

## ***Xwnt-5A*: a maternal *Wnt* that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis***

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### **SUMMARY**

To contribute to an understanding of the roles and mechanisms of action of *Wnts* in early vertebrate development, we have characterized the normal expression of *Xenopus laevis Wnt-5A*, and investigated the consequences of misexpression of this putative signalling factor. *Xwnt-5A* transcripts are expressed throughout development, and are enriched in both the anterior and posterior regions of embryos at late stages of development, where they are found primarily in ectoderm, with lower levels of expression in mesoderm. Overexpression of *Xwnt-5A* in *Xenopus* embryos leads to complex malformations distinct from those achieved by ectopic expression of *Xwnts -1, -3A, or -8*. This phenotype is unlikely to result from *Xwnt-5A* acting as an inducing agent, as overexpression of *Xwnt-5A* does not rescue dorsal structures in UV-irradiated embryos, does not

induce mesoderm in blastula caps, and *Xwnt-5A* does not alter the endogenous patterns of expression of *goosecoid, Xbra, or Xwnt-8*. To pursue whether *Xwnt-5A* has the capacity to affect morphogenetic movements, we investigated whether overexpression of *Xwnt-5A* alters the normal elongation of blastula cap explants induced by activin. Intriguingly, *Xwnt-5A* blocks the elongation of blastula caps in response to activin, without blocking the differentiation of either dorsal or ventral mesoderm within these explants. The data are consistent with *Xwnt-5A* having the potential activity of modifying the morphogenetic movements of tissues.

Key words: *Xenopus, Wnt, embryonic development, pattern formation, growth factors, morphogenesis*

### **INTRODUCTION**

There is considerable interest in the molecules and mechanisms that underlie pattern formation, and the discovery that *Wnt-1 (int-1)* is the vertebrate ortholog of the *Drosophila* segment polarity gene *wingless* (Rijsewijk et al., 1987) led to speculation that *Wnt-1* is involved in pattern formation in vertebrates. Any hypotheses on the involvement of *Wnts* in pattern formation need to accommodate the fact that *Wnt-1* is a secreted protein, which interacts with the plasma membrane (Papkoff and Schryver, 1990) and extracellular matrix (Bradley and Brown, 1990) of secreting and adjacent cells. *Wnts* may act in an autocrine or paracrine fashion through a receptor-mediated pathway to influence intercellular communication (van den Heuvel et al., 1989; Olson et al., 1991; Jue et al., 1992), though neither the receptors for *Wnt-1* nor the signal transduction pathways activated by *Wnt-1* are currently known. The recent cloning of additional members of the *Wnt* gene family from mouse (reviewed by Nusse and Varmus, 1992; McMahon, 1992) and *Xenopus* (reviewed by Moon, 1993), and the demonstration that these *Wnts* are expressed in both neural and non-neural tissues,

raises the possibility that diverse processes in vertebrate development are influenced by the local expression of specific *Wnts*.

The activities and likely functions of specific *Wnts* are best understood for *Drosophila* and murine *Wnt-1* (reviewed by McMahon, 1992; Nusse and Varmus, 1992), and a *Xenopus Wnt (Xwnt)*, *Xwnt-8* (Christian and Moon, 1993a,b). Direct evidence that *Wnt-1* participates in the formation of the vertebrate nervous system has been provided by disruption of the *Wnt-1* gene in mice by homologous recombination, leading to embryos deficient in specific regions of the brain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Supporting the idea that *Wnts* are involved in processes outside of the nervous system, there is now strong evidence that endogenous *Xwnt-8* functions downstream of mesoderm inducing growth factors to promote formation of ventral mesoderm, and to alter cellular responses to dorsalizing signals from the gastrula organizer (Christian and Moon, 1993a,b). Prior to the expression of endogenous *Xwnt-8*, a maternal *Xwnt* or *noggin* activity may operate in the Nieuwkoop center, by synergizing with mesoderm inducing growth factors to

promote the localized formation of dorsal mesoderm, and hence the gastrula organizer (reviewed by Moon and Christian, 1992; Christian and Moon, 1993a; Sive, 1993).

*Wnt-5A* is a *Wnt-1*-related gene identified in mouse (Gavin et al., 1990), *Xenopus* (Christian et al., 1991a), and zebrafish (Krauss et al., 1992). Its sequence and pattern of expression have been best characterized in mouse (Gavin et al., 1990), where it is expressed throughout development. Elevated expression of *Wnt-5A* is correlated with posterior position rather than with specific cell lineage in embryos younger than 9.5 days, though transcripts are also found throughout the embryo. In older embryos, *Wnt-5A* expression is elevated in regions of the face, and in a graded manner in developing limb buds (Gavin et al., 1990). In *Xenopus*, *Xwnt-5A* is expressed as a maternal transcript, which persists through development (Christian et al., 1991a). In the present study we have initiated our investigation of the potential functions of *Xwnt-5A* in *Xenopus* embryos, as well as the potential cellular responses to *Xwnt-5A* signals, by examining the spatial pattern of *Xwnt-5A*, and the consequences of overexpression of this *Xwnt*.

## MATERIALS AND METHODS

### cDNA isolation and sequencing of *Xwnt-5A*

A cDNA partially encoding *Xwnt-5A* (Christian et al., 1991a) was used to screen (Maniatis et al., 1982)  $1.2 \times 10^6$  independent bacteriophage from a *Xenopus* oocyte cDNA library in *gt10* (Rebagliati et al., 1985). Three bacteriophage rescreened positive, though two later proved to be identical cDNAs. Bacteriophage DNA was isolated from the unique clones G5 and G6, and the cDNA inserts were isolated from low melting point agarose and ligated into pGem 2 (Promega). Restriction fragments of these cDNAs were subcloned into pGem4 and fully sequenced on both strands using the dideoxy chain termination method (Sanger et al., 1977), employing SP6 and T7 promoter primers (Promega), as well as unique primers (17 nt). The composite DNA sequence was analyzed with a DNA Inspector IIe program (Textco).

### In vitro transcription and microinjection

To prepare the construct G6EcoR1/SP64T, an *EcoR1* insert from the G6 clone (approximately 2 kb) was blunted with Klenow fragment, cloned into the unique *BglIII* site of SP64T (Krieg and Melton, 1984), and transformed into *E. coli* strain DH5. The construct was linearized with *XbaI*, followed by in vitro transcription in the presence of cap analogue (Moon and Christian, 1989). The integrity and size of all transcribed RNA was verified by separation on formaldehyde agarose gels. The ability of the RNA to be translated to yield a polypeptide of the predicted size was established by in vitro translation in a rabbit reticulocyte lysate and separation of the translation products on SDS polyacrylamide gels. The coding region of *Xwnt-5A* was also cloned into a vector under the control of a cytoskeletal actin promoter, as described by Christian and Moon (1993b).

Synthetic RNAs or DNAs were microinjected into fertilized *Xenopus* eggs, or into blastomeres of the developing embryo (see text), as previously described (Moon and Christian, 1989). In some experiments beta-galactosidase RNA was transcribed from a plasmid (a gift of R. Harland) and mixed with the *Xwnt-5A* RNA. In other experiments, fertilized eggs were UV-irradiated early in the first cell cycle (Christian et al., 1991), prior to microinjection of *Xwnt-5A* RNA. Following injection, RNA was extracted from 5-10 embryos at selected times (Moon and Christian, 1989), and

separated on formaldehyde agarose gels next to standard curves of synthetic *Xwnt-5A* RNA. The gels were blotted to nitrocellulose and probed with  $^{32}\text{P}$ -labeled random primed (Feinberg and Vogelstein, 1984) *Xwnt-5A* DNA probes.

### Isolation and culture of blastula caps in the presence or absence of activin

To assess the effects of *Xwnt-5A* on the differentiation of isolated blastula caps, the animal poles of both blastomeres of two cell embryos were injected with 25-75 pg *Xwnt-5A* RNA, and blastula caps corresponding to approximately the upper one quarter to one fifth of stage 7-8 blastula were obtained (Christian et al., 1992). Blastula caps were cultured in the presence or absence of 10 ng/ml recombinant human activin A (a gift of Genentech) until control embryos reached stage 40. Explants were then fixed, embedded, sectioned (Christian et al., 1992, Christian and Moon, 1993b) and scored morphologically for various mesodermal cell types (Green et al., 1990).

### Histology and whole-mount immunocytochemistry

To assess developmental defects attributable to overexpression of *Xwnt-5A*, embryos were fixed, embedded in paraffin, sectioned, and stained (Kelly et al., 1991). Embryos were also processed for whole-mount immunocytochemistry (Klymkowsky and Hanken, 1991), employing the neural specific monoclonal antibody 2G9 (Jones and Woodland, 1989), the muscle-specific monoclonal antibody 12/101 (Kintner and Brockes, 1984), or commercial (BioRad) antibodies to beta-galactosidase.

### Embryo microdissection and RNase protection assay

At selected stages (see text), RNA was isolated from embryos and digested with DNase I (Moon and Christian, 1989). At stage 40, embryos were microdissected into four regions: the head, tail, mid-dorsal region, and mid-ventral region, then RNA was isolated as above.

For RNase protection assays, the PCR-derived *Xwnt-5A* clone described previously (Christian et al., 1991a) was linearized with *BamHI*,  $^{32}\text{P}$ -labeled antisense RNA was transcribed in vitro with SP6 RNA polymerase then samples were processed for RNase protection using standard protocols (Ausubel et al., 1988). As a control, equivalent proportions of RNA were electrophoresed on 1.3% agarose-formaldehyde gels, blotted to nitrocellulose, and probed with a  $^{32}\text{P}$ -labeled EF1 probe.

To quantify the levels of *Xwnt-5A* in development, 100  $\mu\text{g}$  of total RNA from each developmental stage was processed in the RNase protection assay. Synthetic target *Xwnt-5A* RNA was mixed with carrier tRNA and used to generate a standard curve, with tRNA as a negative control. Protected bands on the autoradiogram were scanned with an Isco densitometer, and the relative peak areas were determined by weight. The level of endogenous *Xwnt-5A* transcripts was in part calculated on the assumption of approximately 5  $\mu\text{g}$  of total RNA for early embryos (Gurdon and Wickens, 1983).

