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Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo

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SUMMARY

The posterior marginal zone (PMZ) of the chick embryo has Nieuwkoop centre-like properties: when transplanted to another part of the marginal zone, it induces a complete embryonic axis, without making a cellular contribution to the induced structures. However, when the PMZ is removed, the embryo can initiate axis formation from another part of the remaining marginal zone. Chick Vg1 can mimic the axis-inducing ability of the PMZ, but only when misexpressed somewhere within the marginal zone. We have investigated the properties that define the marginal zone as a distinct region. We show that the competence of the marginal zone to initiate ectopic primitive streak formation in response to cVg1 is dependent on Wnt activity. First, within the Wnt family, only *Wnt8C* is expressed in the marginal zone, in a gradient decreasing from posterior to anterior. Second, misexpression of Wnt1 in the area pellucida enables this region to form a primitive streak in response to cVg1. Third, the Wnt antagonists Crescent and Dkk-1 block the primitive streak-inducing ability of cVg1 in the marginal zone. These findings suggest that Wnt activity defines the marginal zone and allows cVg1 to induce an axis. We also present data suggesting some additional complexity: first, the Vg1 and Wnt pathways appear to regulate the expression of downstream components of each other's pathway; and second, misexpression of different Wnt antagonists suggests that different classes of Wnts may cooperate with each other to regulate axis formation in the normal embryo.

Key words: Marginal zone, Induction, Primitive streak, Gastrulation, Nieuwkoop centre, Vg1, Wnt, Lef1, β-catenin, JNK, Crescent, Dickkopf, Frizzled 8, Chick

INTRODUCTION

Unlike amphibian embryos, where polarity is established by the third cleavage division through the localisation of maternal determinants (reviewed in Harland and Gerhart, 1997; Arendt and Nübler-Jung, 1999), polarity in the chick embryo remains plastic until the beginning of gastrulation, when the embryo already has 20,000-60,000 cells (Spratt and Haas, 1960b). Up to the time of appearance of the primitive streak, the blastoderm can be cut into several fragments, each of which can spontaneously initiate the formation of a complete axis (Spratt and Haas, 1960b). In the cut fragments, the new axis tends to arise from the edge of the area pellucida, a region called the marginal zone. Furthermore, the frequency of axis formation decreases in a posterior-to-anterior direction around the marginal zone (Spratt and Haas, 1960b).

Subsequent investigators have established that a small posterior domain within the marginal zone (the posterior marginal zone; PMZ) is particularly important. When this domain is transplanted to an ectopic site of the marginal zone of a host embryo, it induces the formation of a second axis (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989). In fact, the PMZ can act in a manner analogous to the Nieuwkoop centre of amphibians, by inducing a complete axis that includes the organiser, but without making a direct cellular contribution to it (Bachvarova et al., 1998).

Misexpression of the TGFβ family member chick Vg1 can mimic the activity of the PMZ: when misexpressed in the anterior marginal zone, it will also induce a complete embryonic axis and organiser (Seleiro et al., 1996, Shah et al., 1997). cVg1 misexpression, like grafts of the PMZ, only induce such an axis if they are placed within the marginal zone of the host embryo, and not in the area pellucida. What makes the marginal zone unique?

In *Xenopus* (Sokol and Melton, 1992; Steinbeisser et al., 1993; Cui et al., 1995; Watabe et al., 1995; Kessler, 1997; Crease et al., 1998; Zorn et al., 1999), as well as during later stages of chick development (Joubin and Stern, 1999), the Vg1/Activin and Wnt signalling pathways can synergise to induce organiser genes, which has prompted us to investigate whether differences in Wnt activity could explain the special properties of the marginal zone. We have found that *Wnt8C* is expressed in the marginal zone, where it describes a gradient that is highest posteriorly. When cVg1 is misexpressed in the

anterior marginal zone together with the Wnt antagonists Crescent or Dkk1, axis induction is inhibited. Furthermore, ectopic Wnt expression is able to overcome the inability of cVg1 to induce an axis in the area pellucida. Based on these results, we propose that *Wnt8C* defines the marginal zone and limits the ability of regions of the embryo to respond to cVg1. We also present data to suggest that the Vg1 and Wnt pathways regulate the expression of downstream components of each other's pathway. Finally, we show that different Wnt antagonists have distinct effects, suggesting that different classes of Wnts cooperate to regulate axis formation in the normal embryo.

MATERIALS AND METHODS

Embryo culture

Fertile White Leghorn hens' eggs (SPAFAS, CT) were incubated for 1-20 hours to obtain embryos between stage X EG&K (Roman numbers for pre-primitive streak stages; Eyal-Giladi and Kochav, 1976) and stage 5 HH (Arabic numerals for later stages; Hamburger and Hamilton, 1951). Embryos were explanted in modified New culture as described previously (New, 1955; Stern and Ireland, 1981). Aggregates of COS cells or RatB1a fibroblasts were grafted onto the marginal zone or area pellucida of the embryo using a micropipette. The posterior end of the embryo was marked with carbon particles (Carbon Lampblack, Fisher). Embryos were incubated at 38°C in a humidified chamber for different periods of time, fixed, and processed for whole-mount in situ hybridisation and immunohistochemistry.

Whole-mount in situ hybridisation

Embryos were fixed in 4% formaldehyde, 2 mM EGTA in phosphate-buffered saline (PBS, pH 7.0) for 1 hour at room temperature or overnight at 4°C, and then stored in 100% methanol at –20°C. In situ hybridisation using digoxigenin-labelled riboprobes was performed at 68°C as described previously. The molecular markers studied in this work are *cBra* (Ch-T, a gift from J. C. Smith; Kispert et al., 1995, Knezevic et al., 1997), *chordin* (Streit et al., 1998), *cFGF8* (a gift from J. C. Izpisúa-Belmonte), *goosecoid* (Izpisúa-Belmonte et al., 1993), *cLef1* (a gift from J. C. Izpisúa-Belmonte; Kengaku et al., 1998), *cNodal* (a gift from M. Kuehn; Jones et al., 1995), *cVg1* (Shah et al., 1997), *Wnt8C* (a gift from J. Dodd; Hume and Dodd, 1993), and *cWnt1, cWnt3, cWnt3a, cWnt4, cWnt5a, cWnt5b, cWnt6, cWnt7b, cWnt8, cWnt10* and *cWnt11* (gifts from A. McMahon; Hollyday et al., 1995).

Myc or HA immunohistochemistry

After in situ hybridisation, embryos were processed for immunohistochemistry as described previously (Streit et al., 1997) using monoclonal anti-Myc (9E10, Evans et al., 1985) or anti-HA (Mono HA.11, Berkeley Antibody Co.) antibodies at a final concentration of 1:4 or 1:1000, respectively. A goat anti-mouse IgG HRP-linked secondary antibody (Jackson Immunoresearch) was used at a final concentration of 1:2000 and the peroxidase reaction carried out using 3,3′-diaminobenzidine (Sigma, MO) as a substrate (Streit et al., 1997). After in situ hybridisation and immunohistochemistry, embryos were transferred to a chamber slide and photographed using bright field optics on Fuji 64T film. Some embryos were embedded in Paraplast and sectioned at 10 μm.

β-catenin immunohistochemistry and confocal microscopy

Detection of β-catenin followed a previously described protocol (Schneider et al., 1996) using a rabbit anti β-catenin antiserum (a gift of R. Moon). Embryos were fixed for 10 minutes in 4% formaldehyde in PBS and stored overnight in 80% methanol:20% DMSO at –20°C. After rehydrating the embryos in 50% methanol:50% PBS, embryos were washed in PBS and incubated in blocking buffer (BB; 20% bovine serum albumen in PBS) for 1 hour at 4°C. Rabbit antiserum was then added to a final concentration of 1:500 and the embryos incubated overnight at 4°C. After five 1 hour washes in PBS at room temperature, embryos were incubated in BB for 1 hour at 4°C. Cy5-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch) was then added to a final concentration of 1:500 and the embryos incubated overnight at 4°C. After five 1 hour washes in PBS at room temperature, embryos were incubated for 5 minutes in 10 μg/ml propidium iodide (Sigma) in PBS and then washed extensively in PBS before being transferred to a chamber slide and mounted in Gelvatol (Stern and Holland, 1993). The epiblast layer of the embryos was examined using a Zeiss LSM 410 confocal laser-scanning system with a 15 mW Argon-Krypton laser attached to a Zeiss Axiovert 100TV inverted microscope, and a 63× water immersion objective with a numerical aperture of 1.2. Propidium iodide and Cy5 were excited at 568 and 647 nm, respectively. Optical sections 1 and 5 μm apart were taken and analysed individually and as a stack using Scion Image 1.62 and Adobe Photoshop 5.5.

