Wnt/frizzled-2 Signaling Induces Aggregation and Adhesion among Cardiac Myocytes by Increased Cadherin-β-Catenin Complex

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Abstract. Wingless is known to be required for induction of cardiac mesoderm in Drosophila, but the function of Wnt family proteins, vertebrate homologues of wingless, in cardiac myocytes remains unknown. When medium conditioned by HEK293 cells overexpressing Wnt-3a or -5a was applied to cultured neonatal cardiac myocytes, Wnt proteins induced myocyte aggregation in the presence of fibroblasts, concomitant with increases in β-catenin and N-cadherin in the myocytes and with E- and M-cadherins in the fibroblasts. The aggregation was inhibited by anti-N-cadherin antibody and induced by constitutively active β-catenin, but was unaffected by dominant negative and dominant positive T cell factor (TCF) mutants. Thus, increased stabilization of complexed cadherin–β-catenin in both cell types appears crucial for the morphological effect of Wnt on cardiac myocytes. Furthermore, myocytes overexpressing a dominant negative frizzled-2, but not a dominant negative frizzled-4, failed to aggregate in response to Wnt, indicating frizzled-2 to be the predominant receptor mediating aggregation. By contrast, analysis of bromodeoxyuridine incorporation and transcription of various cardiogenetic markers showed Wnt to have little or no impact on cell proliferation or differentiation. These findings suggest that a Wnt-frizzled-2 signaling pathway is centrally involved in the morphological arrangement of cardiac myocytes in neonatal heart through stabilization of complexed cadherin-β-catenin.

Key words: Wnt • frizzled • cardiac myocytes • cadherin • β-catenin

Introduction

Wnt genes encode glycoproteins that, when secreted, act as autocrine or paracrine factors (Burrus and McMahon, 1995) affecting morphogenetic events during embryonic and postembryonic development (for review see Mool et al., 1997). In addition, their activation by mouse mammary tumor virus (MMTV) proviral DNA has implicated Wnt genes in the ontogenesis of MMTV-induced mammary tumors (for review see Nusse and Varmus, 1992). More broadly, Wnt genes have been grouped into functional classes based on assays performed in both X. laevis embryos (Du et al., 1995) and mammalian cell lines (Wong et al., 1994). For example, ectopic expression of X. laevis Wnt (Xwnt)-1, -3a, -8, and -8b induces formation of a secondary axis, whereas ectopic expression of Xwnt-5a, -4, and -11 induces morphological movement during gastrulation without altering cell fate (Mool et al., 1993; Cui et al., 1995; Du et al., 1995). Mouse Wnt genes are grouped in a similar manner: Wnt-1, -3a, -7a, and -8 transform mammary C57MG cells, whereas Wnt-4 and -5a lack transforming activity (Wong et al., 1994; Shimizu et al., 1997).

The first insights into the mechanism of Wnt signal transduction came from the discovery of several fly genes exhibiting mutant phenotypes consistent with defects in wingless (Wg) signaling, and from studies of vertebrate counterparts that shed light on the biochemical relationship between gene products (for review see Cadigan and Nusse, 1997; Brown and Mool, 1998). For instance, association of Wnt-1, the vertebrate homologue of Wg, and members of the frizzled protein family leads to activation of Dsh protein. Activated Dsh inhibits glycogen synthase kinase-3β (GSK-3β), increasing cytosolic β-catenin levels as a consequence of decreased GSK-3β-mediated degradation. β-Catenin can then interact with...
members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of architectural transcription factors in the nucleus, inducing transcription of Wnt-responsive genes (for reviews see Nusse 1997; Miller et al., 1999). This type of Wnt signal transduction is required for Wnt-induced cell transformation, although alternative Wnt and frizzled signaling pathways also have been observed recently (Rocheleau et al., 1997; Slusarski et al., 1997; Strutt et al., 1997; Sheldahl et al., 1999).

Cardiac development has been studied extensively and found to be regulated in a highly integrated manner. In Drosophila hearts, Wg is absolutely necessary for induction of cardiac but not visceral mesoderm; however, no vertebrate Wnt genes have been shown to be directly involved in cardiac development to date (Wu et al., 1995; for review see Bodmer and Venkatesh, 1998). Nonetheless, despite numerous morphological differences between vertebrate and invertebrate species, many molecular mechanisms orchestrating individual developmental processes are remarkably conserved. Transcriptional analysis revealed expression of mRNA encoding Wnt-5a in rat neonatal cardiac myocytes, and encoding frizzled-2 and frizzled-4 in both cardiac myocytes and fibroblasts (this study), making it likely that Wnt genes are involved in vertebrate cardiac development.

In this report, we describe the use of cultured cells isolated from rat neonatal hearts as a model system to assess the role of Wnt genes in the growth and behavior of cardiac myocytes. When Wnt-3a and -5a, which differ in their capacity to transform cells, were applied, both induced aggregation of cardiac myocytes that was dependent on the presence of fibroblasts, suggesting two possible effects of Wnt proteins on fetal hearts: morphological movement and proliferation of cardiac myocytes. By interfering with the activity of these genes through the use of specific antibodies and dominant negative constructs, we obtained evidence that in both myocytes and fibroblasts Wnt-5a signaling mediated by frizzled increases levels of intracellular β-catenin, which in turn stabilizes cadherin at intercellular junctions necessary for cell–cell adhesion. On the other hand, proliferation and cardiomyogenesis of so-called precursor cells in neonatal hearts is unaffected. Thus, the morphological effects of Wnt on cardiac myocytes are apparently related to the enhanced homophilic adhesion mediated by cadherin.

**Materials and Methods**

**Cultured Cells and Cell Lines**

Rat neonatal cardiac myocytes were prepared as previously described (Iwaki et al., 1990). In brief, the hearts were isolated from 1-d-old HLA-Wistar rats, the ventricles were minced, and the cells were dispersed by digestion with 0.1% collagenase at 37°C. The dispersed cells were suspended in high glucose DMEM supplemented with 10% FCS and preplated onto culture dishes for 30 min to remove fibroblasts. Nonadhesive cells were collected, concentrated in PBS (1.2 × 10⁶/ml), and plated on the top of a discontinuous Percoll gradient (1.000/1.086 g/ml) made up in buffer containing 116 mM NaCl, 20 mM Hepes, 1 mM NaH₂PO₄, 5.5 mM glucose, 54 mM KCl, and 0.8 mM MgSO₄ (pH 7.35). Centrifugation at 400 g for 30 min yielded myocardial cultures containing 95% myocytes, as assessed by immunofluorescent assay using an anti-cardiac myosin heavy chain antibody β. Fibroblasts on culture dishes were passaged, diluting them fourfold, every four days. A firter three passages, resultant cultures were ~100% fibroblasts, as assessed by immunofluorescent assay using an anti-Vimentin antibody. For 24 h before each experiment, isolated cardiac myocytes (1 × 10⁵ cells/ml) were cultured in serum-free medium (1000/1.086 g/ml) on glass coverslips coated with poly-L-lysine, collagen, fibronectin, or I-laminine (Sigma-Aldrich) in high glucose DMEM supplemented with 10% FCS and maintained at 37°C under an atmosphere of 5% CO₂/95% air. The cultures were then washed and incubated in either conditioned medium containing Wnt protein or FCS free medium.

