**Wnt-11 is expressed in early avian mesoderm and required for the differentiation of the quail mesoderm cell line QCE-6**

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**SUMMARY**

The beginning of mesodermal development involves the aggregation of newly gastrulated cells into epithelial fields, as a prelude to organ formation. To analyze the molecular regulation of this initial patterning, we have focused on the Wnt family of secreted signaling proteins, molecules which have been shown to promote embryonic patterning by regulating cell-cell associations. In this study, we show that the Wnt-11 gene is expressed by newly gastrulated mesoderm cells within avian embryos. The expression pattern of Wnt-11 also suggests that it may be involved in formation of the cardiogenic fields and somites. Subsequently, we utilized the quail mesoderm cell line QCE-6 as a culture model for examining the influence of Wnt-11 on early mesoderm cell differentiation. This cell line has been shown to be representative of early nondifferentiated mesoderm cells and has the potential to differentiate into cardiomyocytes, endothelial or red blood cells. Similar to early mesoderm cells, QCE-6 cells express Wnt-11. We have engineered stable transfectants of these cells that produce either diminished or enhanced levels of Wnt-11 protein. Our studies show that Wnt-11 regulates cellular interactions of QCE-6 cells, as demonstrated by alterations in contact-inhibited growth, tight and gap junction formation and plakoglobin expression. Both the morphology and growth factor-induced differentiation of QCE-6 cells are regulated in a cooperative fashion by Wnt-11 and fibronectin. These results, described in detail below, demonstrate the uniqueness of QCE-6 cells as a culture system for analyzing Wnt activity. In particular, QCE-6 cells are the first cell line that has demonstrated: (1) Wnt-dependent differentiation; (2) concentration-variable responses to Wnt protein; and (3) altered cell phenotypes as a direct response to Wnt-5a class proteins (e.g. Wnt-4 and Wnt-11).

Key words: Wnt gene, QCE-6, mesoderm, plakoglobin, heart, differentiation, quail.

**INTRODUCTION**

The Wnt family of secreted signaling proteins are important regulators of embryonic patterning (McMahon, 1992; Moon, 1993; Nusse and Varmus, 1992) and have been implicated in the development of the brain (Hollyday et al., 1995; Parr et al., 1993), limbs (Christiansen et al., 1995; Dealy et al., 1993; Parr et al., 1993), kidney (Herzlinger et al., 1994; Stark et al., 1994), heart (Park et al., 1996), muscle (Christiansen et al., 1995; Takada et al., 1994) and mammary glands (Weber et al., 1994). Transgenic Wnt gene knockout mice have demonstrated a variety of phenotypes, including brain truncations, mirror-image duplications along the dorsal-ventral axis in limb, and decreases in mesenchymal cell epithelialization in kidney. As many as 17 distinct Wnt genes may be present in the vertebrate genome (Christiansen et al., 1995; Du et al., 1995; Hollyday et al., 1995; Parr et al., 1993; Sidow, 1992). These genes encode a set of glycoproteins that range from 38-46 ×10³ M₉ (McMahon, 1992; Nusse and Varmus, 1992). Recent studies have identified cell membrane receptors for these signaling proteins (Bhanot et al., 1996). A major obstacle in studying Wnt signaling has been the difficulty of obtaining soluble Wnt protein, purified from either endogenous or recombinant sources, that can be added directly to culture (Bradley and Brown, 1990; Smolich et al., 1993).

Two molecules implicated as downstream transmitters of Wnt signaling are the related cell junction proteins β-catenin and plakoglobin (Bradley et al., 1993; Funayama et al., 1995; Hinck et al., 1994; Karynosky and Klymkowsky, 1995). These proteins interact with the cytoplasmic domain either of cadherin or of both cadherin and desmosomal cell-cell adhesion molecules, respectively. In addition to roles in intercellular adhesion, both β-catenin and plakoglobin may have significant regulatory activities within the cytoplasm and/or nucleus (Behrens et al., 1996; Funayama et al., 1995; Karynosky and Klymkowsky, 1995; Papkoff et al., 1996). Wnt activity is thought to be mediated by influencing the cellular localization of these proteins (Bradley et al., 1993; Hinck et al., 1994; Peifer et al., 1994).

Insights into the function(s) and mechanism(s) of Wnt signaling have been provided by studies using Xenopus embryos (Moon, 1993). Injection of either Wnt-1, Wnt-3a or Wnt-8 mRNA into ventral blastomeres of blastula stage embryos produces an anterior duplication of the embryonic axis. Injection of these mRNAs into dorsal blastomeres has no effect on embryonic development. In contrast, microinjection...
of either Wnt-4, Wnt-5a or Wnt-11 mRNA into dorsal blastomeres provokes complex tail and head deformities. Yet, if these latter mRNAs are injected on the ventral side, embryos will develop normally (Du et al., 1995; Moon et al., 1993).