DNA constructs

To facilitate detection of Crescent using an anti-Myc monoclonal antibody (9E10; Evans et al., 1985), a full-length Crescent (Pfeffer et al., 1997) cDNA (a gift from J. C. Izpisúa-Belmonte) was digested with *Not*I and *Nae*I, removing the last 13 amino acids of the protein. A 944 bp fragment was purified and cloned in frame into pcDNA3.1(–)/Myc-His A (Invitrogen) that had been digested with *Not*I and *Eco*RV. The integrity of the construct was confirmed by sequencing.

Isolation of Wnt cDNA clones

A RT-PCR approach was used to identify novel Wnt factors expressed in the pre-primitive streak stage chick embryo. The degenerate primers designed to recognise conserved amino acid stretches present in multiple Wnt family members were fWnt (amino acid sequence, CKCHG), 5′-TGY AAR TGY CAY GGN NT-3′; and *bWnt* (amino acid sequence, CRFHWC), 5′-CAC CAR TGR AAN NBR CA-3′ (Gavin et al., 1990). A third internal degenerate primer was designed to recognise an amino acid stretch conserved in all Wnts except the *Wnt8* and *Wnt10* subclasses (Gavin et al., 1990) – *iWnt* (amino acid sequence, LL/MCCGRG), 5′-CCN CKN CCR CAR CAN A-3′.

Total RNA was isolated from chick embryos at stages X-XIII (Ausubel et al., 1995). First-strand cDNA was synthesized from 1 μg of total RNA using 10 pmoles of bWnt primer and Superscript II RT (Gibco/BRL; Ausubel et al., 1995). After phenol:chloroform extraction and ethanol precipitation, the cDNA was PCR-amplified using 2 pmoles of bWnt and fWnt primers and Taq polymerase (Promega) in the presence of 1.5 mM $MgCl_2$. The conditions for PCR amplification were 25 cycles of 90°C for 1 minute, 55°C for 2 minutes, 72°C for 2 minutes, with a final extension of 10 minutes at 72°C. A second PCR was carried out using 0.5 μl of the initial PCR reaction and fWnt and iWnt primers, under identical amplification conditions. PCR products were cloned into pGEM-T (Promega) and sequenced. All products isolated encoded Wnt family members with significant homology to *Xenopus* and Zebrafish *Wnt3*, *Wnt5a* and *Wnt5b* genes. Probes generated from these clones gave expression patterns identical to *cWnt3a*, *cWnt5a* and *cWnt5b*, respectively, by in situ hybridisation.

Cell culture and protein production

1.5×10^5 COS cells on a 35 mm dish were transfected with 1 μg of plasmid and Lipofectamine Plus (Gibco BRL), according to the manufacturers' instructions. Constructs used for cell transfection were *dsl-myc-cVg1* (Shah et al., 1997) in pMT23, *Crescent-myc-his* in pcDNA3.1, *hDkk1* in pCS2++ (gift from E. Laufer) and *XFz-N8* in

pCS2++ (gift of P. Klein; Deardorff et al., 1998). As negative controls, transfections were carried out without including DNA ('mock'). For conditioned media, transfected cells were grown in 1 ml of serum-free medium for 2 days and the medium then collected as described previously (Shah et al., 1997). To make cell aggregates, 24 hours after transfection, cells were trypsinised and counted. Aliquots of 1000 cells were allowed to aggregate overnight in hanging 20 μl drops of medium (Shah et al., 1997). These small aggregates were rinsed in serum-free medium and grafted to different regions in the embryo. The efficiency of cell transfection and protein secretion was monitored by immunoblot and immunohistochemistry as described above for whole embryos, except that antibody incubation times were reduced to 1 hour. On average, about 75% of the cells expressed high levels of the protein 24 hours after transfection.

A stable RatB1a fibroblast cell line transfected with *mWnt1-HA* in pLNCX was used to misexpress Wnt1 (gift of J. Kitajewski; Shimizu et al., 1997). For immunoblotting, the cells were lysed as described previously (Shimizu et al., 1997). To make cell aggregates, 3000 RatB1a cells were allowed to aggregate overnight in hanging 20 μl drops. The medium was supplemented with 1 mM Sodium Butyrate (Sigma) to induce high levels mWnt1 expression (Shimizu et al., 1997). RatB1a cells stably transfected with empty pLNCX vector were used as negative controls ('mock').

Immunoblots

Secretion of processed cVg1 (Shah et al., 1997), Wnt1 (Shimizu et al., 1997) and Crescent protein was monitored by immunoblot (not shown). The protein concentration present in supernatants of cells expressing *dsl-myc-cVg1*, *Crescent-myc* or *mWnt1-HA* was initially determined using a modified Bradford assay (BioRad). Samples containing 40 μg of protein were electrophoresed in 10% SDS-PAGE. Gels were electroblotted onto PROTRAN nitrocellulose (Schleicher & Schüll). Blots were probed with either anti-Myc (9E10, Evans et al., 1985) or anti-HA (Mono HA.11, Berkeley Antibody Co.) antibody. Goat HRP-coupled anti-mouse IgG (Jackson Immunoresearch) was used as secondary antibody. Blots were developed using LumiGlo chemiluminescence substrate (Kirkegaard & Perry).

RESULTS

The area pellucida can respond to cVg1, but does not form an ectopic primitive streak

It has previously been reported that cVg1 induces an ectopic axis when misexpressed in the marginal zone, but not in the area pellucida (Shah et al., 1997). This is surprising, because cVg1 has the ability to induce organiser markers in the area pellucida of much later embryos (Joubin and Stern, 1999). Does the young area pellucida not yet have a competence to respond to cVg1, or does cVg1 misexpression in this region induce only some of the required downstream events? To address this question, we misexpressed cVg1 in the anterior third of the area pellucida and analysed the time-course of expression (6, 15, 24 and 36 hours) of a series of molecular markers for embryonic polarity before and during gastrulation (*cVg1, cNodal, cFGF8, Wnt8C, chordin, goosecoid* and *cBra*). No ectopic expression of any of these genes was detected after 6 hours. Between 15-24 hours, *cVg1*, *cNodal*, *chordin* (Fig. 1) and *goosecoid* (not shown) appeared, but no expression of *cFGF8, Wnt8C* or *cBra* was seen (Fig. 1), even after 36 hours (*cBra*; Fig. 1). These results suggest that, although the area pellucida cannot form a primitive streak in response to cVg1 misexpression, it does respond to cVg1 by expressing several markers of embryonic polarity.

Misexpression of cVg1+Wnt1 in the area pellucida induces an ectopic primitive streak

The above result suggests that some property of the marginal zone is required for cVg1 to induce a primitive streak. One candidate is the Wnt pathway, because: (1) *Wnt8C* is expressed throughout the marginal zone at pre-streak stages (Fig. 2A; Hume and Dodd, 1993), and (2) activation of the Wnt pathway has been shown to synergise with Vg1/Activin signalling in the induction of organiser markers in *Xenopus* (Sokol and Melton, 1992; Steinbeisser et al., 1993; Cui et al., 1995; Watabe et al., 1995; Kessler, 1997; Crease et al., 1998; Zorn et al., 1999) and chick (Joubin and Stern, 1999).

Is the inability of the area pellucida to form a streak in response to cVg1 misexpression due to the absence of Wnt activity in this region? We were unable to achieve efficient secretion of Wnt8C from transfected COS cells. However, as Wnt1 and Wnt8 belong to the same functional subclass of Wnt proteins in several different *Xenopus* assays (the tests include mesoderm dorsalisation, alterations in gap junctional communication and direct binding to Frzb-1; Olson et al., 1991; Sokol et al., 1991; Torres et al., 1996; Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b), we used instead a stable fibroblast cell line secreting Wnt1 (Shimizu et al., 1997). We grafted cVg1-secreting COS cells together with Wnt1-secreting fibroblasts in the anterior third of the area pellucida of pre-streak embryos. Embryos were allowed to develop for

Table 1. Effects of misexpression of cVg1 and Wnt1 in different regions of the early chick embryo

Region	Stage	Factor	Ectopic streaks/total (%)
Anterior area pellucida	X-XIII	cVg1	1/21 (5)
		Wnt1	1/24 (4)
		cVg1 + RatB1 cells	1/26 (4)
		Wnt1 + COS cells	0/17 (0)
		cVg1 + Wnt1	29/58 (50)**
Anterior marginal zone	X-XIII	cVg1	38/62 (61)
		Wnt1	3/49 (6)
		cVg1 + Wnt1	14/34 (41)
	2-3	cVg1	1/31 (4)
		Wnt1	0/14 (0)
		cVg1 + Wnt1	0/15 (0)

The frequency of induction of an ectopic streak by a graft of cells secreting the factors indicated is shown on the right. Note that misexpression of cVg1+Wnt1 in the anterior area pellucida of pre-streak embryos (X-XIII) generates an ectopic axis, while neither cVg1 alone nor Wnt1 alone does so (**, $P<0.004$). By contrast, cVg1, Wnt1 and a combination of the two failed to induce a primitive streak in older (stage 2-3) embryos.

