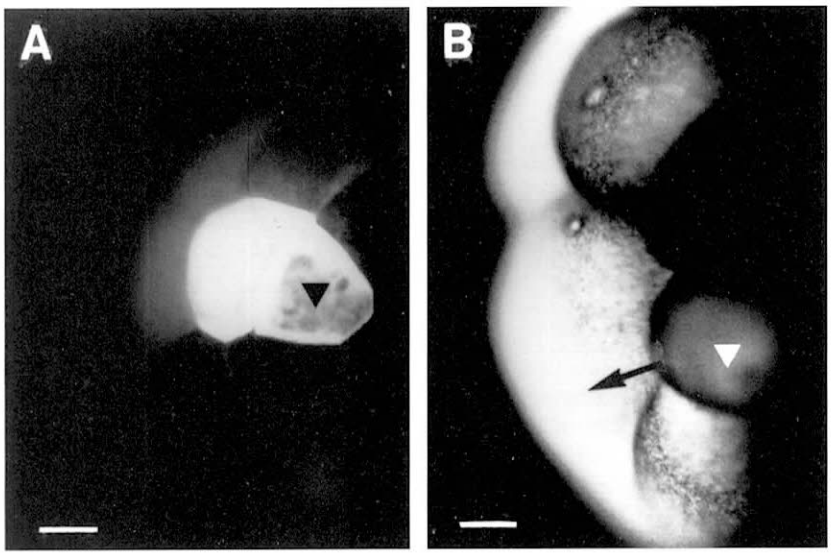


Fig. 5. Effects of *Xwnt*-8 and *Xwnt*-5A on gap junctional permeability. *Xwnt*-5A or control RNAs injected into fertilized eggs do not enhance transfer of Lucifer yellow dye in ventral blastomeres of the 32-cell embryo (A), whereas *Xwnt*-1 and *Xwnt*-8 markedly enhance gap junctional permeability (B). Cells injected with Lucifer yellow are denoted by an arrowhead, and the transfer of dye from the injected cell is indicated by the arrow in B. Scale bar, 0.12 mm (A) and 0.09 mm (B).

suppression of IP_3 levels (reviewed by Berridge, 1993), this clearly suggests that *Xwnt*-8 may work in a similar manner. In preliminary experiments (Busa and Moon, unpublished), we have found that *Xwnt*-8 does indeed suppress IP_3 levels in embryos, providing direct evidence for a signalling pathway modulated by *Wnt* activity.

One might expect that since *Xwnt*-8 and *Xwnt*-5A have distinct effects on embryos and in blastula caps (Table 2) these *Xwnts* must necessarily activate distinct signalling pathways. While this is a tenable hypothesis, until these pathways are elucidated it will remain equally plausible that these *Xwnts* activate the same signalling pathway, but the activation differs in its magnitude or duration.

EXPLOITING THE DIFFERENTIAL RESPONSIVENESS OF EMBRYOS TO *Xwnt*-8 AND *Xwnt*-5A TO IDENTIFY A DOMAIN LIKELY TO SPECIFY *Wnt* SIGNALLING