The principal culture system for analyzing Wnt activity has been mammmary epithelial cell lines, which undergo morphological transformation in response to overexpression of some, but not all, Wnt genes (Wong et al., 1994). Other cell lines have been employed to study Wnt activity (Hinch et al., 1994; van Leeuwen et al., 1994), only one of which demonstrates altered differentiation in response to changes in Wnt expression: the pheochromocytoma cell line PC12 (Bradley et al., 1993; Shackleford et al., 1993). These cells undergo neural differentiation in response to either bFGF or NGF. However, the capacity of stably infected Wnt-1-producing PC12 cells to respond to these growth factors is diminished (Shackleford et al., 1993). Thus, Wnt expression disrupts neural differentiation of PC12 cells.

A candidate model system for Wnt regulation is the quail mesoderm cell line QCE-6 (Eisenberg and Bader, 1995, 1996). This cell line was derived from mesoderm of Hamburger and Hamilton (HH; 1951) stage 4 quail embryos. Previous studies have shown that QCE-6 cells possess the potential to differentiate into three cell types: cardiomyocytes, endothelial and red blood cells (Eisenberg and Bader, 1996; Eisenberg and Markwald, unpublished data). This multi-potentiality of QCE-6 cells is in accordance with what others have reported for early mesoderm (Eichmann et al., 1993; Lee et al., 1994).

In this report, we characterize the expression of Wnt-11 by early mesoderm cells within the avian embryo. Accordingly, we show that QCE-6 cells also express this Wnt gene. Stable transfectants of these cells have been established, with either diminished or enhanced levels of Wnt-11 expression. Characterization of the stable transfectants has shown that Wnt-11 affects QCE-6 cells profoundly, as demonstrated by morphological and phenotypic changes that are exhibited. These results, described in detail below, demonstrate the uniqueness of QCE-6 cells as a model system for analyzing Wnt activity. In particular, that QCE-6 cells are the first cell line to demonstrate: (1) differentiation that is Wnt-dependent; (2) differential responses to variable concentrations of Wnt protein; and (3) altered cell phenotypes as a direct response to proteins of the Wnt-5a class (e.g. Wnt-4 and Wnt-11).

**MATERIALS AND METHODS**

**RNA isolation and cDNA library construction**

Total cellular RNA was isolated from QCE-6 cells treated with TRIzol reagent (Gibco BRL). Subsequently, poly(A)-RNA was purified using Oligotex-dT (Qiagen), according to the manufacturer’s instructions.

To create the QCE-6 cDNA library, poly(A)-RNA (~3 µg) was treated with MMLV reverse transcriptase in the presence of oligo(dT) primer and methylated dNTPs. The second strand was produced by incubation with *E. coli* DNA polymerase and ribonuclease H. Following treatment with T4 DNA polymerase, directional EcoRI/HindIII linkers were ligated to the cDNA and digested with EcoRI and HindIII restriction enzymes. cDNAs were size-separated on SizeSep400 columns (Pharmacia) and ligated into the λExloX cloning vector (Novagen). Phage particles were subsequently produced using Phagemaker extract (Novagen), to give a primary cDNA library with a titer of 2.3 × 10^9 PFU/ml.

**Identification and cloning of Wnt-11**

Single-strand cDNA, generated by reverse transcription of QCE-6 poly(A)-RNA, was used as a template for polymerase chain reaction (PCR) studies. cDNA was amplified using degenerate primers generic for Wnt sequences: 5’-GGGGATTAAAAGGAAG(T/G)CTTAAATGTG(C/T)CAT3’ and 5’-AAATCTTAGAAGCA(A/G)-CACCA(A/G)TG(A/G)AA3’. PCR reactions were performed as described previously (Eisenberg et al., 1992). Final reaction products of approximately 320 bp were isolated from Nusieve agarose gels (FMC) and ligated into Bluescript plasmid vector (Stratagene).

Sequence analysis of multiple cloned PCR fragments identified Wnt-11 as the predominant amplification product. Thereafter, Wnt-11 PCR fragments were labeled with fluorescein and used to screen the QCE-6 cDNA library. Hybridized clones were detected by chemiluminescence (Renaissance kit; NEN). Positive clones were isolated as plasmids, by infection of the PI cre recombinase-positive *E. coli* strain BM25.8 (Novagen). The largest clone contained a 2050 bp cDNA insert, which included the entire Wnt-11 coding region. The complete sequence of this cDNA was determined using Sequenase (Amersham), in the presence of either biotinylated primers or nucleotides. To resolve compressions, some sequencing reactions were performed in the presence of dTTP. To facilitate sequencing of the cDNA, nested deletions were generated by exonuclease III digestion.

**In situ hybridization**

Whole-mount in situ hybridizations of quail embryos were carried out as described by Barth and Ivarie (1994). The transcription template pWnt-11/BS was prepared by subcloning the Wnt-11 cDNA insert into Bluescript. Labeled anti-sense and sense Wnt-11 RNA probes were produced by in vitro transcription of pWnt-11/BS with either T3 or T7 polymerase in the presence of digoxigenin (DIG)-11-UTP (Boehringer Mannheim). As a further control, anti-sense RNA was prepared from a Wnt-2 cDNA template. Hybridized probe was revealed using alkaline phosphatase-coupled anti-DIG antibodies (Boehringer Mannheim). Stained embryos were mounted in PBS/glycerol and examined using a Zeiss Stemi SV 6 stereomicroscope. Stained embryos were sectioned following polyacrylamide embedding, as described by Germroth et al. (1995).