**Construction of cDNAs**

Wnt-3a and Wnt-5a cDNAs. Full-length mouse Wnt-3a (amino acids 352; Roelink and Nusse, 1991) and Wnt-5a cDNAs (amino acids 379; Gavin et al., 1990) were amplified by reverse transcriptase (RT)-PCR using mRNA isolated from mouse lungs and hearts respectively. To create C-Myc-tagged constructs, an antisense PCR primer for the COOH-terminal domain was designed that deleted the endogenous stop codon and replaced it with the XbaI restriction site at the 5′ end. When ligated into pcDNA 3.1/V5-HisA expression vector (Invitrogen), the resultant sequence at the COOH terminus of Wnt-5a contained GPKPINPL-LGLDST, which was later detected using anti-V5 mouse monoclonal antibody (Invitrogen). A firter verifying the nucleotide sequences, the cDNA’s encoding C-Myc-tagged Wnt-3a and -5a were ligated into the pcDNA3 mammalian expression vector (Invitrogen).

Frizzled-2 and Frizzled-4 cDNAs and Frizzled-glycophosphatidylinositol-Chimeric cDNAs. The frizzled gene family, homologues of Drosophila gene frizzled, have been identified as the receptors for Wg and Wnt (Bhanot et al., 1996). Full-length rat frizzled-2 cDNA (amino acids 570; Chan et al., 1994) and mouse frizzled-4 cDNA (amino acids 537; Wang et al., 1996) were amplified by RT-PCR using mRNA isolated from mouse hearts. To create FLAG-tagged constructs, an antisense PCR primer for the COOH-terminal domain was designed that deleted the endogenous stop codon and replaced it with a FLAG sequence. The resultant sequence at the COOH terminus of the constructs was GSYDKDDDDK, an epitope for mouse monoclonal anti-FLAG IgG antibody (Eastman Kodak Co.).

The frizzled gene sequence predicts a protein with an extracellular NH₂-terminal cysteine-rich domain (CRD) and seven transmembrane segments. It has been proposed that the CRD constitutes all or part of the ligand-binding domain (Wang et al., 1996), and cell-surface expression of isolated CRD was found to confer Wnt protein binding activity (Bhanot et al., 1996). Using the protocol described by Bhanot et al. (1996), we used PCR to create frizzled-glycophosphatidylinositol (GPI) chimeras, composed of the CRD (the first 372 amino acids of frizzled-2 or the first 365 amino acids of frizzled-4), a C-Myc epitope, the COOH-terminal 40 amino acids of decay-activating factor, and a GPI-anchored protein (Caras and Wiedell, 1989). A firter verifying the nucleotide sequences, the cDNA’s encoding FLAG-tagged frizzleds and frizzled-GPI chimeras were ligated into pcDNA3 for subsequent transfection.

**N-Cadherin and Deleted Cadherin cDNA**

Cadherins are Ca²⁺-dependent adhesion molecules, which in association with α- and β-catenin constitute the major components of adherent junctions in vertebrates. Full-length N-cadherin cDNA (amino acids 907; Tamura et al., 1998) was amplified by RT-PCR using mRNA isolated from mouse hearts. Cadherin is composed of an extracellular domain that contains the Ca²⁺-sensitive, homophilic binding sites, a transmembrane domain, and a cytoplasmic domain that interacts with α- and β-catenin (for review see Nagafuchi et al., 1993). In mammalian cells responding to Wnt-1, the increased steady-state levels of catenins is primarily due to a selective increase in the amount of uncomplexed, monomeric β-catenin (Papkoff et al., 1996). To detect uncomplexed β-catenin, we used PCR to create a construct consisting of the last 158 amino acids of N-cadherin, including the entire cytoplasmic domain, fused with GST epitope (GST-ΔN-cad), which could be used to precipitate the uncomplexed β-catenin. A firter verifying the nucleotide sequences, the PCR fragment was ligated, in-frame, downstream of the GST coding region in pGEX-3X (A meshrham Pharmaici Biotech).

The role of cell–cell adhesion in cardiac development has been in-
vestigated previously using antibody raised against cadherin (Linask and Lash, 1988; Soler and Kudson, 1994; Linask et al., 1997). To test whether anti-cadherin antibodies directly affect cell adhesion by blocking N-cadherin at the cell surface, we performed antibody-inhibition experiments using GST-fused cadherin. In accordance with information from the manufacturer (Santa Cruz Biotechnology, Inc.) indicating that anti-N-cadherin antibody was produced using amino acids 163-181 of the N-cadherin, we used PCR to create a construct covering amino acids 163-181 of the N-cadherin fused with GST epitope (GST-N-cad), which could then be used to block formation of the antibody-antigen complex. After verifying the nucleotide sequences, the PCR fragment was ligated, in-frame, downstream of the GST coding region in pGEX-3X.

β-Catenin and Deleted β-Catenin cDNAs. β-Catenin is the most downstream component of the Wnt-1 signal transduction pathway (Ossulic and Pfeffer, 1996; Miller and Moon, 1997). D. electron of the first 90 amino acids of β-catenin, lacking GSK-3β phosphorylation sites, results in its accumulation and in activation of signal transduction (Y ost et al., 1996; Zhu and Watt, 1999). Thus, to construct a constitutively active β-catenin (Δβ-catenin), an NH2-terminal domain lacking the first 90 amino acids was created by PCR. After verifying the nucleotide sequences, the full-length cDNAs encoding β-catenin or Δβ-catenin were ligated into the pEGFP-N3 mammalian expression vector.

TCF and Mutant TCF cDNAs. TCF (LEF) transcription factors mediate signaling from Wnt/β-catenin proteins by recruiting β-catenin to serve as a transcriptional coactivator (for review see Nusse, 1997). A β-Catenin-fusing PCR product was used to block formation of the antibody-antigen complex. After verifying the nucleotide sequences, the PCR product was ligated, in-frame, downstream of the GST coding region in pGEX-3X.

β-Catenin and Deleted β-Catenin cDNAs. β-Catenin is the most downstream component of the Wnt-1 signal transduction pathway (Ossulic and Pfeffer, 1996; Miller and Moon, 1997). D. electron of the first 90 amino acids of β-catenin, lacking GSK-3β phosphorylation sites, results in its accumulation and in activation of signal transduction (Y ost et al., 1996; Zhu and Watt, 1999). Thus, to construct a constitutively active β-catenin (Δβ-catenin), an NH2-terminal domain lacking the first 90 amino acids was created by PCR. After verifying the nucleotide sequences, the full-length cDNAs encoding β-catenin or Δβ-catenin were ligated into the pEGFP-N3 mammalian expression vector.