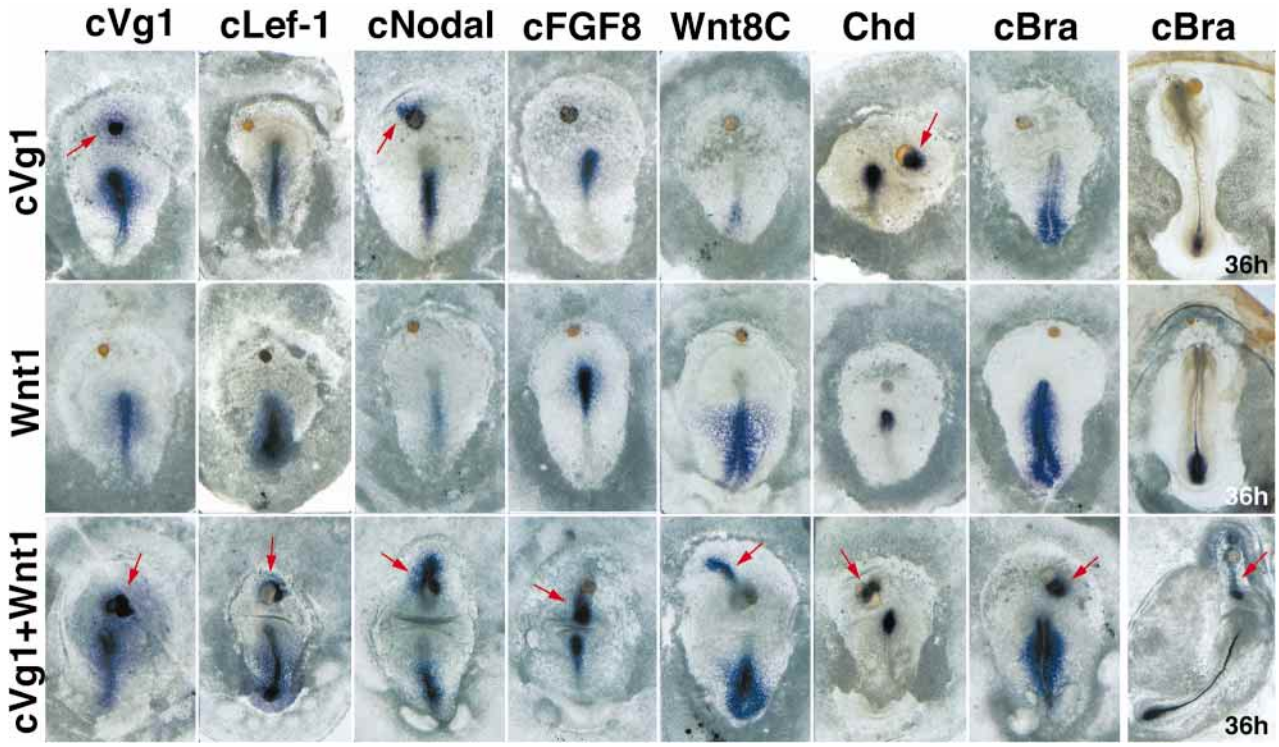

Fig. 1. Comparison of the inducing ability of cVg1, Wnt1 and cVg1+Wnt1 in the area pellucida of stage X-XIII embryos. Cells secreting the factors indicated on the left were grafted into the anterior third of the area pellucida of host embryos, and these were cultured for 24 or 36 hours (right-hand column) before in situ hybridisation (purple) for the genes indicated at the top. Anti-Myc (top row) or anti-HA (middle and bottom rows) immunohistochemistry (brown) was used to identify the cell aggregates and confirm protein synthesis. Red arrows indicate ectopic expression of the gene in the host. The probe used to detect expression of *cVg1* in the embryo also hybridises with the *cVg1*-transfected COS cells in the top and bottom rows of the first column.

24, 36 or 48 hours and the expression of *cVg1*, *cNodal*, *cFGF8*, *Wnt8C*, *chordin* and *cBra* analysed. Misexpression of neither cVg1 nor Wnt1 alone induced an ectopic streak (Table 1), and Wnt1 did not induce expression of any of the markers tested at 24 or 36 hours (Fig. 1). By contrast, when cVg1 and Wnt1 were misexpressed together, an ectopic primitive streak developed (29/58, 50%; Table 1, Fig. 1). At 24 hours, expression of *cVg1* (6/6), *cNodal* (2/2), *chordin* (4/5), *cFGF8* (2/2), *Wnt8C* (2/2) and *cBra* (5/7) was induced in the host. At 36 hours, the induced streak expressed *cBra* and *gsc* (Fig. 1 and not shown), suggesting that it contains axial mesoderm. Some embryos were grown for 48 hours to assess whether they formed a complete axis; none of these produced later axial derivatives, as assessed by morphology and the expression of *cBra* and *gsc* (not shown).

These results suggest that the difference between the area pellucida and the marginal zone in their responses to cVg1 misexpression may be due to differences in Wnt signalling in the two regions.

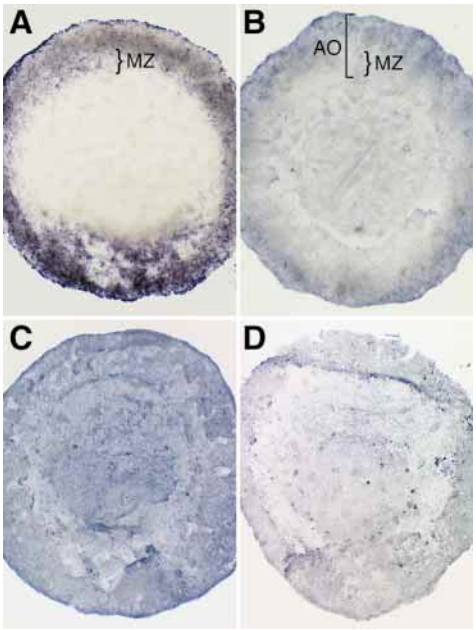

Fig. 2. Wnt signalling in the early chick embryo. (A) Expression of *Wnt8C* at stage XII. Expression is seen all around the marginal zone (MZ) and is strongest posteriorly. Some expression is also seen in the peripheral area opaca. (B) Expression of *Wnt5a* at stage XIII. Weak expression is detected throughout the outer half of the area opaca (AO), but none in the area pellucida or marginal zone (MZ). (C) Expression of *Wnt11* at stage XII. Weak expression is detected throughout the epiblast and hypoblast of the area pellucida and in the epiblast and deep layers of the marginal zone and area opaca. (D) *Wnt3a* is not expressed at stage XIII or at any other stage that precedes primitive streak formation. Its expression starts to be detected by stages 3^{+}-4 (not shown).