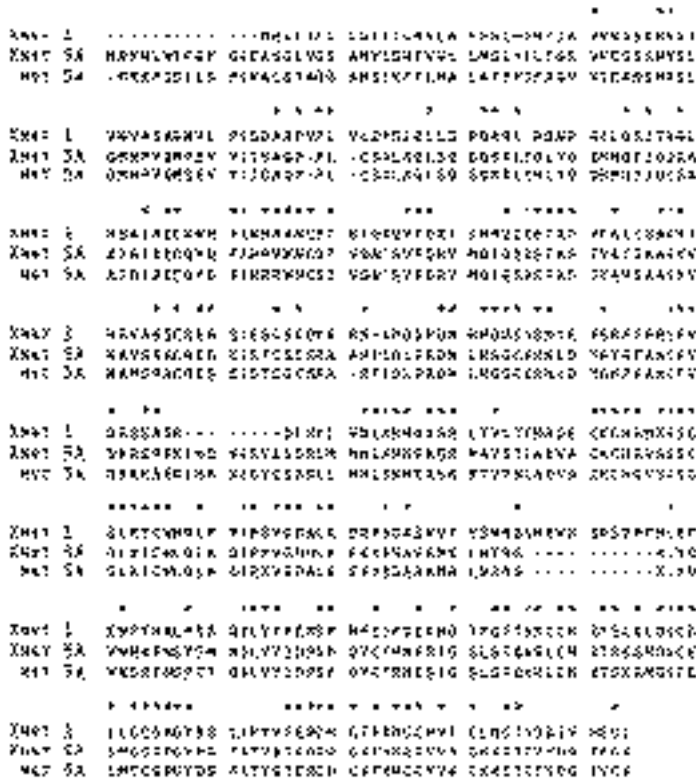
### Whole-mount in situ hybridization

Digoxigenin-labeled sense and antisense probes for *Xwnt-5A* RNA, *Xwnt-8* (Christian and Moon, 1993b), *goosecoid* (Christian and Moon, 1993b), and *Xbra* (Smith et al., 1991) were size-reduced and hybridized to fixed embryos (Harland, 1991). Following photography of the whole mounts, embryos were embedded, sectioned and photographed (Kelly et al., 1991).

## RESULTS

### Sequence analysis of *Xwnt-5A*

The predicted amino acid sequence of *Xwnt-5A* is shown in



**Fig. 1.** Comparison of the predicted amino acid sequences of *Xwnt-5A* (Genbank accession number L19716), murine *Wnt-5A* (Gavin et al., 1990), and *Xwnt-1* (Noordermeer et al., 1989). Identical residues are marked by asterisks, and gaps indicated by dashes were introduced to yield the highest degree of identity.

Fig. 1, aligned with the amino acid sequences of *Xenopus Wnt-1* (*int-1*; Noordermeer et al., 1989) and murine *Wnt-5A* (Gavin et al., 1990). Sequencing of 260 nt 5' of the putative start site did not establish an alternative open reading frame (data not shown). *Xwnt-5A* has 85% amino acid identity with *Wnt-5A*, only 42% identity with *Xwnt-1* (Fig. 1), and similarly low identity to other murine *Wnts* (data not shown). Significantly, the positions of 23 of 24 cysteine residues are absolutely conserved between *Xwnt-5A* and *Wnt-5A* (Fig. 1), with the sole exception at position 36. This high degree of conservation of the positions of cysteines is diagnostic for members of the *Wnt* family (Gavin et al., 1990; Christian et al., 1991a). The predicted hydrophobic leader sequence preceding the two tryptophans at positions 47-48 are not conserved between *Xwnt-5A* and *Wnt-5A*, although both are equivalently hydrophobic (data not shown). Four potential glycosylation sites are completely conserved between *Xwnt-5A* and *Wnt-5A* (NCS, NTS, NES, and NKT, starting at amino acid positions 117, 123, 326, and 340, respectively, in Fig. 1).

**Expression of *Xwnt-5A* transcripts during early development**

Having established that the cDNAs did indeed encode the *Xenopus* ortholog of murine *Wnt-5A*, we first used RNase protection assays to quantify the level of endogenous *Xwnt-5A* transcripts during early development. RNase protection assays revealed that *Xwnt-5A* transcripts are detectable in oocytes, and in all stages of developing embryos (Table 1), consistent with RNA blot analysis (Christian et al., 1991a). The absolute level of *Xwnt-5A* transcript declines about 10-fold during early development, from about 1 pg transcript per cleavage stage embryo to 0.1 pg per embryo by gastrula stage. Levels of *Xwnt-5A* transcript then increase 20- to 30-

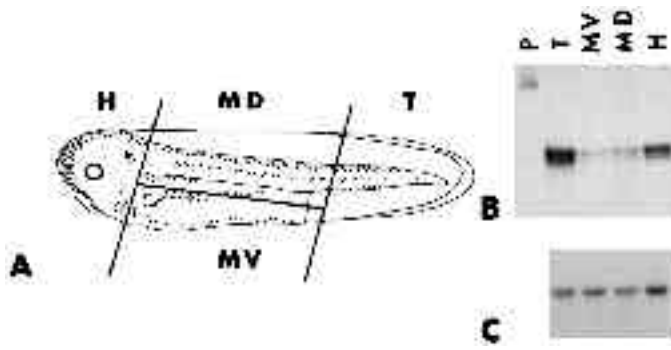
**Table 1. Expression and overexpression of *Xwnt-5A* in *Xenopus* embryos**

	Oocyte	Cleavage	Blastula	Gastrula	Neurula	Tadpole	Phenotypes (%) <sup>†</sup>
<i>Xwnt-5A</i> RNA* (pg per embryo)							
Endogenous	1.6	1.3	0.5	0.1	2.3	2.9	
Injected	-	75	ND	55	13	ND	
(Injection survival (%)) <sup>‡</sup>							
Control (n=150)	-	-	100	ND	93	66	head 6 tail 0
RNA injected							
High dose (n=152)	-	-	100	ND	88	66	head 84 tail 16
Low dose (n=133)	-	-	100	ND	92	60	head 58 tail 8
Plasmid injected							
Sense (n=87)			100	ND	83	ND	head ~90
Antisense (n=72)			100	ND	100	ND	head 0

\*The endogenous levels of *Xwnt-5A* RNA (in pg) at each developmental stage were estimated by RNase protection assay, using synthetic *Xwnt-5A* RNA to derive a standard curve. Amounts of synthetic *Xwnt-5A* injected into eggs, and its persistence in development, was determined in separate experiments by RNA blot analysis. All levels of *Xwnt-5A* are in pg per embryo. ND, not determined.

‡Eggs were injected with no RNA (control, see also prolactin and inactive *Wnt* injection controls in text), or with a high dose of *Xwnt-5A* RNA (approximately 75 pg), or a low dose of *Xwnt-5A* RNA (approximately 20 pg/embryo). In addition, 4-cell embryos were injected in the dorsal marginal zone with 100-200 pg of plasmid containing *Xwnt-5A* in the sense or antisense orientation under the control of a cytoskeletal actin promoter. Embryos were cultured to the stages indicated, and N denotes the number of initial embryos in each culture. Numbers at each developmental stage represent the percentage of initial embryos surviving to this stage. Data for the RNA injections are combined from two similar experiments.

†Percentage of embryos at the tadpole stage with a head or tail defect, with some embryos scored for both defects.



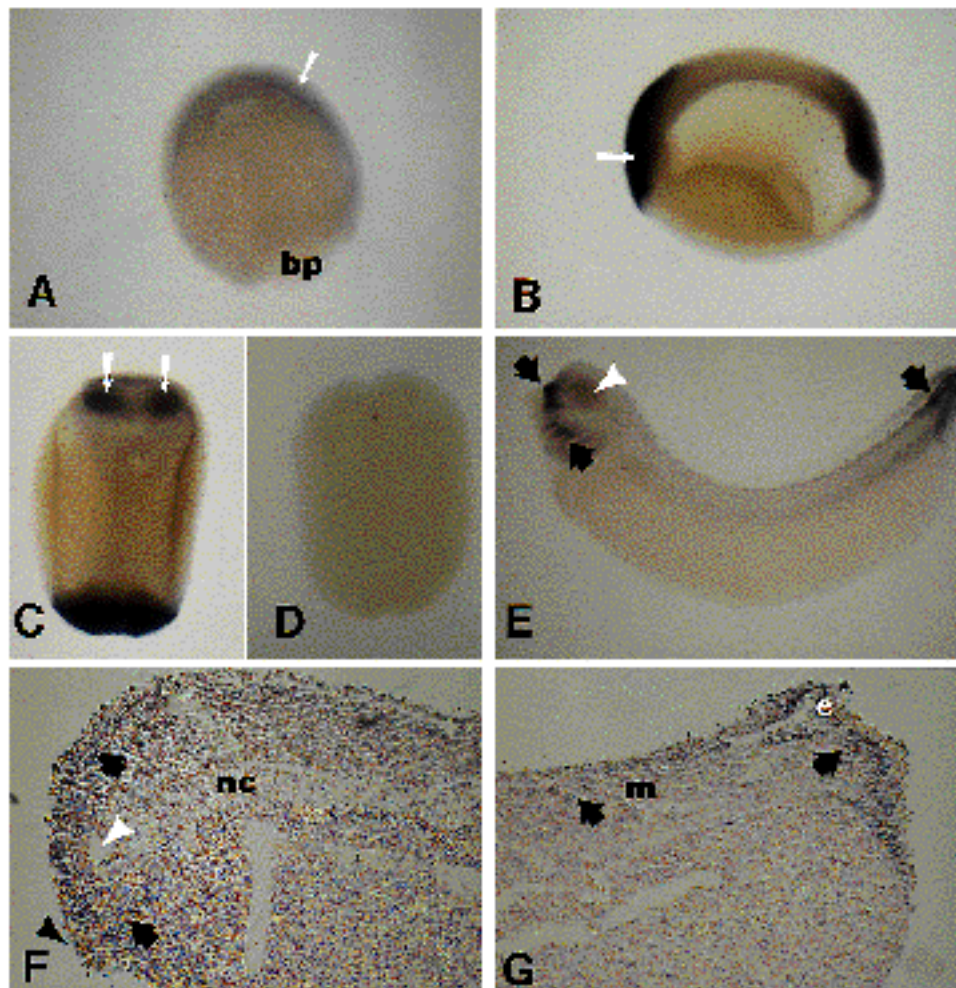
**Fig. 2.** Localization of *Xwnt-5A* transcripts in tailbud embryos by RNase protection. (A) Tailbud stage embryos were dissected into head (H), mid-dorsal (MD), mid-ventral (MV) and tail (T) regions as indicated by the solid lines. (B) RNA was isolated from the dissected regions, and subjected to an *Xwnt-5A*-specific RNase protection assay. The P lane contains undigested probe, and the remaining lanes, labeled as in A display protected fragments of the predicted size. (C) RNA employed in the RNase protection assay in B was employed in an RNA blot analysis with an EF1 probe to provide a reference for the relative levels of expression of *Xwnt-5A*.

fold by neurula and tadpole stages (Table 1). As embryonic transcription commences at mid-blastula transition our

analysis of the spatial distribution of *Xwnt-5A* transcripts (below) is likely based on the localization of newly synthesized transcripts.