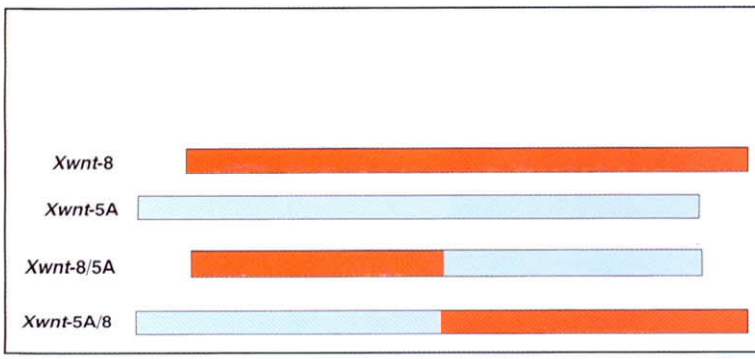
At present there has been no publication of likely Wnt receptors, though there have been a number of candidates under consideration. Moreover, there are no data indicating whether a particular region of a Wnt may be involved in evoking cellular responses, or whether the entire sequence is indispensible. Assuming that Xwnts trigger changes in gap junctional permeability and phenotypes by activation of receptor-mediated signal transduction pathways, one should be able to exploit the different effects of Xwnt-8 and Xwnt-5A to identify potential receptor-activating domains. We have initiated such a study through construction of chimeric Xwnts. As summarized in Fig. 6, a chimera consisting of the amino-terminal half of Xwnt-5A and the carboxy-terminal half of Xwnt-8 elicits Xwnt-8 effects in terms of both gap junctions and phenotypes, suggesting that the carboxy-terminal half is sufficient for a Xwnt-8 response. Consistent with the carboxy-terminal region of a Xwnt dictating the cellular response, the reciprocal chimera, consisting of the amino-terminal half of Xwnt-8 and the carboxy-terminal half of Xwnt-5A evokes responses indistinguishable from native Xwnt-5A. While these data look surprisingly clear, we have prepared additional constructs, which attempt to narrow down the region in the carboxy-terminal end required for evoking either a Xwnt-8 or Xwnt-5A response, and found that too many constructs cloud the verdict. That is, in some constructs, the amino-terminal region affects the embryonic responses. While these studies are continuing, the data presented in Fig. 6 represent the first hint of which region of a Xwnt polypeptide may be necessary for evoking a specific response, presumably via receptor activation.

MESODERM INDUCTION AND PATTERNING MAY INVOLVE AT LEAST TWO DISTINCT *Xwnt* ACTIVITIES - A MATERNAL *Xwnt* ACTING AS A COMPETENCE MODIFIER, AND *Xwnt*-8 ACTING IN A PATHWAY OF VENTRAL MESODERM FORMATION

We and others have revised the three signal model of Smith and Slack to accommodate the data on ectopic expression of *Xwnt*-8, as well as new data on other agents, such as bone morphogenetic proteins, noggin, and retinoic acid. Given the

Table 2. Distinct activities of *Xwnt*-5A and *Xwnt*-8

	Xwnt-5A	*Xwnt*-8
In embryo		
Rescue UV-treated embryo	no	yes (pre-MBT)
Alter expression of mesoderm markers	no	yes (pre- and post-MBT)
Enhance gap junctional permeability	no	yes (pre-MBT)
Blastomere sensitivity	dorsal	ventral (pre-MBT) dorsal (post-MBT)
In blastula cap explants		
Sufficient for mesodermal differentiation	no	post-MBT
Effects on dorsal mesoderm induction by activin	none	enhance (pre-MBT) diminish (post-MBT)
Effects on activin-mediated elongation	inhibit	enhance (pre-MBT)



Phenotype	Transfer of Lucifer Yellow		Gap Junction Activity
	N	%T	
Control	68	9%	–
Xwnt-8	171	44%	+
Xwnt-5A	86	9%	–
Xwnt-5A	33	12%	–
Xwnt-8	38	37%	+

Fig. 6. Effects of chimeric Xwnts on phenotype and gap junctional permeability. RNAs encoding Xwnt-8 or Xwnt-5A, or chimeras consisting of the amino terminus of one Xwnt and the carboxy terminus of the other were transcribed and injected into fertilized eggs. Embryos were scored on the basis of phenotype, or the transfer of Lucifer Yellow as a marker for gap junctional permeability (N denotes sample size, and %T denotes percentage of the embryos displaying transfer of dye to more than one adjacent blastomere).