**Northern blotting**

Poly(A)-RNA samples (1 µg/lane) were separated by electrophoresis in 1% agarose, 0.4 M formaldehyde gels and transferred to nylon membranes. Blots were hybridized with either antisense or sense Wnt-11 RNA, labeled with fluorescein-12-UTP (NEN), at 65°C in 0.25 M NaH_2PO_4, 5% SDS, 1 mM EDTA and 0.5% casein. Afterwards, blots were washed several times with 0.025 M NaH_2PO_4, 0.5% SDS, 0.01 mM EDTA at 65°C. A positive signal was revealed by incubating hybridized blots with alkaline phosphatase-coupled anti-fluorescein antibodies, followed by CSPD chemiluminescent reagent (Tropix).

**Generation of stable transfectants and immunofluorescent staining**

Stable transfectants of QCE-6 cells were established with either enhanced or diminished Wnt-11 expression. Transgenes consisted of full-length Wnt-11 cDNA inserted into the eukaryotic expression vector pCDNA3 (Invitrogen), in either sense or antisense orientations. After transfecting QCE-6 cells with either plasmid in the presence of LipofectAMINE (Gibco BRL), subclones of QCE-6 cells that incorporated the transfected DNA were selected by their resistance to neomycin. For cell mixing experiments, β-galactosidase-expressing cells were produced by subcloning cells stably infected with CXL retrovirus (Gourdie et al., 1995). Immunostaining of cell cultures was according to protocols described previously (Eisenberg and Bader, 1996). Passaged cells were plated at approx. 50% confluency in either poly-D-lysine (20 µg/cm²) or fibronectin-coated (2.0 µg/cm²) 8-well Lab-Tek Chamber Slides (Nunc) for a minimum of 24 hours. To induce cell differentiation, cells were
plated at 25% confluence into precoated Lab-Tek Chamber Slides, with medium containing 2 × 10⁻⁸ M all-trans retinoic acid (Sigma), 250 ng/ml human recombinant basic fibroblast growth factor (bFGF), 10 ng/ml porcine transforming growth factor beta 2 (TGFβ2) and chicken recombinant transforming growth factor beta 3 (TGFβ3) from R & D Systems. After culture termination, cells were fixed in 100% methanol and rehydrated with PBS. Slides were blocked with PBS/0.1% bovine serum albumin (BSA) and incubated with either cingulin (Citi et al., 1989), connexin43 (Zymed), N-cadherin (Sigma), plakoglobin (AbProte International), β-catenin (Hagel et al., 1995) or sarcomeric myosin heavy chain (MF20; Bader et al., 1982) antibodies. Secondary antibodies were either fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse or anti-rabbit IgG (Organon Teknika-Cappel). Nuclei were stained with TOTO-3 (Molecular Probes). Control stainings were performed by substituting PBS/BSA for primary antibody. After cell staining, slides were mounted with 90% glycerol/PBS containing 100 mg/ml diazobicyclooctane, and viewed using either confocal (Bio-Rad MRC-600) or conventional phase-fluorescence (Leitz Dialux) microscopy. Photographs of the latter images were digitized on a Umax scanner. Figures were compiled using Adobe Photoshop.

**Generation of antibodies to Wnt-11 and immunoblotting**

Wnt-11-reactive antibodies were generated by immunization of rabbits with the protein-specific peptide VRHPMgTRKYLVPKDIDIRP (Fig. 1). Serum from immunized rabbits was fractionated using 50% ammonium sulfate, and column-purified against immobilized peptide (AminoLink Plus, Pierce). Total cell protein was isolated from 100 mM culture plates of QCE-6, Wnt-11-minus and Wnt-11-ox cells. Cells were scraped from dishes containing RIPA buffer (150 mM NaCl, 25 mM Tris pH 7.5, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 2 μg/ml Leupeptin, 1 μg/ml Pepstatin A) and disrupted with a Dounce homogenizer. Lysates were centrifuged at 15000 g, and the supernatant collected. 20 μg protein samples were diluted in Laemmli buffer (120 mM Tris pH 6.8, 4% SDS, 10% β-mercaptoethanol, 10% glycerol), boiled and separated on 7.5% polyacrylamide gels. Following electrophoretic transfer to PVDF, filters were incubated with blocking buffer (PBS, 0.1% Tween-20, 4% dried milk). Interspersed by extensive washing with blocking buffer, blots were incubated in sequence with purified Wnt-11 peptide antibody, fluorescein-labeled goat anti-rabbit IgG, anti-fluorescein antibody-coupled alkaline phosphatase, and with CSPD chemiluminescent reagent. After exposing blots to autoradiography film, blots were stripped of antibody using 0.2 M glycine pH 2.2, 0.1% SDS, 1% Tween 20 and reprobed with anti-actin monoclonal antibody IF8 (kindly provided by Dr Tony Capehart). For peptide inhibition studies, primary antibody was pre-absorbed with 50 μg/ml purified Wnt-11 peptide in blocking buffer, before adding to protein blots.