We next used the RNase protection assay as an initial approach for asking whether *Xwnt-5A* was expressed uniformly throughout embryos, or in a spatially restricted manner. Tadpole embryos at approximately stage 40 were chosen for analysis, as *Xwnt-5A* is more abundant later in development (Christian et al., 1991a; Table 1). Embryos were microdissected into head, tail, mid-dorsal region, and mid-ventral region (Fig. 2A), RNA was isolated, and equivalent amounts of RNA were tested in the RNase protection assay. As shown in Fig. 2B, *Xwnt-5A* transcripts were detectable in all four regions of the embryo, but were present at higher levels in the head and tail regions compared to the other two regions, upon normalization to an elongation factor 1 probe (Fig. 2C).

The second approach used for investigating the localization of expression of *Xwnt-5A* employed whole-mount in situ hybridization. As shown in Fig. 3A, hybridization of the antisense probe to late gastrula stage embryos demonstrates that *Xwnt-5A* transcripts are detectable in a diffuse pattern, enriched in the ectoderm. By the late neurula stage *Xwnt-5A* transcripts are enriched in the anterior and posterior of the embryo, relative to the middle of the embryo (Fig. 3B,C). This pattern persists in the tadpole, at which stage hybridiz-



**Fig. 3.** Localization of *Xwnt-5A* transcripts by whole-mount in situ hybridization. Developing embryos at (A) the late gastrula (approximately stage 11 1/2); (B, lateral view, C, dorsal view) late neurula (stage 20); and (E) tadpole (stage 34) were hybridized with an antisense *Xwnt-5A* probe. A dorsal view of control neurula stage embryo hybridized with a sense *Xwnt-5A* probe is shown in D. Random tadpole embryos were then embedded and sectioned to analyze further the expression of *Xwnt-5A* in the head (F) and tail (G). White arrows in B and C denote anterior *Xwnt-5A* hybridization signals. Black arrows (E,F,G) denote specific *Xwnt-5A* hybridization signals. For reference, the eye is denoted by a white arrowhead in E and F, the cement gland is indicated by a black arrowhead in F, and the blastopore (bp), notochord (nc), tail ectoderm (e), and skeletal muscle (m) are noted.



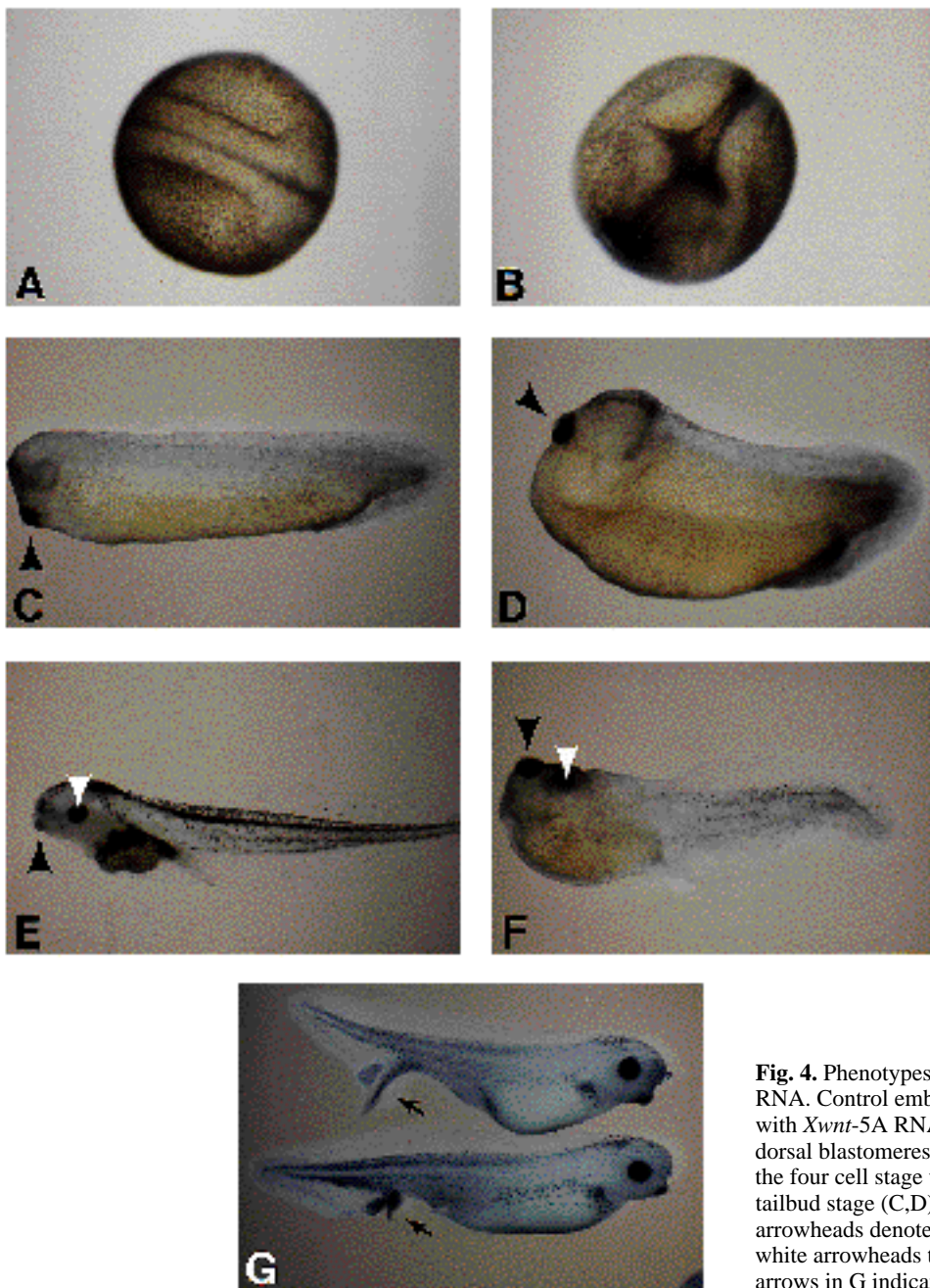
ation of the probe is also detected at lower levels in dorsal tissues throughout the embryo (Fig. 3E). This expression of *Xwnt-5A* throughout tailbud and tadpole embryos, with highest levels at the anterior and posterior ends, is consistent with the above data from the RNase protection assay (Fig. 2).

Following whole-mount in situ hybridization, selected embryos were dehydrated, embedded in paraffin, sectioned, stained with eosin, and examined by bright-field microscopy. At the neurula stage, the ectoderm and mesoderm are the primary tissue layers expressing *Xwnt-5A* (data not shown). In the head of tadpole stage embryos, *Xwnt-5A* is expressed at highest levels in both neural and non-neural ectoderm (Fig. 3F, large black arrows), with neg-

ligible expression in the notochord. As in mouse (Gavin et al., 1990), *Xwnt-5A* is expressed in facial processes, such as branchial arches (Fig. 3E, lower arrow). Expression of *Xwnt-5A* in mesodermal cells is evident in the trunk and tail, with specific hybridization in somitic mesoderm indicated in Fig. 3G by the left arrow. The expression of *Xwnt-5A* transcripts in the tail includes both mesodermal and ectodermal cells (Fig. 3G). However, the nature and fate of *Xwnt-5A* expressing cells is not clearly established.

**Effects of overexpression of *Xwnt-5A* on embryonic development**

Fertilized eggs were injected with *Xwnt-5A* RNA and the embryos were analyzed for the persistence of the injected



**Fig. 4.** Phenotypes of embryos injected with *Xwnt-5A* RNA. Control embryos (A,C,E), and embryos injected with *Xwnt-5A* RNA into the upper marginal zone of dorsal blastomeres (B,D,F) or ventral blastomeres (G) at the four cell stage were cultured to neurula stage (A,B), tailbud stage (C,D), or tadpole stages (E,F,G). Black arrowheads denote the location of the cement gland, and white arrowheads the position of the eye. The black arrows in G indicate supernumerary tails.

RNA at selected stages, and for their phenotype. In representative experiments, embryos were injected with approximately 20 pg *Xwnt-5A* RNA (low dose) or 75 pg RNA (high dose) (Table 1). Approximately 75% (55 pg) of the high dose persisted to the gastrula stage, and about 17% (13 pg) persisted to the neurula stage (Table 1).

We next analyzed the phenotypes of embryos injected with *Xwnt-5A* RNA. Embryos injected with 20-75 pg of *Xwnt-5A* RNA developed with abnormal phenotypes, which were classified into head and/or tail malformations (Table 1, Fig. 4). In representative experiments, 84% of injected embryos developed complex head abnormalities, and 16% of embryos developed supernumerary tails or had other tail defects (Table 1). Shortening of the tail was not scored, though it was apparent to varying degrees in most embryos. A small number of the embryos analyzed in Table 1 had both head and tail malformations. The duplication of the embryonic axis produced by injection of 10 pg of *Xwnt-8* RNA (Christian et al., 1991b, 1992) was not observed even after injection of 1 ng of *Xwnt-5A* RNA (data not shown), suggesting that these *Xwnts* elicit distinct responses in the embryo. To provide negative controls for injection, we injected up to 1 ng of RNA that encoded either *Wnt-1* with a cysteine mutation (McMahon and Moon, 1989), or bovine prolactin (a gift of Peter Walter, UCSF), and did not observe any developmental abnormalities (data not shown).