extensive recent consideration of this issue (Kimelman et al., 1992; Moon and Christian, 1992; Christian and Moon, 1993b; Sive, 1993) it will be mentioned only briefly here, focusing exclusively on the potential roles of *Xwnts*. The first role for a *Xwnt* in mesoderm induction and patterning may occur before midblastula transition, in the localized formation of the Nieuwkoop center activity, which is involved in dorsal mesoderm induction. The duplication of the embryonic axis by *Xwnt*-1, -3A, and -8 and the effects of *Xwnt*-8 RNA in UV-irradiated embryos are consistent with these *Xwnts* mimicking the activity of the Nieuwkoop center. However, this probably does not reflect the normal role of any of these *Xwnts*. Nieuwkoop center activity and mesoderm induction commence by the 16- to 32-cell stage, and must rely on maternal components. As none of these *Xwnts* appear to be maternally expressed, we have developed several scenarios (Christian et al., 1992; Christian and Moon, 1993b). One possibility is that many *Xwnts* are functionally redundant, hence ectopic expression of *Xwnt*-1, -3A, or -8 may activate the signalling pathway normally used by an undiscovered endogenous maternal *Xwnt*. Related to this, it is possible that the maternal *Xwnt*-11 participates in Nieuwkoop center activity but requires an additional activity not present in the UV-irradiated egg assay (Ku and Melton, 1993). A second, equally plausible possibility, is that an unrelated endogenous ligand-receptor system normally activates the same second messenger system activated by these Xwnts, in which case these *Xwnts* would be a useful tool for studying this pathway, but are not involved in the normal ligand-receptor system employed in Nieuwkoop center activity. Supporting this possibility, noggin is an unrelated maternal factor which can mimic Nieuwkoop center activity when expressed from injected RNA (Smith and Harland, 1992). Our recent finding that *Xwnt*-8 expressed after midblastula transition is sufficient for formation of ventral mesoderm (reviewed below) supports yet another possible explanation for the duplication of the axis following injection of *Xwnt*-8 RNA. Perhaps ectopic expression of Xwnt-8 by injection of RNA leads to downregulation of the actual endogenous Xwnt-8 receptors. Then, after midblastula transition when the endogenous *Xwnt*-8 gene is first transcribed, there would be no available

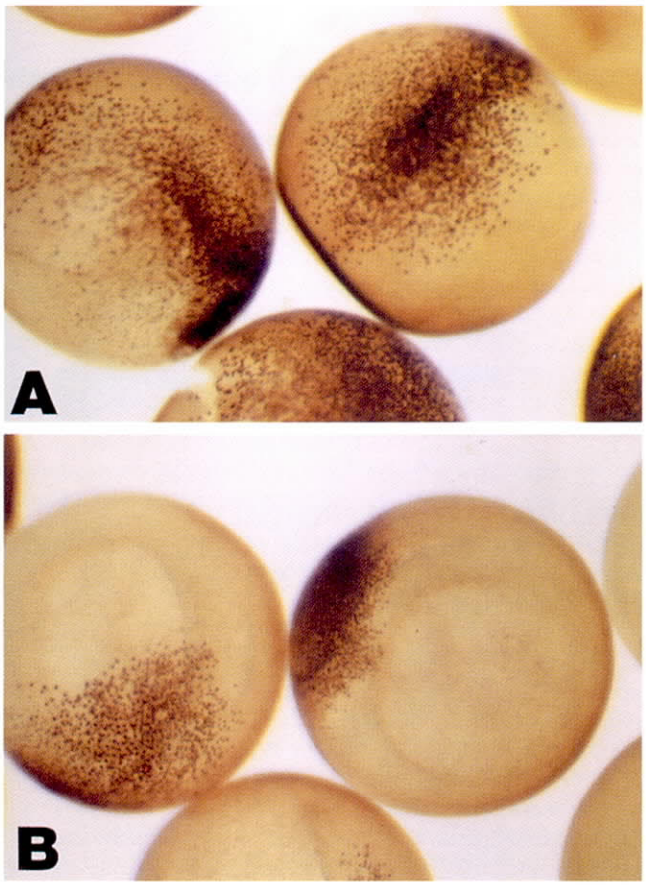


Fig. 7. Effects of Xwnt-5A on the distribution of ectodermal cells during gastrulation. Single animal pole blastomeres of 8-cell embryos were microinjected with either RNA encoding β-galactosidase (A) or a mixture of RNAs encoding β-galactosidase and Xwnt-5A (B). At the completion of gastrulation, embryos were processed for immunolocalization of β-galactosidase, with brown spots in each panel representing individual cells expressing this marker protein. In the presence of Xwnt-5A, cells expressing β-galactosidase are less widely distributed through the embryo, suggesting that Xwnt-5A may have effects upon cell adhesion and cell mixing. These preliminary results require further and more direct studies.