**RESULTS**

**Wnt-11 is expressed by early mesoderm**

Wnt genes are believed to influence embryonic patterning by affecting cell-cell associations. Thus, a logical candidate for Wnt gene expression would be newly gastrulated mesoderm, as subsequent tissue formation is dependent on specificity of cell interactions. To characterize Wnt genes expressed by these cells, we used QCE-6 cells as a convenient and abundant source of early mesoderm. Sequence analysis of RT-PCR fragments generated from QCE-6 cell RNA identified the Wnt-11 gene. Subsequently, cDNAs containing the complete coding region for quail Wnt-11 were obtained by screening a QCE-6 cell cDNA library. Fig. 1 shows the putative amino acid sequence of the quail protein, along with a direct comparison with Wnt-11 characterized from other species.

Whole-mount in situ hybridization on quail embryos was performed (Fig. 2A-1) to analyze the spatiotemporal pattern of Wnt-11 gene expression. Quail embryos used for this study were staged by analogy to that described for the chick by Hamburger and Hamilton (1951). In parallel to hybridizations with Wnt-11 antisense RNA, embryos were hybridized to either Wnt-11 sense or Wnt-2 antisense RNAs as controls. In the earliest embryos we analyzed (HH stage 3+), Wnt-11 was expressed at Hensen’s node (Fig. 2A). As gastrulation continued, Wnt-11 expression spread into the mesoderm. At stages 4 and 5, high levels of Wnt-11 staining was exhibited as a semicircular pattern of mesoderm cells, with Hensen’s node at the apex (Fig. 2C,E). Moreover, this crescent of high level Wnt-11 expression was superimposed upon a domain of low level expression among mesoderm cells lateral to the anterior portion of the streak. Sectioning of these embryos demonstrated that Wnt-11 staining was specific to mesoderm (Fig. 2F). The significant overlap between the position of Wnt-11-positive mesoderm cells at these stages and the emerging cardiac fields, may indicate that this gene plays a role in defining the mesoderm region for quail Wnt-11.

**Fig. 1.** Sequence of quail Wnt-11 and comparison with homologs from chick, mouse and frog. Putative amino acid sequence of quail Wnt-11 was deduced from cloned QCE-6 cell cDNAs. The quail translation product consists of 354 amino acids and demonstrates 99.4%, 83.3% and 64.6% sequence identity with the chick, mouse and frog Wnt-11 homologs, respectively (Christiansen et al., 1995; Ku and Melton, 1993; Tanda et al., 1995). Filled circles represent positions of sequence identity, with amino acid number indicated on the right hand column. Underlined sequences indicate amino acids corresponding to oligonucleotides used for PCR studies. Double underlining denotes the peptide sequence used to generate Wnt-11 specific antibody. The accession number for quail Wnt-11 is X97549.
cells that give rise to the heart. As development proceeded, Wnt-11 expression in the cardiogenic fields faded. However, at stage 6 two symmetrically distributed patches of Wnt-11 staining were observed just anterior to the retreating Hensen’s node (Fig. 2G), at positions corresponding to the emerging somites. During earliest somite stages, Wnt-11 staining continued to be exhibited and restricted by cells within newly formed somites (Fig. 2H). The specificity of the Wnt-11 staining was demonstrated by control hybridizations for these early stages (stages 3+ to 8+). Neither Wnt-11 sense nor Wnt-2 antisense probes produced a positive signal (Fig. 2B,D,I), although the latter prominently stained later stage embryos (data not shown).

Generation of QCE-6 cell stable transfectants with altered Wnt-11 expression

As QCE-6 cells were used to identify expression of Wnt-11 in early mesoderm, we confirmed the expression of this gene among these cells by northern hybridizations (Fig. 3). Thus, we decided to utilize this cell line as a culture system to assess the impact of Wnt-11 on early mesoderm cells. To ascertain both the function(s) and mechanism(s) of Wnt-11 regulation of mesoderm cell differentiation, QCE-6 cell stable transfectants were established with either enhanced or diminished Wnt-11 expression. The transgenes consisted of full-length Wnt-11 cDNA inserted downstream of a cytomegalovirus promoter, in either sense (to overproduce the protein) or antisense (to diminish gene expression) orientations. QCE-6 cell subclones that incorporated the transfected DNA, were isolated and used for subsequent studies. Northern hybridizations of mRNA isolated from these cells verified the Wnt-11 genotype (Fig. 3A). Wnt-11-sense stable transfectants produced an additional sense mRNA of ~3900 nt, in comparison to QCE-6 and Wnt-11-antisense cells. Accordingly, only antisense stable transfectants expressed antisense Wnt-11 RNA.