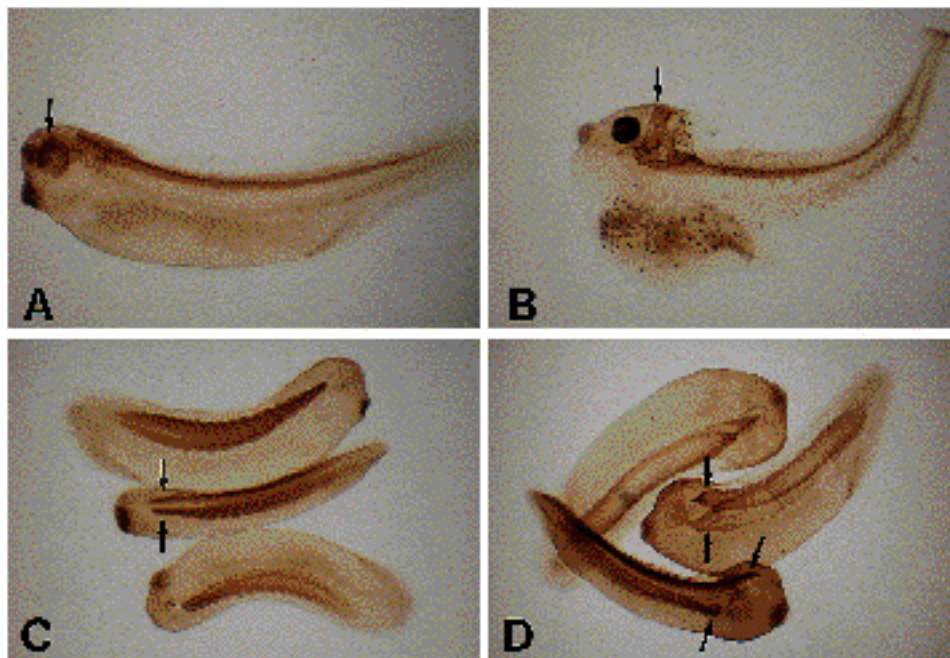
We next directed the *Xwnt-5A* RNA to the upper marginal zone of either dorsal or ventral blastomeres at the four cell stage to investigate how this would affect the incidence of the observed phenotypes. In pooled data from three experiments, injection of *Xwnt-5A* RNA into both dorsal blastomeres produced the head abnormalities described above at higher frequency (95%,  $n=92$  embryos). No duplications of the tail were observed, though the single tail was generally shortened compared to controls. In contrast, injection of *Xwnt-5A* RNA into both ventral blastomeres at the four cell stage produced primarily wild-type embryos (80%,  $n=90$

embryos), with a low incidence of embryos with a defective tail (14%) and a greatly reduced incidence of embryos with head abnormalities (6%).

Embryos injected dorsally with *Xwnt-5A* RNA often display incomplete closure of the anterior neural tube (Fig. 4B compared to controls, 4A). When control embryos hatch (Fig. 4C), injected embryos display a greatly shortened anterior-posterior axis. At the posterior end, they are near-normal, though the tails are somewhat shortened. At the anterior end, head structures appear compressed with the cement gland rotated towards the dorsal side (Fig. 4D). Even after control embryos reach the tadpole stages (Fig. 4E), embryos injected dorsally with *Xwnt-5A* RNA are still considerably shorter (Fig. 4F). Finally, a low percentage of embryos injected at the one cell stage (Table 1), or injected ventrally at the four cell stage, develop with a supernumerary tail (Fig. 4G).

Significantly, dorsal injection of *Xwnt-5A* plasmid under the control of a cytoskeletal actin promoter, which is not expressed until after midblastula transition (Christian and Moon, 1993b), yields a similar though somewhat less pronounced phenotype as the injected RNA (Table 1). Therefore, the primary developmental abnormalities associated with overexpression of *Xwnt-5A*, the complex head malformations and shortening of the tail, are likely to result from the activity of *Xwnt-5A* after midblastula transition.

Whole-mount immunocytochemistry with muscle-specific and neural-specific monoclonal antibodies was then employed to further our analysis of the phenotypes arising from overexpression of *Xwnt-5A*. Embryos injected with *Xwnt-5A* RNA in the upper marginal zone of both dorsal blastomeres at the four cell-stage, and cultured to tadpole stages, stained with the pan-neural monoclonal antibody 2G9 (Jones and Woodland, 1989) and revealed an absence of neural tissue anterior to the eyes. Curiously, the anterior neural axis displays a dorsal-ward shift in these embryos (Fig. 5B) when compared with controls (Fig. 5A). In



**Fig. 5.** Whole-mount immunocytochemistry of tailbud and tadpole embryos injected with *Xwnt-5A* RNA. Control embryos (A,C) and embryos injected with *Xwnt-5A* RNA in the upper marginal zone of both dorsal blastomeres at the 4-cell stage (B,D) were stained with the pan-neural mAb 2G9 (A,B), or the skeletal muscle-specific mAb 12/101 (C,D). Arrows in A and B denote the anterior limit of neural staining in dorsally-injected embryos. Arrows in D denote the anterior widening between somitic mesoderm in embryos dorsally injected with *Xwnt-5A* relative to controls (C).

addition, eyes often form more medially than in controls. Staining of *Xwnt-5A*-injected embryos at the tadpole stage with the muscle-specific monoclonal antibody 12/101 (Kintner and Brockes, 1984) revealed a broadening of, and a subtle bifurcation of, dorsal-anterior structures (compare embryos in Fig. 5D with control embryos, in Fig. 5C). The anterior staining pattern with mAb 12/101 is not always symmetrical relative to the dorsal midline of the embryo after injection of *Xwnt-5A* RNA (Fig. 5D).

### Histological analysis of embryos injected with *Xwnt-5A* RNA

#### (1) Pattern anomalies along the anterior-posterior axis

Following injection of *Xwnt-5A* RNA into the upper marginal zone of both dorsal blastomeres at the four cell stage, random embryos were fixed, embedded and sectioned to extend the above analyses of the abnormal phenotype. Significantly, in all embryos examined, head formation was disrupted, though to differing degrees. In addition, although tails appear superficially normal in these embryos (Table 1, Fig. 4), histological examination generally revealed defects. The first category of developmental defect is the malformation of the axial mesoderm and the overlying nerve cord at specific anteroposterior levels. For example, in the head (Fig. 6D) the nasal placode has fused into a single structure of greater width than that of control embryos (Fig. 6C). In such embryos there is also a corresponding reduction in the size of the telencephalon, along with a dorsal-ward shift of the forebrain (see also Fig. 5B versus control, 5A). This shift placed the nasal placode into the same plane of section as the notochord and somites. Although in this instance the eyes and the diencephalon appear fairly normal (Fig. 6D), in others the diencephalon was much smaller and the eyes fused medially (Fig. 6J versus control Fig. 6I). When the eyes fuse, the telencephalon could not be identified morphologically and the nasal placode does not form (data not shown). Also in those embryos where the eyes fuse, the anterior end of the notochord appears as a bulbous swelling (Fig. 6F), though the somitic mesoderm lateral to the swelling is relatively normal (see also Fig. 5D). Most embryos injected dorsally with *Xwnt-5A* RNA develop notochords that vacuolate but are malformed at the anterior end (Fig. 6D,F). Posteriorly, most embryos injected dorsally with *Xwnt-5A* RNA have well defined midbrain-hindbrain junctions (data not shown), a near-normal rhombencephalon, and normal otic vesicles (ot, in Fig. 6D,F).

The severity of the abnormalities found in the tail varied widely. In more extreme cases, the tail end of the animal consists of a mass of dissociated cells, comprised of endoderm, mesoderm, and neural ectoderm. This disorganization appears gradually, and is preceded by progressively more and more dilated somites (Fig. 6H). In those cases where the tail has formed normally, an unusual position-specific disruption of the axial tissue array is evident. For example, the embryo in Fig. 6H exhibits degeneration of three somite segments and an accompanying notochord malformation in those segments. The body ectoderm, overlying neural tube and underlying endoderm at this position in the tail all appear abnormal.

#### (2) Pattern anomalies along the mediolateral axis

A second category of pattern anomalies arising by overexpression of *Xwnt-5A* is most evident in the trunk region. In approximately 20% of the embryos there was an evident shift in the position of the neural tube with respect to the notochord (Fig. 6B,G), though there was no apparent local truncation or degeneration of axial tissues. In these embryos, the somitic mesoderm on the side containing the notochord appears fairly normal, while the neural plate is enlarged on the opposite side (e.g., Fig. 6B). In addition, over 90% of these animals had an intermittent longitudinal groove along the middle of the notochord (Fig. 6D). In longitudinal sections, such grooves appear as 'dark' septa along the length of the notochord. In two out of ten cases, such dark septa were continuous with true bifurcations of the notochord either anteriorly or posteriorly (Fig. 6E).

The pattern abnormalities along both the antero-posterior and medio-lateral axes were observed in multiple experiments, and at both high and low doses of *Xwnt-5A* RNA. In 10 embryos injected dorsally with plasmid-expressing *Xwnt-5A* in the sense orientation, similar though less pronounced malformations were observed. Embryos injected with antisense orientation *Xwnt-5A* plasmids developed normally. Thus, the complex phenotypic effects of overexpression of *Xwnt-5A* likely arise through its action after mid-blastula transition, as supported by experiments described later.