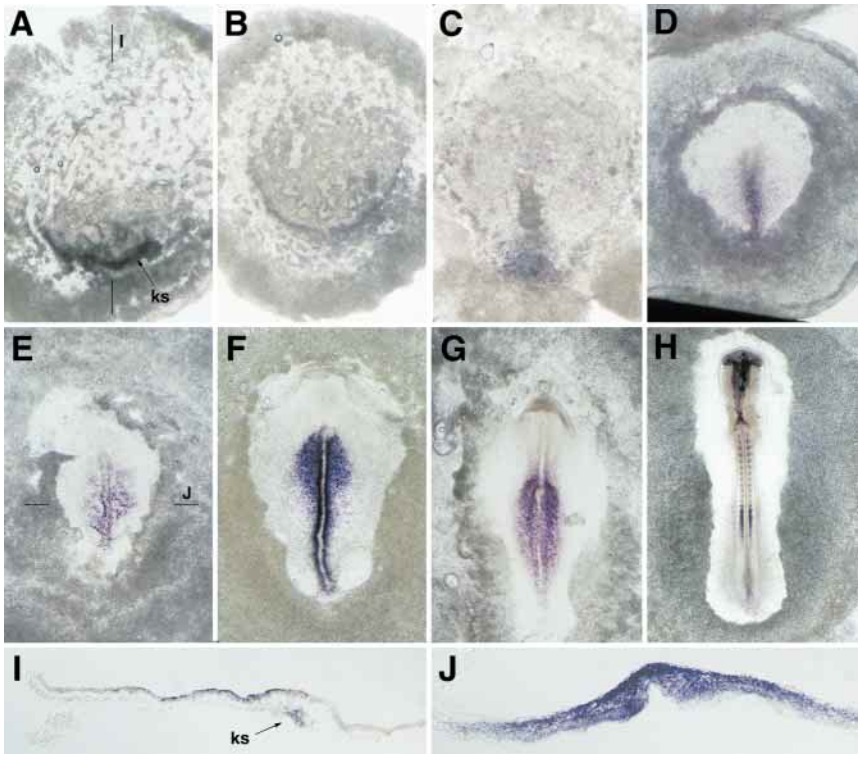

Fig. 3. Expression of *cLef1*. (A) At stage XI, *cLef1* is expressed in Koller's sickle (ks) and posterior marginal zone (which is partly obscured by the overlying germ wall, see section shown in I). (B) At stage XIII, *cLef1* expression continues in the posterior marginal zone and Koller's sickle. (C) By stage 2^+, *cLef1* transcripts are restricted to the primitive streak. (D) At stage 3^+, expression is restricted to the posterior two-thirds of the primitive streak. (E) In stage 4 embryos, expression accompanies the emerging lateral mesoderm (see section shown in J). F. By stage 4^+, *cLef1* expression is strong in the primitive ridges and in mesoderm emerging from the streak. (G) At stage 6, *cLef1* transcripts are found in the forming paraxial mesoderm and primitive streak. (H) At stage 11, *cLef1* expression is seen in the remnants of the streak, the anterior segmental plates and the rostral half of the forming somite. cLef1 is rapidly downregulated from the somites after their formation, but in older (more rostral) somites it appears in the caudal half. cLef1 is also expressed in the heart and brain (see Kengaku et al., 1998). (I) Sagittal section of the embryo in A at the level indicated, showing expression of *cLef1* in Koller's sickle (ks) and in the epiblast of the posterior marginal zone. (J) Transverse section of the stage 4 embryo shown in E at the level indicated, showing expression in the primitive streak and lateral mesoderm.

Expression of Wnts and members of their pathway before primitive streak formation

The above findings implicate Wnt signalling in the difference between the marginal zone and area pellucida in their reactivity to Vg1. Which members of the Wnt family are expressed appropriately to explain this difference in reactivity? We

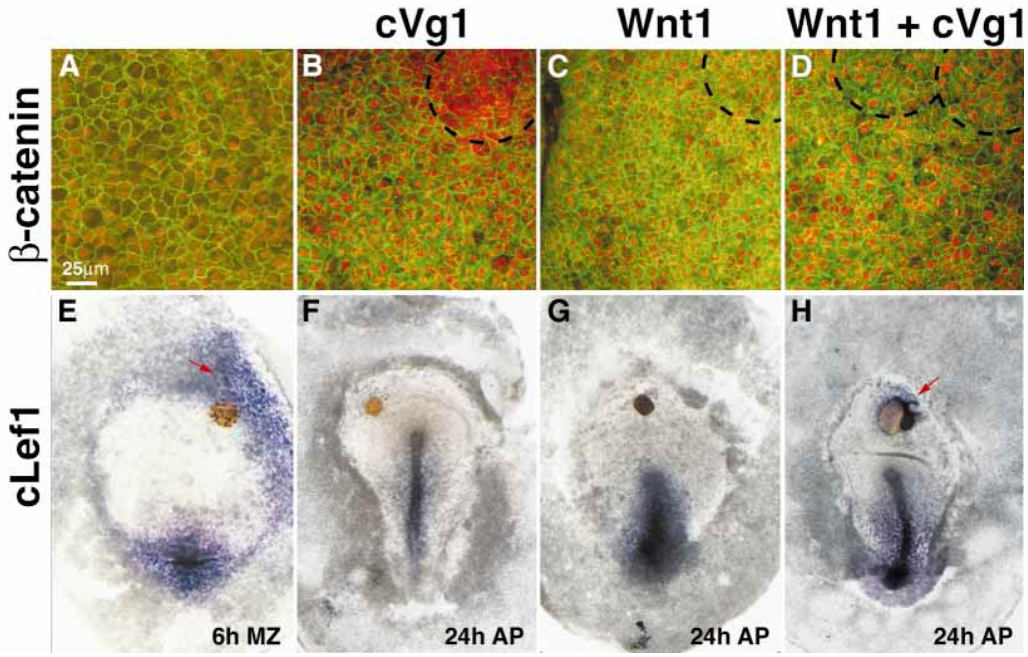

Fig. 4. Regulation of intracellular β-catenin and induction of cLef1 by cVg1+Wnt1. (A-D) Subcellular localisation of β-catenin. In all panels, the green signal corresponds to β-catenin immunofluorescence and the red signal to propidium-iodide stained nuclei. (A) Throughout the area pellucida, most of the immunoreactivity is concentrated at cell membranes. (B) After grafting cVg1-secreting cells (position outlined by broken line), downregulation of β-catenin expression is seen near the graft (outlined). (C) A graft of Wnt1-secreting cells (outlined) increases the level of β-catenin throughout the cell; where it overlaps with the nucleus, the signal appears yellow. (D) When a pellet of Wnt1-secreting cells (indicated on top of left outline) is grafted together with a pellet of cVg1-secreting cells (indicated on top of right outline), the pattern seen is a combination of those in B and C. β-catenin is upregulated in the proximity of the Wnt1 cells and downregulated near the cVg1 cells. Scale bar: 25 μm for A-D; 5 μm for E-H. Expression of *cLef1* after transplantation of cells secreting cVg1 (E,F), Wnt1 (G) or cVg1+Wnt1 (H) in the anterior marginal zone (E) or area pellucida (F-H). (E) cVg1 induces *cLef1* when misexpressed in the anterior marginal zone (arrow). Note that induction of *cLef1* is restricted to the marginal zone and area opaca. In the area pellucida, neither cVg1 (F) nor Wnt1 (G) induces *cLef1*, but a combination of the two factors does (arrow, H).

examined the expression of *cWnt1, cWnt3, cWnt3a, cWnt4, cWnt5a, cWnt5b, cWnt6, cWnt7b, cWnt8, cWnt8C, cWnt10* and *cWnt11* by in situ hybridisation at stages X-4 (Fig. 2). We found that *Wnt8C* was expressed in the marginal zone, most strongly in posterior regions and decreasing laterally and anteriorly (Fig. 2A; Hume and Dodd, 1993), *Wnt5a* was expressed weakly in area opaca and in edge cells of the epiblast (which attach the embryo to the vitelline membrane; New, 1959; Downie and Pegrum, 1971; Downie, 1976; Fig. 2B) and *Wnt11* was expressed ubiquitously at low levels (Fig. 2C). No other Wnts were detectable before gastrulation by in situ hybridisation (Fig. 2D and data not shown). As a complementary approach, we analysed the expression of other Wnts in pre-streak embryos by RT-PCR using degenerate primers designed to amplify fragments of all known Wnt family members except *Wnt8, Wnt8C* and *Wnt10* (see Materials and Methods). Using this approach we cloned new fragments of *Wnt3a, Wnt5a* and *Wnt5b* (see Materials and Methods); however, neither *Wnt3a* (Fig. 2D) nor *Wnt5b* (not shown) was detectable by in situ hybridisation.

It is also possible that the marginal zone and area pellucida differ in their sensitivity to cVg1 because of differential expression of downstream members of the Wnt pathway. We therefore examined the distribution of the Wnt pathway components, *cLef1* and β-catenin, in pre-streak- and primitive-streak-stage embryos. *cLef1* was expressed in the posterior marginal zone and Koller's sickle between stages X and XIII (Fig. 3A,B,I); occasionally, very low levels of expression were detected throughout the marginal zone of stage X embryos (not shown). *cLef1* was also expressed at later stages in a variety of sites (Fig. 3 and Kengaku et al., 1998). We analysed the subcellular distribution of β-catenin by immunofluorescence. In agreement with previous studies (Roeser et al., 1999), we found that β-catenin was concentrated in the nucleus of cells in the germ wall and hypoblast of embryos at stages X-XIII (not shown). Throughout the epiblast, most β-catenin immunoreactivity was associated with cell membranes (Fig. 4A). However, we did not observe either any marked nuclear localisation in any region of the epiblast or the downregulation of β-catenin expression in anterior regions at stages XII-XIII, which have been reported previously (Roeser et al., 1999).