**Fig. 2.** Wnt-11 expression in gastrula stage avian embryos. Whole-mount in situ hybridization of HH stages 3+ (A,B), 4 (C,D), 5 (E,F), 6 (G) and 8+ (H,I) quail embryos using Wnt-11 antisense (A,C,E,F,G,H), Wnt-2 antisense (B,I) or Wnt-11 sense (D) RNA probes. Wnt-11 RNA was detected within Hensen’s node (arrow) at stage 3+ (A). Stage 4 and 5 embryos exhibited Wnt-11 staining (C,E) that spread into the mesoderm in a crescent pattern (arrows); these areas coincided with the cardiogenic fields. This high level expression of Wnt-11 was superimposed over a lower level expression domain of anterior mesoderm cells. Sectioning of hybridized stage 5 embryos confirmed that Wnt-11 expression localizes to the mesoderm (F); arrows indicate regions of high level Wnt-11 staining. By stage 6 (G), Wnt-11 had disappeared from the cardiogenic fields, but was now exhibited at low levels in emerging somites (arrows). During subsequent stages, Wnt-11 became highly expressed in the somites (H). Note that control hybridizations (B,D,I) did not stain the embryos, including regions that were positive for Wnt-11 (arrows). Scale bar, 160 μM (A,B); 200 μM (C,D); 80 μM (F); 300 μM (E,G,I).
To verify that the transgenes altered levels of Wnt-11 protein produced by the cells, antibodies to Wnt-11 were generated by immunizing rabbits with a protein-specific peptide (Fig. 1). Immunoblot analysis of total protein isolated from cultures of the parental and stably transfected QCE-6 cells was used to determine relative levels of Wnt-11 protein expression (Fig. 3B). The major protein recognized by this antibody was of approx. $10^3 \text{Mr}$, which is in the expected range for vertebrate Wnt proteins. The immunoblots demonstrated that Wnt-11 protein was produced by QCE-6 cells. In comparison, Wnt-11 sense and antisense stable transfectants demonstrated either significantly greater or non-detectable levels of this protein, respectively. That the recognized protein was Wnt-11 was indicated by the removal of antibody reactivity by preabsorption with purified Wnt-11 peptide (Fig. 3B). Thus, Wnt-11 sense and antisense transfectants of QCE-6 cells will be referred to as Wnt11-OX (OX = overexpress) and Wnt11-minus cells, respectively.

**Responses of QCE-6 cells to altered levels of Wnt-11 expression**

Preliminary characterization of the stable transfectants was carried out on poly-D-lysine coated dishes, as QCE-6 cells adhere poorly to tissue culture plastic. As shown in Fig. 4, Wnt-11 profoundly affects QCE-6 cell morphology. QCE-6 cells possess an epithelial phenotype, which is characteristic of early mesoderm. These cells grew as a monolayer and began to group at low confluency. Growth of QCE-6 cells was fully contact-inhibited. In contrast, Wnt11-minus cells showed decreases in intercellular adhesion and displayed a mesenchymal morphology at subconfluent concentrations. Cell associations among these cultures were delayed in comparison to QCE-6 cells. Moreover, Wnt11-minus cells no longer exhibited contact-inhibited growth. Conversely, Wnt11-OX cells manifested more pronounced cell-cell associations than QCE-6 cells, and arranged into three-dimensional patterns in culture. The configuration of these latter cultures was suggestive of pretubular morphologies (Drake and Little, 1995; Vernon et al., 1995).

To investigate the molecular basis for Wnt-11 regulation of differential cell-cell interactions, we immunostained the three cell populations for various cell junction proteins (Fig. 5). Formation of tight junctions was analyzed using antibodies to cingulin (Citi et al., 1989). As shown in Fig. 5A, Wnt11-minus cells displayed scattered cingulin staining at the cell membrane. In contrast, both QCE-6 (Fig. 5B) and Wnt11-OX cells (Fig. 5C) exhibited tight junctions that circumscribed the apical portion of the cells. Similar expression patterns were revealed for N-cadherin, as cell membrane staining among Wnt11-minus cells was discontinuous (Fig. 5D), while both QCE-6 and Wnt11-OX
cells displayed uninterrupted cell border staining (Fig. 5E,F). Wnt-11 also promoted gap junction formation, as indicated by connexin43 expression. While antibodies to this protein minimally stained Wnt11-minus cells (Fig. 5G), QCE-6 cells displayed both cytoplasmic and cell membrane connexin43 (Fig. 5H). Furthermore, significant enhancement of gap junction formation was observed among Wnt11-OX cells (Fig. 5I).

Wnt11-minus, QCE-6 and Wnt11-OX cells were also stained for both β-catenin and plakoglobin (Fig. 6), cell junction molecules that are known downstream targets of Wnt signaling. Surprisingly, Wnt-11 did not appear to influence β-catenin expression. High levels of specific β-catenin staining were observed at the cell borders for all three cell types (Fig. 6A-C). In contrast, plakoglobin demonstrated a dynamic expression pattern in response to Wnt-11. Although plakoglobin staining of Wnt11-minus cells was very faint (Fig. 6D), high level expression of this protein was observed at the cell borders of QCE-6 cells (Fig. 6E). Moreover, cell membrane expression of plakoglobin was enhanced further among Wnt11-OX cells (Fig. 6F). Thus, the intensity of plakoglobin immunostaining was proportional to the level of Wnt-11 produced by these cells.