### Determination of blastomere sensitivity to overexpression of *Xwnt-5A*

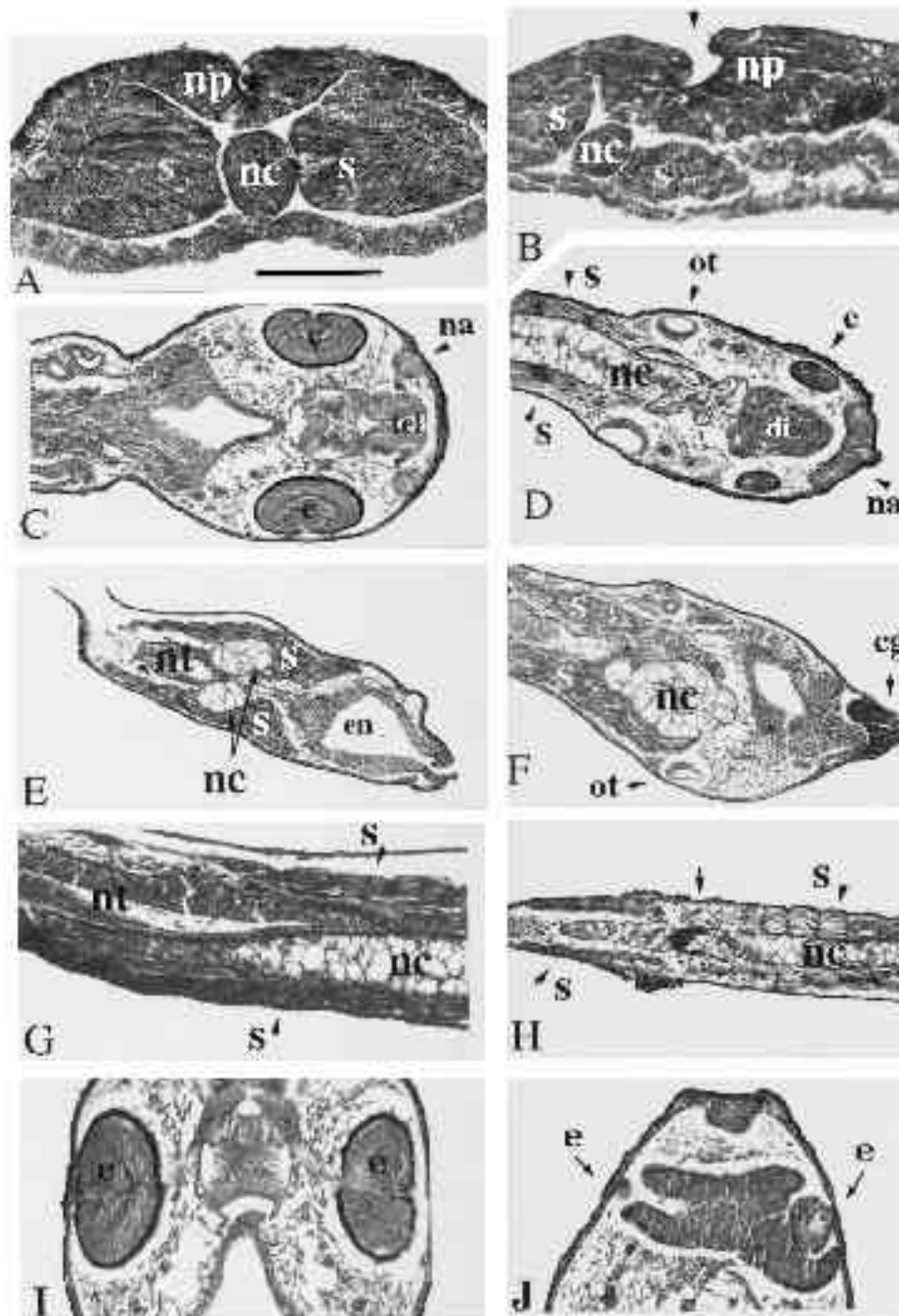
We investigated the sensitivity of different dorsal blastomeres to overexpression of *Xwnt-5A*. In preliminary experiments, embryos were injected at the 4-cell stage with mixed beta-galactosidase and *Xwnt-5A* RNAs, cultured to neurula or hatching stages, processed for immunocytochemistry with the anti-beta-galactosidase antibody, embedded in paraffin, and sectioned. In embryos displaying an abnormally formed and/or positioned notochord, the anterior neural structures contained detectable beta-galactosidase, whereas the notochord generally did not (arrow, Fig. 7A). Thus, the shift in neural structures, and the displacement of the notochord, are likely to be the consequences of overexpression of *Xwnt-5A* in neural ectoderm, rather than a specific effect only on the notochord. As noted above, the head and tail were perturbed to a greater extent than the trunk in embryos overexpressing *Xwnt-5A*. Thus, in the trunk region of embryos co-injected with beta-galactosidase and *Xwnt-5A* RNA, we often observed normal neural tubes and/or notochords staining for beta-galactosidase (Fig. 7B).

At the 32-cell stage of development, specific dorsal blastomeres have a high probability of contributing to specific neural or mesodermal structures (Dale and Slack, 1987; Moody, 1987). Therefore, to assess in greater detail which dorsal structures of the 32-cell embryo were most sensitive to overexpression of *Xwnt-5A*, we mixed *Xwnt-5A* RNA, at a level sufficient to cause defects when injected dorsally at the four-cell stage, with beta-galactosidase RNA, to confirm which blastomeres were injected, but without implying that beta-galactosidase is a strict lineage marker. The mixture of RNAs was injected into two dorsal midline



blastomeres of tier one, two, or three of 32-cell embryos, using embryos from the same female. In two representative experiments, the frequency of head abnormalities was 6% after tier one injection ( $n=64$ ), 74% after tier two injections ( $n=78$ ), and 31% ( $n=98$ ) after tier three injections (Fig. 7). As expected, based on fate maps of the early

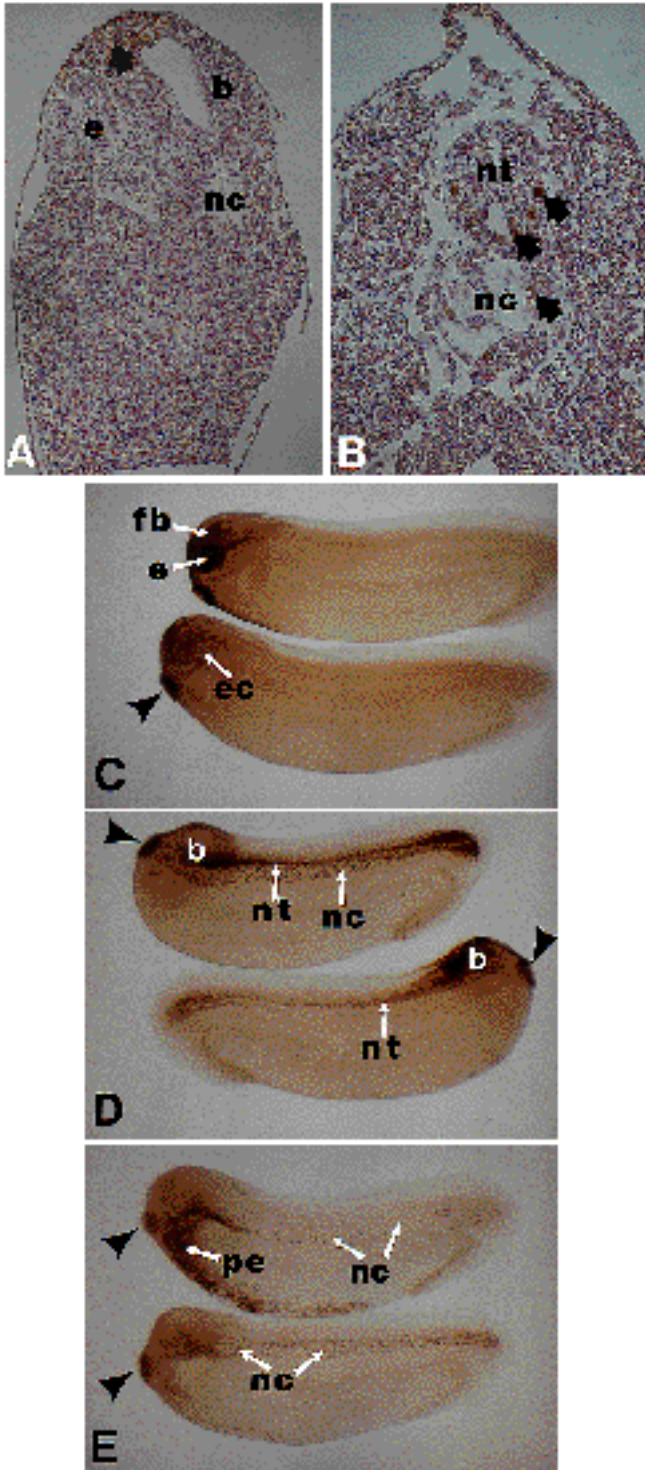
embryo (Dale and Slack, 1987; Moody, 1987) beta-galactosidase was detected in tier one-injected embryos (Fig. 7C) primarily in anterior ectoderm, face structures, and anterior brain. The tier two injections (Fig. 7D) labeled the brain to a greater extent relative to the tier one injections, as well as labeling the notochord. Tier three injections



**Fig. 6.** Histological analysis of embryos injected with *Xwnt-5A* RNA in the upper marginal zone of both dorsal blastomeres at the 4-cell stage. (A) Control embryo (stage 18-19) sectioned transversely through the trunk region. (B) *Xwnt-5A*-injected (high dose) embryo (stage 18-19) sectioned transversely through the trunk region showing the morphology and positional relationship between the notochord, somites, and neural plate. A pointer marks the position of the dorsal midline with respect to the neural axis. (C) Horizontal section of a control embryo (stage 34-36). (D) Horizontal section through a *Xwnt-5A*-injected (high dose) embryo (stage 34-36). This embryo shows a mild phenotype of fused nasal placode, malformed forebrain, and malformed anterior notochord. A subtle bifurcation can also be seen in the notochord, indicated by the dark septum down the middle of the notochord. (E) In some *Xwnt-5A*-injected embryos the notochord clearly bifurcates, as evident in this cross section through the tail of an embryo at stage 34-36. (F) In most *Xwnt-5A*-injected embryos the notochord does not duplicate as in E, rather, it appears as an enlarged bulb anteriorly. (G) This embryo demonstrates that the phenotype observed in B persists at stage 34-36, in horizontal section. (H) This *Xwnt-5A*-injected embryo demonstrates that occasionally there is a position specific disruption of axial development in the tail. In this stage 34-36 embryo, sectioned horizontally, three somite segments along with the notochord and neural tube in these segments are seriously disrupted (downward

arrow). Axial tissue rostral (upward arrow) and caudal (downward arrowhead) to this disrupted area appear normal. (I) Horizontal section through control embryo at stage 34-36 to denote interocular distance. (J) Horizontal section through *Xwnt-5A*-injected embryo at stage 34-36 to denote the reduction in interocular distance relative to the sibling control in I. Abbreviations: e, eye; en, endodermal cavity; cg, cement gland; di, diencephalon; np, neural plate; nc, notochord; na, nasal placode; nt, neural tube; ot, otic vesicle; s, somite; tel, telencephalon. The bar in A serves as a reference to the following scales: A and B, 28  $\mu$ m; C-H, 88  $\mu$ m; G, 105  $\mu$ m; and I and J, 65  $\mu$ m.