Immunofluorescent Analysis

Cells grown on glass coverslides were fixed for 10 min in 3% paraformaldehyde and then permeabilized for an additional 10 min with 0.1% Triton X-100. After blocking with 5% bovine serum albumin in PBS for 30 min, the cells were incubated for 2 h with primary antibody against the respective target proteins. M. mouse monoclonal anti-cardiac myosin heavy chain α subunit (MHC) and goat polyclonal anti-human vimentin antibodies (Chemicon International Inc.) were used to identify cardiac myocytes and fibroblasts, respectively. A. anti-cardiac MHC antibody–antigen complexes were visualized by incubation for 1 h with FITC-conjugated, affinity-purified anti-mouse IgG (Zymed Laboratories), while anti-vimentin antibody–antigen complexes were visualized using rhodamine-conjugated, affinity-purified anti-goat IgG (Chemicon International Inc.). M. mouse monoclonal anti-FLAG antibody was used to localize FLAG-tagged frizzleds; mouse monoclonal anti-α-MHC antibody was used to localize frizzled-GPI chimeras; rabbit anti-GFP antisera was used to localize β-catenins; anti-V5 antibody was used to localize TCFs; and mouse monoclonal anti-bromodeoxyuridine (BrDU) antibody (Boehringer) to assess BrDU (Sigma-Aldrich) incorporation into DNA. Each of the primary antibody-antigen complexes was visualized using an FITC-conjugated secondary antibody against the corresponding primary antibody. After washing with PBS, cells on coverslides were mounted in Permafluor aqueous mounting medium (ImmunoTech) and photographed on a Olympus Provis AX 80 microscope equipped with the appropriate filters.

Luciferase Assays

An oligonucleotide containing three copies of the TCF consensus sequence (CTCTTTGTACG) or a mutant thereof (CTCTTTGGCC), cloned into a minimal HSV-tk promoter-luciferase vector yielded modified tk-TOP and modified tk-FOP according to the previous studies (van de Wetering et al., 1997; Vleminckx et al., 1999). For transient transfection, cardiac myocytes were cotransfected by electroporation with the various combinations of plasmids: 1.0 μg of TCF4 construct or β-catenin construct in pCDNA3; 0.3 μg of tk-TOP or tk-FOP; and 0.03 μg of pRL-TK vector as a renilla luciferase control vector. Luciferase activity was determined using a dual luciferase reporter assay system (Promega). Cells were harvested 24 h after the transfection, and lysed in lysis buffer. The firefly luciferase activities of each of tk-TOP and tk-FOP and the renilla luciferase activities of pRL-CMV were measured in the same sample by a luminometer. Transfection efficiency of each sample was normalized by the activity of renilla luciferase activity.

Pulse–Chase Labeling

Preconfluent cells were starved by incubation in labeling medium that lacked methionine and cysteine for 1 h at 37°C. Cells were then incubated with fresh labeling medium containing 150 μCi/ml 35S-methionine and -cysteine (specific activity >1,180 Ci/mmol; ICN Biomedicals) for 30 min at 37°C. The labeling medium was removed and the cells were washed three times with ice-cold PBS. For the 0 h time point, cells were lysed immediately. For the chase period, normal growth medium supplemented with 5 mM cysteine and methionine (Sigma-Aldrich) was added to the cells. Equal amounts of protein lysates were precipitated with anti-β-catenin antibodies, and separated by 10% SDS-polyacrylamide gels. The gels were then incubated for 30 min with Amplify (A mersham Pharmacia Biotech), dried, and exposed to Kodak X-O MAT x-ray film (Eastman Kodak Co.) at −80°C.

Generation of Cell Lines Overexpressing Wnt-3a and Wnt-5a

HEK293 cells and C2C12 cells were transfected with the pcDNA3 vector containing either the Wnt-3a or Wnt-5a cDNA using the calcium phosphate precipitation technique; stable cell lines overexpressing either Wnt-3a or Wnt-5a were established by subsequent selection with 800 mg/ml G418 (Sigma-Aldrich). The transfectants were grown for selection in D ME containing 400 μg/ml G418. Each selected clone was analyzed by Northern blot and immunoblot analyses.
Generation of Cardiac Myocytes Overexpressing the Frizzled–GPI Chimera

Cardiac myocytes were cotransfected with vector containing CD 4 (Clark et al., 1987) and a vector containing one of the frizzled, frizzled–GPI chimera, β-catenin mutant, or TCF mutant cDNAs using electroporation methods with a Gene Pulser Transfection Apparatus (Bio-Rad Laboratories). The molar ratio of CD 4 to the cotransfected constructs was optimized to be 1:5. Cells expressing CD 4 were isolated by immunomagnetic separation using mouse CD 4 Dynabeads (Dynal), and then detached from the beads by using mouse CD 4 DETACHBEAD (Dynal). In brief, 12 h after transfection, transfectants (~10^7 cells/ml) were suspended in PBS and incubated for 20 min at 4°C with the Dynabeads at a final concentration of 4 × 10^8 beads/ml. Cells bound to the beads were collected by magnetic separation, washed with several units of DETACHBEAD, a polyclonal antibody that reacts with the Fab fragments of monoclonal anti-CD 4 antibodies, was then incubated with the beads for 45 min at 20°C, which detached the cells from the beads, yielding a population of cells expressing CD 4 (~10^7 cells/ml). When the localization of the transfected constructs within the isolated cardiac myocytes was assessed by immunofluorescence microscopy, it was found that almost all of isolated CD 4+ cardiac myocytes expressed the cotransfected constructs at a ratio of 1:5. Immediately after immunomagnetic separation, the isolated cardiac myocytes were cocultured with fibroblasts with or without Wnt proteins.

Cell Proliferation Assay

Cultured cells were labeled with 20 μM BrdU (Sigma-Aldrich) for 1 or 12 h, and then fixed in paraformaldehyde. A fiter denaturing the DNA in 2 M HCl, cells were neutralized with 0.1 M borate buffer (pH 8.5), and BrdU was detected by using a monoclonal anti-BrdU antibody, followed by an FITC-conjugated anti-mouse IgG.

Expression and Purification of GST-fused Proteins

Control GST and GST-cadherin proteins were expressed in bacteria induced with 0.1 mM IPTG at 37°C. The cells were then pelleted, resuspended in cold PBS with 1% Triton X-100 and lysed by mild sonication. The lysates were centrifuged at 10,000 r/min and isolated with washing several times with PBS. Glutathione beads (Amersham Pharmacia Biotech). After extensive washing with PBS, the GST-fused proteins were eluted with 5 mM of glutathione. These GST-fused proteins were used for the antibody-inhibition experiment using cultured cells and affinity binding assay.

Affinity Binding Assay

The GST-cadherin proteins that bound to the glutathione Sepharose 4B beads were extensively washed, first with PBS and then with binding buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 5% BSA, and protease inhibitor mixture), until no further protein was eluted. A fiter transfer to nitrocellulose membranes, immunoblot analysis was performed using mouse monoclonal anti-β-catenin IgG. Primary antibody-antigen complexes were visualized using enhanced chemiluminescence assay kit (Amersham Pharmacia Biotech) and peroxidase-conjugated secondary antibodies against mouse IgG antibodies.

Northern Blot Analysis

A fiter total RNA was extracted from cardiac myocytes, the mRNA was isolated using an Oligotex-DT 30 mRNA purification kit (Takara), electrophoretically separated in a 1.2% agarose-formaldehyde gel, and then capillary blotted onto nitrocellulose. The blots were then hybridized with 32P-labeled probes and examined by autoradiography.