Together, our results suggest that only *Wnt8C, Wnt5a* and *Wnt11* are expressed at significant levels in the pre-streak stage blastoderm, and of these, only *Wnt8C* is expressed appropriately throughout the marginal zone (Fig. 2A; Hume and Dodd, 1993) to account for the difference in cVg1-reactivity between marginal zone and area pellucida. The Wnt pathway components *cLef1* and β-catenin are both expressed at appropriate stages and locations to be involved in primitive streak initiation.

Both cVg1 and Wnt1 regulate intracellular levels of β-catenin and the expression of *cLef1*

The above results point to synergism between Wnt activity and cVg1 in initiating primitive streak formation. As Wnt1 misexpression in the area pellucida does not have any consequence unless cVg1 is also present (Fig. 1, Table 1), does this imply that cVg1 is required for cells to respond to Wnt protein? To address this question, we examined the subcellular localisation of β-catenin in the epiblast of embryos exposed to cVg1, Wnt1 or cVg1+Wnt1 for 3 hours. In untreated embryos,

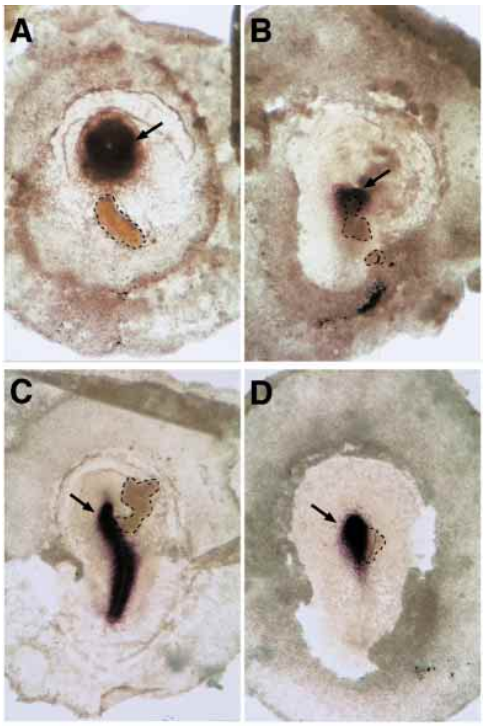

Fig. 5. The broad-spectrum Wnt antagonist Fz-N8 impairs primitive streak formation in stage X (A,B) but not stage XIII (C,D) embryos. (A,B) When cell aggregates secreting Fz-N8 are grafted in the posterior border of the area pellucida of stage X embryos, primitive streak formation is inhibited (A) or seriously impaired (B). After 15 hours' incubation, *cBra* (A) and *chordin* (B) expression are strongly downregulated (white arrowheads, compare with C,D). (C,D) Stage XIII embryos receiving four Fz-N8-secreting cells in the posterior border of the area pellucida develop a normal primitive streak, expressing *cBra* (C) and *chordin* (D). (Stage X embryos receiving mock-transfected cell aggregates develop a normal primitive streak identical to those in C,D.) Black outlines indicate the position of the grafted cell aggregates; black arrowheads indicate *cBra* and *chordin* expression.

β-catenin was predominantly associated with the cell membrane (Fig. 4A). After implantation of cVg1-secreting cells, a pronounced downregulation of β-catenin expression was seen in the vicinity of the graft (Fig. 4B). When Wnt1 was misexpressed alone, a substantial increase in the intensity of staining was seen in the epiblast, not only in the membrane but throughout the cell (Fig. 4C). This suggests that epiblast cells do respond directly to Wnt1, even when cVg1 is not supplied. When cVg1 and Wnt1 were misexpressed together, a combination of the two results was obtained: in the vicinity of the Wnt pellet, the level of β-catenin was increased throughout the cell; near the cVg1-pellet, β-catenin was predominantly membrane associated (Fig. 4D). These findings suggest not only that epiblast cells can respond to Wnt in the absence of exogenous cVg1, but also that Vg1 may dampen the upregulation of β-catenin caused by Wnt1.

The finding that cVg1 misexpression affects intracellular levels of β-catenin raises the possibility that other components of the Wnt pathway may also be regulated by Vg1/Activins. We turned our attention to *cLef1* because it is expressed in the posterior marginal zone (PMZ; Fig. 3A,I), a region that expresses *cVg1* (Seleiro et al., 1996; Shah et al., 1997). We

misexpressed cVg1 in the anterior marginal zone and analysed the expression of *cLef1* at 3 hour intervals for the next 15 hours. Ectopic *cLef1* expression was first seen 6 hours after transplanting the cVg1-secreting cells (4/5 embryos, Fig. 4E), in the marginal zone and area opaca but not in the area pellucida (Fig. 4E). Therefore cVg1 can regulate the expression of *cLef1*.

The finding in this experiment that cVg1 misexpression induces *cLef1* in the marginal zone and area opaca (which express *Wnt8C* and *Wnt5A*, respectively; Fig. 2A,B), could indicate that *cLef1* expression may also be regulated by Wnt signalling. To test this, we misexpressed cVg1, Wnt1 or cVg1+Wnt1 in the anterior area pellucida and analysed the expression of *cLef1* after 9, 15 and 24 hours' incubation. Neither cVg1 nor Wnt1 induces *cLef1* expression in the area pellucida at any time point (Fig. 4F,G), but a combination of cVg1+Wnt1 does so (3/4 embryos at 24 hours; Fig. 4H). This finding is consistent with the posterior restriction of *cLef1* expression at stages XI-2 (Fig. 3A-C) in regions that express both *cVg1* and *Wnt8C*.

Wnt signals are essential for cVg1 to initiate primitive streak formation

The results presented above, in the context of previous findings (Shah et al., 1997), suggest that the difference in reactivity of the marginal zone and area pellucida to cVg1 misexpression could be due to activation of the Wnt pathway in the marginal zone. If the Wnt pathway is indeed required for cVg1 to initiate primitive streak formation, we would expect that inhibition of Wnt signalling should block the ability of cVg1 to induce an ectopic streak. To test this, we used the secreted Wnt antagonists Crescent (Frzb family member) and Dkk1, which can specifically bind Wnt8/-8C and block their activity (Krupnik et al., 1999; Marvin et al., 2001; Schneider and Mercola, 2001), as well as a truncated form of the Xenopus Frizzled-8 receptor, Fz-N8, which is a broad-spectrum Wnt inhibitor (Deardorff et al., 1998; Itoh and Sokol, 1999). When cVg1- and Crescent-secreting cells were grafted together in the anterior marginal zone, the frequency of primitive streak induction by cVg1 dropped from 38/62 (61%) with cVg1 alone to 5/20 (25%) with cVg1+Crescent (P<0.004; Table 2). A similar reduction in frequency of primitive streak induction by cVg1 was observed when Dkk-1 was used instead of Crescent (2/12, 17%, P<0.004; Table 2). Surprisingly, however, misexpression of the broad-spectrum inhibitor Fz-N8 did not significantly inhibit ectopic streak formation by cVg1 (10/17, 59%; Table 2).

These results suggest that Wnt signals sensitive to inhibition by Crescent and Dkk1 are essential for cVg1 to initiate the formation of an ectopic primitive streak, and accounts for the difference in cVg1-reactivity between the area pellucida (where *Wnt8C* is not expressed) and marginal zone.

Primitive streak formation in the normal embryo requires Wnt signals

The conclusion that Wnt signals are required to mediate the induction of an ectopic primitive streak after misexpression of cVg1 leads to the question of whether Wnt activity is similarly required for initiation of the endogenous primitive streak in normal embryos. To test this, we misexpressed the three Wnt inhibitors described above in the posterior area pellucida, close to the site of initiation of primitive streak formation at stages X-XIII. We found that neither Crescent nor Dkk-1 affected the development of the primitive streak at any stage (Table 2). By contrast, the broad-spectrum inhibitor Fz-N8 impaired primitive streak formation when misexpressed at stages X-XI (16/21, 76%, P<0.04; Table 2; Fig. 5A,B) but not at later stages (20/20, 100%; Table 2; Fig. 5C,D). Fz-N8-treated embryos in which the primitive streak failed to form were analysed for *chordin* and *cBra* expression. All the embryos examined expressed these markers at reduced levels (*chordin* 6/6; *cBra*, 8/8; Fig. 5A,B). Together, these results indicate that Wnt signalling is involved in primitive streak initiation in normal development, but that the Wnts involved may be more sensitive to Fz-N8 than to Crescent or Dkk1.