**Fig. 7.** Determination of blastomere sensitivity to overexpression of *Xwnt-5A*. Mixed *Xwnt-5A* and beta-galactosidase RNAs were injected into both dorsal blastomeres at the 4-cell stage (A, B), or into two dorsal midline tier one (C), tier two (D), or tier three (E) blastomeres at the 32-cell stage. Hatched embryos were processed for whole-mount immunocytochemical localization of beta-galactosidase and photographed (C,D,E), or embedded and sectioned through anterior (A) or trunk (B) regions. Arrows denote examples of specific beta-galactosidase staining, and the black arrowheads (C,D,E) denote the position of the cement gland. Abbreviations: b, brain; e, eye; ec, ectoderm; fb, forebrain; nc, notochord; nt, neural tube; pe, pharyngeal endoderm.

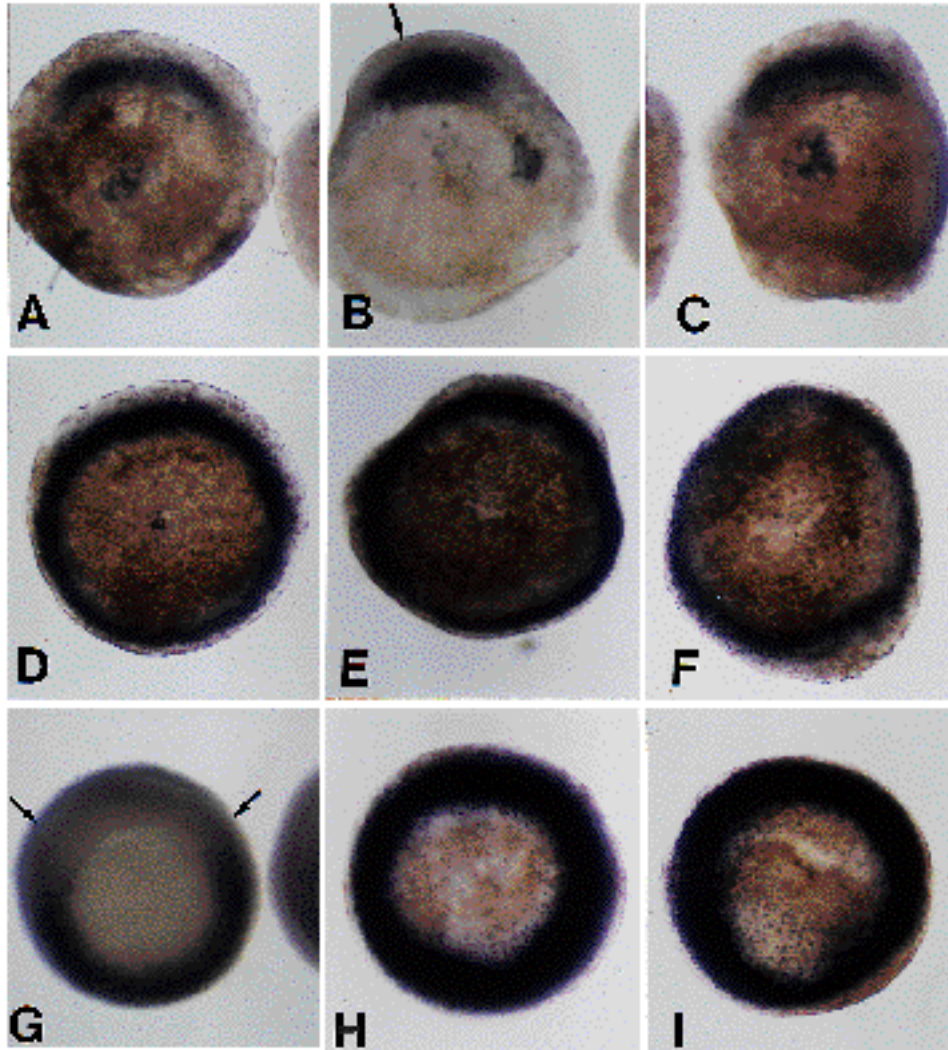
displayed head defects. Based on the visual examination (e.g., of representative embryos in Fig. 7), overexpression of *Xwnt-5A* in dorsal tier two blastomeres, and to a lesser extent tier three blastomeres, produced the highest incidence of the head abnormalities.

#### Effects of overexpression of *Xwnt-5A* on the endogenous patterns of expression of *gooseoid*, *Xbra* and *Xwnt-8*

As ectopic expression of *Xwnt-1*, -3A, and -8 have the capacity to mimic the Nieuwkoop signalling center (reviewed by Christian and Moon, 1993a), we next investigated whether *Xwnt-5A* could also mimic this activity, and thereby alter the expression of genes in the marginal zone of the gastrula embryo. *Gooseoid* is a putative transcription factor expressed in the gastrula organizer (Fig. 8A; Cho et al., 1991), and it is expressed in response to injection of *Xwnt-1* RNA in the ventral marginal zone (Christian and Moon, 1993b). Injection of *Xwnt-5A* RNA into the marginal zone of both dorsal blastomeres of 4-cell embryos did not alter the expression of *gooseoid* (Fig. 8B), nor did injection into ventral marginal zone (Fig. 8C) promote expression at an ectopic site. We have previously shown that the expression of endogenous *Xwnt-8* in the marginal zone (Fig. 8G) is negatively regulated by Nieuwkoop center activity of the blastula, and extends throughout the marginal zone following UV-irradiation (Fig. 8H; Christian and Moon, 1993b). This pattern of expression in UV-irradiated embryos is unaffected by microinjection of *Xwnt-5A* RNA into the marginal zone of irradiated eggs (Fig. 8I) and, taken with the lack of effect of *Xwnt-5A* on *gooseoid* expression, support the conclusion that *Xwnt-5A* lacks the ability to mimic Nieuwkoop center activity. The *Xenopus brachyury* (*Xbra*) transcript is expressed in prospective dorsal and ventral mesoderm (Fig. 8D, Smith et al., 1991). Since *Xwnt-8* expressed from plasmids promotes formation of ventral mesoderm (Christian and Moon, 1993b), we used expression of *Xbra* as one indication of whether *Xwnt-5A* had any affect on the formation of mesoderm. Injection of *Xwnt-5A* RNA into the marginal zone of both dorsal (Fig. 8E) or ventral (Fig. 8F) blastomeres at the 4-cell stage had no pronounced effect on this pattern.

Interestingly, manual removal of the gastrula stage embryos from their vitelline envelopes prior to fixation for in situ hybridization resulted in the spherical embryos becoming more pear-shaped in *Xwnt-5A* dorsally injected embryos (Fig. 8B, arrow). Microdissection of unfixed

(Fig. 7E) in one of the experiments labeled primarily endoderm in 31% ( $n=15$ ) of embryos, endoderm plus mesoderm (either head, notochord, or both) in 31% ( $n=15$ ) of embryos, only the notochord in 15% ( $n=7$ ) of embryos, and the brain as well as the ectoderm or notochord in 24% ( $n=11$ ) of embryos. In 10 of the 11 tier three-injected embryos displaying beta-galactosidase in the brain, head abnormalities of varying degrees were observed, whereas none of the other tier three-injected embryos overtly



**Fig. 8.** Effects of overexpression of *Xwnt-5A* on the endogenous patterns of expression of *goosecooid*, *Xbra*, and *Xwnt-8*. *Goosecooid* is expressed exclusively in the gastrula organizer of control embryos (A), as well as in embryos injected with *Xwnt-5A* RNA in the marginal zone of both dorsal (B) or ventral (C) blastomeres at the 4-cell stage. Arrow in B denotes slight bulging of the dorsal side. The pattern of expression of endogenous *Xbra* (D) is similarly unaffected by dorsal (E) or ventral (F) injection of *Xwnt-5A* RNA. *Xwnt-8* is expressed in future ventral and lateral mesoderm of control embryos, and is excluded (between arrows) from the gastrula organizer field (G). UV irradiation of eggs ventralizes the embryo, leading to expression of *Xwnt-8* throughout the marginal zone (H), a pattern that is unaffected by injection of *Xwnt-5A* RNA into the marginal zone (I).

gastrula revealed that the dorsal mesodermal cells were noticeably more adherent to one another. Thus, at the onset of gastrulation, overexpression of *Xwnt-5A* has not affected the expression of markers for mesoderm, or the gastrula organizer, but it may have effects on cell adhesion.

#### Effects of overexpression of *Xwnt-5A* in UV-irradiated embryos

To test further whether *Xwnt-5A* had any measurable capacity to alter cell fate, and whether the effects of ectopic expression of *Xwnt-5A* were indeed distinct from the effects of *Xwnt-1*, *-3A*, and *-8*, we ventralized embryos by UV-irradiation, and injected doses of *Xwnt-5A* which were sufficient to generate the head abnormalities in non-irradiated embryos. Embryos were cultured until controls had reached stage 20, then analyzed histologically. As summarized in Table 2, both uninjected UV-irradiated embryos, and *Xwnt-5A*-injected UV-irradiated embryos, developed into ventralized embryos lacking striated muscle, notochords, or neural tubes. Thus, the effects of *Xwnt-5A* on UV-irradiated embryos are quite distinct from the effects of *Xwnt-8* (reviewed by Christian and Moon, 1993a) or *Xwnt-9* (Ku and Melton, unpublished data).