Analysis of RNA by RT-PCR

RNA was extracted from cardiac myocytes and fibroblasts. To control for DNA contamination, we omitted reverse transcriptase from the synthesis reaction. Transcripts were detected by semi-quantitative RT-PCR. The PCR protocol consisted of 25 cycles at 96°C for 30 s, 54–62°C for 15 s, depending on the melting temperature of the primers, and 72°C for 1 min. The specificity of the amplified product was confirmed by Southern blot and comparison to a corresponding cDNA or internal control gene with probe. Moreover, cDNA was used in each PCR reaction was shown to be in the linear range of the signal by generating a dose–response curve carried for each set of primers. The following pairs of primers were designed: 5'-GGC GAT GGC TCC TCT CGG-3' and 5'-CTT AAG CCT TAC CAG-3' for Wnt-3a (sequence data available from EMBL/Genbank/DBJ under accession number M89798); 5'-ATG CAC CTC GAG GCC CGC-3' and 5'-GAG GAC CGG GCC CTC GAG-3' for frizzled-2 (sequence data available from EMBL/Genbank/DBJ under accession number X50842); 5'-CTC GCC ATG AAG CAG CCC-3' and 5'-CAA CCA GTC CGG AGG CAG-3' for Wnt-5a (sequence data available from EMBL/Genbank/DBJ under accession number M22992); 5'-ATG TCT GCC ATG AAG CAG-3' and 5'-GCT TCT GTA TAG TGA-3' for TCF-1 (sequence data available from EMBL/Genbank/DBJ under accession number X61385); 5'-ATG TGC TGG AAG CAG CAC ACG-3' and 5'-CTT TGT TGT GTC TCT GCT GAG-3' for TCF-3 (sequence data available from EMBL/Genbank/DBJ under accession number A223069); 5'-TGA ACG GCC GGC GAG GAC-3' and 5'-CGG GGT GAA GTG TTC ATT-3 for TCF-4 (sequence data available from EMBL/Genbank/DBJ under accession number A223070); 5'-TCT TAT GAA ATG GTC TCT-3' and 5'-GCT TAT GGT GAA GTG-3 for TCF-7L (sequence data available from EMBL/Genbank/DBJ under accession number M10094); 5'-TGG TGA AAG CAC ACA AGG-3' and 5'-ATG TCT GGA-3 for TCF-9 (sequence data available from EMBL/Genbank/DBJ under accession number A223070); 5'-GAT ACG GCG ATG AGC-3' and 5'-ATG TCT GCT GAG-3 for TCF-10 (sequence data available from EMBL/Genbank/DBJ under accession number X74513); 5'-CAG TGA TGG GAA CAC ACG-3' and 5'-ATG TCT GCG TCC-3 for TCF-11 (sequence data available from EMBL/Genbank/DBJ under accession number Y11352); 5'-GAT TGG ACG ACT AAC GAA-3' and 5'-ATG TCT GGA-3 for TCF-12 (sequence data available from EMBL/Genbank/DBJ under accession number X74515). For the estimation of the expression of TCF-1, we used the following primers: 5'-ATG TCT GGT AAG CAG GAC-3' and 5'-ATG TCT GGC GAG GAC-3' for TCF-2 (sequence data available from EMBL/Genbank/DBJ under accession number X61385); 5'-ATG TGC TGG AAG CAG CAC ACG-3' and 5'-CTT TGT TGT GTC TCT GCT GAG-3' for TCF-3 (sequence data available from EMBL/Genbank/DBJ under accession number A223069); 5'-TGA ACG GCC GGC GAG GAC-3' and 5'-CGG GGT GAA GTG TTC ATT-3 for TCF-4 (sequence data available from EMBL/Genbank/DBJ under accession number A223070); 5'-TCT TAT GAA ATG GTC TCT-3' and 5'-GCT TAT GGT GAA GTG-3 for TCF-7L (sequence data available from EMBL/Genbank/DBJ under accession number M10094); 5'-TGG TGA AAG CAC ACA AGG-3' and 5'-ATG TCT GGA-3 for TCF-9 (sequence data available from EMBL/Genbank/DBJ under accession number A223070); 5'-GAT ACG GCG ATG AGC-3' and 5'-ATG TCT GCT GAG-3 for TCF-10 (sequence data available from EMBL/Genbank/DBJ under accession number X74513); 5'-CAG TGA TGG GAA CAC ACG-3' and 5'-ATG TCT GCG TCC-3 for TCF-11 (sequence data available from EMBL/Genbank/DBJ under accession number Y11352); 5'-GAT TGG ACG ACT AAC GAA-3' and 5'-ATG TCT GGA-3 for TCF-12 (sequence data available from EMBL/Genbank/DBJ under accession number X74515).

Estimation of the Morphological Effect of Wnt on Cardiac Myocytes

We found Wnt on cardiac myocytes and found that cardiac myocytes coexpressed with fibroblasts aggregate in the presence of Wnt proteins. To compare this effect among various experiments, we defined criteria with which to assess aggregation. Cardiac myocytes were considered to be “aggregation-plus” if they formed wide-based, polyp-like structures that were sharply demarcated from other cell masses. On the other hand, myocytes were considered to be “aggregation-minus” if they appeared in a flat monolayer lacking demarcation, even if the cells were sometimes clustered. Whether cells were aggregation-plus or -minus could be assessed readily under the microscope without immunostaining. To quantify our findings, we dissected out the demarcated aggregates, dispersed each aggregate in one well of a 96-well plate, and then stained with anti-cardiac MHC antibody followed by FITC-labeled anti-mouse IgG. Numbers of FITC-positive cells in each well were then counted.

Results

Expression of Wnt and Frizzled Gene Families in Cardiac Myocytes and Fibroblasts

Expression of Wnt-5a and frizzled-2 and -4 is known to take place in mouse hearts (for review see
Brown and Moon, 1998), whereas X Lef-1 and Lef-3 were identified in Xenopus hearts (Molenaar et al., 1998). To determine which cell type expresses these genes, transcription of the Wnt, frizzled, and TCF genes was analyzed by RT-PCR using mRNA from neonatal cardiac myocytes and fibroblasts. Wnt-5a was detected in cardiac myocytes, but not in fibroblasts, whereas frizzled-2 and -4, and Tcf-3 and -4 were detected in both cell types (Fig. 1 A).

Morphological Effects of Wnt-3a and -5a on Cardiac Myocytes

To obtain biologically active Wnt proteins, we established stable cell lines expressing Wnt-3a and -5a in HEK 293 and C2C12 cells. After incubating the cells (~2 × 10^7 cells/ml) in the absence of FCS, they were removed by centrifugation at 2,000 g, and concentrated 10-fold using a Centriprep 10 column (Amicon). Although there have been reports that Wnt proteins secreted from cultured cells are present in the extracellular matrix (ECM) rather than in the culture medium (Bradley and Brown, 1990; Papkoff and Schryver, 1990), our immunoblot analysis showed c-Myc–tagged Wnt proteins to be in the supernatant (Fig. 1 B). To test whether biologically active Wnt proteins could be detected in the conditioned medium, we incubated HEK 293 cells with either Wnt-3a or -5a containing medium (Fig. 1 C). Levels of β-catenin were immediately increased after 30 min incubation of both media, and then reached the maximum levels after 180 min, whereas the presence of anti-c-Myc antibodies in the conditioned medium blocked the accumulation of β-catenin. Therefore, we decided to use conditioned medium in this study, as other investigators have successfully done in the past (van Leeuwen et al., 1994; Bradley and Brown, 1995).