**Restoration of parental phenotype to Wnt11-minus cells**

Wnt gene mutations are cell nonautonomous since Wnt proteins are secreted molecules. Thus, Wnt11-minus cells should revert to the parental cell phenotype if cultured together with Wnt-11-producing cells. To determine if Wnt11-minus cells could be rescued from its altered phenotype, these cells were co-cultured with QCE-6 cells. Wnt11-minus cells were labeled with a β-galactosidase expressing retrovirus to distinguish them from QCE-6 cells. Wnt11-minus and QCE-6 cells were cultured at a 1:9 ratio for 48 hours and triple stained for cingulin, β-galactosidase and TOTO-3 (to visualize nuclei). As shown in Fig. 7, co-culture with QCE-6 cells restored circumferential cingulin staining among Wnt11-minus cells. Individual Wnt11-minus cells adjacent to QCE-6 cells exhibited prominent tight junctions along the cell periphery, even at cell borders shared between two Wnt11-minus cells (Fig. 7A,B). This prominent tight junction staining was also displayed by cells contained within larger clusters of Wnt11-minus cells (Fig. 7C). However among these cell clusters, Wnt11-minus cells that were several cell diameters distanced from QCE-6 cells displayed either spotty staining or the lack of antibody reactivity. Thus, Wnt11-minus cells reverted to the parental phenotype only if they were in proximity to Wnt-11-producing cells.

**Fibronectin engagement modifies QCE-6 cell responses to Wnt-11**

The results described above were generated from cultures plated

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**Fig. 5.** Effect of Wnt-11 on cell junction protein expression. From left to right are shown Wnt11-minus, QCE-6 and Wnt11-OX cells; from top to bottom are shown cells stained for cingulin, N-cadherin and connexin43. While cingulin staining among Wnt11-minus cells was sparse (A), both QCE-6 (B) and Wnt11-OX cells (C) showed this protein being expressed around the entire cell periphery. N-cadherin staining was discontinuous among cultures of Wnt11-minus cells (D), but was exhibited around the entire cell perimeter of both QCE-6 (E) and Wnt11-OX cells (F). Connexin43 was not exhibited by Wnt11-minus cells (G), but was displayed both in the cytoplasm and at the cell membrane of QCE-6 cells (H). Cell membrane connexin43 expression was significantly enhanced among Wnt11-OX cells (I). Note the dotted pattern of cell membrane connexin43 (arrows), which is typical for gap junction staining. All of these cells were plated on a poly-D-lysine substrate. Scale bar, 30 μM (A-C), 50 μM (D-F), 17 μM (G-I).
Wnt-11 regulation of mesodermal development

QCE-6 cell differentiation is dependent on Wnt-11 expression

QCE-6 cells will differentiate and express a myocardial phenotype, in response to the combination of retinoic acid, bFGF, TGFβ2 and TGFβ3 (Eisenberg and Bader, 1996). To ascertain whether Wnt-11 expression is a requisite for QCE-6 cell differentiation, the parental and stable transfectant cells were treated with these four factors. After 6 days of culture, cells were stained with anti-muscle myosin heavy chain antibody as a marker for differentiation (Fig. 10). As expected, treated cultures of QCE-6 cells were myosin positive. Likewise, sarcomeric myosin expression was exhibited by Wnt11-OX cells. In contrast, Wnt11-minus cells did not display myosin staining. Thus, it appears that Wnt11-minus cells are unable to undergo myocardial differentiation, at least under conditions that have been established for QCE-6 cells. These data indicate that QCE-6 cells are the first cell line whose differentiation has been reported to be Wnt-dependent.

DISCUSSION

Wnt-11 is expressed by early avian mesoderm

In this study, we have identified Wnt-11 as a candidate regulatory gene for early mesoderm development. The mesoderm arises from epiblast cells that ingress through the primitive streak during gastrulation. The gastrulating cells, which furnish the mesodermal layer, initially form a loose network of mesenchymal cells. In rapid fashion, these cells begin to coalesce and produce epithelial fields, a necessary prelude to organogenesis. As Wnt genes have been shown to regulate cellular interactions, we believe these early events in mesodermal development are candidate processes for Wnt regulation.

In Xenopus, Wnt-11 has been shown to be a maternally expressed gene and present in pregastrula embryos. Later, Wnt-11 was expressed at the dorsal lip, where gastrulation occurs, but appeared to be present in ectoderm only (Ku and Melton, 1993). Previous studies of both the mouse and chick have shown that Wnt-11 is expressed in the developing somites and limb bud (Christiansen et al., 1995; Tanda et al., 1995). However, pre- and early somite stages were not analyzed in those studies.
The expression pattern of Wnt-11 in the early embryo is suggestive of its importance for early mesoderm development. To ascertain this gene’s function, we utilized the quail mesoderm cell line QCE-6 as a cell culture system for examining Wnt-11 regulation of early mesoderm cells. Previous studies have shown that these cells are representative of early mesoderm stem cells, and have the potential to differentiate into myocardial, endodermal and red blood cells (Eisenberg and Bader, 1996; Eisenberg and Markwald, unpublished data). In this study, we demonstrate that QCE-6 cells retain the expression of Wnt-11 exhibited by the tissue from which these cells were derived, i.e. HH stage 4 quail mesoderm. Thus, QCE-6 cells provide us with a culture model for analyzing the function of Wnt-11 in regulating the diversification of nondifferentiated mesoderm.