receptors for the endogenous *Xwnt*-8 protein, and in the absence of Xwnt-8 signaling, the embryo would become defective in formation of ventral mesoderm. Thus, dorsal mesoderm would predominate, leading to the observed phenotype.

The second proposed role for a *Xwnt* occurs after mid-blastula transition (Christian and Moon, 1993a,b), and clearly meets the criteria of a Xwnt being expressed in the right place and time relative to its demonstrated activities. Xwnt-8 is normally expressed at midblastula transition, and this expression is restricted to future ventral and lateral mesoderm. Xwnt-8 expression can be induced in blastula caps treated with either bFGF or activin. This localization and growth factor responsiveness suggest that endogenous Xwnt-8 acts downstream of these mesoderm inducing agents. As Xwnt-8, expressed from injected plasmids in blastula caps in the absence of exogenous mesoderm inducing agents, directly promotes formation of ventral mesoderm, this suggests that Xwnt-8 is indeed in a pathway leading to formation of ventral mesoderm. Furthermore, Xwnt-8 directed to the gastrula organizer region by targeted injection of plasmid leads to formation of somitic mesoderm from the organizer cells, rather than notochord, suggesting that Xwnt-8 can actively attenuate dorsalizing signals within the organizer. Thus, Xwnt-8 may direct tissue towards ventral mesoderm in the absence of the dorsalizing signal, noggin, and may function to sharpen the spatial boundaries of dorsal and ventral mesoderm in embryos by modulating the distance of action of noggin (Moon and Christian, 1992; Christian and Moon, 1993a,b; Sive, 1993).

FUTURE DIRECTIONS IN MESODERM INDUCTION AND PATTERNING

Clearly, additional studies are necessary to dissect further the respective roles of various factors in mesoderm induction and patterning. For example, several questions remain unresolved regarding the formation of ventral and lateral mesoderm. It is presently unclear whether the ability of Xwnt-8 (expressed from plasmid) to direct blastula caps towards differentiation as ventral mesoderm requires the participation of other factors in the cap. In addition, it is unclear whether *Xbra* (Cunliffe and Smith, 1992) operates in the same pathway as *Xwnt*-8. Regarding the potential dorsalizing signal of the gastrula organizer, *noggin* (Smith et al., 1993), it is unclear whether *noggin* acts in the dorsal marginal zone within the gastrula organizer to alter cell fate in a cell autonomous or non-autonomous manner, though its action upon ventral mesoderm is likely to be non cell autonomous. It is also unknown whether notochord would still form in the dorsal marginal zone in absence of *noggin* activity. Moreover, one might expect that *Xwnt*-8 plasmid in blastula caps could be employed to induce ventral mesoderm (Christian and Moon, 1993a), and that noggin co-expressed in these blastula caps from a separate plasmid (Smith et al., 1993) could then pattern this ventral mesoderm as skeletal muscle, though this is currently unknown. Finally, as dominant negative fibroblast growth factor (Amaya et al., 1991) and activin (Hemmati-Brivanlou and Melton, 1992) receptors, and a dominant mutant *ras* (Whitman and Melton, 1992) can block mesoderm formation, it is clear that the door is open for pursuing signal transduction during mesoderm induction and patterning.

Xwnt-5A: A MODULATOR OF MORPHOGENETIC MOVEMENTS?