#### Effects of overexpression of *Xwnt-5A* on the differentiation of isolated blastula caps

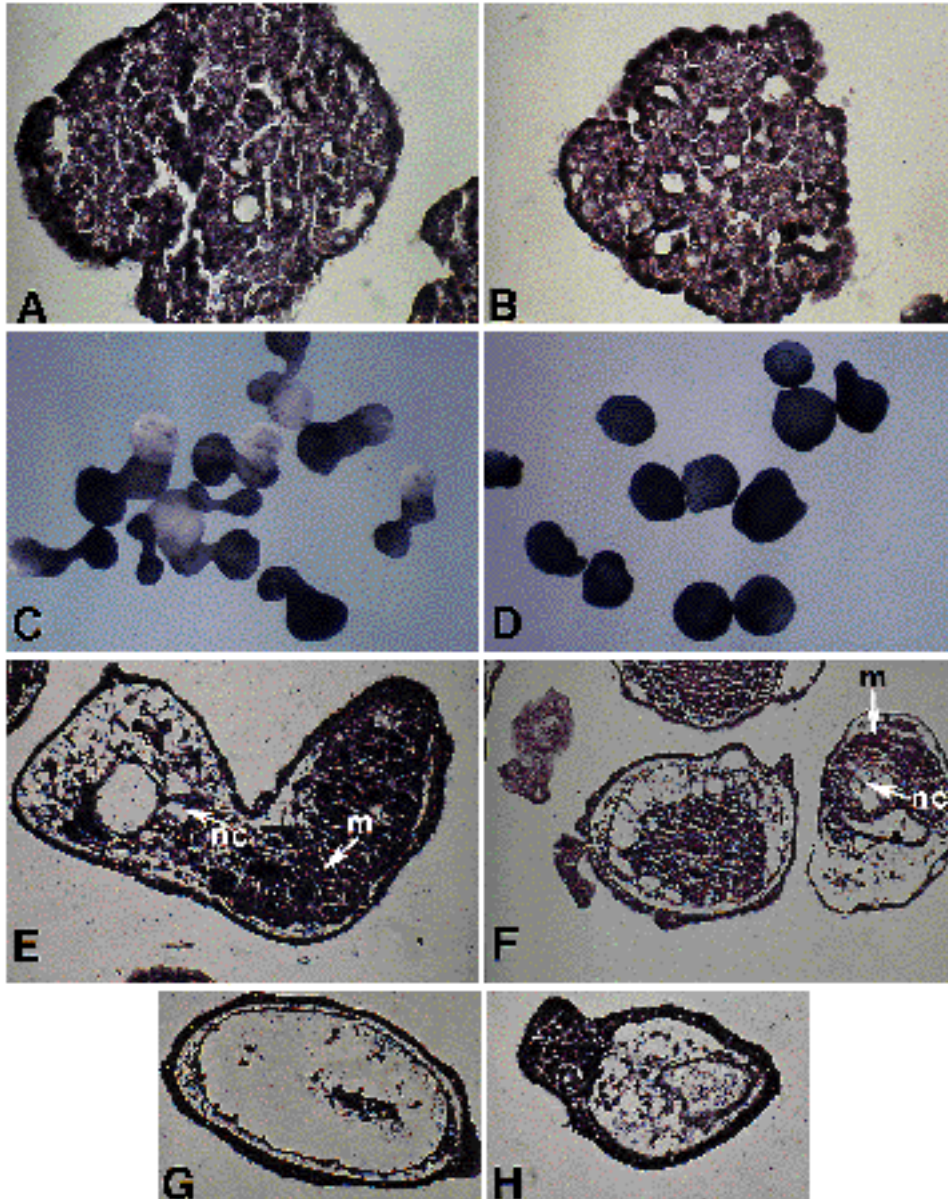
We then investigated the effect of overexpression of *Xwnt-5A* on the differentiation of blastula cap explants. *Xwnt-5A* RNA was injected into the animal poles of both blastomeres of 2-cell embryos, and blastula caps were isolated and cultured in vitro until control embryos reached stage 40. Histological examination of these blastula caps (Fig. 9B) revealed that *Xwnt-5A* led to differentiation of the caps as

**Table 2.** Effects of overexpression of *Xwnt-5A* on UV-irradiated embryos

	Structures detected by histology		
	Notochord	Neural tube	Striated muscle
Control embryos ( $n=20$ )	95	100	90
UV embryos ( $n=20$ )	10	0	5
UV + <i>Xwnt-5A</i> embryos ( $n=25$ )	8	0	4

Uninjected embryos (control), UV-irradiated controls (UV embryos) or UV-irradiated embryos injected with *Xwnt-5A* RNA (UV+*Xwnt-5A*) were cultured to stage 20, fixed, embedded, and sectioned. Numbers represent percentages of embryos displaying the indicated structures, except for  $n$ , which denotes the sample size.





**Fig. 9.** Effects of overexpression of *Xwnt-5A* on the differentiation of isolated blastula caps. Histological examination of blastula caps from control embryos (A) and embryos injected with *Xwnt-5A* RNA in the animal poles of both blastomeres at the 2-cell stage (B) reveals formation of atypical epidermis. Treatment of control explants with recombinant activin A induces extensive elongation of the explants (C), whereas parallel treatment of explants from *Xwnt-5A*-injected embryos does not induce elongation (D). Histological examination of dorsal halves of control blastula caps (E) reveals skeletal muscle (m) and notochord (nc) in the explants treated with activin A, similar to the mesodermal types in the activin A-treated dorsal halves of caps from *Xwnt-5A*-injected embryos (F). Ventral halves of blastula caps from control embryos (G) and from *Xwnt-5A*-injected embryos (H) treated with activin A display similar ventral mesodermal types (shown here), as well as skeletal muscle (in explants not shown).

atypical epidermis, indistinguishable from control blastula caps (Fig. 9A). Therefore, *Xwnt-5A* is not a mesoderm inducing growth factor in this assay.

Surprisingly, only 10% of blastula caps from embryos injected with *Xwnt-5A* RNA elongated when blastula caps were isolated and immediately cultured in 10 ng/ml recombinant activin A (Fig. 9D), whereas 83% of blastula caps from control embryos underwent the normal elongation associated with activin-mediated mesoderm induction (Fig. 9C; Table 3). In subsequent experiments, isolated blastula caps were dissected into dorsal or ventral halves, and cultured in the presence or absence of activin A. None of the explants from control embryos elongated in the absence of activin, and treatment of the control explants with activin A resulted in strong elongation in dorsal (60%) and ventral (73%) explants. Consistent with the above analysis of entire blastula caps, few dorsal (3%) or ventral (0%) blastula caps from embryos injected with *Xwnt-5A* RNA exhibited

elongation in response to activin A. We then examined dorsal and ventral blastula caps histologically to ascertain whether *Xwnt-5A* affected the mesodermal cell types normally induced by activin A (Table 3). Dorsal explants from both control (Fig. 9E) and *Xwnt-5A*-injected embryos (Fig. 9F) exhibited notochords in 67% vs 53% of explants, respectively, contained skeletal muscle in at least 90% of explants, and contained ventral mesodermal cell types in 50% or greater of explants. Activin A-treated ventral explants from both control (Fig. 9G) and *Xwnt-5A*-injected embryos (Fig. 9H) contained a comparably lower incidence of notochord formation compared to dorsal explants. The ventral explants from both control and *Xwnt-5A*-injected embryos also contained skeletal muscle and erythroid-like or mesenchymal cells (Table 3) indicative of ventral mesoderm (Green et al., 1990). In summary, overexpression of *Xwnt-5A* leads to reduced elongation movements in response to activin in isolated blastula caps, without dra-

**Table 3. Effects of overexpression of *Xwnt-5A* on the differentiation of isolated blastula caps**

	<i>n</i>	Elongation at stage 18	<i>n</i>	Tissues induced at stage 40			
				epi	not	e/mt	mus
<b>Entire Cap</b>							
Control	16	0	18	89	0	11	0
+ activin	18	83		-	-	-	-
<i>Xwnt-5A</i>	8	0	17	94	0	12	0
+ activin	20	10		-	-	-	-
<b>Dorsal half</b>							
Control	9	0	6	83	0	17	-
+ activin	10	60	6	0	67	50	100
<i>Xwnt-5A</i> + activin	29	3	19	0	53	100	90
<b>Ventral half</b>							
Control	10	0	7	86	0	14	0
+ activin	11	73	8	0	12	88	88
<i>Xwnt-5A</i> + activin	26	0	21	0	5	95	43

The upper one quarter to one fifth of stage 7-8 blastula caps were excised from control or *Xwnt-5A*-injected embryos and, in some experiments, cut into dorsal or ventral halves. Explants were cultured to the indicated stage in the presence or absence of 10 ng/ml recombinant human activin A, then scored for elongation, or processed for histology and scored for tissue types. Numbers refer to percentages except for *n*, representing the sample size. Abbreviations: epi, atypical epidermis; not, notochord; e/mt, erythroid-like and/or mesothelial cells; mus, striated muscle.

matically altering the types of mesoderm induced in the explants.

## DISCUSSION

In the present study we have examined the sequence, expression, and consequences of overexpression of a maternal *Wnt*, *Xwnt-5A*, which is a member of a family of at least 12 genes in *Xenopus* (reviewed by Moon, 1993). The intriguing result of this study is that overexpression of *Xwnt-5A* has no discernible effects upon early development, until the movements of gastrulation commence. The data discussed below are consistent with the possibility that *Xwnt-5A* has the activity of a modulator of morphogenetic movements, rather than the activity expected of a factor that directly alters cell fate. Cell fate may be affected indirectly by *Xwnt-5A*, in that morphogenetic movements can affect the positioning or delivery of factors that modulate cell fate.

### Expression of *Xwnt-5A*

Any understanding of the functions of *Xwnt-5A* must in part be predicated on an understanding of its spatial patterns of expression. By the neurula stage, *Xwnt-5A* transcripts are detected primarily in the ectoderm and mesoderm, a pattern that persists into tailbud and tadpole stages. Moreover, by both RNase protection of RNA from microdissected tailbud embryos, and by in situ hybridization, higher levels of *Xwnt-5A* transcripts are detected in the head and tail, with lower levels in the middle of the embryo. Analysis of thick sections of the embryos processed for whole-mount in situ hybridization demonstrated that *Xwnt-5A* expression in the head includes both neural and non-neural ectoderm but no expression was noted in the notochord. Similarly, expression of *Wnt-5A* in mouse embryos is elevated in facial processes (Gavin et al., 1990). While most *Xwn*ts are expressed primarily if not exclusively in neural tissue (reviewed in Moon, 1993), our data indicate that *Xwnt-5A*

is detectable in posterior and somitic mesoderm, as well as in ectoderm.

### Effects of overexpression of *Xwnt-5A* on embryonic development

To provide insights into which developmental processes are sensitive to levels of *Xwnt-5A*, we overexpressed *Xwnt-5A* by injection of synthetic RNA into fertilized eggs. 60-80% of the injected embryos developed with head defects, while a low percentage of embryos develop normally, or with supernumerary tails. As the frequency of the head defects could be increased to 95% by directing the RNA to the dorsal blastomeres at the 4-cell stage, we examined these embryos histologically to come to some understanding of the effects of overexpression of *Xwnt-5A* on *Xenopus* development. Importantly, at a gross level we find a correlation between the normal pattern of expression of *Xwnt-5A*, and the regions of the embryos affected by overexpression of *Xwnt-5A*. That is, head and tail formation were disrupted in all embryos examined histologically. We then asked whether the phenotype arose with equivalent frequency after injection of the top three tiers of blastomeres at the 32-cell stage. We found that the head abnormalities generally correlated with overexpression of *Xwnt-5A* in neural ectoderm, primarily when the RNA was targeted to tier 2 blastomeres, though this analysis does not directly test the involvement of mesoderm versus neural ectoderm in the abnormal phenotype. Nevertheless, this analysis, in conjunction with other histological and immunocytochemical approaches, suggests that overexpression of *Xwnt-5A* perturbs the organization of a variety of tissues, without dramatically enhancing or reducing the formation of mesodermal or neuronal structures.