To study the effect of Wnt-11 on mesodermal development, we attempted to alter levels of Wnt-11 protein produced by QCE-6 cells. Quail Wnt-11 cDNA was inserted immediately downstream of a viral promoter, in either antisense or sense orientations, and stably transfected into QCE-6 cells. Multiple clones were produced from both transfections, and the antisense and sense genotypes confirmed by northern analysis. Initial experiments indicated that clones generated from each transfection exhibited similar properties. In this paper, we report our results with a representative clone containing either the antisense and sense transgene, designated as Wnt11-minus and Wnt11-OX cells, respectively.

Immuno blotting with antibodies to a Wnt-11 peptide demonstrated that the antisense and sense transgenes significantly decreased or increased, respectively, levels of Wnt-11 protein produced by QCE-6 cells. Surprisingly, the antisense transgene did not affect levels of endogenous Wnt-11 message. Although other investigators have demonstrated that introduction of antisense RNAs may cause a decrease in levels of specific endogenous mRNAs (Steinbeisser et al., 1995), this was not observed for any Wnt-11 antisense stable transfectant. Since Wnt11-minus cells showed decreases in Wnt-11 protein expression, we speculate that antisense inhibition in QCE-6 occurs at the translational level.

The effects of altered Wnt-11 expression were profound, as indicated by changes in cell morphology, cell junction formation, plakoglobin expression and competence to respond to growth factor-induced differentiation. A dramatic consequence of differential Wnt-11 expression was the changes in cell morphology on a poly-D-lysine substrate. QCE-6 cells exhibit an epithelial morphology and contact-inhibited growth. The effect of diminished Wnt-11 expression, as shown by Wnt11-minus cells, was to decrease cell-cell adhesion. Tight and gap junction formation was dramatically reduced and contact-inhibition lost. If a function of Wnt-11 is to promote the epithelialization of early mesoderm, then Wnt11-minus cells may represent cells that have reverted to a newly gastrulated phenotype. Wnt-11 expression among early mesoderm cells may then provoke cellular interactions that are necessary for further differentiation.

**Fig. 7.** Wnt11-minus cells revert to parental phenotype when co-cultured with QCE-6 cells. Mixed cultures of 10% β-galactosidase-labeled Wnt11-minus and 90% QCE-6 cells were triple stained for cingulin (green), β-galactosidase (red) and the nuclear marker TOTO-3 (blue). (A and B) Representative clusters of Wnt11-minus cell clusters that were surrounded by QCE-6 cells. Wnt11-minus cells, adjacent to Wnt-11 producing cells, displayed strong cingulin staining along the cell circumference. Notice that tight junctions were exhibited between shared borders of Wnt11-minus cells (arrows). (C) A larger cluster of Wnt11-minus cells. While Wnt11-minus cells exhibited prominent tight junction staining when in proximity to QCE-6 cells (arrow), those farther away from the latter cell type showed either minimal or spotty cingulin staining (arrowhead). Scale bar, 20 μM.

Our analysis of gastrula stage avian embryos revealed Wnt-11 expression within the mesoderm. Within the earliest stage embryos we analyzed, HH stage 3+, Wnt-11 RNA was expressed around Hensen’s node. At stages 4 and 5, Wnt-11 expression spread convexly to the mesodermal layer. This high level crescent of Wnt-11 RNA was superimposed over a pattern of lower level expression within cells lateral to the primitive streak. The distribution of Wnt-11 RNA at these stages coincides with the cardiogenic fields (DeHaan, 1965; Rawles, 1943). By stage 6, Wnt-11 expression within heart mesoderm disappeared. However, two symmetrically opposed patches of staining were observed just anterior to Hensen’s node; these were areas corresponding to somite-forming regions. During subsequent stages (stages 7 to 8+), Wnt-11 was expressed at high levels within and restricted to the somites. Together, these results suggest that Wnt-11 is important for early mesodermal patterning. Specifically, Wnt-11 may play a role in defining and/or specifying both the heart fields and somites.
Overexpression of Wnt-11 also produced profound changes in QCE-6 cells. In comparison to the parental cells, Wnt11-OX cells exhibited increased cellular associations. This is demonstrated most dramatically by the three-dimensional arrangements manifested by Wnt11-OX cells on poly-D-lysine, which are reminiscent of pretubular morphologies (Drake and Little, 1995; Vernon et al., 1995). These morphologies may reflect an important function of Wnt genes in eliciting cellular organization into pretubular structures, as has been shown for Wnt-4 in the developing kidney (Herzlinger et al., 1994; Stark et al., 1994). The higher levels of Wnt-11 produced by Wnt11-OX cells also resulted in increased gap junction formation and plakoglobin expression. Two possible explanations for the differences observed between QCE-6 and Wnt11-OX cells are that they represent: (1) simply linear increases in cell-cell adhesion, or (2) altered differentiation potential. Although overexpression of Wnt-11 may increase cellular associations further, this may not have any consequence for cellular differentiation. However, there is precedence for cell diversification being sensitive to levels of Wnt expression (Hoppler and Benz, 1995). Presently, we are examining Wnt11-OX cells for altered differentiation potential, including the ability to form tubular structures.