Does endogenous *Xwnt*-5A play a role in mesoderm induction or patterning? While one can employ an ectopic expression analysis to elucidate the potential activities of a factor, this gain-of-function approach does not reveal the normal function of the endogenous gene product. However, from the data reviewed above, it appears that overexpression of *Xwnt*-5A has no discernible effect on any aspect of mesoderm induction or patterning and, unlike *Xwnt*-1, 3A, and -8, has no apparent ability to alter cell fate, or to act as an inducing agent. While overexpression of *Xwnt*-5A has no effect upon the differentiation of blastula caps in the presence or absence of activin, it does have the capacity to block the elongation of the caps in response to activin (Moon et al., 1993a). These results led us to test further whether *Xwnt*-5A modulates other cell movements in the embryo. We have investigated whether *Xwnt*-5A has effects upon the morphogenetic movements of gastrulation by excising the dorsal lip, and forcing the movements to occur in two rather than three dimensions - a technique developed by the laboratory of Ray Keller. Preliminary results from this assay reveal that *Xwnt*-5A profoundly affects the movements of gastrulation (Shih and Moon, unpublished). These consequences of overexpression of *Xwnt*-5A may be attributable to enhanced cellular adhesion, a possibility raised by the experiments discussed below.

During gastrulation, cells of the *Xenopus* embryo undergo migration and mixing with non-sister cells. Using the assay of injecting β-galactosidase RNA into single animal pole cells at the 8-cell stage, fixing the embryos at the end of gastrulation, and staining for β-galactosidase one can readily observe the end-point of cell mixing, though not the process. This assay has been employed to show that the progeny of cells injected with β-galactosidase RNA disperse, whereas cells injected with a mixture of β-galactosidase and N-cadherin RNA remain adherent to one another, consistent with the hypothesis that N-cadherin-expressing cells display increased cell adhesion (Detrick et al., 1990). To begin to investigate whether the above-mentioned effects of Xwnt-5A on the activin-induced movements of blastula caps, and upon the explants of gastrula embryos, may be due to a similar increase in cell adhesion, we have undertaken an analysis using the methods of Detrick et al. (1990), now using β-galactosidase RNA with or without *Xwnt*-5A RNA (Moon et al., unpublished). As shown in Fig. 7, this sensitive but subjective assay reveals that Xwnt-5A reduces the dispersion of β-galactosidase-expressing cells, much like the effects of N-cadherin. In multiple experiments to date, *Xwnt*-8 RNA has not had the effect of reducing cell mixing, nor does prolactin RNA, indicating some level of specificity. While these results point to future research directions, it is important to note that to monitor cell mixing requires real-time rather than end-point measurements.

The above data, the observation that both *Xwnts* and

changes in cell adhesion can alter gap junctional permeability, and the identification of homologs to the *armadillo* gene in vertebrates, strongly argue that *Xwnts* may affect cell adhesion, presumably via activation of a receptor mediated signal transduction pathway (reviewed by Moon et al., 1993b). It is curious, however, that while *Xwnt*-5A transcripts are present in the oocyte and early embryo, overexpression does not seem to perturb any aspect of early development until the movements of gastrulation commence. Thus, the normal functions of *Xwnt*-5A in *Xenopus* embryos may remain elusive until its activity can be blocked.

CONCLUSIONS

The data reviewed above suggest that multiple *Xwnt* activities participate in the lengthy process of mesoderm induction and patterning, and that cellular responses to *Xwnt*-8-like signals can include changes in IP_3 levels, gap junctional permeability, and competence to respond to bFGF, activin, and dorsalizing signals. These and undiscovered cellular responses may give *Xwnt*-8 and *Xwnts* with similar activites the capacity to alter cell fate. In contrast, in assays to date, *Xwnt*-5A has no ability to alter cell fate, but displays the activity of modulating morphogenetic movements. Given the high conservation in *Wnt* family members between species, and conservation in their patterns of expression, it is likely that an understanding of the developmental processes that involve *Wnts*, and the signalling pathways employed by *Wnts*, will continue to come from investigations of these interesting factors in diverse species.

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