Conditioned medium containing Wnt-3a or -5a was then added to cardiac myocytes plated on poly-L-lysine-coated coverslips in the presence or absence of fibroblasts (Fig. 2). Although application of conditioned medium evoked no morphological changes in either cardiac myocytes or fibroblasts plated alone (data not shown), when myocytes were cocultured with fibroblasts (~10^5 cells/ml each), they began aggregating within 1 d after application, forming ellipsoid bodies (up to 2–3 mm) on top of the fibroblast sheet within 3–4 d. When the effect was quantified by dissecting the demarcated aggregates, dispersing them in respective wells of a 96-well plate, and staining with anti-cardiac MHC antibody followed by FITC-labeled anti–mouse IgG, 50–70 FITC-labeled cells were found in each aggregate (Fig. 3 B). Addition of anti-c-Myc antibody (200 µg/ml) to the conditioned medium blocked the aggregation of cardiac myocytes, confirming that the observed effects were mediated by the tagged Wnt proteins secreted into the conditioned medium. The aggregated myocyte mass beat synchronously at 60–80/min, in contrast to the asyn-
chronous beating of control cells, which suggests formation of gap junctions between aggregated myocytes.

Role of Fibroblasts in the Morphological Changes Induced by Wnt Proteins in Cardiac Myocytes

Having shown that the presence of fibroblasts promotes Wnt-induced aggregation of cardiac myocytes, we next assessed which factors might be important for this morphological effect. We found that aggregation was unaffected by the material coating the coverslips on which the myocytes were grown. As with poly-L-lysine, cardiac myocytes plated on collagen, laminin, or fibronectin only aggregated when cocultured with fibroblasts (data not shown). Moreover, incubating cardiac myocytes in Wnt-containing medium further conditioned for 12 h by fibroblasts did not induce formation of aggregated masses (Fig. 3 A). Therefore, it appears that direct contact with fibroblasts is key for promoting aggregation of cardiac myocytes under the influence of Wnt proteins.

It has been proposed that cell–cell adhesion mediated by cadherins as well as cell–ECM interactions mediated by integrins are important for morphogenesis during development (for review see Gumbiner, 1996; Radice et al., 1997), and indeed anti-cadherin antibody has been used previously to study cardiac development (Linask and Lash, 1988; Soler and Knudsen, 1994; Linask et al., 1997). Similarly, we added antibodies raised against N-, E-, M-, and P-cadherins and integrin-β to respective samples of Wnt-containing medium and then applied them to 2-d-old cardiac myocyte/fibroblast cocultures. We found that only anti-N-cadherin antibody caused aggregated myocyte masses to revert to clusters of flattened cells; the others had little or no effect on the myocyte clusters (Fig. 3 A and B). The effect of anti-N-cadherin antibody was dose-dependently blocked by the GST-fused extracellular region of N-cadherin (GST-ΔNcad), which presumably competed with endogenous N-cadherin for binding antibody (Fig. 3 A), indicating that formation of cardiac myocyte aggregates is specifically dependent on N-cadherin-mediated cell–cell adhesion, and interactions with ECM were not involved.
Subsequent immunoblot analysis showed that N-cadherin is the predominant cadherin in cardiac myocytes, while E-cadherin predominates in fibroblasts (Fig. 4). Furthermore, incubation of cardiac myocytes and fibroblasts in Wnt-containing medium for 2 d induced distinct patterns of cadherin expression in the two cell types: protein level of N-cadherin was increased in cardiac myocytes, whereas those of E- and M-cadherin were increased in fibroblasts (Fig. 4A). Denstometric estimation of three independent experiments indicated a 2.0 ± 0.4-fold increase in N-cadherin levels of cardiac myocytes, and 2.2 ± 0.4-fold and 5.9 ± 1.0-fold increase in E- and M-cadherin levels of fibroblasts, respectively. Nevertheless, Northern blots showed that levels of mRNA encoding these cadherins were not affected by incubation with Wnt (Fig. 4B), suggesting that Wnt increases the stability of cadherins in both cell types without inducing gene transcription, which is consistent with the effect of Wnt-1 on mammalian cell lines (Hinck et al., 1994). Since cell–cell adhesion is established by homophilic binding between cadherins, the increased levels of distinct sets of cadherin in cardiac myocytes and fibroblasts leads to the strengthening of interaction of cardiac myocytes with one another.

**Intracellular Signal Transduction of Wnt Proteins in Cardiac Myocytes**

Frizzled-2 and -4 are the predominant frizzled genes expressed in both cardiac myocytes and fibroblasts. To test whether a particular frizzled protein functions as an endogenous Wnt-5a receptor, we created dominant-negative constructs of frizzled-2 and -4. Because we were unable to generate stable cultures of cardiac myocytes overexpressing a frizzled-GPI chimera, a transient expression strategy was used. Cardiac myocytes were cotransfected with vectors containing CD4 or frizzled–GPI chimera at a molar ratio of 1:5. At this ratio, 20–30% of live cardiac cells expressed frizzled-GPI, and then the FITC-positive cells were counted. 20 aggregates were quantified in three independent experiments. Note that only anti-N-cadherin antibody had an effect on aggregated cardiac myocytes.
chimeras compete with the extracellular CRD of endogenous frizzleds for the binding of Wnt, increased frizzled expression might be expected to facilitate association of Wnt with cell surfaces. Wnt-5a-V5 cDNA was transiently transfected into cardiac myocytes overexpressing either frizzleds or frizzled-GPI chimeras, and the localization of expressed Wnt proteins then was detected by immunostaining. Wnt proteins were detected at the surface and in the nucleus of cells overexpressing frizzled-2 or frizzled-2-GPI chimera, whereas Wnt proteins were detected mainly in the nucleus of cells overexpressing frizzled-4 or frizzled-4-GPI chimera (Fig. 5 B, top). This result suggested that the signals at the cell surface of cells expressing frizzled-2 and frizzled-2-GPI chimera represent the specific binding of Wnt-5a-V5 proteins to the extracellular CRD of frizzled-2. To clarify this notion, we used antibody against secreted Wnt-5a-V5 to inhibit its binding to the cell surface receptors. As shown in Fig. 5 B, bottom, incubation of anti-V5 antibody eliminated Wnt proteins localized at the surface of cells overexpressing frizzled-2 or frizzled-2-GPI chimera, although incubation with anti-V5 antibody did not affect the Wnt proteins in the nucleus in all types of cells. This result indicated that overexpression of frizzled-2 or frizzled-2-GPI chimera, but not frizzled-4 or frizzled-4-GPI, stabilized Wnt-5a at, or recruited it to, the cell surface. Since endogenous frizzled-2 should also bind Wnt-5a, differences in Wnt-5a signaling between cells expressing frizzled-2-GPI and frizzled-4-GPI should correspond to the differences in bound Wnt-5a between cells expressing only endogenous frizzled-2 and those expressing endogenous frizzled-2 plus frizzled-2-GPI. Thus, the frizzled-2-GPI chimera lacking the cytoplasmic domain, which is essential for the Wnt signal transduction, may function as a dominant-negative construct.