Cooperativity between fibronectin and Wnt-11 signaling

The responses of QCE-6 cells to differential Wnt-11 expression is significantly influenced by culture substrate. On poly-D-lysine, QCE-6 cells exhibited dramatically different...
morphologies as a function of Wnt-11 expression levels. Yet, these morphological differences provoked by Wnt-11 were diminished when cells were plated on fibronectin. When cultured on fibronectin, QCE-6, Wnt11-minus and Wnt11-OX cells all displayed an epithelial phenotype and grew as a contact-inhibited monolayer. The major morphological difference among these fibronectin-containing cultures was that Wnt-11 appeared to promote a flatter, more substrate-adhesive phenotype. Surprisingly, the culture substrate did not appear to affect the pattern of tight or gap junctions, nor the cell membrane distribution of either N-cadherin or β-catenin. Yet, the culture substrate did influence the plakoglobin expression pattern. More importantly, the growth factor-induced cardiomyocyte differentiation of QCE-6 cells required both Wnt-11 and fibronectin, as shown by the stimulation of sarcomeric protein expression. This latter result provides further support for the importance of Wnt-11 for early cardiac development, considering the prominent expression of this gene in the early cardiogenic fields. Thus, it appears that Wnt-11 and fibronectin act cooperatively in regulating QCE-6 cell responses, as indicated by: (1) changes in cell morphology; (2) changes in plakoglobin expression; and (3) the joint requirement for myocardial differentiation.

Cell interactions with fibronectin, but not with poly-D-lysine, involve integrin ligation. Transgenic mouse studies have shown that fibronectin-integrin interactions play an essential role (George et al., 1993; Hynes, 1994) during early mesodermal development. Thus, Wnt and integrin signaling pathways may converge in jointly regulating early mesoderm cell differentiation. Moreover, these findings may have a more general relevance, as a previous study has indicated that Wnt and integrin co-regulate development in the Drosophila foregut (Pankratz and Hoch, 1995). A possible point of convergence among these signaling pathways may be the regulation of plakoglobin localization. The cellular distribution of plakoglobin is affected by its phosphorylation at serine, threonine and tyrosine residues. The current paradigms are that Wnts influence serine and threonine phosphorylation of plakoglobin, while integrin ligaton stimulates tyrosine phosphorylation. An analysis of plakoglobin distribution within QCE-6 cells as a function of protein phosphorylation should provide important information on Wnt-integrin co-regulation of mesodermal development.

**Wnt-11 signaling pathway**

An important observation is that vertebrate Wnt genes can be grouped into two broad classes based on their biological activities (Du et al., 1995; Torres et al., 1996). Wnt-1, 5a and 8 (Wnt-1 Class) induce a complete secondary axis when injected into ventral blastomeres of Xenopus embryos. Wnt-4, 5a and 11 (Wnt-5a Class) produce anterior truncations when injected into dorsal blastomeres (Du et al., 1995). To date, it is not known how the signal transduction pathways of the two classes of Wnts differ. Various studies have established that Wnt activity is elaborated via regulation of the cell junction protein armadillo and its vertebrate homologs, β-catenin and plakoglobin. We have not observed any obvious influence by Wnt-11 on β-catenin expression, as this cell junction protein is strongly exhibited at the cell membrane of QCE-6, Wnt11-minus and Wnt11-OX cells. Yet, plakoglobin is greatly influenced by the level of Wnt-11 expression. Why Wnt-11 appears to differentially affect these two sister proteins is currently being investigated. It is possible that an answer to this question may relate to different signaling properties of the two classes of Wnt proteins. To date, little information is available about the signaling pathway of Wnt-5a Class proteins. In this regard, it would be interesting to see what the result of expressing a Wnt-1 Class gene (e.g. Wnt-8) would have on QCE-6 cell differentiation and expression of β-catenin and plakoglobin.

It has been suggested that Wnt-5a Class proteins act by inhibiting cellular movements (Moon et al., 1993). This may occur by inhibiting the convergence or extension of epithelial sheets, as has been observed by injection of Wnt-4 mRNA into Xenopus or zebrafish embryos (Ungar et al., 1995). Alternatively, this class of Wnts may cause cells to coalesce into epithelial sheets. This is exhibited in the developing kidney, as Wnt-4 promotes aggregation of mesenchymal cells into pre-tubular structures (Herzlinger et al., 1994; Stark et al., 1994). Both the Wnt-11 expression pattern within early mesoderm and regulation of QCE-6 cell morphology suggest that this gene also is involved in epithelialization of mesenchymal cells. The expression of Wnt-11 in QCE-6 cells promoted cell-cell adhesion and formation of tight and gap junctions. Previous studies in Xenopus have shown that elevated Wnt expression could enhance gap junctional communication (Moon, 1993). Our study is the first to demonstrate that increasing cellular levels of Wnt protein promote gap junction assembly. The formation of gap junctions may be essential for the development of an organized epithelium (Becker and Davies, 1995), such as the early cardiogenic mesoderm. Wnt regulation of gap junction assembly may thus provide a mechanism by which early tissue compartments are formed, confining the sharing of


(Accepted 1 November 1996)