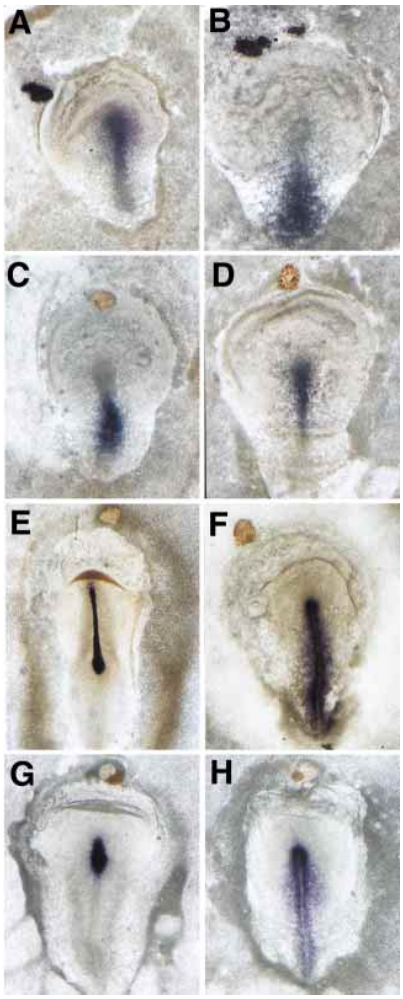

Fig. 6. The competence of the marginal zone to respond to cVg1+Wnt is lost at the primitive streak stage. Cell aggregates secreting cVg1 were grafted alone (A-F) or in combination with Wnt1-secreting cells (G,H) into the anterior margin of stage 2-3 host embryos. After 6 (A-D) or 24 (E-H) hours of incubation, embryos stained for *cVg1* (A), *cLef1* (B), *cNodal* (C), *cFGF-8* (D), *chordin* (E,G) or *cBra* (F,H) by in situ hybridisation. Anti-Myc (A-F) or anti-HA (G,H) immunohistochemistry identifies the cell aggregate and confirms synthesis of the factor. Both cVg1 (A-F) and cVg1+Wnt1 (G,H) are unable to induce any of the markers tested (the dark colour in the grafts in panels A and B is nonspecific).

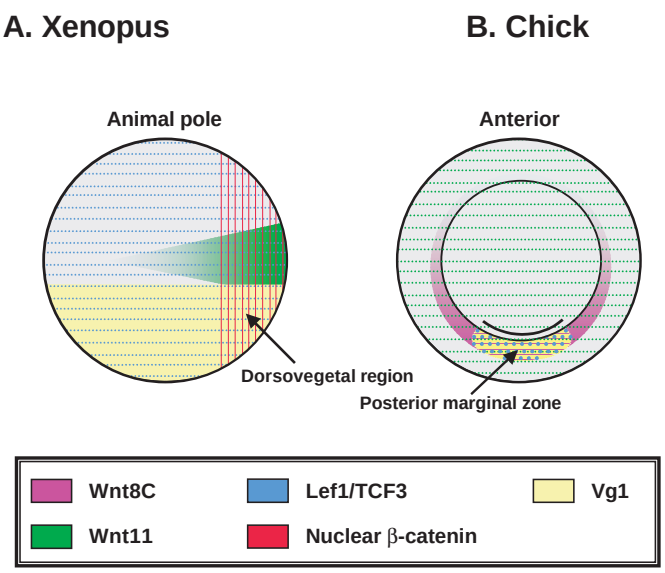

Fig. 7. Distribution of Vg1 and various Wnt signalling pathway components in amphibian and avian pre-gastrula embryos. (A) Lateral view (animal pole up) of a stage 9 (Nieuwkoop and Faber, 1967) *Xenopus* embryo. Vg1 protein is localised to the vegetal pole of the embryo (yellow; Tannahill and Melton, 1989), whereas β-catenin is concentrated in the nucleus of prospective dorsal cells (red; Schneider et al., 1996), even though *cLef1/TCF3* is expressed throughout the embryo (blue; Molenaar et al., 1996; Molenaar et al., 1998). *Wnt11* transcripts (green; Ku and Melton, 1993) are restricted to the marginal zone, in a dorsal-to-ventral gradient. (B) Ventral view of a stage X (Eyal-Giladi and Kochav, 1976) chick embryo (anterior to the top). *Wnt8C* expression in the marginal zone describes a gradient highest posteriorly (purple; Fig. 2A; Hume and Dodd, 1993), where *cVg1* (yellow; Seleiro et al., 1996; Shah et al., 1997) and *cLef1* (blue, Fig. 3) transcripts are also detected. In contrast to *Xenopus*, chick *Wnt11* is ubiquitously expressed at low levels throughout the embryo (green, Fig. 2C). In both species, the region where Vg1 and Wnt activities overlap (the dorsal-vegetal part in *Xenopus* and the posterior marginal zone in the chick embryo) is where the organiser-inducing centre resides (Bachvarova et al., 1998; Harland and Gerhart, 1997, Heasman, 1997, Moon and Kimelman, 1998).

Changes in the Wnt pathway do not account for the loss of competence to cVg1 misexpression at primitive streak stages

In the previous section, we showed that the ability of cVg1 to induce an ectopic primitive streak requires Wnt activity. By stage 2, cVg1 misexpression in the anterior marginal zone can no longer induce an ectopic primitive streak (Shah et al., 1997). At this stage, expression of *Wnt8C* has become restricted to the forming primitive streak (Hume and Dodd, 1993). Is the loss of competence to cVg1 misexpression due to the disappearance of Wnt activity from the marginal zone? To address this question, we misexpressed cVg1, Wnt1 or cVg1+Wnt1 in the anterior marginal zone of stage 2-3 embryos. We found that none of these treatments could induce an ectopic axis, nor the expression of any marker tested (*cVg1*, *cLef1*, *cNodal*, *cFGF8*, *chordin* or *cBra*) (Table 1; Fig. 6). These results suggest that the loss of competence to form a streak in response to cVg1 misexpression at stages 2-3 is not due to the absence of Wnt protein.

DISCUSSION

Synergism between Vg1 and Wnt pathways during induction of an ectopic primitive streak

The ability of cVg1 to induce an ectopic primitive streak is limited to the marginal zone (Shah et al., 1997), a region that expresses *Wnt8C* (Hume and Dodd, 1993). We now show that the area pellucida can also form an ectopic primitive streak in response to cVg1, provided that a source of Wnt is also supplied. In addition, inhibition of endogenous Wnt activity with Crescent or Dkk1 in the marginal zone blocks the axis-inducing ability of cVg1. However, both cVg1 and Wnt1 alone elicit molecular responses even in regions where neither can induce an ectopic streak. How do the Vg1 and Wnt pathways interact to induce a primitive streak? This synergism may occur at the level of transcriptional activation of target genes or at other levels in the two pathways.

Synergism at the level of transcriptional activation

Analysis of the promoter region of genes induced by members of the Wnt and TGFβ families in several species has led to the idea that synergism between the Wnt and TGFβ pathways can occur at the transcriptional level. In *Drosophila*, expression of the homeotic/selector gene *Ultrabitorax* (*Ubx*) in posterior endoderm is regulated by the Wnt and TGFβ family members *wingless* (*wg*) and *decapentaplegic* (*dpp*), respectively (Bienz, 1996), via separate Wg and Dpp response elements in the *Ubx* promoter (Riese et al., 1997). In amphibians, high levels of *siamois*, *twin* and *goosecoid* expression also depend on cooperation between these two pathways acting through separate Wnt and TGFβ response elements (Sokol and Melton, 1992; Steinbeisser et al., 1993; Cui et al., 1995; Watabe et al., 1995; Laurent et al., 1997; Crease et al., 1998; Nishita et al., 2000). Molecular characterisation of the mouse *goosecoid* promoter has also revealed the presence of Wnt and TGFβ-regulatory elements similar to those found in *Xenopus goosecoid*, suggesting that the mechanisms of *goosecoid* regulation have been conserved during evolution (Watabe et al., 1995; Labbe et al., 1998). The chick *goosecoid* promoter has not yet been analysed and it will be interesting in future to determine whether the promoter regions of this and other organiser markers contain comparable regulatory elements.