When the isolated transfectants were cocultured with fibroblasts in Wnt-5a-containing medium, cardiac myocytes expressing the frizzled-2-GPI chimera failed to aggregate, in contrast to myocytes expressing the frizzled-4-GPI chimera (Fig. 5 C). The cell counts were 20–30 per aggregate among cultures of transfectants subjected to immunomagnetic isolation in the Wnt-containing medium (Fig. 5 C). The cell counts were 20–30 per aggregate among cultures of transfectants not subjected to immunomagnetic isolation (Fig. 3 B). Although the reason for this difference is unclear, we speculate that immunomagnetic isolation may disturb the mobility of the transfected cells. Therefore, we compared cell counts per aggregate among groups subjected to the same experimental protocol and found that in Wnt-5a-expressing cardiac myocytes and fibroblasts in Wnt-containing medium, overexpression of frizzled-2-GPI inhibited aggregation, as compared to cultures only expressing endogenous frizzleds (Fig. 6). Thus, interaction between Wnt-5a/Wnt-3a and frizzled-2 appears crucial for the morphological effect of Wnt-5a on cardiac myocytes.

Given that cell–cell adhesion mediated by N-cadherin plays a key role in the morphological effect of Wnt, interaction of cadherin and β-catenin or plakoglobin (γ-catenin) should be a component of this effect. Immunoblot analysis showed that β-catenin is the predominant catenin in cardiac myocytes and fibroblasts. Furthermore, incubating cardiac myocytes and fibroblasts in Wnt-containing medium for 2 d increased protein levels of β-catenin in both cell types, and levels of γ-catenin were slightly increased (Fig. 7 A). Since β-catenin and γ-catenin mRNA levels were unaffected by Wnt proteins (Fig. 7 B), the observed increases in β- and γ-catenin should have been the product of increased protein stability, rather than increased protein synthesis. Incubation of cardiac myocytes with Wnt proteins increased levels of β-catenin complexed with cadherin, which was detected by immunoprecipitation using anti-N-cadherin antibody, and increased levels of free β-catenin, which was detected by affinity binding assay using the cytoplasmic β-catenin binding region of N-cadherin fused to GST (Fig. 7 C). Incubating fibroblasts with Wnt proteins yielded similar results (Fig. 7 A, B, and
The finding that levels of both free N-cadherin and N-cadherin–complexed β-catenin were increased by Wnt proteins confirmed the idea that Wnt proteins strengthen cell–cell adhesion by increasing formation of cadherin–catenin complexes at those sites (Bradley et al., 1993; Hinck et al., 1994).

We also examined the effect of introducing a constitutively active form of β-catenin (ΔN-catenin) into cardiac myocytes. The NH$_2$ terminus of β-catenin contains several GSK-3β phosphorylation sites, which facilitate the rapid degradation of β-catenin (Rubinfeld et al., 1996; Yost et al., 1996). Wild-type β-catenin has a very short half-life and
deletion or mutations of the GSK-3β phosphorylation sites at the NH₂ terminus results in accumulation of stable β-catenin in the cytoplasm (Gat et al., 1998; Zhu and Watt, 1999). To determine the half-life of β-catenin and Δβ-catenin in the transfected cells, transfected cells were starved for 1 h, pulse-labeled with [35S]cysteine and [35S]methionine for 30 min, and chased in radioactive-free medium for up to 4 h (Fig. 8 C). As predicted, endogenous and transfected β-catenin had a half-life of less than 1 h, whereas Δβ-catenin was stable throughout the time period examined. Cardiac myocytes were cotransfected with CD4 and β-catenin constructs and then immunomagnetically isolated. β-Catenin and Δβ-catenin were mainly localized at cell–cell interfaces and in a few nuclei (Fig. 8 A), indicating that both proteins are involved in the formation of cadherin-catenin complexes. Overexpression of Δβ-catenin in cardiac myocytes induced their aggregation, even in the absence of Wnt proteins (Fig. 8 B); the cell counts were 15 ± 3 per aggregate, which is significantly higher than in the absence of Wnt (Fig. 6).

Δβ-Catenin lacks the first 90 amino acids but should nonetheless bind to both N-cadherin and α-catenin (Orsolic and Peifer, 1996). To confirm that Δβ-catenin participates in the formation of cadherin-catenin complexes, lysates of cardiac myocytes expressing β-catenin or Δβ-

Figure 6. Histogram summarizing the data collected by counting cardiac myocytes in each aggregate. Isolated cardiac myocytes and fibroblasts were incubated in control medium (white bar), Wnt-3a-containing medium (hatched bar), or Wnt-5a-containing medium (black bar) for 3 d. Judgement of aggregation-plus or -minus in each experiment was performed under microscopy without immunostaining. To count cells in aggregate, each aggregate of cardiac myocytes was excised with a fine glass pipette, dispersed in one well of a 96-well plate, and immunostained. Numbers of FITC-positive cells were counted in each well. 20 aggregates were quantified in three independent experiments.

Figure 7. Wnt proteins increased β-catenin levels in cardiac myocytes and fibroblasts. (A) Total cell protein was extracted from cardiac myocytes and fibroblasts incubated in control or Wnt-containing medium. Levels of β- and γ-catenin were determined by immunoblotting equivalent amounts of total cell protein with anti-β-catenin and −γ-catenin antibodies, respectively. (B) Levels of β- and γ-catenin mRNA were determined by Northern blot using equivalent amounts of mRNA with corresponding 32P-labeled oligonucleotide probes. (C) For determination of β-catenin–cadherin complex, equivalent amounts of total cell protein were immunoprecipitated with antibody against N- or E-cadherin; the precipitate was subjected to SDS-PAGE, followed by immunoblotting with anti-β-catenin antibody. For determination of free β-catenin, equivalent amounts of total cell protein were affinity precipitated using a GST-fusion protein containing the cytoplasmic domain of N-cadherin; the precipitate was subjected to SDS-PAGE, followed by immunoblotting with anti-β-catenin antibody. Molecular mass standards are shown on the right.
catenin were immunoprecipitated with anti-GFP antibodies, and the associated proteins were immunoblotted with antibodies against β-catenin, N-cadherin, or α-catenin. We found that in cells expressing Δβ-catenin, association of N-cadherin and α-catenin was greater than in cells expressing β-catenin (Fig. 8 D). Densitometric estimation of three independent experiments indicated a 2.2 ± 0.3-fold increase in N-cadherin and 1.8 ± 0.4-fold increase in α-catenin. These results suggested that stabilization of β-catenin promotes myocyte aggregation by increasing formation of N-cadherin–catenin complexes.