As injected *Xwnt-5A* RNA was shown to persist above endogenous levels through neurula stage, it is possible that the abnormal phenotypes described above arise by the overexpressed *Xwnt-5A* acting at any time during the period of overexpression. To address the effects of delaying overexpression of *Xwnt-5A* until after mid-blastula transition,



*Xwnt-5A* was expressed from microinjected plasmids, under the control of a cytoskeletal actin promoter. This resulted in embryos with phenotypes similar to those described above, achieved by targeting RNA to dorsal blastomeres. These data suggest that the developmental abnormalities attributed to overexpression of *Xwnt-5A* are due to the activity of *Xwnt-5A* after mid-blastula transition. Whether *Xwnt-5A* polypeptides are translated from the endogenous maternal *Xwnt-5A* transcripts, or have any activity prior to mid-blastula stage, is unknown.

### **Xwnt-5A does not mimic Nieuwkoop center or gastrula organizer activities**

Several experiments were conducted to test whether *Xwnt-5A* altered mesodermal cell fate, or mimicked Nieuwkoop center or gastrula organizer activities. We found that injection of *Xwnt-5A* RNA into the marginal zone of either dorsal or ventral blastomeres at the 4-cell stage had no effect on the expression of the gastrula organizer-specific gene, *gooseoid*. Thus, the activity of *Xwnt-5A* is distinct from *Xwnt-1*, which leads to expression of *gooseoid* following ventral injection of synthetic RNA (Christian and Moon, 1993b). Since *gooseoid* is expressed in the gastrula organizer in response to signals from the Nieuwkoop center (blastula signalling center) (Christian and Moon, 1993b), these data establish that *Xwnt-5A* does not mimic the Nieuwkoop center. Similarly, signals downstream of the Nieuwkoop center, probably *gooseoid*, negatively regulate expression of endogenous *Xwnt-8* (Christian and Moon, 1993b). We found that *Xwnt-5A* RNA has no effect on the endogenous expression of *Xwnt-8*, further supporting the conclusion that *Xwnt-5A* cannot mimic Nieuwkoop center activity. If *Xwnt-5A* could mimic the gastrula organizing center, then ventral injection of *Xwnt-5A* RNA should lead to a secondary dorsal lip during gastrulation, and to a secondary embryonic axis, neither of which is observed.

UV irradiation of fertilized eggs is a useful approach for blocking formation of the Nieuwkoop center and thus gastrula organizer activities, resulting in an embryo with ventral properties (reviewed by Christian and Moon, 1993a). This provides a background into which one can introduce factors to test their ability to rescue dorsal mesodermal, or neural structures. As *Xwnt-8* RNA injection into the vegetal hemisphere or marginal zone of UV-irradiated embryos rescues normal development, without injected blastomeres necessarily contributing to dorsal axial structures (Smith and Harland, 1991), *Xwnt-8* mimics the Nieuwkoop center activity (reviewed by Christian and Moon, 1993a). *Xwnt-9* partially rescues UV-irradiated embryos to form somitic mesoderm and a spinal cord, but they do not form notochord (Ku and Melton, unpublished data). *Xwnt-5A* is distinct from either of these *Xwnts* in this assay - it has no ability to rescue formation of a notochord, somitic mesoderm, or a spinal cord.

### **Xwnt-5A modulates tissue movements but not mesoderm induction**

Blastula caps provide a highly useful cellular environment in which one can test whether the *in vitro* differentiation of these explants as atypical epidermis can be diverted into

pathways leading to other cell types. In the present study, we found that overexpression of *Xwnt-5A* in blastula cap explants by injection of synthetic RNA did not alter the differentiation of these explants into atypical epidermis, suggesting that *Xwnt-5A* is not a mesoderm inducing agent. Taken with the inability of *Xwnt-5A* to alter the expression of mesodermal genes in the marginal zone, and the lack of muscle, notochord, or neural tissue in UV irradiated embryos injected with *Xwnt-5A* RNA, we conclude that *Xwnt-5A* is not likely to be an inductive agent.

As incubation of blastula caps with mesoderm-inducing growth factors such as basic fibroblast growth factor (bFGF) or activin A leads to formation of dorsal or ventral mesoderm (reviewed in Kimelman et al., 1992), this provides a useful system for asking whether a *Xwnt* can alter the growth factor responsiveness of the blastula cap. Previously, it was reported that *Xwnt-8* RNA enhances the dorsal response of blastula caps to bFGF (Christian et al., 1992) and activin (Sokol and Melton, 1992), such that they undergo greater physical elongation, and express greater levels of muscle actin. Surprisingly, in the present study we found that *Xwnt-5A* has a very distinct effect on the activin responsiveness of blastula cap explants. The explants were blocked in elongation, which has been described as resembling the morphogenetic movements of gastrulation (Symes and Smith, 1987). Interestingly, sectioning the explants revealed that at the one concentration of activin tested, the activin had induced the normal distribution of dorsal and ventral mesodermal cell types. These data, though based on only one concentration of activin, demonstrate that in this context *Xwnt-5A* has the capacity to alter the morphogenetic movements of tissues, without altering their cell fate. As an aside, these data also indicate that dorsal mesoderm formation can occur in the absence of the morphogenetic movements resulting in the elongation of the blastula caps in response to activin.

Is *Xwnt-5A* unique in its effect on the activin responsiveness of blastula caps? A dominant inhibitory mutant *ras* can also block the activin mediated elongation of blastula caps (Whitman and Melton, 1992). However, this effect differs from *Xwnt-5A* insofar as the mutant *ras* also blocks mesoderm induction. The effects of *Xwnt-5A* are also distinct from the effects of bone morphogenetic protein 4 (BMP-4, Dale et al., 1992; Jones et al., 1992). BMP-4 similarly reduces elongation of blastula caps in response to activin but, unlike *Xwnt-5A*, BMP-4 ventralizes the tissue types observed, so that muscle and notochord are rarely formed.

Dorsal and ventral halves of blastula caps have distinct responses to activin (Sokol and Melton, 1991), and expression of *Xwnt-8* RNA in the ventral half of blastula caps enhances the competence of the cap to respond to growth factors, so that the ventral half responds like the dorsal half (reviewed by Kimelman et al., 1992; Christian and Moon, 1992). We report that *Xwnt-5A* does not alter the normally greater responsiveness to activin of dorsal relative to ventral halves of blastula caps, suggesting that *Xwnt-5A* does not synergize with activin to yield a greater dorsal response to mesoderm induction, and suggesting that *Xwnt-5A* is not normally involved in the competence of tissue to respond to mesoderm inducing growth factors.

### Ectopic expression of *Xwn*ts suggest multiple *Wnt* signalling pathways

Although over a dozen *Wnts* have been identified, there have been few data addressing whether *Wnts* in any species are functionally redundant, or activate distinct pathways. Several experiments in *Xenopus* bear on this issue. First, injection of RNA encoding *Xwnt-8* (Sokol et al., 1991; Smith and Harland, 1991), *Xwnt-5A* (present study), and *Xwnt-9* (Ku and Melton, unpublished data) into UV-irradiated embryos produce three distinct phenotypes. Second, *Xwnt-5A* and *-8* have distinct effects on modulating gap junctional permeability in early embryos (Olson et al., 1991), and on the growth factor responsiveness of blastula caps. Third, *Xwnt-5A* is distinct from *Wnt-1* in its inability to transform C57 mammary epithelial cells (Olson et al., unpublished). Fourth, *Xenopus* blastomeres differ in their sensitivity to overexpression of *Xwn*ts - dorsal blastomeres are sensitive to injection of *Xwnt-5A* RNA but not *Xwnt-8* RNA, whereas the converse is true for ventral blastomeres. Thus, it is likely that there are multiple signal transduction pathways activated by *Xwn*ts. Alternatively, a single pathway may be activated by all *Xwn*ts, but the amplitude or duration of activation differ in response to distinct *Xwn*ts. Indicating that some *Xwn*ts can activate the same pathways, the *Xwnt-8* response is likely to be indistinguishable from the effects of *Xwn*ts-1 and -3A (Wolda et al., 1993; reviewed by Moon, 1993).

### Speculation on the functions of *Xwnt-5A*

We speculate that *Xwnt-5A* activates a receptor-mediated signal transduction pathway, which ultimately leads to changes in cell adhesion, and to changes in the morphogenetic movements of tissues. Three lines of evidence support this hypothesis. First, from assays monitoring gap junctional permeability at the 32-cell stage, observing phenotype, and investigating the expression of mesodermal markers at the gastrula stage, overexpression of *Xwnt-5A* has no effect upon the embryo until the morphogenetic movements of gastrulation commence. Importantly, *Xwnt-5A* does affect the movements of gastrulation in Keller explants (Shih et al., unpublished data), consistent with the phenotype of the intact embryo overexpressing *Xwnt-5A*, reported here. Second, *Xwnt-5A* blocks the elongation of blastula caps in response to activin, without altering the types of mesoderm formed within the explants. Third, preliminary evidence suggests that *Xwnt-5A* reduces the normal mixing of ectodermal cells during gastrulation (Moon et al., unpublished data), much as Detrick et al. (1990) found in response to overexpression of N-cadherin.

There is accumulating evidence that *Wnts* may act at a cellular level to alter cell adhesion (reviewed by Moon et al., 1993). In part, this is based on data establishing that the *armadillo* gene, involved in the *Wnt-1* signalling pathway in *Drosophila*, is homologous to vertebrate plakoglobin and to the E-cadherin-associated protein, beta-catenin. As *Xenopus* embryos express both beta-catenin and plakoglobin (DeMarais and Moon, 1992), and as *Xwnt-5A* has effects upon cell movements, there are new opportunities for investigation of how *Wnt* signalling may affect cell adhesion.

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