More complex interactions between Vg1- and Wnt pathways

The Wnt and TGFβ signalling pathways have been characterised extensively in *Drosophila*, *Xenopus* and mammals (reviewed in Cadigan and Nusse, 1997; Massagué and Chen, 2000). Activation of the 'canonical' Wnt pathway leads to the stabilisation of β-catenin (Cadigan and Nusse, 1997; Moon and Kimelman, 1998), which then forms a complex with Lef1/TCF transcription factors to activate target gene expression (Behrens et al., 1996; Molenaar et al., 1996). TGFβ family members, on the other hand, lead to the assembly of a Smad complex, which then recruits co-activators or co-repressors to regulate the expression of target genes (Massagué and Chen, 2000). At first sight, therefore, the TGFβ and Wnt pathways are quite distinct. However, recent experiments in *Xenopus* led to the surprising finding that Lef1 and β-catenin interact directly with Smad4 to activate *twin* transcription (Nishita et al., 2000), suggesting that some components of the

Table 2. Effects of misexpression of the Wnt antagonists Crescent, Dkk1 and Fz-N8 on normal and cVg1-induced primitive streak development

Region	Stage	Factor	Primitive streaks/total (%)
Anterior marginal zone	X-XIII	cVg1	38/62 (61)
		Crescent	0/20 (0)
		Dkk1	0/10 (0)
		Fz-N8	0/16 (0)
		cVg1+COS cells	9/17 (56)
		cVg1+Crescent	5/20 (25)**
		cVg1+Dkk1	2/17 (17)**
		cVg1+Fz-N8	10/17 (59)
Posterior area pellucida	X-XI	COS cells	16/16 (100)
		Crescent	22/22 (100)
		Dkk1	5/5 (100)
		Fz-N8	16/21 (76)*
	XII-XIII	Fz-N8	20/20 (100)

The frequency of induction of an ectopic streak by a graft of cells secreting cVg1 (top section) or the development of a normal primitive streak by the host embryo (bottom) in the presence of the factors indicated is shown on the right. Note that misexpression of Crescent or Dkk1, together with cVg1 reduces the frequency of induction in the marginal zone of stage X-XIII embryos from 61% to 25% or 17%, respectively (**, $P<0.004$). In this assay, Fz-N8 does not have any effect. However, Fz-N8 impairs normal primitive streak formation when misexpressed at the posterior border of the area pellucida in stage X embryos (*, $P<0.04$).

signal transduction machinery in the Wnt and TGFβ pathways can interact directly. Our finding that cVg1 regulates the expression of components of the Wnt pathway reveals some additional complexity. First, cVg1 misexpression in the area pellucida (where *Wnt8C* is not expressed) causes a downregulation of β-catenin levels (Fig. 4B). Second, misexpression of cVg1 in the marginal zone (a region that does express *Wnt8C*) induces *cLef1* expression (Fig. 4E). Third, when cVg1 is misexpressed together with Wnt1 in the area pellucida, *cLef1* expression is induced, which is not seen when either factor is misexpressed alone (Fig. 4F-H). Together, these findings suggest that many components of the Vg1/TGFβ and Wnt pathways may be intertwined.

In addition, both TGFβ and Wnt signals can be transduced by mechanisms that do not involve the 'canonical' Smad and β-catenin pathways. A growing body of evidence suggests that TGFβ family proteins can regulate expression of target genes using the JNK pathway (reviewed by Massagué and Chen, 2000) via TAK1 (TGFβ activated kinase) and NLK (Nemo-like kinase), inducing a variety of responses depending on the cell type (Afti et al., 1997; Shibuya et al., 1998; Hocevar et al., 1999; Yamaguchi et al., 1999). Interestingly, in both *Xenopus* and *C. elegans*, activation of the TAK-NLK-JNK pathway causes phosphorylation of Lef1/TCF/POP-1 and subsequent inhibition of β-catenin-mediated responses (Ishitani et al., 1999; Meneghini et al., 1999); this mechanism could explain the downregulation of β-catenin levels caused by cVg1 misexpression in our experiments (Fig. 4B).

Likewise, Wnt signals can be transduced using pathways other than β-catenin, and two such pathways are known (Cox and Peifer, 1998; Boutros and Mlodzik, 1999; Noselli and Agnes, 1999). One involves activation of the Rho/Rac/Cdc42 family of small GTPases and JNK-type kinases (Strutt et al., 1997; Boutros et al., 1998; Boutros and Mlodzik, 1999; Noselli and Agnes, 1999). The second alternative pathway involves activation of the phosphoinositol (PI) pathway and increase in intracellular Ca^{2+} (Slusarski et al., 1997a; Slusarski et al., 1997b). The β-catenin, Rho/JNK and PI/PKC/Ca^{2+} pathways may serve different functions: activation of the β-catenin pathway in *Xenopus* and zebrafish causes axial duplications (reviewed in Moon and Kimelman, 1998), whereas activation of the PI/PKC/Ca^{2+} pathway disrupts morphogenetic movements (Moon et al., 1993; Du et al., 1995; Torres et al., 1996) and the Rho/Rac/JNK pathway has been implicated in the regulation of cell movements and cell shape through the actin cytoskeleton (Nobes and Hall, 1995; Nobes and Hall, 1999).

Taken together, these considerations suggest that the cVg1 and Wnt pathways can interact at several different levels, and that the choice of alternative transduction pathways may regulate different aspects of cell behaviour that are crucial to gastrulation, such as cell fate decisions and morphogenetic movements.

The marginal zone as a special region of the early embryo, defined by Wnt activity

Unlike amphibians, where polarity is determined by localisation of maternal determinants before the beginning of zygotic transcription (Harland and Gerhart; 1997; Heasman, 1997; Moon and Kimelman, 1998; Arendt and Nübler-Jung, 1999), avian embryos can re-establish their polarity up until the time when primitive streak formation begins, when the embryo may already contain as many as 20,000-60,000 cells (Spratt and Haas, 1960b). Spratt and Haas showed that when embryos are cut into fragments, each fragment can spontaneously generate an embryonic axis, provided both that the fragment contains a portion of the marginal zone and that they are obtained from embryos younger than the primitive streak stage (Spratt and Haas, 1960b). The frequency of primitive streak formation in these fragments decreases in a posterior-to-anterior direction, but even the most anterior fragments are capable of initiating an axis. These observations led them to propose that 'the marginal zone of an unincubated blastoderm exhibits a gradient in embryo forming potentiality…the highest point of which is in the prospective posterior median position' (Spratt and Haas, 1960b).

Within the marginal zone, only the posterior part (the PMZ) can induce an axis (including Hensen's node) when transplanted to the anterior side of a host embryo fragment (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989)

without making a cellular contribution to the induced structures (Bachvarova et al., 1998). In this sense, the PMZ is the avian equivalent of the Nieuwkoop centre of amphibians (see Bachvarova et al., 1998). In *Xenopus*, this centre is thought to arise from interaction between the Wnt and TGFβ pathways (Fig. 7). The Wnt pathway is activated (as revealed by nuclear localisation of β-catenin; Schneider et al., 1996; Yost et al., 1996; Larabell et al., 1997) throughout the prospective dorsal side of the embryo (Wylie et al., 1996), while *Vg1* RNA is preferentially localised in the vegetal half of the blastula (Rebagliati et al., 1985; Weeks and Melton, 1987; Dale et al., 1989; Tannahill and Melton, 1989; Fig. 7). The two activities overlap in the dorsal-vegetal part of the embryo, where the Nieuwkoop centre resides (Harland and Gerhart, 1997; Heasman, 1997; Moon and Kimelman, 1998; Fig. 7). In the chick, *Wnt8C* is expressed throughout the marginal zone (Fig. 2A; Hume and Dodd, 1993) and *cVg1* only in the posterior region (Seleiro et al., 1996; Shah et al., 1997), making the PMZ the only region of the pre-primitive streak stage embryo that expresses both *cVg1* and a member of the Wnt 1 subfamily (Fig. 7). Therefore, despite the differences between avian and amphibian embryos in the mode of polarity determination, similar pathways appear to be involved, and it is tempting to speculate that the overlap of *cVg1* and *Wnt8C* activity in the PMZ is responsible for the Nieuwkoop centre-like properties of this region (Fig. 7).

We now provide both loss-of-function and gain-of-function results that support this notion. Inhibition experiments with Crescent and Dkk-1 strongly suggest that Wnt activity is essential for the formation of an ectopic primitive streak in response to cVg1. We also show that the area pellucida can be rendered competent to respond to cVg1 and initiate a primitive streak if an exogenous source of Wnt activity is supplied. These latter results are consistent with the finding that misexpression of Wnt1 can enhance the axis-inducing activity of Activin (a TGFβ family member closely related to Vg1; Cooke et al., 1994), although we were not able to reproduce the finding (Cooke et al., 1994) that Wnt1 misexpression alone can re-position the host axis (not shown).