Although increased free β-catenin can contribute to increased stability of the cadherin/catenin complex (Hinck et al., 1994), when associated with TCF free β-catenin can also serve a signal transduction function in response to Wnt (for review see Nusse, 1997). To test the possibility that the morphological effects of Wnt require TCF target gene expression, dominant negative and dominant positive TCF mutants were constructed. The transactivation activity of TCF constructs were analyzed by measuring luciferase activity of the tk-TOP or tk-FOP reporter plasmids cotransfected into cardiac myocytes (Fig. 9). Luciferase expression of tk-TOP is driven by three copies of a consensus TCF motif and a minimal HSV-tk promoter, while tk-FOP contains mutated, non-functional TCF motif. Upon overexpression of TCF-4, tk-TOP activity increased by ~2.8-fold, probably because TCF-4 could recruit endogenous β-catenin. In contrast, no stimulation was seen in ΔN-TCF, confirming the previous result that the NH₂-terminal domain is essential for the transactivation of TCF (Behrens et al., 1996; Roose et al., 1998). Overexpression of TA-TCF resulted in a 17.5-fold stim-
ulation of luciferase activity, indicating TA-TCF as a dominant positive construct. None of the various TCF constructs activated the tk-FOP activity. In comparison, overexpression of β-catenin increased tk-TOP activity by 2.3-fold, whereas overexpression of Δβ-catenin increased by 7-fold, indicating that overexpressed β-catenin and Δβ-catenin recruit endogenous TCF to activate the tk-TOP activity; the differences of activation levels may correspond to the differences of stability between β-catenin and Δβ-catenin as shown in Fig. 8 B. When the cardiac myocytes were co-transfected with ΔN-TCF and Δβ-catenin at the molar ratio of 5:1, ΔN-TCF suppressed the Δβ-catenin-induced activation of the tk-TOP activity from 7-fold to 2.5-fold. Since ΔN-TCF retains the DNA-binding high mobility group domain, this result indicated that ΔN-TCF functions as a dominant negative construct to compete with endogenous TCF for the TCF-binding motif of tk-TOP gene.

The transfected TCF proteins in the cardiac myocytes were detected in the nuclei of the transfectants (Fig. 10 A). Introduction of ΔN-TCF did not inhibit aggregation of cardiac myocytes in Wnt-containing medium, and expression of TA-TCF did not induce their aggregation in the absence of Wnt (Fig. 10 B). Although it is not clear whether TA-TCF functions as the dominant-positive construct on all target genes in cardiac myocytes, when considered together with the findings that incubation with Wnt had no effect on transcription of cadherin or β-catenin mRNA in cardiac myocytes or fibroblasts, it is plausible that TCF target gene expression may be not involved in the morphological effect of Wnt on cardiac myocytes.

### Effects of Wnt Proteins on the Proliferation and Differentiation of Cardiac Myocytes

By analyzing BrdU incorporation, we also examined whether Wnt affects the proliferation or differentiation of cardiac myocytes or fibroblasts by regulating gene expression. Whether cardiac myocytes were incubated with BrdU for 1 or 12 h, no nuclear staining was observed, with or without Wnt proteins (Fig. 11 A). In contrast, numbers of BrdU-labeled nuclei increased time-dependently in fi-
broblasts, although Wnt had no effect on the number of BrdU-labeled nuclei (Fig. 11 B), indicating that Wnt did not induce cell proliferation.

To test whether Wnt induces differentiation of so-called precardiac cells in the neonatal heart, semiquantitative RT-PCR of selected cardio-specific genes was performed. In contrast to other experiments in which a discontinuous Percoll gradient was used to purify cardiac myocytes from other cell types, we separated cells according to their ability to adhere to culture plates, hoping to retain precardiac cells, if any were present, in one or the other cell pool. Eventually, the nonadherent pool contained mainly cardiac myocytes, and the adherent pool contained mainly fibroblasts. Semiquantitative RT-PCR of the gene transcripts expressed early in cardiogenesis, including mRNAs encoding GATA-4 (Heikinheimo et al., 1994), Nkx2.5 (Lints et al., 1993) and MEF-2C (Lints et al., 1993; Edmondson et al., 1994) showed that, although all three markers were constitutively expressed in the cardiac myocyte-rich pool, none were affected by Wnt proteins (Fig. 12). A trace amount of GATA-4 mRNA was detected in the fibroblast-rich pool, but it, too, was unaffected by Wnt proteins. Similarly, terminal differentiation markers, including transcripts for contractile proteins cTnC and β-MHC and for the peptide hormone, BNP, were exclusively expressed in the cardiac myocytes-rich pool and were unaffected by Wnt proteins (Fig. 12). Although we cannot rule out the possibility that small differences between gene transcripts could be masked by RT-PCR, Wnts probably have little if any effect on the transcriptional regulation of cardio-specific genes in cells derived from fetal hearts.

Discussion

Heart development in vertebrates and Drosophila is initiated by bilaterally symmetrical primordia that may be of equivalent embryological origin: the anterior lateral plate mesoderm in vertebrates and the dorsal mesoderm in arthropods. These mesodermal progenitors then merge, forming a heart tube at the ventral midline in the case of the former or in the dorsal midline in the case of the latter. In Drosophila, Wg is known primarily for its ectoderm patterning function (for review see Nusse and Varmus, 1992; Perrimon, 1994) and for specifying neuroblast iden-
In addition, at the stage that dorsal mesoderm is subdivided into somatic, visceral, and cardiac mesoderm, Wg is required for induction of cardiac, but not visceral, mesoderm (Wu et al., 1995; Park et al., 1996, 1998). The product of decapentaplegic (dpp), a member of the TGF-β superfamily secreted from ectoderm, was also found to induce visceral and cardiac mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995). The concerted actions of Wg and dpp thus appear to be required for determination of cardiac cell fate in Drosophila. On the other hand, no vertebrate Wnt genes involved in cardiogenesis have yet been found, even though some have been detected in the developing mammalian heart (Monkley et al., 1996). Therefore, we hypothesize that in mammalian hearts the central role of Wnt may be to exert a morphological rather than a cardiogenetic effect.

We observed that rat cardiac myocytes secrete Wnt-5a, which promotes their aggregation in the presence of fibroblasts. Using dominant negative constructs of frizzled-2 and -4, we further showed that the receptor mediating the morphological effects of Wnt-3a and -5a was frizzled-2. There is ample evidence that the biological effects of Wnt can be mediated by coexpressed frizzled functioning as the Wnt receptor. For instance, in Xenopus before midblastula transition, overexpression of Wnt-1, -3a, -8, and -8b induces axis duplication by stabilizing β-catenin (Moon et al., 1993; Cui et al., 1995; Du et al., 1995), whereas overexpression of Wnt-5a does not, and may even antagonize induction of axis duplication by Wnt-1 (Torres et al., 1996). But when human frizzled-5 is coexpressed, it serves as a receptor for Xwnt-5, mediating axis duplication (He et al., 1997). After midblastula transition, the same overexpression of Wnt-5a stabilizes β-catenin (Larabell et al., 1997), which might be explained by the presence of newly synthesized Wnt receptors allowing Wnt-5a to activate an intracellular pathway that stabilizes β-catenin. Moreover, in zebrafish embryos, frizzled-2 causes an increase in the release of intracellular Ca²⁺ which is enhanced by Xwnt-5a (Slusarski et al., 1997). Thus, Wnt-5a and other Wnts may couple to multiple frizzleds that may in turn signal along multiple pathways (for review see Miller et al., 1999).

The biological effects of Wg are also regionally regulated by the distribution and level of frizzled expression in Drosophila (Cadigan et al., 1998), which limits the effective range of diffusion of secreted Wg proteins to, at most, 20 cell diameters from their secretion site (Zecca et al., 1996). Regarding the functional role of the Wnt/frizzled signaling pathway in mammalian heart, it is noteworthy that frizzled-2 is induced in infarcted and hypertrophied hearts in rodents (Blanksteijn et al., 1996, 1997). In that
context, our findings suggest that Wnt-5a may play a role in remodeling injured hearts through binding to increased frizzled-2.

The interaction between catenins and cadherin is known to be crucial for the formation and strength of Ca\(^{2+}\)-dependent, cell–cell adhesions (for review see Kemler, 1992; Nagafuchi et al., 1993). \(\beta\)-Catenin is a component of adhesion junctions and has been shown to physically associate with the cadherin cytoplasmic domain. We observed that modulation of \(\beta\)-catenin by Wnt-3a and -5a was in concert with similar changes in the abundance of cadherin in both cardiac myocytes and fibroblasts. Increases in \(\beta\)-catenin, immunoprecipitated from these cells using antibodies against N- and E-cadherin, confirmed that \(\beta\)-catenin and cadherin colocalize at cell–cell interfaces; that is, the adhesion between cells was cadherin-based. Consistent with that conclusion, anti–N-cadherin antibodies prevented the morphological effects of Wnt proteins. In earlier studies using suspensions of PC-12, C57MG, and AtT20 mammalian cell lines, overexpression of Wnt-1 induced Ca\(^{2+}\)-dependent reaggregation in parallel with increased expression of cadherins and \(\beta\)-catenin (Bradley et al., 1993; Hinck et al., 1994). Neither study, nor ours, showed an increase in mRNA of cadherin nor \(\beta\)-catenin, indicating the posttranscriptional stability of two proteins by Wnts. Thus, cell–cell adhesion between cardiac myocytes is apparently strengthened by Wnt-evoked stabilization of cadherin–\(\beta\)-catenin complexes, thereby inducing aggregation of cardiac myocytes. This notion is supported by the finding that overexpression of constitutively active \(\beta\)-catenin promoted aggregation of cardiac myocytes, even without Wnt.

Catenins also function in cadherin-independent signaling pathways that regulate differentiation and cell proliferation (for review see Cadigan and Nusse, 1997). Free \(\beta\)-catenin is known to interact with TCF-1/LEF1 transcription factors and to activate target genes (Behrens et al., 1996; Molenar et al., 1996; van de Wetering et al., 1997). For example, Wnt-1 increases levels of homeobox genes such as mouse en- gravelled-1, which is important for vertebrate neural development (Danielian and McMahon, 1996). Furthermore, injection of TCF mutants into Xenopus embryo blocks both endogenous axis formation and the ability of ectopic \(\beta\)-catenin to induce a secondary axis formation (Molenar et al., 1996). By contrast, in this study, overexpression of neither a dominant-negative nor a dominant-positive TCF mutant affected aggregation, indicating that the \(\beta\)-catenin/TCF gene activation pathway has little or no impact on Wnt-induced stabilization of the cadherin–\(\beta\)-catenin complex.

We also examined whether Wnt proteins might induce proliferation and/or differentiation via a \(\beta\)-catenin/TCF gene activation pathway in neonatal heart cells. However, BrdU-labeling and semiquantitative RT-PCR analysis of markers of early cardiogenesis (GATA-4, Nkx2.5, and MEF-2C) and terminal differentiation (Troponin-C, \(\beta\)-MHC, and BNP) both indicated that Wnt has little if any effect on the transcriptional regulation of cardio-specific genes in cells derived from fetal hearts and suggested that there are few if any precardiac cells capable of differentiating in response to Wnt.

The morphological changes evoked by Wnt in cardiac myocytes required the presence of fibroblasts. Plating cardiac myocytes on various matrices, including l-laminine, fibronectin, and collagen, or culturing the cells in fibroblast-conditioned, Wnt-containing medium had no effect on aggregation, suggesting that direct contact between myocytes and fibroblasts is necessary for the induction of aggregation. Although the molecular mechanism remains unknown, one possible explanation may be derived from the finding that different cadherin isoforms are induced in cardiac myocytes and fibroblasts. Since Wnt proteins induced N-cadherin in cardiac myocytes, and E- and M-cadherin in fibroblasts, homotypic cell–cell adhesion between myocytes and fibroblasts should be strengthened by increasing homophilic binding of N- and E-cadherin, respectively. It may be that strengthening homotypic cell–cell adhesion distinguishes cardiac myocytes from fibroblasts, enabling their subsequent aggregation on the surface of fibroblasts.

Although the specific role of Wnt-mediated cell adhesion in the formation of various organ compartments is not known, an effect of Wnt on the regulation of intercellular gap junctions has been demonstrated in Xenopus embryos, in vivo (Olsson et al., 1991). Cadherin-mediated cell adhesion appears to be a prerequisite for gap junction formation in several cell types (Jongen et al., 1991; Gumbiner et al., 1988), and the abundance of gap junctions can be regulated by both cadherin (Meyer et al., 1997; van der Heyden et al., 1998). In our study, electrophysiological experiments carried out in our
laboratory have thus far shown gap junctional conductance to be unaffected by the morphological changes induced in cardiac myocytes by Wnt (data not shown). Tissue formation requires coordinated cell proliferation and morphogenetic movement of groups of cells (for review see Gerhart and Keller, 1986). During these events, the integrity of cell groups is maintained by cell–cell adhesion, but at the same time, the cells must proliferate and must slide past or dissociate from other groups of cells (for review see Gumbiner, 1996). The induction of differentiation during the formation of skeletal muscle is necessarily preceded by aggregation of precursor cells (Edwards et al., 1983; Skerjanc et al., 1994). This requirement for close contact between similar cells during skeletal muscle myogenesis is known as the community effect, which is important for differentiation of somites, cell lines, and embryonic stem cells into skeletal muscle (for review see Gurdon et al., 1993; Skerjanc et al., 1994; Cosso et al., 1995). Cadherin-mediated adhesion has been implicated in the community effect, as well as in skeletal muscle differentiation (for review see Gurdon et al., 1993; George-Winston et al., 1997). Similarly, at an early stage of vertebrate heart development, the N-cadherin/β-catenin complex is involved in demarcating the boundary separating ventral and dorsal mesoderm, and it is within the resultant clusters of N-cadherin-containing dorsal mesoderm that the commitment and phenotypic differentiation of cardiac myocytes proceeds (Linask, 1992; Linask et al., 1997). The developmental stage of these events is analogous to the stage at which Wg induces cardiac mesoderm in Drosophila (Wu et al., 1995; Park et al., 1996). Therefore, we hypothesize that by modulating the interaction of cadherin and catenin, Wnt proteins play a central role in the morphogenetic translocation of cardiac precursor cells during development of the heart-forming region.

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