Together with the complexity of the interactions between Wnt and Vg1 signals already discussed, we can now provide a partial molecular explanation for the results of Spratt and Haas (Spratt and Haas, 1960b): we propose that Wnt activity defines the marginal zone as a whole, within which the expression of cVg1 defines the PMZ (Fig. 7). Only the region where cVg1 is present will express *cLef1*, transduce the Wnt signal and initiate the formation of a primitive streak. When embryos are bisected into anterior and posterior halves, the posterior half retains the PMZ and continues to form a primitive streak at high frequency. By contrast, the anterior half retains the graded expression of *Wnt8C* in the marginal zone, with two points of high *Wnt8C* expression in lateral marginal zones. High levels of *Wnt8C* expression may bias the stochastic induction of *cVg1* (by an unknown factor) in one of the two lateral marginal zones, which initiates formation of the axis.

Unexpected effects of Wnt antagonists

The above conclusions are based in part on the ability of the Wnt antagonists Crescent and Dkk-1 to block the induction of an ectopic axis by cVg1. However, we also show that the same antagonists do not block the formation of the normal primitive streak when placed posteriorly in the embryo. Conversely, the broad-spectrum Wnt antagonist Fz-N8 blocks formation of the normal streak, but has no effect on the axis induced by cVg1 misexpression in the marginal zone. How can these results be explained?

Functional studies in *Xenopus* have shown that while Dkk-1, Frzb-1, Fz-N8 and Crescent can antagonise signalling by the Wnt1/Wnt8 family of proteins (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b; Deardorff et al., 1998; Itoh and Sokol, 1999; Krupnik et al., 1999; Marvin et al., 2001; Schneider and Mercola, 2001), only Dkk-1 and Fz-N8 can block signalling by Wnt2b, Wnt3a and Wnt5a (Wang et al., 1997b; Itoh and Sokol, 1999; Krupnik et al., 1999; Marvin et al., 2001; Schneider and Mercola, 2001) even though Frzb-1 can bind Wnt5a in co-immunoprecipitation assays (Lin et al., 1997). Furthermore, indirect evidence also suggests that Crescent and Fz-N8 might inhibit Wnt11 activity as well (Deardorff et al., 1998; Pera and De Robertis, 2000). Therefore, the different Wnt ligand specificities shown by the various antagonists tested may partly account for the results obtained in our assays.

Recently, it has been shown that recombinant secreted Frizzled-related protein 1 (sFRP1) exerts a biphasic effect on Wg activity in a β-catenin stabilization assay, increasing β-catenin levels at low concentrations, but reducing them at higher concentrations (Üren et al., 2000). This has lead to the idea that Frzb-1 type of molecules (including Crescent) may have a dual function as Wnt agonist or antagonist in a dose-dependent manner (Üren et al., 2000; see Pfeiffer and Vincent, 1999). In the *Xenopus* assays discussed above, injection of mRNA is likely to produce high concentrations of Wnt antagonists, probably comparable with the doses of sFRP1 that result in Wnt inhibition (Üren et al., 2000). This contrasts with our experimental conditions, where local sources of antagonists are used and the levels of protein is likely to decrease rapidly with increasing distance from the source. The ability of Crescent and Dkk-1 to block primitive streak induction by cVg1 in the anterior marginal zone can be explained by their ability to inhibit endogenous Wnt8-related factors expressed at low levels in this region. Close to the posterior marginal zone, however, the relative levels of Dkk-1 or Crescent may not be high enough to antagonise Wnt8-related signalling, but instead may facilitate presentation of the Wnt molecule to its receptor. Why, then, does the broad-spectrum Wnt antagonist Fz-N8 does not block axis induction by cVg1, while Crescent and Dkk-1 do?

When Fz-N8 is misexpressed in the posterior area pellucida it blocks the formation of the endogenous primitive streak but does not inhibit *chordin* or *brachyury* expression (Fig. 5). The expression of *chordin* in the area pellucida suggests that organiser fates have been established. This differs from the consequences of misexpression of another primitive streak inhibitor, BMP4: in this case, the embryos do not express *cNot1* (an organiser maker) or *brachyury* (Streit et al., 1998). Therefore it is likely that BMP4 prevents primitive streak formation by blocking the specification of particular cell fates, while misexpression of Fz-N8 at the posterior end of the embryo does not affect cell fate specification but rather some other aspect of primitive streak formation, such as cell movements.

The recent finding that a zebrafish homologue of *Wnt11*

(*Silberblick*) is required for the proper convergence/extension movements of gastrulation, acting through a β-catenin-independent pathway (Heisenberg et al., 2000, see also Tada and Smith, 2000; Makita et al., 1998) is consistent with this idea. Misexpression of Fz-N8 in the posterior area pellucida could prevent primitive streak formation by inhibiting Wnt11 signalling that disrupts the Polonaise movements (Gräper, 1929; Spratt and Haas, 1960a; Stern, 1990; Hatada and Stern, 1994) that precede gastrulation, without blocking the expression of organiser markers.

The molecular basis of competence for primitive streak initiation by cVg1

When cVg1 is misexpressed in the marginal zone of embryos that have initiated gastrulation, neither *chordin* nor *cBra* expression is induced, and no ectopic primitive streak develops. Although *Wnt8C* is downregulated in the marginal zone at the start of gastrulation (Hume and Dodd, 1993), this is not responsible for the loss of competence that occurs at this stage, because misexpression of Wnt1 does not rescue the ability of these older embryos to respond to cVg1. Possible candidates to explain this loss of competence are the 'ventralising' factors of the TGFβ family *BMP-2*, *BMP-4*, and *BMP-7*, which are appropriately expressed at the periphery of the embryo (Streit et al., 1998; Joubin and Stern, 1999). Misexpression of the BMP antagonist Noggin at the edge of the area pellucida allows cVg1+Wnt1 to induce expression of organiser markers at stages 3^{+}-4 (Joubin and Stern, 1999). Therefore, before gastrulation, the competence of the marginal zone to respond to cVg1 requires Wnt activity. Once gastrulation is initiated, other signalling molecules present at the periphery, such as BMPs, may make the marginal zone refractory to cVg1 and Wnt signals.

However, some aspects of the competence for ectopic primitive streak formation cannot be explained by cVg1 and Wnt activity alone. When a PMZ is transplanted to the anterior half of a host embryo (Bachvarova et al., 1998) or to the lateral side of embryos that have been deprived of their own PMZ (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989), a primitive streak is induced, but only if the host embryo is at stage XI or younger. By contrast, cVg1 can induce a complete axis even if misexpressed in the marginal zone of stage XIII embryos (Seleiro et al., 1996; Shah et al., 1997), and can induce an ectopic organiser when misexpressed with Wnt1 in the area pellucida up to stage 3^{+} (Joubin and Stern, 1999). The molecular mechanisms that underlie this early window of competence to the PMZ remain unknown.

Likewise, mechanisms other than Wnt signalling seem to be required for the primitive streak to continue its development and give rise to an axis. In the area pellucida of pre-streak embryos, cVg1 alone can induce organiser markers but not an ectopic streak, while cVg1+Wnt induce expression of organiser markers as well as a primitive streak. However, even these ectopic primitive streaks do not give rise to axial derivatives like a notochord or prechordal mesoderm. This result is reminiscent of the finding that local misexpression of Chordin in the marginal zone or area pellucida induces an ectopic streak containing an organiser, but again no further axial derivatives are formed (Streit et al., 1998; Streit and Stern, 1999). Together, these observations suggest that the competence of the area pellucida to give rise to axial structures derived from the streak is limited either by the lack of some additional permissive signals, or by the presence of inhibitors.

Conclusions

We have shown that the ability of cVg1 to induce an ectopic primitive streak in the anterior marginal zone of the chick embryo requires Wnt activity of the Wnt1/Wnt8-related class. However, our results also point to an involvement of other Wnts in axis formation, perhaps members of the Wnt11 class. Our gain- and loss-of-function results explain why an ectopic axis is induced when cVg1 is misexpressed in the marginal zone but not in the area pellucida. The differences in sensitivity to Wnt antagonists between the normal axis-forming region and of the ectopic axes induced by Vg1 suggest that there may be a diversity of cVg1- and Wnt transduction pathways, which appear to interact with each other in complex ways. We propose that different transduction pathways are responsible for controlling different aspects of primitive streak formation, such as the acquisition of cell fates and the regulation of cell movements and adhesion.

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